A Polyphenolic Fraction from Grape Seeds Causes Irreversible Growth Inhibition of Breast Carcinoma MDA-MB468 Cells by Inhibiting Mitogen-activated Protein Kinases Activation and Inducing G1 Arrest and Differentiation

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ABSTRACT

In recent years, significant emphasis is being placed on identifying naturally occurring cancer preventive and interventive agents. In this regard, a polyphenolic fraction isolated from grape seeds (hereafter referred as GSP) has recently been shown by us and others to prevent tumorigenesis in mouse skin models. Chemical analysis of GSP has shown that it is largely constituted with procyanidins that are strong antioxidants. Breast cancer is the most common invasive malignancy and the second leading cause of cancer-related deaths in United States women. Accordingly, here we investigated the effect of GSP on mitogenic signaling and regulators of cell cycle and apoptosis as molecular targets for the growth arrest, apoptotic death, and/or differentiation of estrogen-independent MDA-MB468 human breast carcinoma cells. Treatment of cells with GSP (at 25-, 50-, and 75-μg/ml doses for 1–3 days) resulted in a highly significant inhibition (90% to complete, P < 0.001) of constitutive activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase1/2 in a dose-dependent manner after 72 h of treatment. Whereas GSP treatment of cells did not show a conclusive effect on MAPK/JNK1 activation, a moderate to highly significant inhibition (15–70%, P < 0.1–0.001) of constitutive activation of MAPK/p38 was also observed in a dose-dependent manner as early as 24 h of GSP treatment. GSP-treated cells also showed a strong induction (1.7–2.7 fold, P < 0.001) of cyclin-dependent kinase inhibitor Cip1/p21 and a decrease (10–50%, P < 0.1–0.001) in cyclin-dependent kinase 4. Consistent with these findings, GSP-treated cells resulted in their accumulation in G1 phase of the cell cycle in a dose-dependent manner. An irreversible growth inhibition (44–88%, P < 0.001) was also observed in 50 and 75 μg/ml GSP-treated cells in a time-dependent manner. Additional studies assessing the biological fate of GSP-treated cells showed that they do not undergo apoptotic death, as evidenced by a lack of DNA fragmentation, poly (ADP ribose) polymerase cleavage, and apoptotic morphology of the cells. A morphological change suggestive of differentiation was observed in GSP-treated cells that was further confirmed by a significant induction (1.7–2.6 fold, P < 0.001), in both a dose- and time-dependent manner, in cytokeratin 8 protein level, a marker of differentiation. An irreversible growth-inhibitory effect of GSP possibly via terminal differentiation of human breast carcinoma cells suggests that GSP and the procyanidins present therein should be studied more extensively to be developed as preventive and/or interventive agents against breast cancer in humans.

INTRODUCTION

Breast cancer is the most commonly diagnosed invasive malignancy and a leading cause (after lung cancer) of cancer deaths in American women (1). One approach to control this malignancy is its prevention. In most of the cancer prevention clinical trials including breast cancer, the term “prevention or intervention” is also frequently used to chemopreventive suppression or reversal of premalignant lesions although the lesion is not permanently eliminated (2, 3). Several studies in animal tumor models including rat mammary tumors, followed by epidemiological studies or vice versa, have convincingly established that consumption of yellow-green vegetables and fruits is associated with a decreased risk of several malignancies, including breast cancer (4–12). Accordingly, there has been an increased effort to define the mechanisms by which fruits and vegetables afford protection against cancer and to identify the nutritive and nonnutritive components of the fruits and vegetables that exert such effects. One group of cancer preventive phytochemicals from fruits and vegetables that is receiving increasing attention in recent years is polyphenolic antioxidants (13–18).

