Journal of Cellular Biochemistry 78:73–84 (2000)

Inhibition of UV Irradiation–Induced Oxidative Stress and Apoptotic Biochemical Changes in Human Epidermal Carcinoma A431 Cells by Genistein

Wen-Hsiung Chan and Jau-Song Yu*

Department of Cell and Molecular Biology, Institute of Basic Medicine, Medical College of Chang Gung University, Tao-Yuan, Taiwan, Republic of China

Abstract Ultraviolet (UV) light is a strong apoptotic trigger that can induce a caspase-dependent biochemical change in cells. We previously showed that UV irradiation can elicit caspase-3 activation and the subsequent cleavage and activation of p21-activated kinase 2 (PAK2) in human epidermal carcinoma A431 cells. We report that genistein, an isoflavone compound with known inhibitory activities to protein tyrosine kinases (PTKs) and topoisomerase-II (topo-II), can prevent UV irradiation–induced apoptotic biochemical changes (DNA fragmentation, caspase-3 activation, and cleavage/activation of PAK2) in A431 cells. Surprisingly, two typical PTK inhibitors (tyrphostin A47 and herbimycin A) and three known topo-II inhibitors (etoposide, daunorubicin, and novomycin) had no effect on UV irradiation–induced apoptotic biochemical changes, suggesting that the inhibitory effect of genistein is not dependent on its property as a PTK/topo-II inhibitor. In contrast, azide, a reactive oxygen species (ROS) scavenger, could effectively block the UV irradiation–induced apoptotic cell responses. Flow cytometric analysis using the cell-permeable dye 2′,7′-dichlorofluorescin diacetate as an indicator of the generation of ROS showed that UV irradiation caused increase of the intracellular oxidative stress and that this increase could be abolished by azide, suggesting that oxidative stress plays an important role in mediating the apoptotic effect of UV irradiation. Importantly, the UV irradiation–induced oxidative stress in cells could be significantly attenuated by genistein, suggesting that impairment of ROS formation during UV irradiation is responsible for the antiapoptotic effect of genistein. Collectively, our results demonstrate the involvement of oxidative stress in the UV irradiation–induced caspase activation and the subsequent apoptotic biochemical changes and show that genistein is a potent inhibitor for this process. J. Cell. Biochem. 78: 73–84, 2000.

Key words: ultraviolet irradiation; apoptosis; oxidative stress; genistein; caspase-3; p21-activated kinase 2

Exposure of mammalian cells to ultraviolet (UV) light leads to damage of cellular constituents and results in a complicated cell response, including induction of genes and perturbation of a variety of signaling pathways [for reviews, see Bender et al., 1997; Schwarz, 1998]. Cells that cannot recover from severe damage of its constituents and perturbation of intracellular signals will ultimately die. One of the major death processes of UV-irradiated cells is apoptosis. Apoptosis, which is widely observed in different cells of various organisms, has a unique morphologic cell death pattern characterized by chromatin condensation, membrane blebbing, and DNA fragmentation [Kerr et al., 1972; Ellis et al., 1991]. A variety of physical and chemical signals can trigger apoptosis, indicating the existence of a core death program in cells. Considerable evidence has shown that the caspases, a cysteine protease family identified in recent years, play crucial roles in this core program for apoptotic execution [for reviews, see Martins and Earnshaw, 1997; Nicholson and Thornberry, 1997]. Caspases exist as proenzymes and can be activated at the onset of apoptosis via proteolytical processing to two smaller subunits, which then form functionally active protease that can act on many proteins and enzymes involved in di-

Grant sponsor: National Science Council of Taiwan, ROC; Grant numbers: NSC88-2314-B-182-036 and NSC89-2316-B-182-005); Grant sponsor: Chang Gung University, Tao-Yuan, Taiwan, ROC; Grant number: CMRP-724.

*Correspondence to: Jau-Song Yu, Department of Cell and Molecular Biology, Institute of Basic Medicine, Medical College of Chang Gung University, Tao-Yuan, Taiwan, ROC. E-mail: yusong@mail.cgu.edu.tw

Received 9 November 1999; Accepted 30 December 1999

This article published online in Wiley InterScience, April 2000.

verse cell functions. Among the caspases, caspase-3 (also known as CPP32/Yama/apopain) has been implicated as a downstream effector protease that can be activated by other upstream caspases [Fernandes-Alnemri et al., 1994; Enari et al., 1996].

Reactive oxygen species (ROS) are oxygen-containing molecules having either unpaired electrons or the ability to abstract electrons from other molecules. UV irradiation, like many of the chemical and physical treatments capable of inducing apoptosis, also provokes oxidative stress by generating ROS in cells [Halliwell and Gutteridge, 1990], suggesting a close relation between oxidative stress and apoptosis. Direct evidence establishing oxidative stress as a mediator of apoptosis came from observations that addition of oxidants such as H_2O_2 can trigger apoptosis, whereas addition of antioxidants block this effect [for review, see Buttke and Sandstrom, 1994].

Genistein is a natural isoflavone compound found in soy products, with inhibitory activities toward both protein tyrosine kinases (PTKs) and topoisomerase-II (topo-II) [Akiyama et al., 1987; Markovits et al., 1989]. Multiple effects of genistein on diverse cell functions have been found. It can inhibit tumor cell proliferation and induce tumor cell differentiation [Constantinou et al., 1990; Rauth et al., 1997; Brown et al., 1998]. Furthermore, it can trigger cell cycle arrest and apoptosis in several cell types [Azuma et al., 1993; Bergamaschi et al., 1993; McCabe and Orrenius, 1993; Yanagihara et al., 1993; Kyle et al., 1996; Schao et al., 1998; Zhou et al., 1998] but block apoptosis induced by several death signals [Uckun et al., 1992; Liu et al., 1994; Migita et al., 1994; Bronte et al., 1996; Johnson et al., 1996]. Although multiple biologic effects of genistein have been found, the exact molecular mechanisms are not clear.

We previously showed that environmental stresses including UV irradiation, heat shock, and hyperosmotic shock can induce activation of caspase-3 and the subsequent DNA fragmentation and cleavage/activation of p21-activated kinase 2 (PAK2) [Tang et al., 1998; Chan et al., 1998, 1999]. PAK2 is a member of the PAK family of serine/threonine kinases that can bind to and be activated by small (21 kDa) guanosine triphosphatases (GTPases) Cdc42 and Rac that regulate actin polymerization [for review, see Sells and Chernoff, 1997]. After removal of its N-terminal regulatory region by caspase-mediated cleavage, the activity of the resulting C-terminal catalytic fragment (36 kDa) of PAK2 can easily by detected by an in-gel kinase assay [Tang et al., 1998]. In the present report, we show that genistein can inhibit the UV irradiation–induced, caspase-dependent apoptotic biochemical changes in A431 cells. The inhibitory effect of genistein is unlikely to be due to its intrinsic activity as a PTK and topo-II inhibitor but relies on its novel property to prevent oxidative stress in UV-irradiated cells.

