BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 291–295 (1998)
ARTICLE NO. RC989462

Genistein Induces p21$^{Cip1/WAF1}$ Expression and Blocks the G1 to S Phase Transition in Mouse Fibroblast and Melanoma Cells

Takejiro Kuzumaki,[1] Takashi Kobayashi, and Kiichi Ishikawa
Department of Biochemistry, Yamagata University School of Medicine, Yamagata 990-9585, Japan

Received September 5, 1998

Genistein, the principal isoflavonoid in soybeans, is reported to inhibit cell cycle progression, but the molecular basis for this event is unknown. Here we show that genistein inhibits DNA synthesis and suppresses cyclin E-associated cyclin-dependent kinase-2 (CDK2) activity when quiescent BALB/c 3T3 fibroblasts are stimulated with serum. In these cells, a CDK2 inhibitor, p21$^{Cip1/WAF1}$, is markedly increased by genistein, but another CDK2 inhibitor, p27^{Kip1}, is not increased. In exponentially growing BALB/c 3T3 cells, genistein inhibits proliferation of the cells in a dose-dependent manner. Flow cytometric analysis and measurement of DNA synthesis indicate that genistein blocks the G1 to S phase transition of these cells, which is concomitant with G2-M arrest. In mouse B16-F1 melanoma cells, genistein also blocks the transition of G1 to S phase without arresting at G2-M at low doses. In both cell lines, genistein suppresses cyclin E/CDK2 activity and induces p21$^{Cip1/WAF1}$ expression. These results suggest that genistein affects the restriction point control of the cell cycle by inducing p21$^{Cip1/WAF1}$ expression in mouse fibroblast and melanoma cells.

Genistein is the principal isoflavonoid contained in soybeans (1). Genistein has been reported to have many biochemical activities. Genistein inhibits the activities of several protein-tyrosine kinases, including those of growth-factor receptor tyrosine kinases and pp60^{src} (2). The activities of topoisomerase II and S6 kinase are also inhibited by genistein (3, 4). In addition, genistein was reported to have antioxidant activity, estrogenic/antiestrogenic activities, and antiangiogenetic activity (5-7). Epidemiological studies suggest that high intake of soybean products reduces carcinogenic risk in the tissues of breast, prostate, and colon (5). Moreover, genistein potently inhibits proliferation of cancer cells, and induces cell differentiation and apoptosis in various cancer cells (5, 8, 9). In some cancer cell lines, genistein was observed to arrest the cell cycle at G2-M phase (9-11). Other papers showed that genistein prevented cell cycle progression at multiple steps, including the G1 phase (12, 13). However, the molecular basis for cell cycle arrest has not been determined.

Recently, p21 and p27 have been identified as inhibitors of cyclin D/CDK4, cyclin E/CDK2, and cyclin A/CDK2, which are key regulators for progression of G1 to S phase (14, 15). We previously reported that genistein blocks the G1 to S phase transition in BALB/c 3T3 fibroblasts (16). In this paper, we report that genistein does so by inducing p21 expression and suppressing cyclin E/CDK2 activity in mouse BALB/c 3T3 fibroblasts and B16-F1 melanoma cells in culture.

MATERIALS AND METHODS

Cell culture. Mouse BALB/c 3T3 fibroblasts and mouse B16-F1 melanoma cells were cultured with Dulbecco's MEM medium supplemented with 200 units/ml penicillin G, 0.1 mg/ml streptomycin sulfate, and 10% FCS. BALB/c 3T3 fibroblasts were synchronized to the quiescent state by serum starvation, and the quiescent cells were stimulated with 10% FCS by the procedures previously described (16). BALB/c 3T3 cells were also synchronized at the G1/S boundary by the DNA polymerase-a inhibitor, aphidicolin (16). In short, exponentially growing fibroblasts were exposed to aphidicolin (4 μg/ml) for 15 h, and then cultured without aphidicolin for 10 h. After this treatment, cells were cultured with aphidicolin again for 23 h, and then aphidicolin was removed from the culture medium. At 16 h after aphidicolin removal, genistein was added to the culture medium. At this time, cells were synchronized at the middle G1 phase because duration of S phase is 9-10 h (16). Viable cell density was determined by counting cells that excluded trypan blue with a microscope. Genistein and daidzein were purchased from Funakoshi Chemical Co. (Tokyo, Japan) and dissolved in dimethylsulfoxide. Cultured cells in the control dishes were treated with the same amount of dimethylsulfoxide as that in the dishes treated with the reagents.