Grapes (Vitis vinifera) are one of the most widely consumed fruits in the world. Grapes are rich in polyphenols, and about 60–70% of grape polyphenols exist in grape seeds as dimers, trimers and other oligomers of flavan-3-ols commonly known as procyanidins (or proanthocyanidins; Refs. 19–21). Commercial preparations of grape seed polyphenols are mar
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The multifactorial mechanisms of uncontrolled breast cancer causative, mitogenic and antiapoptotic signaling contributors to targets was based on the fact that they are the major, possibly cycle and apoptosis regulators. The selection of these molecular attention on the effect of GSP on MAPK activation and cell active agents against breast cancer, in this study we focused our cancer prevention and/or intervention.

In an effort to develop GSP and procyanidins as interven-
tive agents against breast cancer, in this study we focused our attention on the effect of GSP on MAPK activation and cell cycle and apoptosis regulators. The selection of these molecular targets was based on the fact that they are the major, possibly causative, mitogenic and antiapoptotic signaling contributors to the multifactorial mechanisms of uncontrolled breast cancer growth. For example, enhanced expression of EGF receptor family members [erbB1 (or EGFR) and Her-2/neu/erbB2] and associated ligands (e.g., transforming growth factor α/EGF) has been shown with high frequency in both estrogen-dependent and -independent breast carcinomas and derived cells (31–36). This high expression of growth factors and receptors leads to an autocrine loop for both mitogenic and antiapoptotic signaling, leading to autonomous growth and metastasis of breast cancer (31–36). The activation of these and other signaling cascades ultimately activate MAPKs that, following their translocation to nucleus, activate transcription factors and command cell cycle regulatory molecules for cell growth, proliferation, and differenti-
tion (37–47). Several studies have shown that MAPKs are constitutively active in human breast carcinomas and derived cell lines as well as in rat mammary tumors (48–52). Together, it can be appreciated that targeting the MAPK signaling pathway should be a useful strategy for the prevention and/or inter-
vention of breast cancer. Using estrogen receptor-negative breast carcinoma MDA-MB468 cells, in this study we demon-
strate the irreversible inhibitory effect of GSP on cell growth and suggest that this effect is possibly via inhibition of MAPK activation and modulation of cell cycle regulators involved in G1 phase. The observed molecular effects of GSP on MDA-
MB468 cells result in a G1 arrest in cell cycle, followed by terminal differentiation, not the apoptotic cell death.

MATERIALS AND METHODS

Cell Line and Other Reagents. The MDA-MB468 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO2 atmosphere. Anti-Cip1/p21 antibody was from Calbiochem (Cambridge, MA). Anti-cytokeratin 8 and anti-CDK4 antibodies were from Neomarkers, Inc. (Fremont, CA). Antibodies to CDK2, cyclin D1, and cyclin E and rabbit antiamouse immunoglobulin- and goat antirabbit immunoglobulin-horseradish peroxidase-conjugated secondary antibod-
ies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho- (and regular) MAPK/ERK1/2, JNK1, and p38 antibodies were from New England Biolabs (Beverly, MA). PARP antibody was from PharMingen (San Diego, CA). The ECL detection system was from Amersham (Arlington Heights, IL). The GSP preparation used was that described in detail recently (29). Approximately 60% (w/w) of this total GSP preparation has been identified and defined by us to contain polyphenols, namely procyanidin B3 (3%), procyanidin B1 (0.7%), catechin (5%), procyanidin B4 (2%), procyanidin B2 (3.5%), epicatechin (6%), procyanidin C1 (6%), procyanidin B5–3′-gallate (15%), and procyanidin B5 (19%), each w/w of total GSP (29). For all of the studies, GSP was dissolved in DMSO as a 10-mg/ml stock solution and diluted as desired directly in the medium. Unless specified otherwise, the final concentration of DMSO in culture medium during GSP treatment did not exceed 0.75% (v/v), and, therefore, the same concentration of DMSO was present in control dishes.