RESULTS

Inhibition of UV Irradiation–Induced Apoptotic Biochemical Changes by Genistein

In searching for compounds that can modulate the UV irradiation–induced apoptosis, we found that genistein has profound effects on this process. Three of the previously established biochemical characteristics of UV irradiation–induced apoptosis, namely DNA fragmentation, proteolytic processing of caspase-3, and cleavage/activation of PAK2 [Tang et al., 1998], were used to examine the dose effect of genistein. Although little effect could be detected at low dosages of genistein, complete blockage of all the UV irradiation–induced apoptotic biochemical changes was clearly observed at a higher dose (>100 μM) of genistein (Fig. 1), providing initial evidence that genistein is a potent inhibitor of the UV irradiation–induced apoptotic cell response. Genistein alone at 25–400 μM has no effect on the viability of A431 cells as assessed by trypan blue exclusion and is not able to induce those apoptotic biochemical changes as UV irradiation does, as described above, under the same assay condition (data not shown), indicating that the observed suppressive effect of genistein is not due to the nonspecific cytotoxic effects of this compound.

Commonly Used PTK and Topo-II Inhibitors Have no Effect on the UV Irradiation-Induced Apoptotic Cell Response

Because genistein is an established inhibitor of both PTKs and topo-II, the blocking effect of genistein on UV irradiation–induced apoptotic cell response may be due to inhibition of the intracellular PTKs and/or topo-II activities.

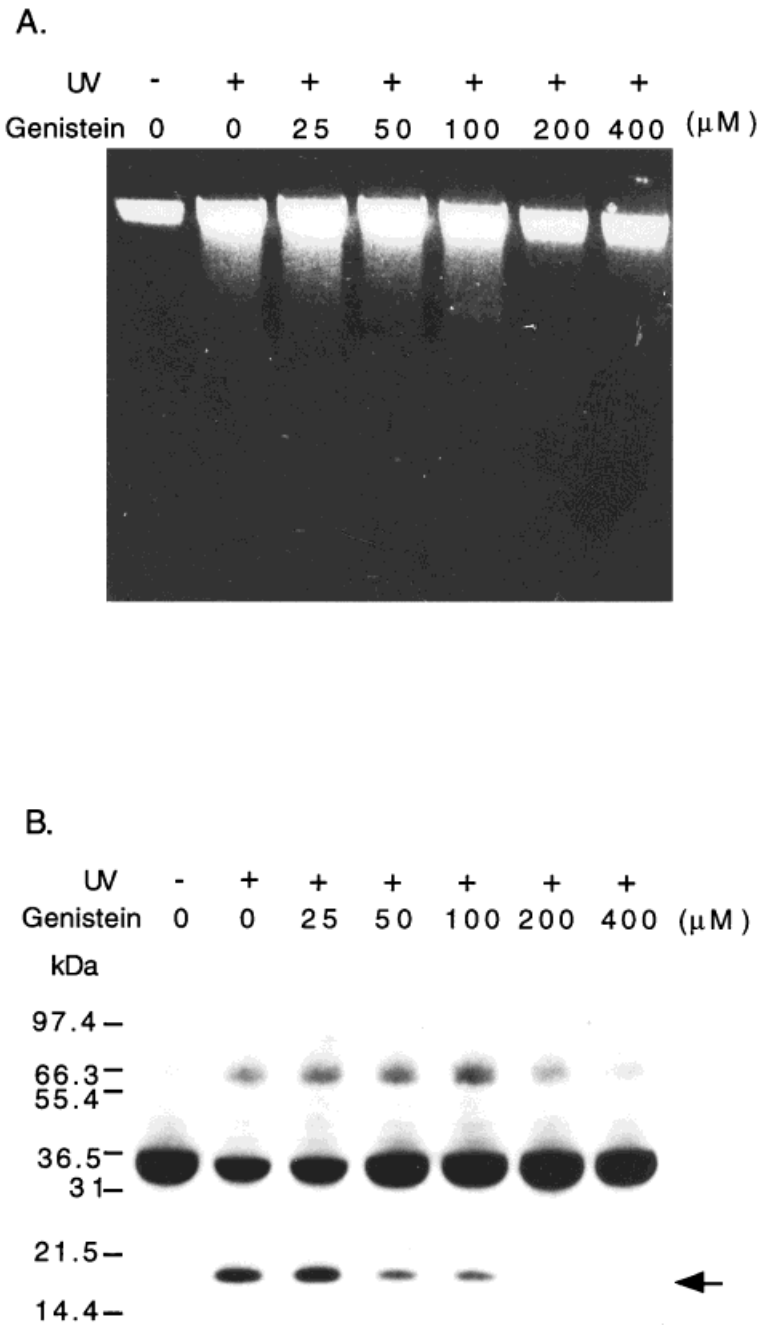

Fig. 1. Dose effects of genistein on ultraviolet (UV) irradiation–induced DNA fragmentation, activation of caspase-3, and cleavage/activation of $p21^{Cdc42/Rac}$-activated kinase 2 (PAK2) in A431 cells. A431 cells were preincubated with different concentrations of genistein as indicated at 37°C for 1 h and then exposed to UV light (200 J/m^2). Cell extracts and genomic DNA were prepared at 2 h later. **A:** The genomic DNA (20 μl/sample) was subjected to 2% agarose gel electrophoresis for DNA fragmentation analysis. Forty micrograms of the cell extracts was separated on a 15% sodium dodecyl sulfate (SDS) gel followed by immunoblot analysis with anti-CPP32/caspase-3 antibody (**B**), separated on a 10% SDS gel followed by immunoblot analysis with αPAK (C19) antibody (**C**), or separated on a 10% SDS gel containing myelin basic protein followed by in-gel kinase assay (**D**). Arrow indicates the position of the proteolyticly processed p17 subunit of caspase-3 (in B) or the 36-kDa catalytic fragment of PAK2 (in C and D), respectively. The experiment was performed four times, and the result is reproducible.

This possibility was evaluated by examining the effects of several commonly used PTK and topo-II inhibitors besides genistein on the apoptotic cell response elicited by UV irradiation. It was found that tyrphostin A47 and herbimycin A, two well-known PTK inhibitors at the dose reported to effectively block intracellular PTKs activities [Levitzki, 1990; Uehara and Fukazawa, 1991], have no effect on the UV irradiation–induced activation of caspase-3 (Fig. 2A, lanes 4 and 5) and cleavage/activation of PAK2 (Fig. 2B,C, lanes 4 and 5). In contrast, daidzein (another isoflavone with a structure analogous to that of genistein but without inhibitory activity to PTKs) [Yokoshiki et al., 1996] could significantly diminish the UV irradiation–induced activation of caspase-3 and cleavage/activation of PAK2 (Fig. 2A–C, lane 6). In addition, pretreatment of the cells with various topo-II inhibitors (etoposide,