Measurement of DNA synthesis. Newly synthesized DNA was detected by labeling with [3H]thymidine by the procedures previ-

[1] To whom correspondence should be addressed. Fax: +236-28-5230. E-mail: kuzumaki@med.id.yamagata-u.ac.jp.

Abbreviations: fetal calf serum, FCS; cyclin-dependent kinase, CDK.

0006-291X/98 $25.00

ously described (17). The number of nuclei labeled with [3H]thymidine was counted with a microscope.

Immunoblot analysis. Immunoblot analysis using total protein extracts from cultured cells was performed as previously described (17). Antibodies specific for p21, cyclin E, and cyclin A were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies specific for p27 and CDK2 were from Transduction Laboratories, Inc. (Lexington, KY).

Immunoprecipitation and cyclin E-associated histone H1 kinase activity. Cyclin E-associated histone H1 kinase activity in the proteins immunoprecipitated with anti-cyclin E antibodies was measured by the same procedures previously described (17).

Northern blot analysis. Total RNAs were extracted by the acid guanidinium thiocyanate-phenol-chloroform method (18). Northern blot analysis was performed as previously described (16). The p21 probe (345 bps) was generated by an amplification of mouse p21 exon 2 by the polymerase-chain-reaction method (19). The p53 probe (0.8 kbps) was prepared from cloned mouse p53 cDNA (20).

Flow cytometry. Cultured cells were fixed with ice-cold 70% ethanol after trypsinization and stored at 4°C. Fixed cells were treated with RNAse A (100 μg/ml) for 30 min at 37°C and then incubated with phosphate-buffered saline containing propidium iodide (25 μg/ml) for 30 min on ice. The DNA histogram was obtained with a flow cytometer (FACSCalibur, Becton Dickinson). Percentage of the cells in each phase of the cell cycle was determined by the ModFit LT software (Becton Dickinson) based on the DNA histogram.

RESULTS AND DISCUSSION

When quiescent BALB/c 3T3 fibroblasts were stimulated with 10% FCS, the cells entered S phase at around 12 h after serum stimulation (Fig. 1A), as previously described (16). As shown in Fig. 1B, over 90% of nuclei were labeled with [3H]thymidine when quiescent cells were stimulated with serum for 22 h. However, only 3–8% nuclei were labeled when cells were exposed to genistein during late G1 and S phases (6–22 h after serum stimulation). As a control, we used daidzein, which is a structurally similar compound to genistein but has no biological activities which genistein has. Daidzein did not suppress DNA synthesis. Since activation of cyclin E/CDK2 is a crucial event for the G1 to S phase transition (14), we measured cyclin E-associated histone H1 kinase activity (Fig. 1C). It was shown that cyclin E-associated histone H1 kinase was activated at the G1/S boundary (10-14 h after serum stimulation), and genistein abrogated this kinase activation, but daidzein did not. Since activity of cyclin E/CDK2 was reported to be suppressed by CDK2 inhibitors p21 and p27, and activated by cyclin E (15), we measured the abundance of the p21, p27, cyclin E, and CDK2 proteins in the cells by immunoblot analysis using specific antibodies. As shown in Fig. 1D, p27 was abundant in quiescent cells and down-regulated by serum stimulation. Genistein did not significantly prevent this down-regulation of p27 abundance. In contrast, p21 was not detected in quiescent cells, and genistein markedly increased the p21 abundance. Daidzein had no effect for the p21 and p27 abundance. Since the abundance of CDK2 and cyclin E