GSP Treatment of Cells and Western Immunoblotting. MDA-MB468 cells were grown in 100-mm dishes, as detailed above, and at 70% confluency were treated with either DMSO alone or varying concentrations of GSP. After 24, 48, and 72 h of treatments, medium was aspirated, cells were washed two times with cold PBS, and cell lysates were prepared as described in detail recently (53). For Western immunoblotting, 40–100 μg of protein lysate per sample was denaturated with 2× sample buffer, samples were subjected to SDS-PAGE on 12% gels, and separated proteins were transferred onto membrane. The levels of phospho- and regular ERK1/2, JNK1, p38, Cip1/p21, CDK4, CDK2, cyclin D1, cyclin E, PARP, and cytokeratin 8 were determined using specific primary antibodies, followed by per-
oxidase-conjugated appropriate secondary antibody and visualiza-
tion by the ECL detection system, as described in detail recently (53).

Cell Cycle Analysis. MDA-MB468 cells at 70% conflu-
ency were treated with either DMSO alone or varying concen-
trations of GSP. After 24, 48, and 72 h of treatments, medium was aspirated, cells were quickly washed two times with cold PBS and tryspinized, and cell pellets were collected. Approximately 0.5 × 10^6 cells in 0.5 ml of saponin/PI solution [0.3% saponin (w/v), 25 μg/ml PI (w/v), 0.1 mM EDTA, and 10 μg/ml

3 The abbreviations used are: GSE, grape seed extract; CDK, cyclin- dependent kinase; CDK1, CDK inhibitor; ERK1/2, extracellular signal- regulated protein kinase 1/2; GSP, a polyphenolic fraction isolated from grape seeds; IGF, insulin-like growth factor; IGF-1R, IGF-1 receptor; MAPK, mitogen-activated protein kinase; PARP, poly(ADP ribose) polymerase; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor; JNK, c-jun-NH2-kinase; FACS, fluorescence-activated cell-sorting; PI, propidium iodide.
RNase (w/v) in PBS] were left at 4°C for 24 h in the dark. Cell cycle distribution was then analyzed by flow cytometry using the FACS Analysis Core Services of the University of Colorado Cancer Center (Denver, CO).

**Cell Growth Assay.** MDA-MB468 cells were plated at 5000 cells/cm² density in 35-mm dishes under the culture conditions detailed above. After 24 h, cells were fed with fresh medium and treated with either DMSO alone or varying concentrations of GSP. The cultures were fed with fresh medium with or without the same concentrations of GSP every alternate day up to the end of the experiment; each treatment and time point had four plates. After 2, 4, and 6 days of treatments, cells were trypsinized, collected, and counted using a hemocytometer. Trypan blue dye exclusion was used to determine cell viability.

In another study, MDA-MB468 cells were plated at 5000 cells/cm² density in 35-mm dishes and, after 24 h, treated with DMSO alone or varying doses of GSP. After 72 h of treatments, total cell growth was determined by cell counting. At this point, in separate dishes, after 72 h of treatments with GSP, cultures were washed several times with medium to remove GSP and grown in fresh medium without GSP for another 24, 48, or 72 h. The cell number was determined at these time periods.

**DNA Ladder Assay.** MDA-MB468 cells were grown in 100-mm dishes, as detailed above, and at 70% confluency were treated with either DMSO alone or varying concentrations of GSP. After 24, 48, and 72 h of treatments, trypsinized cells (together with any floating cells) were collected and the DNA ladder analysis was done as described in detail recently (54).

**Morphological Analysis.** MDA-MB468 cells were grown in 100-mm dishes, as detailed above, and at 50% confluency were treated with either DMSO alone or 50- and 75-µg/ml concentrations of GSP. After 24, 48, 72, and 96 h of treatments, pictures were taken using phase-contrast microscope at ~200 magnification. The cell pellets were then collected for cytokeratin 8 protein expression by Western immunoblotting, as detailed above.

**Densitometric and Statistical Analysis.** Autoradiograms of the Western immunoblots were scanned using Adobe Photoshop. The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed using Scanimage Program. As needed, a two-tailed Student’s t test was used to assess statistical significance of difference between vehicle- and GSP-treated samples. Until and unless specified otherwise, the results shown in each case are representative of three independent experiments with similar findings.