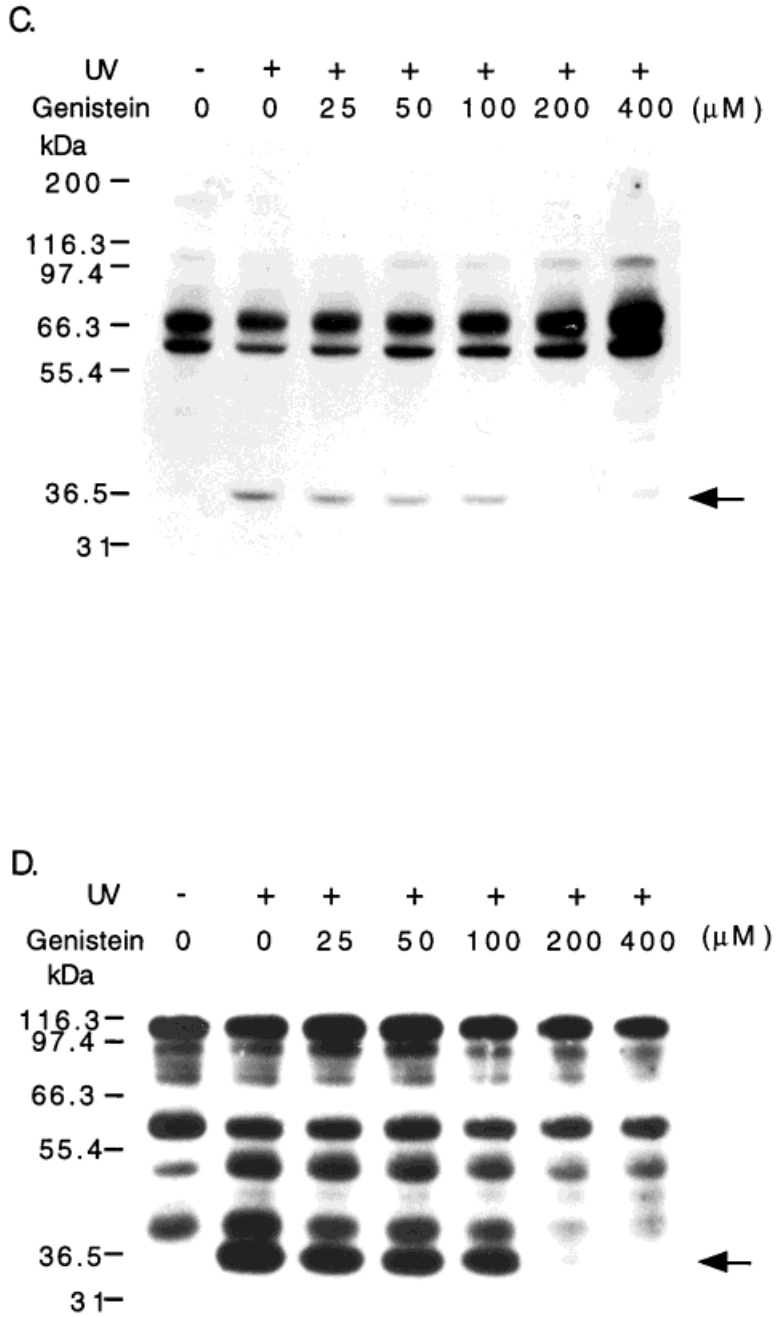

Fig. 1. (Continued.)

daunorubicin, or novomycin) at the dose reported to effectively block intracellular topo-II activity [Constantinou et al., 1989; Hande, 1998; Gewirtz, 1999] also had no effect on the UV irradiation–induced activation of caspase-3 (Fig. 3A) and cleavage/activation of PAK2 (Fig. 3B,C). Together these results indicate that inhibition of intracellular PTKs or topo-II is not sufficient to prevent UV irradiation–induced apoptotic cell response and suggest that the antiapoptotic effect of genistein is not attributed to its inhibitory activity to PTKs or topo-II.

UV Irradiation Results in ROS Formation in A431 Cells

To explore the mechanism responsible for the antiapoptotic effect of genistein, we inves-

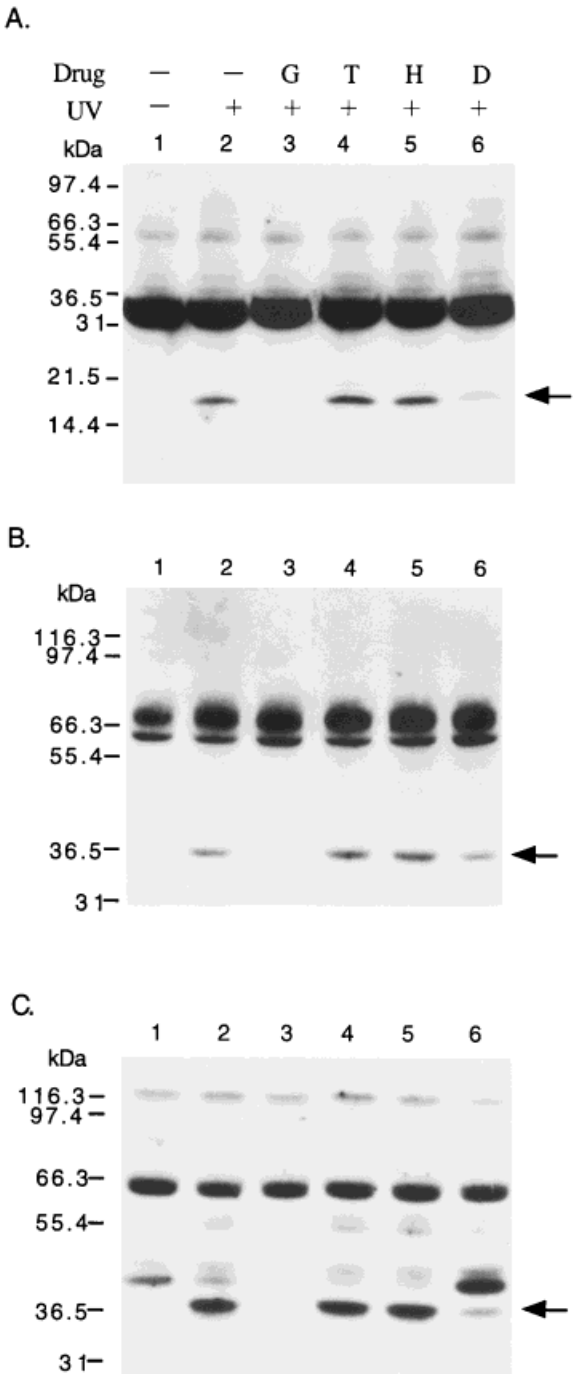

Fig. 2. Effects of protein tyrosine kinase inhibitors on the ultraviolet (UV) irradiation–induced activation of caspase-3 and cleavage/activation of $p21^{Cdc42/Rac}$-activated kinase 2 (PAK2) in A431 cells. A431 cells were incubated without or with genistein (400 μM, **lane G**), tyrphostin A47 (400 μM, **lane T**), herbimycin A (10 μM, **lane H**), or daidzein (400 μM, **lane D**) at 37°C for 1 h and then subjected to UV irradiation (200 J/m^2) followed by incubation at 37°C for another 2 h. Forty microgram of the cell extracts was subjected to analysis for activation of caspase-3 (**A**), cleavage (**B**), and activation (**C**) of PAK2, as described in Figure 1. The experiment was repeated three times, and the result is reproducible.

