Genistein Elicits Pleiotropic Molecular Effects on Head and Neck Cancer Cells

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ABSTRACT

Genistein (4,5,7-trihydroxyisoflavone) has been reported to induce cell cycle arrest and apoptosis in different cancer cell lines in vitro and to show antitumor activity against a variety of tumors in animal models. We have previously reported (S. A. Alhasan et al., Nutr. Cancer, 34:12–19, 1999; S. A. Alhasan et al., Int. J. Oncol., 16:333–338, 2000) that genistein induces cell cycle arrest and apoptosis by up-regulating p21WAF1 and Bax, and down-regulating cyclin B1 and Bcl-2 in a head and neck cancer cell line. However, the precise molecular mechanism(s) by which genistein elicits its effects on head and neck cancer cells still remains to be elucidated. In the present study, we report that genistein induces several specific molecular changes in head and neck cancer cells, such as down-regulation of c-erbB-2 expression, down-regulation of MMP-2 and MMP-9 secretion, inhibition of tumor cell invasion and down-regulation of nuclear factor-κB DNA binding activity. In addition, genistein inhibited the levels of phosphorylated Akt and the expression of 14-3-3 protein. Moreover, genistein induces telomere shortening in treated cells without affecting telomerase activity in vitro. We also observed that genistein inhibits the translocation of telomerase catalytic subunit [human telomerase reverse transcriptase (hTERT)] to the nucleus, which may result in telomere shortening, although the activity of telomerase is unaffected, along with the inhibition of metaphase spread of chromosomes. From these results, together with our previously published reports, (S. A. Alhasan et al., Nutr. Cancer, 34:12–19, 1999; S. A. Alhasan et al., Int. J. Oncol., 16:333–338, 2000) we conclude that genistein elicits pleiotropic molecular changes that resulting in the inhibition of cell growth and the induction of apoptotic cell death of head and neck cancer cells, which suggests that genistein may be useful as a chemotherapeutic and/or chemopreventive agent for head and neck cancer.

INTRODUCTION

Genistein is an isoflavone, believed to be responsible for the lower incidence of cancers in Asians compared with populations in the United States (1, 2). Genistein has a heterocyclic, diphenolic structure similar to estrogen (2, 3) and was shown to be a potent inhibitor of epidermal growth factor receptor tyrosine kinase (3–7). c-erbB-2 belongs to a growth factor receptor family with intrinsic tyrosine kinase activity that can form heterodimers with epidermal growth factor receptor, resulting in increased tyrosine kinase activity (8), which may be important for uncontrolled growth of tumor cells. c-erbB-2 (also known as Her-2/neu) is reported to be overexpressed in HNSCC (9, 10). A transcription factor known as NF-κB, whose DNA binding activity to its consensus sequence, was found to be altered by a specific protein that binds to c-erbB2 gene promoter sequence and, thereby, may influence the expression and function of c-erbB-2 (11). The overexpression of c-erbB-2 is also known to cause an increase in the secretion of MMPs and concomitant aggressive tumor phenotype (12).

Genistein has been shown to be an inhibitor of angiogenesis both in vitro and in vivo (13, 14) and, thus, inhibits the formation of new blood vessels, which carry important nutrients to the tumor cells and provide access to the circulatory system, resulting in tumor cell metastasis (15–18). This complex process is also regulated by the alterations in the expression and activity of MMPs, which suggests a complex interplay between the activation of c-erbB-2, NF-κB, MMPs, angiogenesis, degradation of basement membrane, and tumor metastasis (19–22). MMP secretion by tumors has been indirectly correlated with a lower apoptotic profile, which occurs through a complex regulation of MMPs and their inhibitors (23–26).