**RESULTS**

GSP Inhibits Constitutive Activation of ERK1/2 and p38, But Causes Moderate Effect on JNK1 in MDA-MB468 Cells. First, we focused our attention on the effect of GSP on constitutive activation of MAPKs. Because the MAPK family includes ERKs, JNK (stress-activated protein kinase), and p38 (HOG), which are functional units involved in three distinct signaling pathways (37–47), we assessed the effect of GSP on all of these MAPKs. As shown in Fig. 1A, treatment of MDA-MB468 cells with GSP resulted in a moderate to complete inhibition of constitutive activation of ERK1/2. As shown in Fig. 1B, the effect of GSP on JNK1 was moderate. As shown in Fig. 1C, the effect of GSP on p38 was also moderate.

![Fig. 1](image-url) Effect of GSP on the constitutive activation of MAPK signaling in human breast carcinoma MDA-MB468 cells. Cells were cultured in DMEM with 10% serum and at 70% confluency were treated with either DMSO alone or 25-, 50-, and 75-µg/ml concentrations of GSP for 24, 48, and 72 h. Cell lysates were prepared and subjected to SDS-PAGE, followed by Western blotting, as detailed in “Materials and Methods.” The membranes were probed with antiphospho-ERK1/2, antiphospho-JNK1, and antiphospho-p38 MAPK antibodies and then peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was done using ECL detection system. A, phosphorylation of ERK1/2. B, phosphorylation of JNK1. C, phosphorylation of p38. Treatments are as labeled in the figure. IB, Western immunoblot. In each panel, the densitometric analysis bars represent the mean ± SE values (arbitrary units) of three independent experiments. At each time point of the study, the vehicle-treated control value is presented as 100%.
inhibition of constitutive activation of ERK1/2 in both a dose- and time-dependent manner. The densitometric analysis of three different blots showed that compared with DMSO control, after 24 h of GSP treatment, 25- and 50-µg/ml doses were not effective, but a 75-µg/ml dose showed moderate (although statistically not significant) inhibition (10%, P < 0.1) of constitutive ERK1/2 activation. However, a higher inhibition (20–25%, P < 0.05) was observed at both 50- and 75-µg/ml doses after 48 h of GSP treatment, and after 72 h these doses showed complete inhibition (P < 0.001) of constitutive ERK1/2 activation; even a 25-µg/ml dose resulted in 90% inhibition (P < 0.001) after this treatment time (Fig. 1A). The observed decreases in constitutive ERK1/2 activation by GSP were not due to a change in its protein levels following these treatments (data not shown), suggesting that GSP impairs mitogenic signaling of ERK1/2. These results are specifically important because of a dose and time response where it could be suggested that a longer treatment time with a lower dose(s) produce comparable efficacy to that with higher dose and shorter treatment time.

Unlike its effect on ERK1/2 activation, GSP did not show a conclusive effect on JNK1 activation (Fig. 1B). For example, 24 h of treatment with 25-, 50-, and 75-µg/ml doses did not result in any change in constitutive activation of JNK1, whereas after 48 h of treatment a 75-µg/ml dose showed a strong stimulation (Fig. 2B). Conversely, 50- and 75-µg/ml doses of GSP showed ~35% inhibition (P < 0.05) after 72 h of treatment (Fig. 2B). Similar to ERK1/2, GSP did not show any change in JNK1 protein expression following these treatments (data not shown).