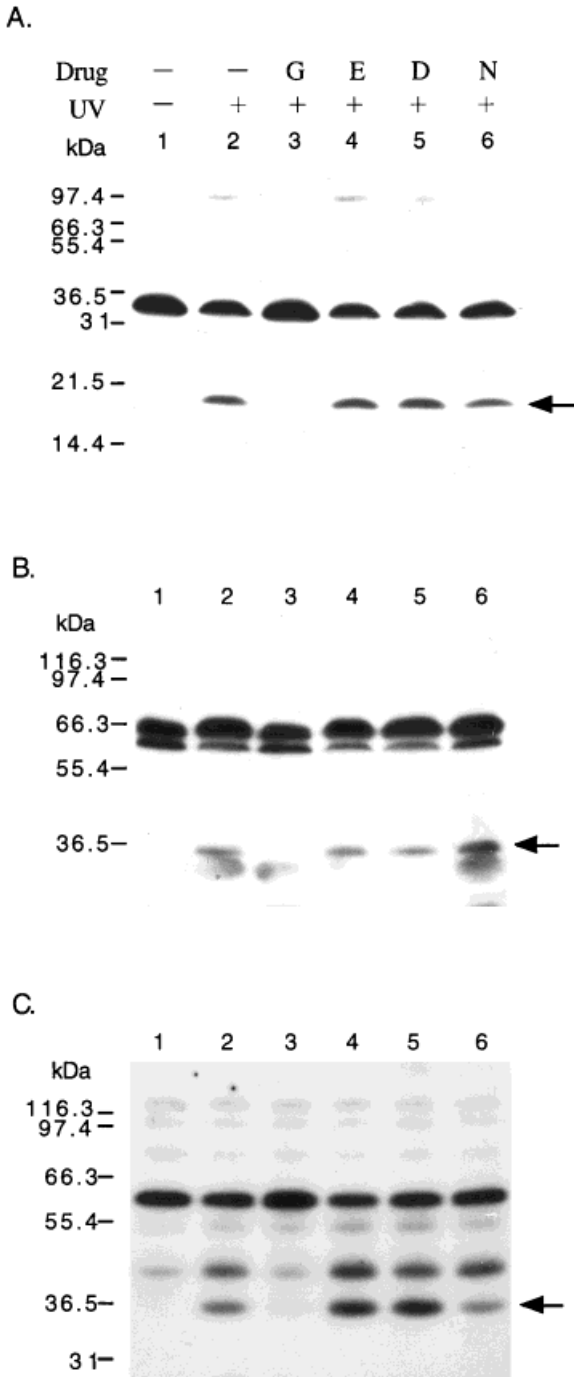

Fig. 3. Effects of topoisomerase-II inhibitors on ultraviolet (UV) irradiation–induced activation of caspase-3 and cleavage/activation of $p21^{Cdc42/Rac}$-activated kinase 2 (PAK2) in A431 cells. A431 cells were incubated without or with genistein (400 μM, **lane G**), etoposide (20 μg/ml, **lane E**), daunorubicin (2.5 μg/ml, **lane D**), or novomycin (200 μM, **lane N**) for 1 h and then exposed to UV light (200 J/m^2) followed by incubation at 37°C for another 2 h. The cell extracts were prepared at 2 h later. Forty micrograms of the cell extracts was then analyzed for activation of caspase-3 (**A**), cleavage (**B**), and activation (**C**) of PAK2 as described in Figure 1. The experiment was repeated three times, and the result is reproducible.

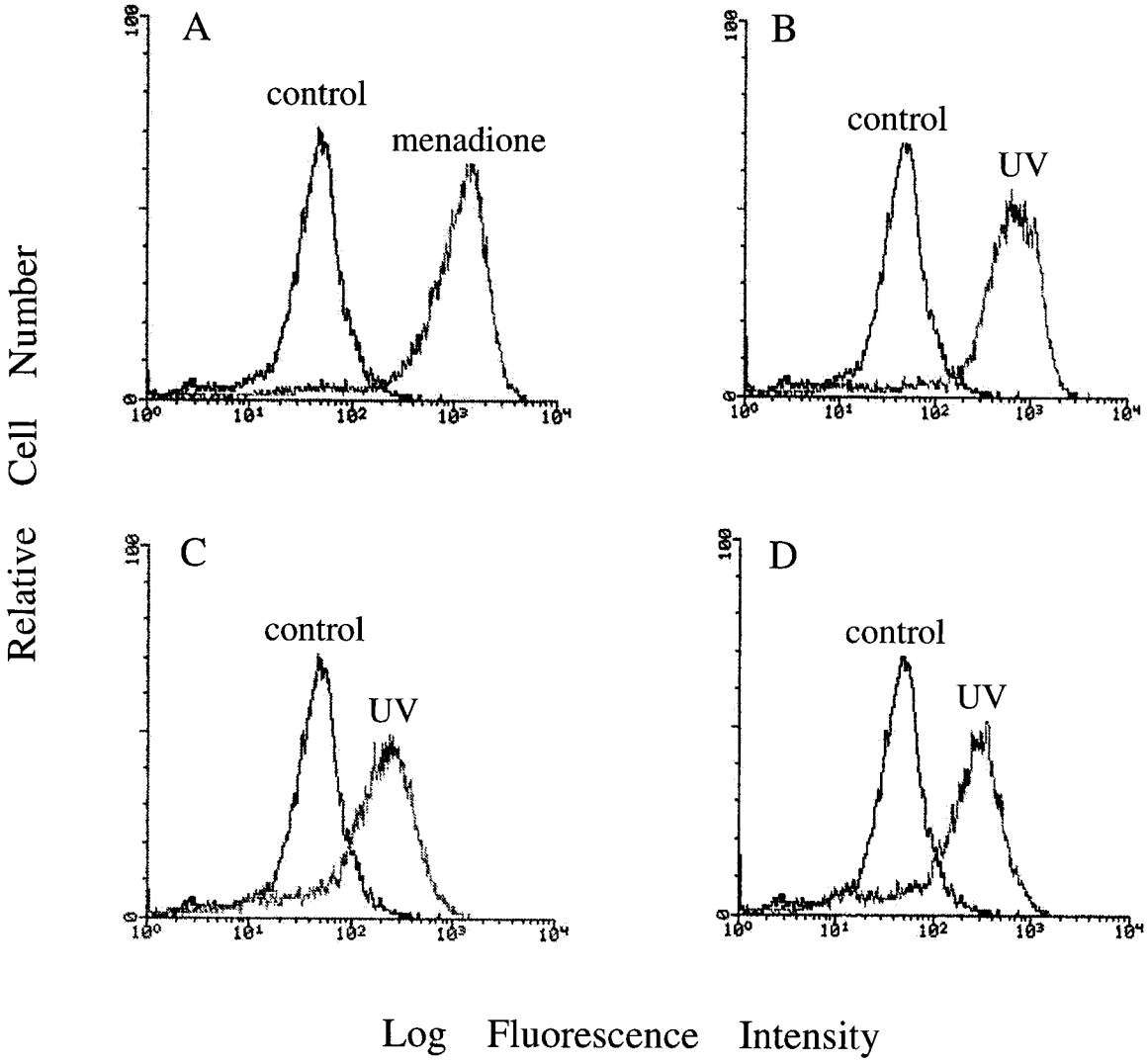

Fig. 4. Flow cytometric analysis of oxidative stress in ultraviolet (UV)-irradiated cells. **A:** A431 cells were left untreated or treated with 50 μM menadione for 3 h and then incubated with 10 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA) for another 1 h. Cells were harvested and immediately analyzed by flow cytometry. A431 cells preloaded with 10 μM DCF-DA for 1 h were left untreated or exposed to UV light (200 J/m^2). Cells were collected at 1 (**B**), 2 (**C**) or 3 (**D**) h after UV irradiation and analyzed by flow cytometry. The experiment was repeated four times, and the result is reproducible.

