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Heme Oxygenase-1 Protects Against Apoptosis Induced by Tumor Necrosis Factor-α and Cycloheximide in Papillary Thyroid Carcinoma Cells

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Abstract Heme oxygenase-1 (HO-1) plays a role in the resistance to apoptosis of several types of cells, but its role in the development of thyroid cancer is unknown. In this study, we investigated the regulation of HO-1 in human papillary thyroid carcinoma cells (KAT5). The results show that HO-1 is significantly induced by hemin and cadmium. In addition to inducing HO-1, hemin and cadmium also cause a rise in the levels of p21, a cyclin-dependent kinase inhibitor. Cells with increased levels of HO-1 and p21 were more resistant to apoptotic stimuli than cells with normal levels. The cells resistant to apoptosis also displayed an increased arrest at the G_0/G_1 phase of the cell-cycle. The induced levels of HO-1 and p21 were significantly reduced by p38 mitogen-activated protein kinase (p38 MAPK) and extracellular-regulated kinase (ERK) inhibitors. More importantly, KAT5 cells regained their sensitivity to apoptotic stimuli after they were treated with these kinase inhibitors, indicating that p38 MAPK and ERK are required for the resistance to apoptosis conferred by HO-1. Furthermore, we demonstrated that increased levels of HO-1 and p21 expression are associated with an increase in the activity of NF-kappaB and that inhibiting NF-kappaB leads to a block in the induction of HO-1 and p21. In summary, this study reveals that an increase in the level of HO-1 markedly reduces the sensitivity of papillary thyroid carcinoma cells to apoptotic stimuli. The HO-1 pathway of apoptosis resistance is associated with an increase in the levels of p21, involves a p38 MAPK and ERK-mediated mechanism and can be suppressed by inhibiting NF-kappaB. J. Cell. Biochem. 92: 1246–1256, 2004.

Key words: thyroid cancer; heme oxygenase-1; p21; apoptosis

Papillary thyroid carcinoma, a thyroid follicle epithelial cell-derived tumor, is the most common thyroid malignancy, accounting for approximately 80% of cases. Although the pathogenesis of papillary thyroid carcinoma is not completely known, it is probably a multifactorial disease. While well described epidemiological studies have shown that exposure to radiation is an etiological factor [Busnardo and De Vido, 2000], a high dietary intake of iodine has been associated with a high incidence of papillary thyroid carcinoma [Bacher-Stier et al., 1997; Harach et al., 2002]. An excessive intake of iodine may therefore contribute to the development of papillary thyroid carcinoma.

Recent animal experiments have supported this epidemiological finding, showing that thyroid cancer develops in irradiated rats who have a high iodine intake but not in those with a normal iodine intake [Boltze et al., 2002]. Increased levels of iodine have also been shown to result in an increase in epidermal hyperplasia and in a reduction in the expression of inducible nitric oxide synthase (iNOS) [Nyska

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et al., 2001]. iNOS is a factor that may function to inhibit the growth of tumor cells [Williams et al., 2003]. A high level of iodine can lead to an increase in the activity of heme oxygenase (HO), as iodine is a quenching molecule that has been shown to markedly reduce the activity of HO inhibitors [Delaney et al., 1988].

HO is the rate-limiting enzyme in the catabolism of heme to bilirubin. Three isoforms, transcribed from separate genes, have been described. HO-2 and HO-3 are primarily constitutive, whereas HO-1 is highly inducible [McCoubrey et al., 1997]. HO-1 has been shown to be associated with cell proliferation and growth. Furthermore, HO participates in the pathogenesis of several types of cancers [Murphy et al., 1993; Maines and Abrahamsson, 1996; Goodman et al., 1997]. An elevated expression or increased activity of HO-1 has been reported to be associated with cellular proliferation in some tumors, such as in prostate cancer and renal adenocarcinoma [Maines and Abrahamsson, 1996; Goodman et al., 1997]. An elevated level of HO-1 correlates with the activity of certain molecules that are involved in cellular transformation, such as estrogen receptors, p53 protein and p21, a cyclin-dependent kinase inhibitor that causes cell-cycle arrest in the G_1 phase in response to DNA damage [Elledge et al., 1994; Takahashi et al., 1994; Deng et al., 1995]. There is currently no report that describes the role of HO in the development of thyroid cancer. The present study was undertaken to investigate the regulation of HO-1 in the human papillary thyroid carcinoma cell line KAT5 [Wang et al., 2001], and to propose a possible role that HO-1 may play in the development of papillary thyroid carcinoma.