With regard to p38, as shown in Fig. 1C, compared with DMSO control, GSP treatment of MDA-MB468 cells resulted in a highly significant inhibition of p38 activation in both a dose- and time-dependent manner. The observed effect of GSP on p38 was much stronger than ERK1/2 activation at both 24 and 48 h at all three doses examined, but was less effective than ERK1/2 after 72 h of treatment (Fig. 1C versus A). Quantitative analysis of the blots showed that 24 h of GSP treatment at a 25-µg/ml dose was not effective, however, 50- and 75-µg/ml doses resulted in 15% (P < 0.1) and 70% (P < 0.001) decrease, respectively, in constitutive p38 activation. After 48 h of GSP treatment of cells, as much as a 15% (P < 0.1), 68% (P < 0.001), and 70% (P < 0.001) decrease was evident in p38 activation by these doses, respectively. No further effect, however, was observed following additional treatment time up to 72 h. An interesting observation was a strong increase in constitutive activation of p38 in control samples after 48 h, as compared with those at 24 and 72 h (Fig. 1C). The reason for this increase remains to be established. Taken together, these findings suggest that GSP treatment of MDA-MB468 human breast carcinoma cells results in a significant decrease in ERK1/2 and p38 activation, the two MAPK pathways associated with cell growth and differentiation (55), but has moderate, if any, effect on JNK1, the MAPK pathway associated largely with cell death (55–57).

**GSP Induces Cip1/p21 Levels and Decreases G1 Phase Regulators CDK4 and Cyclin D1 in MDA-MB468 Cells.** A controlled cell growth, proliferation, differentiation, and/or apoptosis are mediated via cell cycle progression that is governed by cellular signaling (Refs. 58–62 and references therein).
However, cancer cells often display defects in the genes that govern the cellular responses to a growth factor(s)-growth factor receptor(s) interaction (61). Perturbations in cell cycle regulation have been demonstrated to be one of the most common features of transformed cells (61), which could be associated with gain of function for uncontrolled growth due to enhanced expression of growth factor-receptor autocrine loop (31–36), a lack of CDKI or loss in its function (58–63), and so on. Accordingly, next we assessed the effect of GSP on cell cycle regulators involved in G1 phase. As shown by data in Fig. 2A, treatment of cells with GSP resulted in a significant induction of Cip1/p21 and a cell cycle-dependent manner. The densitometric analysis of blots showed that 24 h of GSP treatment caused a 1.7-, 2.3-, and 2.7-fold increase (P < 0.001) in Cip1/p21 at doses of 25, 50, and 75 μg/ml, respectively. Although 48 and 72 h of GSP treatment also showed significant Cip1/p21 induction, it was less profound than that at 24 h and amounted for 1.2–1.6-fold increases (P < 0.1 to 0.001). Similar to its effect on Cip1/p21, GSP treatment of cells also resulted in a significant decrease in CDK4 protein levels (Fig. 2B). In this case, 24 h of GSP treatment caused a 10% (P < 0.1), 30% (P < 0.05), and 50% (P < 0.001) decrease in CDK4 expression at 25-, 50-, and 75-μg/ml doses, respectively. Similar effects of GSP were also observed following its treatment for 48 and 72 h at identical doses. Unlike its effect on Cip1/p21 and CDK4, GSP treatment of cells for 24 h resulted in a marginal increase in cyclin D1 protein levels, however, 48 and 72 h of treatment caused marginal decrease (Fig. 2C); GSP treatment did not show any effect on CDK2 and cyclin E protein expression (data not shown).

**GSP Induces G1 Arrest in Cell Cycle Progression of Human Breast Carcinoma MDA-MB468 Cells.** On the basis of the results showing a strong induction in Cip1/p21 and a decrease in CDK4 by GSP, we next examined its effect on cell cycle progression. As shown in Fig. 3, FACS analysis of DMSO-treated cells showed a cell cycle distribution that followed a proliferation pattern. However, a moderate to strong difference in cell cycle progression was observed following GSP treatment where all of the doses and time points examined showed a G1 arrest in the cell cycle progression of MDA-MB-468 cells largely at the expense of S phase population (Fig. 3). Within 24 h after GSP treatment, compared with DMSO-treated control, 25- and 50-μg/ml doses showed marginal accumulation of cells in G1 phase (55% and 58% of cells in G1, respectively, compared with 53% in control; Fig. 3A). However, as much as 71% of cells accumulated in G1 phase following a 75-μg/ml dose of GSP treatment for 24 h (Fig. 3A). The increase in G1 population by GSP at all of the doses was accompanied by a significant decrease of the cells in S phase (Fig. 3B). An accountable increase in G2-M population of the cells was also observed following a 24-h treatment with GSP at these doses (Fig. 3C). When MDA-MB-468 cells were treated for a longer time (i.e., 48 and 72 h) with GSP, even a lower dose (25 μg/ml) resulted in a strong accumulation of the cells in G1 phase that is moderately affected by increasing the doses to 50 and 75 μg/ml (Fig. 3A). Once again, this increase was mainly due to a decrease in S phase population (Fig. 3B) with moderate alterations in G2-M phase (Fig. 3C). Together, these results were consistent with other findings, showing that (a) maximum effect of GSP on Cip1/p21 was at 24 h (Fig. 2A) and (b) GSP modulates cell cycle regulators associated with G1 phase (Fig. 2).