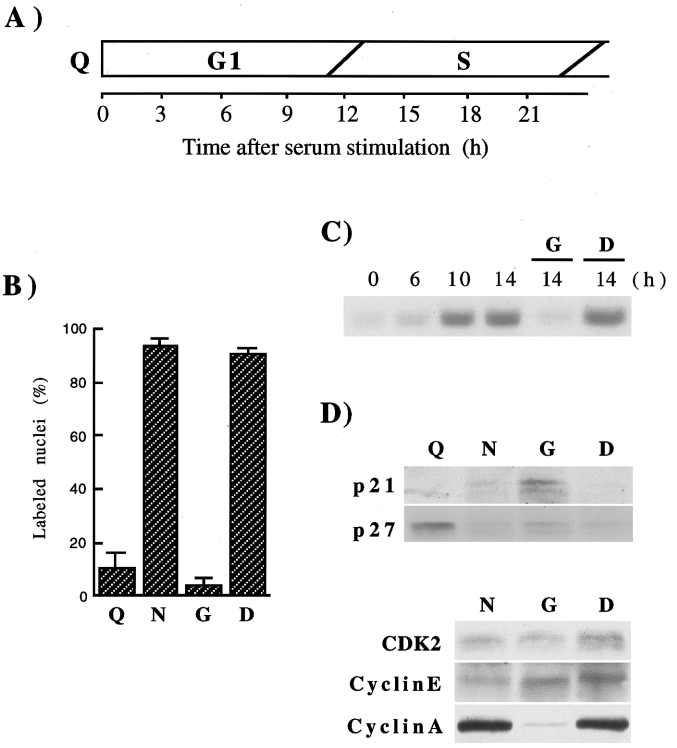

FIG. 1. DNA synthesis and cyclin E-associated histone H1 kinase activity are suppressed, and p21 is increased by genistein when quiescent BALB/c 3T3 fibroblasts are stimulated with serum. (A) A scheme for progression of the BALB/c 3T3 fibroblast cell cycle when quiescent fibroblasts are stimulated with 10% FCS. (B) DNA synthesis. Quiescent BALB/c 3T3 fibroblasts were stimulated with 10% FCS. Genistein, daidzein, and [^{3}H]thymidine were added to the culture medium at 6 h after serum stimulation. Cells were fixed with methanol at 22 h after serum stimulation and labeled nuclei were counted. (C) Cyclin E-associated histone H1 kinase activity. Quiescent fibroblasts were stimulated with 10% FCS. Genistein or daidzein was added to the culture medium at 6 h after serum stimulation. At each time point after serum stimulation, total cellular proteins were extracted. Immunoprecipitates formed with anti-cyclin E antibodies were analyzed for cyclin E-associated histone H1 kinase activity as described in MATERIALS AND METHODS. (D) Immunoblot analysis. Quiescent fibroblasts were stimulated with 10% FCS. Genistein or daidzein was added to the culture medium at 6 h after serum stimulation. Total cellular proteins were extracted at 14 h (for p21, p27, and cyclin E), at 12 h (for CDK2), or at 18 h (for cyclin A) after serum stimulation. Extracted proteins were analyzed by immunoblot analysis as described in MATERIALS AND METHODS. Q, quiescent cells (not stimulated with serum); N, serum-stimulated cells with no further treatment; G, genistein (180 μM); D, daidzein (180 μM).

proteins was not significantly changed by genistein, it is probable that abrogation of cyclin E/CDK2 activity by genistein was mediated by the increase of p21 abundance. Furthermore, it is established that cyclin A is the cyclin which is preferentially expressed in S phase, and this cyclin plays an important role for S phase progression by activating cyclin A/CDK2 (14). The appearance of cyclin A protein at 18 h after serum stimulation (middle of S phase) was significantly suppressed by genistein, which indicates that genistein

disturbs S phase progression of BALB/c 3T3 cells. Daidzein did not suppress the induction of cyclin A.