Previous studies have shown that genistein inhibits the invasion of different tumor cell lines in vitro (27–30) and reduces metastasis of tumors in animal models in vivo (31–32). Genistein also down-regulates MMP secretion in breast cancer cells, as reported by us previously (33). In addition, MMP-9 has been shown to be transcriptionally regulated by different transcription factors in coordination with NF-κB (20). Genistein is also known to induce cell cycle arrest at G0-M and to induce apoptosis in a variety of cancer cells including breast, gastric, lung, head and neck, and prostate (34–39). Moreover, we also reported that genistein-induced cell growth inhibition is probably mediated by the up-regulation of p21WAF1, down-regulation of cyclin B1 and cdc25C, and activation of CPP32, which

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2 The abbreviations used are: HNSCC, squamous cell carcinoma of the head and neck; hTERT, human telomerase reverse transcriptase; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; TBST, Tris-buffered sodium with Tween 20; TRAP, telomeric repeat assay protocol; TNF, tumor necrosis factor.
results in the cleavage of poly-ADP-ribose polymerase (PARP) and induces cellular senescence (34, 40).

During cellular senescence, telomere length is shortened to a critical point, which is regulated by telomerase activity (41, 42). Telomerase is a specialized reverse transcriptase that directs the synthesis of telomeric DNA repeats at the end of the chromosome (43) and is known to be transcriptionally regulated by the activation of NF-κB (44). Telomerase is usually present in the cytoplasm, and translocates into the nuclear compartment by the molecular chaperon molecule, 14-3-3 during the activation process (45). The activation of NF-κB was also reported to require phosphatidylinositol 3-kinase (PI3-kinase) activity (46), which phosphorylates Akt and mobilizes it to the cell membrane (47). In addition, Akt was reported to phosphorylate a motif that is present in all of the 14-3-3 consensus sequences (48). At the same time Akt enhances the activity of telomerase by phosphorylating the catalytic subunit hTERT (47). These reports strongly suggest a complex interplay between c-erbB-2, MMPs, telomerase, 14-3-3, Akt, and NF-κB DNA binding activity in cell growth, differentiation, and apoptosis.

In this study, we report that genistein down-regulates c-erbB-2, inhibits the secretion of both MMP-2 and MMP-9 in a dose- and time-dependent manner. Additionally, we show that genistein inhibits tumor cell invasion of HNSCC, and down-regulates 14-3-3 and phosphorylated Akt. In addition, we also report for the first time that genistein inhibits metaphase spread of chromosomes with concomitant inhibition in the nuclear translocation of hTERT without affecting telomerase activity.

MATERIALS AND METHODS

Tissue Culture. HN4, a HNSCC cell line, was established at Wayne State University and was cultured as described in our previous report (34). All tissue culture supplies were purchased from Life Technologies, Inc., Rockville, MD.

Genistein. Genistein (4,5,7-trihydroxyisoflavone) was purchased from Biomol Research Laboratories, Inc., Plymouth Meeting, PA), dissolved in 0.1 M sodium carbonate, and stored at Wayne State University and was cultured as described

Protein Extraction. HN4 cells were seeded at a density of 7.5 × 10⁵/100-mm dish and allowed to adhere for 24 h. The adherent cells were treated with vehicle only (0.1 M Na₂CO₃) or with 25 or 50 μM genistein for up to 3 days. After incubation with genistein for the indicated time period, HN4 cells were scraped from the culture dishes and centrifuged at 2,000 RPM for 5 min. The cell pellets were washed in 1× PBS and were centrifuged again at 2,000 RPM for 5 min. The cell pellets were suspended in 120 μl of 125 mM Tris-HCl (pH 7.5) and sonicated three times, 15 s each at 30% output, and were lysed by adding a equal volume of 4% SDS. The extracts were boiled in a water bath for 10 min, chilled on ice, and centrifuged at 13,000 RPM for 15 min. The pellets were discarded, and the supernatants were subjected to protein measurement using a protein assay kit from Pierce (Rockford, IL); 50 μg of protein from each sample was used for SDS-PAGE and subjected to Western blot analysis.