tigated the signaling pathway upstream of caspase activation on UV irradiation and examined whether genistein could interfere with this pathway. Because UV irradiation provokes oxidative stress in cells [Halliwell and Gutteridge, 1990; Scharffetter-Kochane et al., 1997] and ROS such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) are effective apoptotic inducers [Buttke and Standstrom, 1994], UV irradiation could elicit formation of ROS in cells and these ROS in turn could trigger downstream apoptotic signaling pathway(s). Therefore, we tested whether formation of ROS could occur in UV-irradiated A431 cells. Intracellular ROS formation was detected by using the permeable dye 2′,7′-dichlorofluorescin diacetate (DCF-DA) as a probe [Keher and Paraidathathu, 1992; LeBel et al., 1992]. As shown in Figure 4A, when the A431 cell was treated with menadione, an established oxidant [Thor et al., 1982; Caricchio et al., 1999], the magnitude of the intracellular ROS content as as-

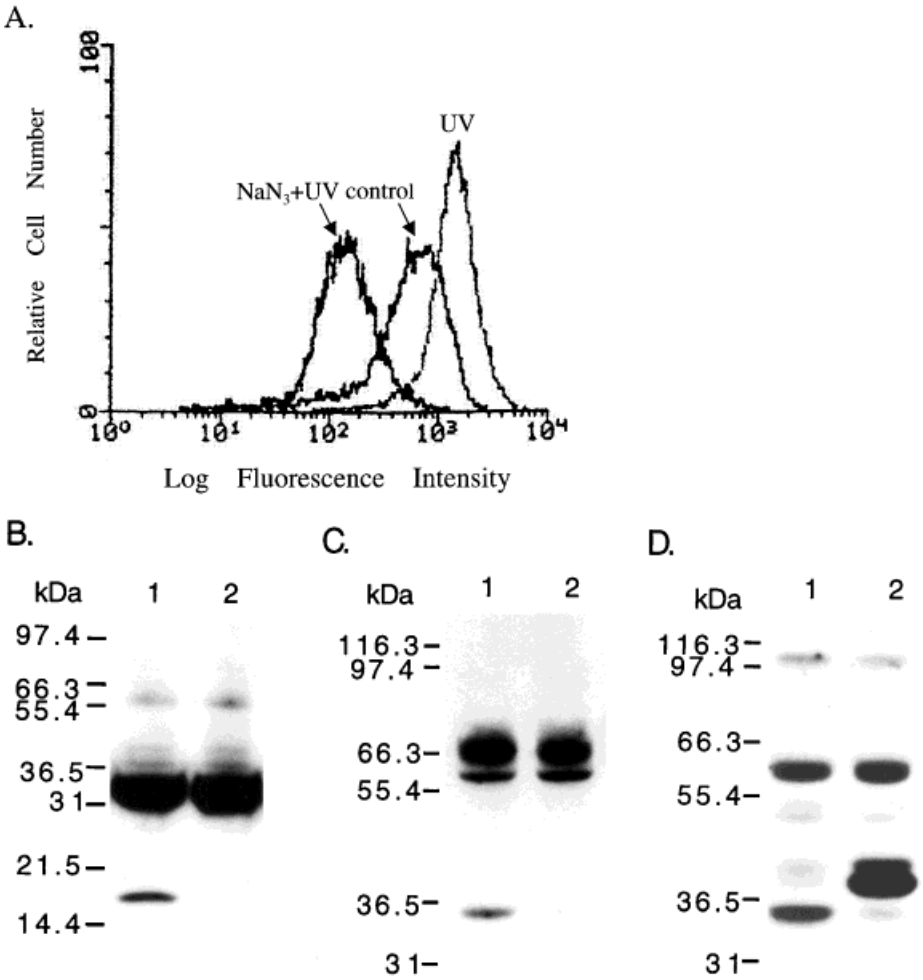

Fig. 5. Effects of azide on ultraviolet (UV) irradiation–induced formation of reactive oxygen species, activation of caspase-3, and cleavage/activation of $p21^{Cdc42/Rac}$-activated kinase 2 (PAK2) in A431 cells. **A:** A431 cells were left untreated or treated with NaN_3 (5 mM) for 1 h and then loaded with 10 μM 2′,7′-dichlorofluorescin diacetate for another 1 h followed by exposure to UV light (200 J/m^2). Cells were harvested and analyzed by flow cytometry. A431 cells were incubated without (**lane 1**) or with (**lane 2**) NaN_3 (5 mM) for 1 h and then subjected to UV irradiation (200 J/m^2) followed by incubation at 37°C for another 2 h. Forty micrograms of the cell extracts was analyzed for activation of caspase-3 (**B**), cleavage (**C**), and activation (**D**) of PAK2, as described in Figure 1. The experiment was repeated three times, and the result is reproducible.

sessed by the intensity of DCF-DA fluorescence was markedly increased, demonstrating the efficacy of this assay system to measure quantitatively the change of intracellular ROS. Using this assay system, the relative intracellular ROS content was measured in A431 cells at different time intervals after exposure to UV light. As shown in Figure 4B–C, UV irradiation caused an increment of intracellular ROS content. Significant increase of intracellular ROS content could be detected 1 h after the cells were exposed to UV light, and this high level of ROS content lasted for at least 3 h under the experimental condition used (Fig. 4B–C).

Oxidative Stress Is Involved in the Regulation of UV Irradiation–Induced Activation of Caspase-3 and Subsequent Cleavage/Activation of PAK2

To elucidate the role of ROS in the UV irradiation–induced apoptotic cell response, the effects of azide (NaN_3), a well-known ROS scavenger [Tsou et al., 1996; Wei et al., 1997], on this process were examined. Pretreatment of the cells with azide completely blocked the UV irradiation–induced formation of intracellular ROS (Fig. 5A). Under this circumstance, the UV irradiation–induced apoptotic biochemical changes including activation of caspase-3

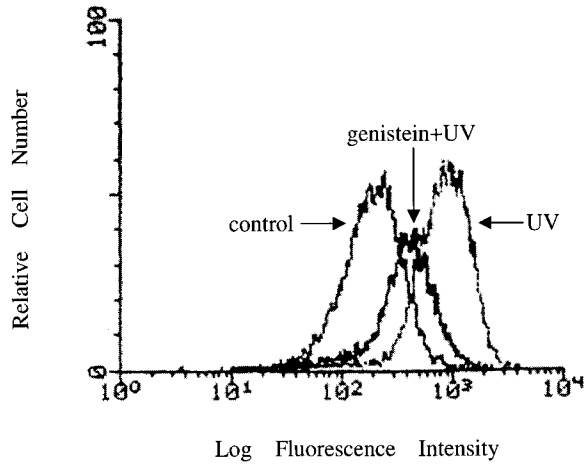

Fig. 6. Genistein attenuates ultraviolet (UV) irradiation–induced oxidative stress in A431 cells. A431 cells were left untreated or treated with genistein (400 μM) for 1 h and then loaded with 10 μM 2′,7′-dichlorofluorescin diacetate for another 1 h followed by exposure to UV light (200 J/m^2). Cells were collected at 2 h after UV irradiation and analyzed by flow cytometry. The experiment was repeated three times, and the result is reproducible.

and cleavage/activation of PAK2 also were inhibited by azide (Fig. 5B–D). The results demonstrate the involvement of oxidative stress in regulating activation of caspase-3 and the subsequent cleavage/activation of PAK2 on UV irradiation and suggest that generation of ROS is a key signaling event upstream of caspase activation during UV irradiation–induced apoptotic cell response.