Genistein suppressed cell proliferation of exponentially growing BALB/c 3T3 fibroblasts in a dose-dependent manner (Fig. 2A). Sixty μM genistein was sufficient for complete block of cell proliferation. Apoptotic cells were not observed in all samples examined. Flow cytometric analysis indicated that genistein increased the number of cells in G2-M and decreased the number of cells in S phase in a dose-dependent manner (Fig. 2B). This change of DNA histogram pattern suggests that genistein arrests the cell cycle at G2-M and concomitantly blocks the transition of G1 to S phase. However, it is not neglected that the decrease of S phase cells is the secondary effect caused by G2-M arrest. To confirm the blockage of the transition of G1 to S by genistein in growing cells more directly, we measured DNA synthesis by using cells synchronized at the G1/S boundary with the DNA polymerase-a inhibitor, aphidicolin. For these synchronized cells, genistein was added to the culture medium at middle G1 phase (16 h after aphidicolin removal) and DNA synthesis for 24 h thereafter was measured. DNA synthesis of the cells exposed to genistein during middle-late G1 and S phases was much suppressed by genistein (Fig. 2C). The result indicates that the blockage of G1 to S phase transition is a direct effect of genistein, not the secondary effect caused by G2-M arrest. In exponentially growing cells, genistein also suppressed cyclin E-associated histone H1 kinase activity (100%, 79%, and 27%, with 0 μM, 20 μM, and 60 μM genistein, respectively) (Fig. 2D), and increased the p21 abundance (100%, 308%, and 483% with 0 μM, 20 μM, and 60 μM genistein, respectively) in a dose-dependent manner (Fig. 2E). However, genistein did not change the p27 abundance.

We further examined the validity of the observation that genistein blocks the G1 to S phase transition in cancer cells. For this purpose, we examined a mouse cancer cell line, B16-F1 melanoma cells. Genistein effectively suppressed cell proliferation of B16-F1 cells at low doses (10-20 μM) (Fig. 3A). These concentrations are physiologically achievable in the plasma with a diet containing high soybean product content (6). Flow cytometric analysis indicated that genistein decreased the number of cells in S phase and increased that in G0-G1 (Fig. 3B). This change of DNA histogram pattern indicates that genistein arrests the cell cycle at G0-G1 by blocking the G1 to S phase transition of B16-F1 melanoma cells. It must be added here that genistein treatment at higher doses caused increase of cell number in G2-M (data not shown), suggesting that G2-M arrest was also took place in addition to the blockage of G1 to S phase transition at high doses in B16-F1 melanoma cells. Genistein also suppressed cyclin E-associated histone H1 kinase activity (100%, 56%, and 43%, with 0 μM, 10 μM, and 20 μM genistein,