Antibodies. Rabbit antitelomerase catalytic subunit (hTERT) was purchased from Calbiochem (La Jolla, CA) and used at 1:750 dilution in 2% BSA in 1× PBS. Texas Red-conjugated goat-antirabbit polyclonal antibodies were purchased from Molecular Probes (Eugene, OR), and used at 1:100 dilutions. Rabbit anti-phospho Akt and rabbit anti-14-3-3 were purchased from Cell Signaling (Beverly, MA), and used at 1:1000 dilutions. Mouse anti-c-erbB-2 was purchased from Oncogene Sciences (MA) and used at 1:500 dilutions. Rabbit anti-β-actin polyclonal antibody was purchased from Sigma Chemical Co. (St. Louis, MO) and diluted 1:5000 in TBST. Horseradish peroxidase-conjugated goat antirabbit and goat antit- mouse antibodies were purchased from Bio-Rad, (Hercules, CA) and used at 1:3000 dilutions in TBST with 5% fat free milk.

SDS-PAGE and Western Blot Analysis. Fifty μg of whole cell extract were separated using 10–12% polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 10% nonfat milk in TBST for 1 h. The membranes were incubated with primary antibodies overnight, washed with 1× TBST for 30 min, and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The membranes were washed three times, 10 min each, with 1× TBST. The membranes were developed using a chemiluminescent detection kit (Pierce) and then exposed to X-ray film. The relative absorbance of β-actin was used as gel-loading control for proteins. The intensity of the band in the Western blot was measured by the Gel Doc system (Bio-Rad), and the values were calculated as means ± SE of three independent experiments, using Graph Pad Prism 2.01, program (San Diego, CA), and the Ps were calculated using Student’s t test.

Metaphase Spread. HN4 cells were cultured and treated with genistein as described earlier. Thirty min before harvesting the cells, one drop of 10 mg/ml colcemid per ml of culture medium were added to the culture dishes and returned to the incubator for 30 min for arresting cells in the metaphase. The cells were then trypsinized and collected in 15-ml conical centrifuge tubes. The cell suspension was centrifuged at 2000 RPM for 5 min. The supernatant was discarded leaving 0.5 ml on top of the pellet. The cells were resuspended in 0.075 M prewarmed (37°C) KCl, first drop-wise and then by 10 drops at a time and mixed rapidly to a total volume of 10 ml. The resuspended cells were incubated in a 37°C water bath for 13 min. One drop of methanol:acetic acid (3:1) fixative was added for each ml of cell suspension, and the tubes were laid flat at room temperature for 1 h. The cells were centrifuged at 1000 RPM for 10 min. The supernatant was discarded leaving 0.5 ml. The pellet was resuspended, and the fixative was added again up to 10–12 ml, and the tubes were laid flat at −20°C for 1 h. The cells were centrifuged at 1000 RPM for 10 min. The supernatant was discarded, the fixative was added as before, and the tubes were laid at room temperature for 30 min. The cells were centrifuged as above, and the pellet was resuspended by adding the fixative up to 10 ml. The tubes were kept at −20°C overnight. The next morning, the cells were centrifuged, and the pellet was resuspended, dropped on glass slides, air-dried, and examined under light microscope. The cells with metaphase spread were counted.

Telomerase Assay. TRAP reagent kit was purchased from Intergen (Purchase, NY), and used according to the manufacturer’s instructions.
Immunostaining and Confocal Microscopy. The cells were grown on glass coverslips and treated with genistein as indicated. The cells were fixed in ice-cold 100% methanol. The coverslips were treated with 2% BSA in PBS (blocking buffer) for 45 min. The cells were then washed with PBS and incubated with rabbit anti-hiTERT antibodies in 2% BSA in PBS for 2 h. The coverslips were washed three times with PBS and then incubated with Texas Red-conjugated goat antirabbit antibodies. The coverslips were washed three times with PBS, fixed for 10 min with ice-cold 100% methanol, and then mounted on clean slides using aqua gel mount with antifading agents. The slides were examined using Zeiss laser scanning inverted confocal microscope system 310, and the red fluorescent images were captured.