Genistein Attenuates the Formation of ROS During UV Irradiation

Like the effect of azide, pretreatment of the cells with genistein also significantly attenuated the increase of intracellular ROS content triggered by UV irradiation (Fig. 6). The result provides initial evidence that the inhibitory effect of genistein in the apoptotic cell response to UV irradiation is due to its ability to prevent intracellular oxidative stress.

DISCUSSION

Multiple intracellular signaling pathways that participate in diverse cell functions are altered by UV irradiation [Bender et al., 1997]. The reason that this simple physical perturbation can cause complicated biologic effects seems to be due to the capability of UV irradiation to interact directly with multiple cell membrane and cytoplasmic targets besides DNA [Schwarz, 1998]. Considerable evidence has indicated that oxidative stress resulting from UV irradiation–triggered formation of intracellular ROS plays a crucial role in this process [Buttke and Sandstrom, 1994; Scharffetter-Kochanek et al., 1997]. Using the permeable dye DCF-DA as a tool, we provide direct evidence that UV irradiation can elicit ROS formation in A431 cells (Fig. 4). Results from the time course study showed that after UV irradiation the amount of intracellular ROS increased drastically at 1 h and then remained at a submaximum level for up to 3 h (Fig. 4). However, we previously showed that activation of caspase-3 occurs initially at 1 h and then gradually increases up to 3 h after UV irradiation under the same conditions [Tang et al., 1998], indicating that generation of ROS precedes the activation of caspase-3 with UV irradiation in A431 cells. The observations and the finding that quenching of intracellular ROS by sodium azide, an established scavenger for ROS, completely blocked the UV irradiation–triggered, caspase-dependent apoptotic biochemical changes in cells (Fig. 5) strongly suggest the involvement of ROS in mediating the signal that leads to caspase activation during UV irradiation–induced apoptosis of A431 cells. At present, how ROS generated by UV irradiation can trigger caspase activation is elusive and remains to be established.

The influence of genistein on apoptotic process is quite ambiguous. As noted, both inhibitory and stimulatory effects could be observed in several cases. For instance, genistein alone at doses of 30–74 μM induces apoptosis of prostate carcinoma cells [Kyle et al., 1996], immature human thymocytes [McCabe and Orrenius, 1993], leukemia cell lines [Bergamaschi et al., 1993], human stomach cancer cells [Yanagihara et al., 1993], and bladder carcinoma cell lines [Zhou et al., 1998]. In contrast, apoptosis of human B lymphocyte precursors induced by ionization radiation [Uckun et al., 1992], human ovarian tumor cell line induced by taxol [Liu et al., 1994], mouse thymocytes induced by anti-CD3 [Migita et al., 1994], human HL-60 cells induced by didemnin B [Johnson et al., 1996], and murine mastocytoma and leukemia cell lines induced by ATP [Bronte et al., 1996] could be inhibited by genistein at doses greater than 74 μM. Our results show that genistein at doses greater than 100 μM

blocks UV irradiation–induced, caspase-dependent apoptotic biochemical changes in A431 cells (Fig. 1). These observations taken together seem to implicate that variation in the dosage of genistein used in experiments can result in a totally distinct outcome. It is likely that genistein at low doses can interfere with a set of intracellular signaling components such as the ones involved in G_2–M cell cycle control [Schao et al., 1998; Zhou et al., 1998] and thereby induce subsequent apoptosis, whereas at high doses genistein may further meddle with another set of signaling events that control the apoptotic machinery so as to inhibit the cell death process induced by a variety of apoptotic triggers.

Inhibition by genistein of apoptosis induced by several apoptotic triggers has been attributed to its inhibitory activity to PTKs because other commonly used PTK inhibitors such as herbimycin A or tyrohostins have also blocked apoptosis in several studies [Migita et al., 1994; Liu et al., 1994; Johnson et al., 1996]. However, we found that herbimycin A or tyrohostin A47 has no effect on the UV irradiation–induced caspase activation and the subsequent biochemical changes (Fig. 2). Similar results could also be observed using the three established topo-II inhibitors (Fig. 3), indicating that the inhibitory effect of genistein would be mediated through mechansim(s) other than inhibition of intracellular PTKs and topo-II. Furthermore, it is unlikely that caspases, which underlie the apoptotic core program in cells, are the direct targets for inhibition by genistein because it has been shown that direct addition of high-dose genistein (>100 μM) into a cell-free caspase activation assay system does not inhibit caspase-3 activation [Mesner et al., 1999].

In addition to the inhibitory activity to both PTKs and topo-II, genistein has been shown to posses other biologic activity. Wei et al. [1996] reported that genistein inhibited the UV irradiation–triggered oxidative damage of calf thymus DNA in vitro. They also demonstrated that genistein could potently scavenge hydrogen peroxide in the medium and superoxide anion generated by xanthine/xanthine oxidase, indicating that genistein can act as an antioxidant in vitro. Indeed, antioxidative activity of soybean isoflavones has been recognized for a long time [Naim et al., 1976]. In the present study, we show that genistein can also significantly inhibit the formation of intracellular ROS on UV irradiation (Fig. 6). Because oxidative stress caused by intracellular ROS is an upstream signal leading to caspase activation in UV-irradiated cells (Figs. 4, 5), it seems likely that the capability to attenuate intracellular ROS formation is responsible for the observed inhibitory effect of genistein on the UV irradiation–induced, caspase-dependent biochemical changes in A431 cells. This notion is consistent with the finding that daidzein, another isoflavone compound having a structure similar to that of genistein but without inhibitory activity to PTKs, could also significantly diminish the UV irradiation–induced, caspase-dependent biochemical changes in A431 cells (Fig. 2). Whether prevention of UV irradiation–induced oxdiative stress by genistein is mediated directly through its ROS-scavenging activity or indirectly through the interference with other cellular ROS-generating systems is unclear and represents an intriguing issue deserving further investigation. Nevertheless, the present study clearly demonstrates the involvement of oxidative stress in the UV irradiation–induced caspase activation and the subsequent apoptotic biochemical changes and shows that genistein is a potent inhibitor for this process.

EXPERIMENTAL PROCEDURES

Materials

[γ-^{32}P]ATP was purchased from Amersham (Buckinghamshire, UK). Polyvinylidene fluoride (PVDF) membrane was obtained from Millipore (Bedford, MA). Dulbecco's modified Eagle's medium, DCF-DA, goat anti-rabbit and anti-mouse IgG antibodies conjugated with alkaline phosphatase were obtained from Sigma (St. Louis, MO). Anti-αPAK (C19) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-CPP32/caspase-3 antibody was obtained from Transduction Laboratories (Lexington, KY). CDP-Star™ (a chemiluminescent substrate for alkaline phosphatase) was obtained from Boehringer Mannheim (Mannheim, Germany).