MATERIALS AND METHODS

Cell Culture

A papillary thyroid carcinoma cell line, KAT5, was cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. To induce HO-1 and p21, KAT5 cells were exposed to hemin (Sigma, St. Louis, MO) or cadmium (Sigma) for 4 h after they were cultured in a complete medium for 48 h and then subsequently in a serum-free medium for 24 h. To inhibit the expression of HO-1 and p21, kinase inhibitors SB203580 and PD098059 were added 1 h prior to the addition of hemin or cadmium.

Determination of HO-1 and p21 Levels

Western blotting was employed to determine the level of expression of HO-1 and p21. Briefly, the total protein of the culture was extracted and samples containing 100 μg of protein were separated on a 12% Tris-HCl gel, and then transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). To detect the HO-1 protein, a rabbit-anti-human polyclonal HO-1 antibody (StressGen Biotechnologics, Victoria, BC, Canada) was employed, followed by a horseradish peroxidase-conjugated goat-anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The p21 protein was analyzed using a rabbit-anti-human polyclonal p21 antibody (Santa Cruz Biotechnology) as the primary antibody and a polyclonal goat-anti-rabbit IgG antibody (Santa Cruz Biotechnology) as the secondary antibody. Anti-human actin antibody (Santa Cruz Biotechnology) was used to detect human actin, which was used as a control for equal loading. Detection of the target protein signal was achieved using a chemiluminescence method (Amersham Biosciences).

Assessment of Cell Death and Apoptosis

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to quantify cell death. Cell death was also determined by the measurement of DNA fragmentation. Briefly, KAT5 cells were cultured in a complete medium for 48 h and subsequently in a serum-free medium for 24 h. Cells were then exposed to hemin or cadmium for 4 h. The kinase inhibitors, SB203580 and PD098059 (both from Promega, Madison, WI), were given 1 h prior to the addition of hemin or cadmium. Following this, the cells were treated with 5 ng/ml of recombinant human tumor necrosis factor-α (rhTNF-α) (Promega) in conjunction with 10 μg of cycloheximide (Sigma) for 3 h. Apoptosis was assessed by a cellular DNA fragmentation ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany).

Fluorescence-Activated Cell Sorter Analysis

Cell-cycle analysis was performed by fluorescence-activated cell sorter analysis (FACS). Approximately 10^6–10^7 cells were harvested by trypsinization and gently pelleted by

centrifugation at 200*g* for 5 min. After centrifugation, cells were washed in cold phosphate-buffered saline (PBS) and re-suspended in 0.5 ml of PBS. The suspended cells were transferred drop-wise into 4.5 ml of 70% ethanol, where they were fixed for more than 2 h. The ethanol-suspended cells were then collected, washed, and resuspended in 1 ml of 20 μg/ml propidium iodide (PI, Molecular Probe, Eugene, OR)/0.1% (v/v) Triton X-100 staining solution with 200 μg/ml RNase A (Sigma) in the dark for 30 min at room temperature. Flow cytometry was performed on a Becton Dickinson FACScan machine, using an excitation wavelength of 488 nm and an emission wavelength of 585 nm. DNA content frequency histogram deconvolution software was employed to analyze data.

Nuclear Protein Extraction and NF-kappaB Activity Assay

Nuclear protein was isolated according to a described procedure [Davis et al., 2001]. Briefly, cells were harvested, washed in PBS, and collected by centrifugation. The cell pellet was resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/0.05% (v/v) Triton X-100 and lysed with 20 strokes in a homogenizer. The homogenate was centrifuged at 10,000*g* for 15 min at 4°C to obtain a nuclear pellet. The nuclear pellet volume was estimated and the pellet resuspended in an equal volume of 10 mM Tris-HCl (pH 7.4)/5 mM $MgCl_2$, followed by the addition of 1 nuclear pellet volume of 1 M NaCl/10 mM Tris-HCl (pH 7.4)/4 mM $MgCl_2$. The lysing nuclei were left on ice for 30 min and then centrifuged at 10,000*g* for 15 min at 4°C. The supernatant (nuclear extract) was removed and 80% glycerol was added so that the final glycerol concentration was 20% (v/v). The concentration of the nuclear protein was determined. NF-kappaB activity was measured by an enzyme immunoassay kit from Oxford Biomedical Research (Oxford, MI), which employs an oligonucleotide containing the DNA binding NF-kappaB consensus sequence. Any NF-kappaB present in the sample specifically binds to the oligonucleotide coated onto the plate and the DNA-bound NF-kappaB selectively recognized by an antibody to NF-kappaB subunits p50 or p65.