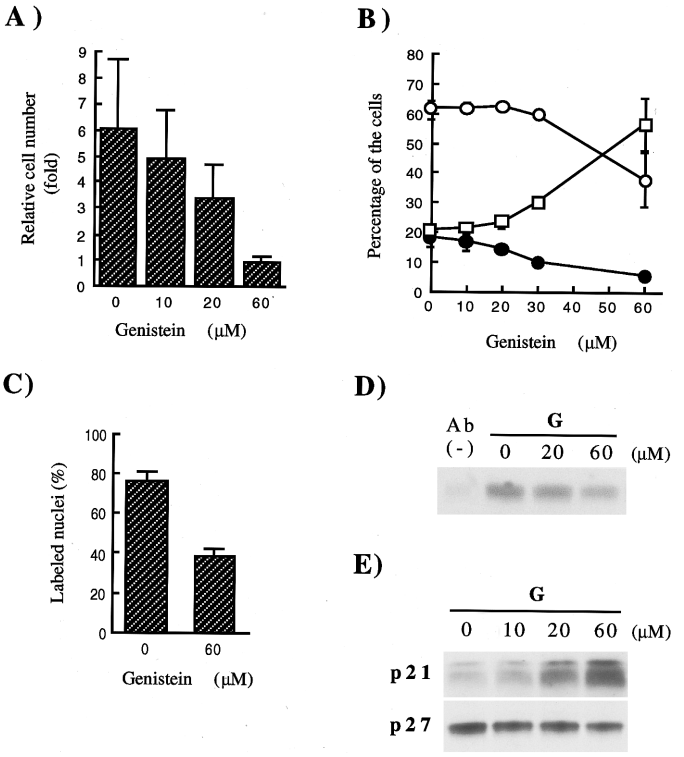

FIG. 2. Genistein blocks the transition of G1 to S phase concomitant with G2-M arrest and increases the p21 abundance in exponentially growing BALB/c 3T3 fibroblasts. (A) Cell proliferation. Exponentially growing BALB/c 3T3 fibroblasts (1×10^5 cells were seeded in 35-mm dish) were exposed to each concentration of genistein for 48 h, and then the number of viable cells was determined. The values represent relative cell numbers in comparison with the cell number at the time genistein was added (0 h) taken as 1. The values represent the means ± S.D. (n=4−5). (B) Percentage of the cells in G0-G1, S, and G2-M phases obtained by flow cytometric analysis. Exponentially growing fibroblasts were exposed to each concentration of genistein for 24 h. DNA histogram of the cells stained with propidium iodide was obtained by flow cytometric analysis as described in Materials and Methods. Percentage of the cells in each phase was calculated based on the DNA histogram. The values represent the means ± S.D. (n=3−4). ○, G0G1 phase; ●, S phase; □, G2-M phase. (C) DNA synthesis. Exponentially growing fibroblasts were synchronized at the G1/S boundary with aphidicolin. Aphidicolin was then removed from the culture medium to allow cells to proceed through the cell cycle. At 16 h after aphidicolin removal (at this time cells were around the middle G1 phase), genistein and [^{3}H]thymidine were added to the culture medium. Cells were fixed with methanol at 24 h after administration of genistein and labeled nuclei were counted. (D) Cyclin E-associated histone H1 kinase activity. Exponentially growing fibroblasts were exposed to each concentration of genistein for 16 h, and total cellular proteins were extracted. Immunoprecipitates formed with anti-cyclin E antibodies were analyzed for cyclin E-associated histone H1 kinase activity as described in Materials and Methods. Ab(-), total cellular proteins (no genistein treatment) were immunoprecipitated without anti-cyclin E antibodies and histone H1 kinase activity was measured. G, genistein. (E) Immunoblot analysis. Exponentially growing fibroblasts were exposed to each concentration of genistein for 6 h. Total proteins were extracted and analyzed by immunoblot analysis as described in Materials and Methods. G, genistein.

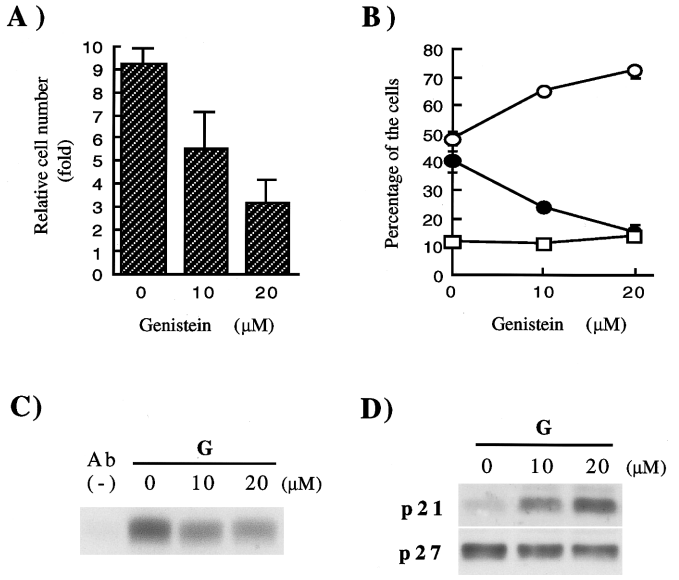

FIG. 3. Genistein blocks the transition of G1 to S phase and increases the p21 abundance in exponentially growing B16-F1 melanoma cells. Exponentially growing B16-F1 cells were treated with genistein and analyzed by the same procedures described in the legend of figure 2. (A) Cell proliferation. (B) Percentage of the cells in G0-G1, S, and G2-M phases obtained by flow cytometric analysis. ○, G0-G1 phase; ●, S phase; □, G2-M phase. (C) Cyclin E-associated histone H1 kinase activity. Ab(-), total cellular proteins (no genistein treatment) were immunoprecipitated without anti-cyclin E antibodies and histone H1 kinase activity was measured. G, genistein. (D) Immunoblot analysis. G, genistein.

respectively) (Fig. 3C) and increased the p21 abundance in a dose-dependent manner (100%, 337%, and 500%, with 0 μM, 10 μM, and 20 μM genistein, respectively) (Fig. 3D). All above results suggest that genistein blocks the transition of G1 to S phase in addition to G2-M arrest by increasing the abundance of p21 in mouse BALB/c 3T3 fibroblasts and B16-F1 melanoma cells in culture.