Protein Purification

Myelin basic protein (MBP) was purified from porcine brain according to the procedure described in a previous report [Yu and Yang, 1994a].

Cell Culture and UV Irradiation

The human epidermoid carcinoma A431 cells were cultured as previously described [Yu and Yang, 1994b]. One day before experiments, cells (~5–6 × 10^6) were plated on 100-mm culture dishes. UV irradiation (254 nm, UVC) was carried out with a UV gene linker (UV Stratalinker, model 1800, Stratagene, La Jolla, CA) equipped with an energy output control. Before irradiation, the medium was removed from the culture dish, and the dish, with the lids off, was exposed to UV light at 200 J/m^2. The medium was added again immediately into the dish after UV irradiation, and the dish was incubated at 37°C in a CO_2 incubator for 2 h. The cells were washed twice with ice-cold phosphate buffer saline (PBS) and lysed in 600 μl of solution A [20 mM Tris-HCl at pH 7.4, 1 mM ethylene-diaminetetraacetic acid (EDTA), 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, and 1 mM sodium orthovanadate] on ice for 10 min. The cell lysates were collected and then sonicated on ice by a Sonicator (model W-380, Heat Systems-Ultrasonics) for 3 × 10 s at 50% power output followed by centrifugation at 15,000*g* for 20 min at 4°C. The resulting supernatants were used as the cell extracts.

Immunoblots

Immunoblot analysis was performed essentially as previously described [Yu and Yang, 1994c]. Anti-α-PAK (C19) antibody (0.2 μg/ml) or anti-CPP32/caspase-3 monoclonal antibody (0.25 μg/ml) was used to immunoblot proteins transferred from sodium dodecyl sulfate (SDS) gel to the PVDF membrane. The proteins of interest were detected by using goat anti-rabbit or anti-mouse IgG antibody conjugated with alkaline phosphatase and CDP-Star™ (chemiluminescent substrate for alkaline phosphatase) according to the procedure provided by the manufacturer.

DNA Fragmentation Analysis

DNA fragmentation was analyzed according to the method of Zhu and Wang [1997]. Briefly, A431 cells (~1 × 10^6 cells) detached from culture dishes by trypsin/EDTA were collected, washed once with ice-cold PBS, and centrifuged. After removing the supernatants, cells were dispersed in 30 μl lysis buffer (10 mM Tris-HCl at pH 7.4, 100 mM NaCl, 25 mM EDTA, and 1% sarkosyl) by gentle vortexing, and 4 μl of proteinase K (10 μg/μl) was then added, and the cell lysates were incubated at 45°C for 2 h. Two microliters of RNase (10 μg/μl) was added, and the cell lysates were incubated for another 1 h at room temperature. The resulting reaction mixtures (20 μl/sample) were subjected to electrophoresis on 2% agarose gels for DNA fragmentation analysis.

In-Gel Kinase Assay

In-gel kinase assay was performed as previously described [Tang et al., 1998]. Briefly, 0.5 mg/ml of MBP was copolymerized in the separating gel of 10% SDS–polyacrylamide gels, and cell extracts (40 μg) were resolved in these MBP-containing SDS gels. After removing SDS from the gels, proteins were renatured, and an in-gel kinase assay was performed by incubating the gel in phosphorylation buffer containing [γ-^{32}P]ATP. The gels were stained, destained, and dried, and ^{32}P-incorporation of MBP was detected by autoradiography.

Fluorescent Measurements of Intracellular Oxidants

DCF-DA was used to detect production of ROS in cells. The fluorescence of this cell-permeable agent significantly increases after oxidation [Kehrer and Paraidathathu, 1992; LeBel et al., 1992]. DCF-DA was dissolved in dimethyl sulfoxide and stored as 50 mM stock at −20°C. A431 cells were incubated with genistein or other compounds for 1–3 h at 37°C and then with 10 μM DCF-DA for 1 h. Cells were harvested at the indicated time points after UV irradiation, washed three times with PBS, and then immediately analyzed (3 × 10^5 cells/sample) by flow cytometry on a FACScan (Becton-Dickinson, Moutain View, CA) using a 488-nm excitation beam.

Analytic Methods

Protein concentrations were determined by using the BCA protein assay reagent from Pierce (Rockford, IL). SDS-polyacrylamide gel electrophoresis was performed by using the method of Laemmli [1970] with 10 or 15% gels.

REFERENCES

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y. 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262:5592–5595.

Azuma Y, Onishi Y, Sato Y, Kizaki H. 1993. Induction of mouse thymocyte apoptosis by inhibitors of tyrosine kinases is associated with dephosphorylation of nuclear proteins. Cell Immunol 152:271–278.

Bergamaschi G, Rosti V, Danova M, Ponchio L, Lucotti C, Cazzola M. 1993. Inhibitors of tyrosine phosphorylation induce apoptosis in human leukemic cell lines. Leukemia 7:2012–2018.

Bender K, Blattner C, Knebel A, Iordanov M, Herrlich P, Rahmsdorf HJ. 1997. UV-induced signal transduction. J Photochem Photobiol B 37:1–17.

Bronte V, Macino B, Zambon A, Rosato A, Mandruzzato S, Zanovello P, Collavo D. 1996. Protein tyrosine kinases and phosphatases control apoptosis induced by extracellular adenosine 5′-triphosphate. Biochem Biophys Res Commun 218:344–351.

Brown A, Jolly P, Wei H. 1998. Genistein modulates neuroblastma cell proliferation and differentiation through induction of apoptosis and regulation of tyrosine kinase activity and N-myc expression. Carcinogenesis 19:991–997.

Buttke TM, Sandstrom PA. 1994. Oxidative stress as a mediator of apoptosis. Immunol Today 15:7–10.

Caricchio R, Kovalenko D, Kaufmann WK, Cohen PL. 1999. Apoptosis provoked by the oxidative stress inducer menadione (vitamin K(3)) is mediated by the Fas/Fas ligand system. Clin Immunol 93:65–74.

Chan WH, Yu JS, Yang SD. 1998. Heat shock stress induces cleavage and activation of PAK2 in apoptotic cells. J Protein Chem 17:485–494.

Chan WH, Yu JS, Yang SD. 1999. PAK2 is cleaved and activated during hyperosmotic shock-induced apoptosis via a caspase-dependent mechanism: evidence for the involvement of oxidative stress. J Cell Physiol 178:397–408.

Constantinou A, Henning-Chubb C, Huberman E. 1989. Novobiocin- and phorbol-12-myristate-13-acetate–induced differentiation of human leukemia cells associated with a reduction in topoisomerase II activity. Cancer Res 49:1110–1117.

Constantinou A, Kiguchi K, Huberman E. 1990. Induction of differentiation and DNA strand breakage in human HL-60 and K-562 leukemia cells by genistein. Cancer Res 50:2618–2624.

Ellis RE, Yuan J-Y, Horvitz HR. 1991. Mechanisms and functions of cell death. Annu Rev Cell Biol 7:663–698.

Enari M, Talanian RV, Wong WW, Nagata S. 1996. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature 380:723–726.

Fernandes-Alnemri T, Litwack G, Alnemri ES. 1994. CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1β-converting enzyme. J Biol Chem 269:30761–30764.