MMP Activity Measured by Gelatin Zymography. Gelatin zymography was performed according to the protocol of Tan et al. (49) to study the effect of genistein on the secretion and activity of MMP-2 and MMP-9. Briefly, HN4 cells were cultured in complete medium in 6-well plates. Duplicate wells were used for each treatment. After 24 h, the medium was aspirated, and the wells were rinsed with serum-free phenol red-free medium. The cells were cultured with serum-free medium and treated with genistein. The conditioned medium was collected, and the cell debris was spun off. The supernatants were concentrated using Amicon (Beverly, MA) spin columns, and the protein content was measured using protein assay kit. Supernatants were combined, and the cell debris was spun off. The supernatants were used for each treatment. After 24 h, the medium was aspirated, and the wells were rinsed with serum-free phenol red-free medium. The cells were cultured with serum-free medium and treated with genistein. The conditioned medium was collected, and the cell debris was spun off. The supernatants were concentrated using Amicon (Beverly, MA) spin columns, and the protein content was measured using protein assay kit.

Invasion Assay. The invasion assay was performed according to the instruction of Becton Dickinson Lab Ware (Bedford, MA), the supplier of Matrigel. Matrigel was diluted in ice-cold DMEM at a final concentration of 400 μg/ml. The Matrigel was thawed on ice in the refrigerator overnight. Pipette tips were stored at −20°C and were used to dispense Matrigel to the invasion chambers. Costar trans-well culture inserts were purchased from Fisher Scientific (Hanover Park, IL). The inserts were coated with 100 μl of Matrigel and incubated at 37°C for 3 h to allow the Matrigel to polymerize. DMEM was allowed to evaporate under sterile conditions overnight. The Matrigel-coated inserts were rehydrated by adding 100 μl of serum-free culture medium and incubating for 2 h at 37°C. The cells were trypsinized and resuspended in serum-free medium. The cells were dispersed in serum-free medium with the indicated concentrations of genistein and added to the culture inserts. Complete medium with genistein was added to the culture wells in such a way that it touched the other side of the membrane. The inserts were incubated in tissue culture incubator for a maximum of 18 h, and the inserts were collected. The culture medium was discarded and the Matrigel was scrapped off the insert surface by using cotton applicators. The cells were fixed in Hema 3 fixative twice, for 3 min each time, and then stained with Hema3/Eosin twice for 3 min each, then Hema3/methylene blue twice for 3 min each. The inserts were washed in distilled water, then air-dried and examined and counted under a light microscope. The percentage inhibition of cell invasion relative to control was plotted using Graph Pad Prism 2.01 software.

Electrophoretic Mobility Shift Assay. The NF-κB binding consensus oligonucleotide was purchased from Promega Corporation (Promega, WI). The oligonucleotide was end labeled with [γ-32P]ATP, and electrophoretic mobility shift assay was performed according to the protocol supplied by Promega Corporation. The gels were dried and exposed to X-ray film.

RESULTS

The Effect of Genistein on c-erbB-2 Expression and Tumor Cell Invasion. When we examined the effect of genistein treatment on the expression of c-erbB-2 by Western blot analysis, we found that c-erbB2 protein was down-regulated in a dose- and time-dependent manner (Fig. 1; all of the data was statistically significant [see figure legend]). As reported earlier, c-erbB-2 is associated with more aggressive and invasive behavior of breast as well as of head and neck cancer cells (12); our results also directly correlate with our previously published report on breast cancer cells (33). On the basis of these results, we investigated whether the down-regulation of c-erbB-2 correlates with the inhibition in the invasive potential of HN4 cells.
We observed a dose- and time-dependent inhibition in the secretion of MMP-2 and MMP-9 (Fig. 2). In addition, we also observed inhibition in the tumor cell invasion through Matrigel-coated membranes by the cells treated with genistein compared with untreated controls (Fig. 3; all of the data was statistically significant [see figure legend]). The down-regulation of c-erbB-2 and MMPs together with the inhibition of tumor cell invasion in genistein-treated cells prompted us to investigate additional molecular mechanisms that might be responsible for the ultimate cellular effects of genistein in head and neck cancer cells.