Finally, we examined by Northern blot analysis whether genistein activates the p21 gene or not. In exponentially growing BALB/c 3T3 fibroblasts, genistein markedly increased p21 protein within 4 h after administration of genistein (Fig. 4A). The p21 mRNA significantly increased by genistein, just preceding the increase of p21 protein (within 2 h after administration of genistein) (Fig. 4B). The transcription of the p21 gene was reported to be activated by the tumor suppressor gene product, p53 (19). However, genistein did not increase the mRNA abundance of p53. This observation suggests that genistein induces p21 gene expression at the transcriptional level, and that genistein has no effect for the p53 gene activation.

Many previous reports indicated by flow cytometric analysis that genistein arrests the cell cycle at G2-M in cancer cell lines, such as Jurkat and HL-60 leukemia cells, gastric cancer cells, melanoma cells, breast cancer cells, and lung cancer cells (9-11, 21-23). In these reports, G1 arrest by genistein was not clearly observed. By use of DNA synthesis measurement, other authors found that genistein prevents cell cycle progression at multiple steps, including the G1 phase in non-transformed cells, such as endothelial and smooth muscle cells (12, 13). We show here that genistein blocks the transition of G1 to S phase by use of both flow cytometric analysis and measurement of DNA synthesis in immortal and cancer cell lines. Further, we obtained evidence that genistein induces p21 expression and suppresses cyclin E/CDK2 activity, thus blocking the transition of G1 to S phase. Apigenin, which is structurally similar to genistein, is one of the principal flavonoid contained in fruits and vegetables. Apigenin is also suggested to play an important role for cancer prevention and was reported to inhibit protein-tyrosine kinases at high doses (2, 23). Apigenin was shown to arrest the cell cycle at G2-M in mouse fibroblast and keratinocyte cell lines (23, 24). In addition, apigenin was shown to arrest the cell cycle at G1 by inducing p21 expression in human normal fibroblast cells, but not in cancer cells (25). To elucidate the action of chemopreventive agents, however, it is important to study their effects in cancer cells. We show here that genistein blocks the transition of G1 to S phase in B16-F1 melanoma cells, one of the typical cancer cell lines. Few report has shown that genistein arrests the cell cycle at G1 in cancer cells, determined by flow cytometric analysis. In cancer cells the machinery of the restriction point control, for example, p53 and pRb,

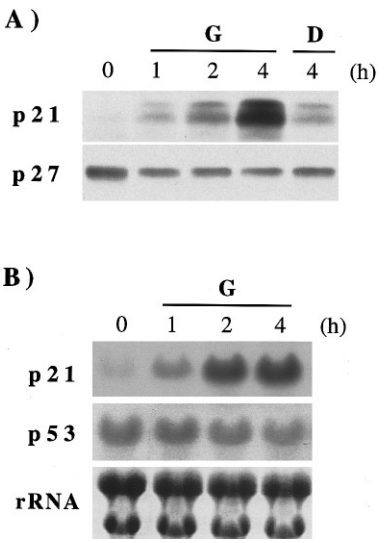

FIG. 4. Genistein increases the abundance of p21 mRNA. A) Immunoblot analysis. Total proteins were extracted from exponentially growing BALB/c 3T3 fibroblasts at each time point after administration of the reagents and analyzed by immunoblot analysis as described in Materials and Methods. B) Northern blot analysis. Total RNAs were extracted from exponentially growing BALB/c 3T3 fibroblasts at each time point after administration of genistein and analyzed by Northern blot analysis as described in Materials and Methods. G, genistein (180 μM); D, daidzein (180 μM).

are frequently mutated. This may be one of the reasons why responses of cancer cells to genistein are various in each of cancer cell lines. It can not be neglected that this variety may affect the degree of genistein effect *in vivo*. At present it is unclear that cell cycle arrest by genistein is directly mediated by tyrosine kinase inhibition (6). More information is needed to understand the molecular basis for suppressing cancer cell growth by genistein.