Gewirtz DA. 1999. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 57:727–741.

Halliwell B, Gutteridge JM. 1990. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 186:1–85

Hande KR. 1998. Etoposide: four decades of development of a topoisomerase II inhibitor. Eur J Cancer 34:1514–1521.

Johnson KL, Vaillant F, Lawen A. 1996. Protein tyrosine kinase inhibitors prevent didemnin B-induced apoptosis in HL-60 cells. FEBS Lett 383:1–5.

Kehrer JP, Paraidathathu T. 1992. The use of fluorescent probes to assess oxidative processes in isolated-perfused rat heart tissue. Free Rad Res Commun 16:217–225.

Kerr J, Wyllie A, Currie A. 1972. Apoptosis: a basis biological phenomenon with wide-ranging implication in tissue kinetics. Br J Cancer 26:239–257.

Kyle E, Bergan R, Neckers L. 1996. Genistein inhibits prostate cancer cell growth by inducing apoptosis. Proc Am Assoc Cancer Res 37:359.

Laemmli UK. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685.

LeBel CP, Ischiropoulos H, Bondy SC. 1992. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5:227–231.

Levitzki A. 1990. Tyrphostins—potential antiproliferative agents and novel molecular tools. Biochem Pharmacol 40:913–918.

Liu Y, Bhalla K, Hill C, Priest DG. 1994. Evidence for involvement of tyrosine phosphorylation in taxol-induced apoptosis in a human oveaian tumor cell line. Biochem Pharmacol 48:1265–1272.

Markovits J, Linassier C, Fosse P, Couprie J, Pierre J, Jacquemin-Sablon A, Saucier JM, Le Pecq JB, Larsen AK. 1989. Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer Res 49:5111–5117.

Martins LM, Earnshaw WC. 1997. Apoptosis: alive and kicking in 1997. Trends Cell Biol 7:111–114.

McCabe MJ Jr, Orrenius S. 1993. Genistein induces apoptosis in immature human thymocytes by inhibiting topoisomerase-II. Biochem Biophys Res Commun 194: 944–950.

Mesner PW Jr, Bible KC, Martins LM, Kottke TJ, Srinivasula SM, Svingen PA, Chilcote TJ, Basi GS, Tung JS, Krajewski S, Reed JC, Alnemri ES, Earnshaw WC, Kaufmann SH. 1999. Characterization of caspase processing and activation in HL-60 cell cytosol under cell-free conditions. Nucleotide requirement and inhibitor profile. J Biol Chem 274:22635–11645.

Migita K, Eguchi K, Kawabe Y, Mizokami A, Tsukada T, Nagataki S. 1994. Prevention of anti-CD3 monoclonal antibody-induced thymic apoptosis by protein tyrosine kinase inhibitors. J Immunol 153:3457–3465.

Naim M, Gestetner B, Bondi A, Birk Y. 1976. Antioxidative and antihemolytic activities of soybean isoflavones. J Agric Food Chem 24:1174–1177.

Nicholson DW, Thornberry NA. 1997. Caspases: killer proteases. Trends Biochem Sci 22:299–306.

Rauth S, Kichina J, Green A. 1997. Inhibition of growth and induction of differentiation of metastatic melanoma cells in vitro by genistein: chemosensitivity is regulated by cellular p53. Br J Cancer 75:1559–1566.

Schao ZM, Alpaugh ML, Fontana JA, Barsky SH. 1998. Genistein inhibits proliferation similar in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by p21WAF1/CIP1 induction, G2/M arrest, and apoptosis. J Cell Biochem 69:44–54.

Scharffetter-Kochanek K, Wlaschek M, Brenneisen P, Schauen M, Blaudschun R, Wenk J. 1997. UV-induced reactive oxygen species in photocarcinogenesis and photoaging. Biol Chem 378:1247–1257.

Schwarz T. 1998. UV light affects cell membrane and cytoplasmic targets. J Photochem Photobiol B 44:91–96.

Sells MA, Chernoff J. 1997. Emerging from the Pak: the p21-activated protein kinase family. Trends Cell Biol 7:162–167.

Tang TK, Chang WC, Chan WH, Yang SD, Ni MH, Yu JS. 1998. Proteolytic cleavage and activation of PAK2 during UV irradiation–induced apoptosis in A431 cells. J Cell Biochem 70:442–454

Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA, Orrenius S. 1982. The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. J Biol Chem 257:12419–12425.

Tsou TC, Chen CL, Liu TY, Yang JL. 1996. Induction of 8-hydroxydeoxyguanosine in DNA by chromium(III) plus hydrogen peroxide and its prevention by scavengers. Carcinogenesis 17:103–108.

Uckun FM, Tuel-Ahlgren L, Song CW, Waddick K, Myers DE, Kirihara J, Ledbetter JA, Schieven GL. 1992. Ionizing radiation stimulates unidentified tyrosine-specific protein kinases in human B-lymphocyte precursors, triggering apoptosis and clonogenic cell death. Proc Natl Acad Sci USA 89:9005–9009.

Uehara Y, Fukazawa H. 1991. Use and selectivity of herbimycin A as inhibitor of protein-tyrosine kinases. Methods Enzymol 201:370–379.

Wei H, Cai Q, Rahn RO. 1996. Inhibition of UV light- and Fenton reaction-induced oxidative DNA damage by the soybean isoflavone genistein. Carcinogenesis 17:73–77.

Wei H, Cai Q, Rahn R, Zhang X. 1997. Singlet oxygen involvement in ultraviolet (254 nm) radiation-induced fromation of 8-hydro-deoxyguanosine in DNA. Free Rad Biol Med 23:148–154.

Yanagihara K, Ito A, Toge T, Numoto M. 1993. Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. Cancer Res 53:5815–2581.

Yokoshiki H, Sumii K, Sperelakis N. 1996. Inhibition of L-type calcium current in rat ventricular cells by the tyrosine kinase inhibitor, genistein and its inactive analog, daidzein. J Mol Cell Cardiol 28:807–814.

Yu J-S, Yang S-D. 1994a. Protein kinase FA/glycogen synthase kinase-3 predominantly phosphorylates the in vivo site Thr^{97}-Pro in brain myelin basic protein. Evidence for Thr-Pro and Ser-Arg-X-X-Ser as consensus sequences motif. J Neurochem 62:1596–1603.

Yu J-S, Yang S-D. 1994b. Okadaic acid, a serine/threonine phosphatase inhibitor, induces tyrosine dephosphorylation/inactivation of kinase FA/GSK-3α in A431 cells. J Biol Chem 269:14341–14344.

Yu J-S, Yang S-D. 1994c. Tyrosine dephosphorylation and concurrent inactivation of protein kinase FA/GSK-3α by genistein in A431 cells. J Cell Biochem 56:131–141.

Zhou JR, Mukherjee P, Gugger ET, Tanaka T, Blackburn GL, Clinton SK. 1998. Inhibition of murine bladder tumorigenesis by soy isoflavones via alterations in the cell cycle, apoptosis, and angiogenesis. Cancer Res 58:5231–5238.

Zhu N, Wang Z. 1997. An assay for DNA fragmentation in apotosis without phenol/chloroform extraction and ethanol precipitation. Anal Biochem 246:155–158.