**GSP Irreversibly Inhibits the Growth of MDA-MB468 Cells.** The studies assessing the biological significance of the observed effects of GSP showed that it causes the inhibition of human breast carcinoma MDA-MB468 cells and that this inhibition was irreversible at higher doses examined. As shown by data in Fig. 4A, GSP treatment of growing cells resulted in a significant to almost complete inhibition of cell growth in both a dose- and time-dependent manner. Compared with DMSO-treated controls, whereas a 10-μg/ml dose was not effective, a 25-μg/ml dose resulted in 20–50% (P < 0.001) inhibition after 2–6 days of treatment (Fig. 4A). A much higher inhibitory effect was observed at 50- and 75-μg/ml doses of GSP, accounting for
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As shown in Fig. 4B, compared with DMSO-treated controls, 72 h of GSP treatment at a 25-μg/ml dose, followed by a washout study for 1–3 days, did not show any change in cell growth. However, treatment of cells with 50 μg/ml GSP for 72 h and then following their growth after 24, 48 and 72 h of washout showed that there was 44%, 46%, and 52% inhibition (P < 0.001) in cell growth, respectively (Fig. 4B). A much stronger inhibition was evident in this washout study where 75 μg/ml GSP led to a 77%, 86%, and 88% inhibition in cell growth after 24, 48 and 72 h of GSP washout, respectively (Fig. 4B). Because another interpretation of the data shown in Fig. 4B leads to the argument that the observed irreversible inhibition is due to the fact that 72 h of GSP treatment at different doses already reduced the total number of cells to begin with in washout studies, we also followed cell growth pattern during 72 h after GSP washout. As shown in Fig. 4B, in case of DMSO-treated and 25 μg/ml GSP-treated cultures, after washout, the cell growth pattern was comparable and was 205%, 172%, and 125% (compared with the previous 24 h) after 24, 48, and 72 h of washout, respectively. However, under similar treatment conditions, these patterns were 140%, 164%, and 110% in case of 50 μg/ml GSP-treated cultures, and 140%, 104%, and 100% in case of 75 μg/ml GSP-treated cultures (Fig. 4B). Together, these results provide convincing evidence that the observed cell growth inhibitory effects of GSP are not just cytostatic, but are irreversible.

Irreversible Growth Inhibitory Effect of GSP Leads to Differentiation of MDA-MB468 Cells. The observed irreversible growth inhibitory effect of GSP on MDA-MB468 cells led to the question that what was the ultimate fate of the growth arrested cells. Both apoptotic cell death and differentiation possibilities, therefore, were explored to answer this question. For the apoptotic cell death studies, cultures were treated with 25-, 50-, and 75-μg/ml doses of GSP for 24, 48 and 72 h, and both floating and attached cells were subjected to several apoptosis-associated assays. In these studies, none of the GSP doses and time points examined showed: (a) DNA ladder; (b) PARP cleavage; (c) morphological changes suggestive of apoptosis; (d) TUNEL staining; and (e) sub-G1 population in cell cycle assay, as compared with positive findings with paclitaxel used as a positive control (data not shown).