ACKNOWLEDGMENTS

We thank the Japanese Cancer Research Resources Bank for supplying the BALB/c 3T3 cell line and the p53 cDNA. Thanks are also given to Professor M. Jacobs-Lorena, Case Western Reserve University, Cleveland, Ohio, for his critical reading of the manuscript.

REFERENCES

1. Anderson, R. L., and Wolf, W. J. (1995) *J. Nutr.* **125,** 581S–588S.
2. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. (1987) *J. Biol. Chem.* **262,** 5592–5595.
3. Okura, A., Arakawa, H., Oka, H., Yoshinari, T., and Monden, Y. (1988) *Biochem. Biophys. Res. Commun.* **157,** 183–189.
4. Linassier, C., Pierre, M., Pecq, J. L., and Pierre, J. (1990) *Biochem. Pharmacol.* **39,** 187–193.
5. Herman, C., Adlercreutz, T., Goldin, B. R., Gorbach, S. L., Hšckerstedt, K. A. V., Watanabe, S., Hämäläinen, E. K., Markkanen, M. H., Mäkelä, T. H., Wähälä, K. T., Hase, T. A., and Fotsis, T. (1995) *J. Nutr.* **125,** 757S–770S.
6. Peterson, G. (1995) *J. Nutr.* **125,** 784S–789S.
7. Fotsis, T., Pepper, M., Adlercreutz, H., Hase, T., Montesano, R., and Schweigerer, L. (1995) *J. Nutr.* **125,** 790S–797S.
8. Barnes, S. (1995) *J. Nutr.* **125,** 777S–783S.
9. Spinozzi, F., Pagliacci, M. C., Migliorati, G., Moraca, R., Grignani, F., Riccardi, C., and Nicoletti, I. (1994) *Leukemia Res.* **18,** 431–439.
10. Matsukawa, Y., Marui, N., Sakai, T., Satomi, Y., Yoshida, M., Matsumoto, K., Nishino, H., and Aoike, A. (1993) *Cancer Res.* **53,** 1328–1331.
11. Rauth, S., Kichina, J., and Green, A. (1997) *British J. Cancer* **75,** 1559–1566.
12. Shimokado, K., Umezawa, K., and Ogata, J. (1995) *Exp. Cell Res.* **220,** 266–273.
13. Hawker, Jr. J. R., and Granger, H. J. (1994) *Am. J. Physiol.* **266,** H107–H120.
14. Sherr, C. J. (1993) *Cell* **73,** 1059–1065.
15. Sherr, C. J. (1994) *Cell* **79,** 551–555.
16. Kuzumaki, T., Matsuda, A., Ito, K., and Ishikawa, K. (1996) *Biochim. Biophys. Acta* **1310,** 185–192.
17. Kuzumaki, T., and Ishikawa, K. (1997) *Biochem. Biophys. Res. Commun.* **238,** 169–172.
18. Chomczynski, P., and Sacchi, N. (1987) *Analytical Biochem.* **162,** 156–159.
19. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Cell* **75,** 817–825.
20. Zakut-Houri, R., Oren, M., Bienz, B., Lavie, V., Hazum, S., and Givol, D. (1983) *Nature* **306,** 594–597.
21. Okabe, S., Suganuma, M., Hayashi, M., Sueoka, E., Komori, A., and Fujiki, H. (1997) *Jpn. J. Cancer Res.* **88,** 639–643.
22. Pagliacci, M. C., Spinozzi, F., Migliorati, G., Fumi, G., Smacchia, M., Grignani, F., Riccardi, C., and Nicoletti, I. (1993) *Eur. J. Cancer* **29A,** 1573–1577.
23. Lepley, D. M., Li, B., Birt, D. F., and Pelling, J. C. (1996) *Carcinogen.* **17,** 2367–2375.
24. Plaumann, B., Fritsche, M., Rimpler, H., Brandner, G., and Hess, R. D. (1996) *Oncogene* **13,** 1605–1614.
25. Lepley, D. M., and Pelling, J. C. (1997) *Mol. Carcinogen.* **19,** 74–82.