It is well known that the overexpression of c-erbB-2 leads to the induction of cell growth, which could be mediated by the activation of NF-κB, because the activity of NF-κB has been shown to be inhibited by genistein in prostate cancer cells in our earlier studies (50). Hence, we conducted subsequent experiments to investigate whether genistein elicits any effect on NF-κB in HN4 cells.

The Effect of Genistein on NF-κB DNA Binding Activity. We found that DNA binding activity of NF-κB was significantly reduced in cells treated with genistein (Fig. 4), supporting our previous results (50). In contrast, we found that there was a significant induction in the DNA binding activity of NF-κB in cells treated with TNF-α, as reported previously (50), and that this activity was abrogated in cells pretreated with genistein, similar to our earlier results in prostate cancer cells (50). It is interesting to note that NF-κB binds to the promoter sequence of MMP-9 and trans-activates the expression of MMP-9 (20). In addition, NF-κB transcriptionally activates telomerase catalytic subunit hTERT (44), which suggests that NF-κB plays a pivotal role in the complex regulation of c-erbB-2, MMPs, and telomerase, and that a cross-talk between these molecules may determine the effects of genistein in HN4 cells.

The Effect of Genistein on Metaphase Spread and Telomerase Activity. In our earlier publication, we reported that genistein induces cell cycle arrest at the S-phase/G2-M phase of the cell cycle (34). We also observed that genistein-treated cells became larger in size showing polyploidy. In this study, we observed a significant reduction in the metaphase chromosomal spread of cells treated with genistein (Fig. 5), which suggested that the genistein-induced G2-M phase cell cycle arrest contributes to the inhibition of metaphase spread of chromosomes and that this effect could also be attributable to the direct effect of genistein on the function and activity of telomerase. Interestingly, we did not find any effect of genistein on telomerase activity as measured by TRAP assay compared with untreated controls (Fig. 6). However, we observed a significant inhibition in the translocation of hTERT from cytoplasm to the nucleus in HN4 cells treated with genistein compared with untreated controls (Fig. 7). It is important to note that cells exposed to colcemid in the absence of genistein showed increased number of metaphase nuclei, which was expected; however, genistein-treated cells exposed to colcemid did not show a statistically
significant difference in metaphase spread, which suggested that genistein-induced G2-M cell cycle arrest plays a significant role in the inhibition of metaphase spread of chromosomes.

**Effect of Genistein on Akt and 14-3-3 Proteins.** It has been shown earlier that Akt plays an important role by phosphorylating hTERT and enhancing its activity (47). In addition, 14-3-3 proteins are believed to act as molecular chaperon in translocating telomerase from cytoplasmic compartment to nuclear compartment (45). It is also interesting to note that Akt phosphorylates a motif present in all variant of 14-3-3 proteins, creating a binding cliff for other proteins to interact with 14-3-3 (48). These observations suggest that phosphorylated hTERT may bind to phosphorylated 14-3-3 and, in turn, may facilitate the translocation of hTERT to the nucleus. Hence, we investigated whether genistein elicits any effects on Akt and 14-3-3 proteins. We observed a significant reduction in the expression of Akt and 14-3-3 proteins in cells treated with genistein (Fig. 8). These results strongly suggest that the inhibition of Akt will lead to the inhibition in the phosphorylation of hTERT and, in turn, inhibit nuclear translocation of hTERT for its activity. In addition, the down-regulation of 14-3-3 inhibits its function as a chaperone for hTERT and, thereby, inhibits nuclear translocation of hTERT. Collectively, our results show convincing evidence for pleiotropic molecular alterations, which may cross-talk in determining the cellular effects of genistein in head and neck cancer.