On the contrary, GSP-treated MDA-MB468 cells showed unique morphological changes. As shown in Fig. 5, compared with DMSO-treated control cultures, cells treated with 50 μg/ml GSP for 4 days were much larger in their size with a change in morphology to spindle shape (Fig. 5, A versus B). A more profound effect of GSP was evident at a 75-μg/ml dose for 4 days where cells became much larger in size with significant changes in morphology because they were both elongated in length and had spindle shape (Fig. 5, C versus A). Although less profound, 48- and 72-h treatment time points at these doses of GSP also showed morphological changes (data not shown).

These morphological changes were similar to that of epithelial cell differentiation, suggesting that GSP induces differentiation of MDA-MB468 cells. Following these morphological changes, the cells started detaching from the culture dishes presumably due to their death, suggesting that the observed morphological changes with GSP are associated with terminal differentiation that ultimately causes cell death. In other studies, as shown in...
we found that GSP treatment of cells also results in a significant induction of cytokeratin 8 protein expression, a marker of epithelial cell differentiation (64). The densitometric analysis of the blot showed that compared with DMSO, both 50- and 75-μg/ml doses of GSP resulted in a 1.7-fold increase ($P < 0.001$) in cytokeratin 8 expression following a 48-h treatment. Whereas no further increase was observed at these doses after 72 h of treatment, 96 h of GSP treatment at these doses showed 2.4- and 2.6-fold increases ($P < 0.001$) in cytokeratin 8 levels, respectively.

**DISCUSSION**

The central finding in the present study is that a polyphenolic fraction isolated from grape seeds that is rich in procyanidins, inhibits constitutive activation of MAPK/ERK1/2 and MAPK/p38, and causes an induction of CDKI Cip1/p21 and a decrease in CDK4. These effects of GSP result in a G1 arrest in cell cycle, followed by an irreversible inhibition of cell growth. The GSP-treated cells unable to grow do not die by apoptosis but undergo terminal differentiation, followed by cell death. Our findings that GSP inhibits constitutive activation of ERK1/2 are specifically significant because constitutive activation of this pathway has been shown to be associated with human breast carcinomas and derived cell lines for uncontrolled growth (48–52).

The MAPK family includes ERKs, JNK (stress-activated protein kinase), and p38 (HOG), which are functional units involved in three distinct signaling pathways (37–47). Activated MAPKs transport to the nucleus where they phosphorylate and activate a series of transcription factors such as Elk-1, cMyc, ATF-2, cJun, CREB, and so on (37–47, 65–67). The differential responses of ERKs, JNKs, and p38 in terms of activation of different transcription factors suggest that these signaling cascades are distinct (37–47). Furthermore, several studies have linked ERKs and p38 signaling to proliferation and differentiation, whereas the JNK pathway is largely associated with apoptosis (55–57). Consistent with these reports, in the present study we found that GSP inhibits constitutive activation of ERK1/2 and p38, but had little (if any) effect on JNK activation. These results were in accord with the biological effects of GSP where it showed growth inhibitory and differentiation inducing potential but did not cause apoptotic death. The observed inhibitory effect of GSP on ERK1/2 activation is similar to those reported with other polyphenolic antioxidants, such as silymarin, genistein, quercetin, and others (54), however, to the best of our knowledge, this is the first study showing the inhibitory effect of a polyphenolic antioxidant fraction on p38 activation.

Earlier, it has been shown that erbB1 and other members of protein levels. Cells at 50% confluency were treated either with DMSO alone or 50- and 75-μg/ml concentrations of GSP for 48, 72, and 96 h. Cell lysates were prepared and subjected to SDS-PAGE, followed by Western blotting, as detailed in “Materials and Methods.” The membranes were probed with anti-cytokeratin 8 antibody, followed by peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was done using ECL detection system. Treatments are as labeled in the figure. IB, Western immunoblot.
the erbB family (Her-2/neu or erbB2) play an important role in human breast cancer (31–36). In addition, IGF-1R has also been shown to play an important role in the growth, proliferation, and metastasis of breast cancer, which is also associated with a decrease or loss of IGF-binding protein-3 that binds to IGF, leading to an inhibition of the binding of this ligand to IGF-1R (35). This impairs mitogenic and antiapoptotic signaling mediated by IGF-1/IGF-1R pathway causing a malignant cell growth arrest and apoptotic cell death (68). These and other signaling pathways ultimately activate MAPKs that then translocate to the nucleus and activate transcription factors for cell growth, differentiation, and proliferation (37–47, 68). Taken together, these reports raise the question whether the observed inhibitory effects of GSP on ERK1/2 and p38 activation are direct effect or due to impairment of upstream signaling involving both receptor tyrosine kinases and cytosolic signaling. This cause and effect is an important issue that is being studied in an ongoing program.