**DISCUSSION**

Genistein is an isoflavone with intrinsic biological activity in a variety of cancer cells as reported earlier by our laboratory and others (27–34). However, the molecular effects of genistein on head and neck cancer have not been clearly elucidated. In our earlier publication, we showed the biological activity of genistein on HN4 cancer cells by cell cycle deregulation with concomitant alterations in several key molecules that are important regulators of cell cycle and apoptosis (34, 40). In this study, we extended our previous observations and showed that genistein down-regulates c-erbB-2. c-erbB-2 was reported to be overexpressed in a significant number of HNSCC (12) and to be associated with a more aggressive and invasive phenotype (12, 49). The down-regulation of c-erbB-2 may cause the tumor cells to revert to a less invasive phenotype, and the down-regulation of c-erbB-2 could be due to a variety of cellular events in genistein-treated cells. We have previously shown that a specific protein named HPBF binds to c-erbB-2 promoter sequence and that this interaction leads to the activation of NF-κB (11), which suggests that the activation of NF-κB may lead to the activation of c-erbB-2 or vice versa. NF-κB was also reported to play a role in the transcriptional regulation of other proteins, such as MMP-9 and telomerase, both of which may play an important role in the progression of cancer cell growth and metastasis (20, 44). In our study, we found that the DNA binding activity of NF-κB was significantly reduced in genistein-treated cells and that the reduction in NF-κB activity may, in turn, inhibit the expression of MMP-9 and, thus, inhibit tumor cell invasion (20–28). The down-regulation of NF-κB could also be significant because it may also inhibit the expression of hTERT, which is believed to be important for cellular immortalization (41–47).

In addition to the inhibition of the synthesis of MMPs, which may be caused by the down-regulation of NF-κB, we also observed a decrease in the secretion of both MMP-2 and MMP-9, which provides strong evidence for the inhibition of tumor cell invasion as observed in this study. However, the
mechanism by which genistein down-regulates the secretion of MMPs requires further in-depth investigation. Nevertheless, these results are very significant because MMP secretion plays an important role in cancer progression, angiogenesis, and metastasis (20–28). Increased MMP secretion was also associated with a low apoptotic profile, and with more invasive phenotypes as well as poor prognosis (19–24). At the same time, inhibitors of MMP secretion are reported to inhibit cell growth and induce apoptosis (23–24). Collectively, our results showed a direct correlation with the down-regulation of NF-κB and inhibition in the secretion of MMPs and tumor cell invasion of HN4 cells.

In our previous study, we reported that genistein inhibits cell growth and induce apoptotic processes in HNSCC cells (34). We also observed an increase in cell size with concomitant increased expression of p21WAF1, the induction of which is known to play a role in cellular senescence (51), which could be overcome by the activation of telomerase activity (52). Our results clearly showed a significant inhibition in the metaphase spread of chromosome by genistein treatment, suggesting that the activity of the telomerase or telomere shortening may contribute to this effect of genistein. Telomere shortening results in the formation of sticky chromosomal ends and, thereby, will inhibit chromosomal spread. However, we could not find any alterations in the activity of telomerase in genistein-treated cells. In contrast, we observed decreased nuclear distribution of telomerase catalytic subunit (hTERT) in genistein-treated cells as documented by immunostaining and confocal microscopy. hTERT was reported to be chaperoned to the nucleus by 14-3-3 (45), which is known to be regulated by Akt (48). Akt was reported to be a substrate for phosphatidyl inositol 3 kinase (46), which can play a decisive role in cell death or cell survival mechanism by enhancing the telomerase activity as a result of phosphorylation of its catalytic subunit, hTERT (47). Our results showed decreased levels of phosphorylated Akt, which is the active form of Akt, together with a reduction in the expression of 14-3-3 at the protein level. These results suggest that although the protein expression of hTERT was not affected by genistein, the availability of hTERT in the nucleus to maintain the telomere length was greatly affected. This supports our hypothesis that the sequestration of hTERT by down-regulating its molecular chaperon contributes to the down-regulation of telomerase activity in vivo; the down-regulation cannot be detected in vitro because the current assay measures all hTERT irrespective of its partitioning into various cellular compartments. The down-regulation in the phosphorylated form of the Akt may also be important because Akt is known to phosphorylate hTERT and 14-3-3 prior to the nuclear translocation of hTERT (47, 48). However, additional in-depth studies are needed to establish cause-and-effect relationships between these molecular alterations with the biological activity of genistein. Nevertheless, our results provide strong evidence for potential...
benefits of genistein as a chemopreventive and/or chemotherapeutic agent for HNSCC.

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