Several studies have shown that mitogenic signaling must be continually renewed, even if at quite low levels, throughout much of the growth stimulating G1 phase of the mammalian cell cycle (58–62). For example, a cell arrest or growth signal determines whether the cells will be in a resting, nondividing “G0” phase or whether they will enter the G1 phase of cell cycle and thereby undergo cell replication and proliferation (58–62). Cyclin D1 and its associated CDK4 (or CDK6) normally control cell cycle events in early G1 phase, and cyclin E coupled with CDK2 is involved in the transition from late G1 to S phase (58–62). Alternatively, impairment of a growth-stimulatory signaling pathway leads to the induction of CDKIs (such as Cip1/p21 and Kip1/p27) that inhibit the activity of cyclin-CDK complexes, leading to cell growth arrest (69). These studies suggest that modulation of cell cycle regulatory molecules either directly or via impairment of mitogenic signaling that regulate them, could be a practical and translation approach for the prevention and/or intervention of human malignancies, including breast cancer. Consistent with these reports, in the present study we found that GSP treatment of breast carcinoma MDA-MB468 cells results in a significant induction of Cip1/p21. We also found that GSP significantly inhibits CDK4 levels and moderately alters cyclin D1 expression. Conversely, GSP was not effective in inhibiting cyclin E and CDK2 levels. Together, these data are consistent with the observed effect of GSP on G1 accumulation and suggest that GSP causes an early effect, leading to arrest of cells in early G1 phase. These effects of GSP on breast carcinoma cells are specifically important because an overexpression of G1 phase cyclin D1 has been shown with high frequency in human breast cancer (61).

An up-regulation of Cip1/p21 strongly correlates with cell growth inhibition that ultimately decides the fate of cell between differentiation and death. For example, inhibition of Cip1/p21 expression through transfection of Cip1/p21 antisense oligonucleotides has been shown to block growth factor-induced differentiation of SH-SY5Y neuroblastoma cells and resulted in their death (70). Conversely, Cip1/p21 induction is shown in the differentiation of a variety of cells such as myogenic, keratinocytic, promyelocytic (HL-60), and human melanoma cells (71–73). Consistent with these findings, we observed that GSP-caused induction of Cip1/p21 and resultant G1 arrest did not induce apoptosis but caused terminal differentiation of cells, followed by their death. An irreversible cell growth inhibition by GSP further supports this observation.

An analysis of the time kinetics of the observed effects of GSP on cell cycle regulators suggests that possibly GSP directly affects Cip1/p21 and CDK4 expression as an early response that leads to cell growth inhibitory effect via G1 arrest. This growth inhibitory effect of GSP then presumably leads to an impairment of autocrine loop, causing a decrease in constitutive activation of MAPK/ERK1/2 as a late response and a terminal differentiation of the cells. This presumption could be supported indirectly by the studies where at least in prostate carcinoma cells it has been shown that an induction in MAPK/ERK1/2 activation is associated with the reversal of cellular differentiation (74). More studies, however, are needed to further establish this cause and effect relationship.

In summary, based on the results of the present study, we conclude that GSP and procyanidins present therein should be studied in more detail to be developed as breast cancer preventive and/or intervention agents. In addition, studies are also needed to establish whether the observed effective doses of GSP in cell culture are achievable physiologically in breast cancer patients and that such doses are both nontoxic and effective for preventive intervention of this deadly malignancy.

REFERENCES


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