Inhibition of NF-kappaB

A recombinant replication-deficient adenovirus, Ad5IkB, contains an IkBα construct in which serines 32 and 36 are mutated to alanines, driven by the cytomegalovirus promoter-enhancer. As the mutant IkBα cannot be phosphorylated, it prevents the activation of NF-kappaB by irreversibly binding to it. Ad5IkB (a generous gift from Dr. D.A. Brenner) was used as previously described. The adenovirus, Ad5LacZ, which contains the *Escherichia coli* β-galactosidase gene, was used as a control. Both viruses were grown in 293 cells and purified as previously described. For adenovirus infection, sub-confluent cells (~80%) were infected with the virus in a serum-free medium at multiplicities of infection (MOI) of 10–500 for 12 h. The virus was then washed off and fresh media containing serum was added to the cells. The cells were cultured for another 12 h prior to the experiments.

Statistical Analysis

All values were expressed as a mean ± standard deviation. Statistical comparisons were analyzed by the Student's *t*-test using the statistical computer software SPSS for Windows (Release 11.0.1, Chicago, IL). A *P*-value of less than 0.05 was taken as statistically significant.

RESULTS

Expression of HO-1 and p21 Are Up-Regulated by Cadmium or Hemin

The level of HO-1 was determined in untreated KAT5 cells. The level of HO-1 was significantly increased when KAT5 cells were treated with either cadmium or hemin (Fig. 1). The highest level of HO-1 expression in the cells was achieved with cadmium at concentrations of 5–10 μM, and hemin at a concentration of not less than 10 μM. The expression of HO-1 plateaued when a concentration of 20 μM of either cadmium or hemin was used. The expression of p21 was undetectable in untreated KAT5 cells (Fig. 1). Cadmium did not induce p21 expression until a concentration of 5 μM was used, and the level of p21 was the highest when a concentration of 10 μM was used. Hemin at concentrations of 2.5–20 μM induced the expression of p21. The expression of p21 did not increase significantly more once the concentration of hemin had reached 10 μM, and a slight decrease in the expression of p21 was seen when the concentration of hemin was greater than 10 μM.

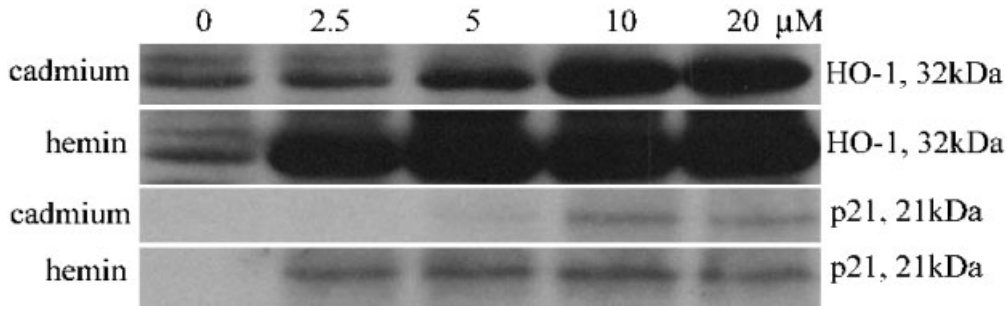


Fig. 1. The expressions of heme oxygenase-1 (HO-1) and p21 are up-regulated by hemin or cadmium in KAT5 cells. KAT5 cells were cultured in a complete medium for 48 h and subsequently in a serum-free medium for 24 h, and were then exposed to hemin and cadmium separately at concentrations from 2.5 to 20 μM for 4 h. The total protein was extracted and the expression of HO-1 and p21 analyzed by Western blotting.

Time Course of Up-Regulation of the Expression of HO-1 and p21

A time course experiment revealed that the levels of both HO-1 and p21 expression were time-dependent (Fig. 2). HO-1 protein was found at all time points, but the highest level was observed at or after 4 h of treatment with either cadmium or hemin. p21 protein expression was not apparent until 3 h of treatment with either cadmium or hemin. When the time course experiments of HO-1 and p21 were compared, the appearance of p21 occurred about 2–3 h after the appearance of HO-1.

Regulation of HO-1 and p21 Expression by Kinase Inhibitors and NF-kappaB Inhibitors

Both hemin and cadmium significantly up-regulated the expression of HO-1 and p21 in KAT5 cells. However, the up-regulation of HO-1 and p21 by hemin was reduced by about 3–4 folds by SB203580 and by about 1–2 folds by PD098059 (Fig. 3). The results indicate that HO-1 and p21 are positively regulated by both p38 mitogen-activated protein kinase (p38 MAPK) and extracellular-regulated kinase (ERK) because SB203580 and PD098059, potent inhibitors of p38 MAPK and ERK respectively, reduced the up-regulation of HO-1 and p21.

NF-kappaB Affects HO-1 and p21 Expression

As shown in Figure 1, cadmium and hemin are powerful inducers of HO-1 expression in KAT5 cells. Figure 4A shows that both cadmium and hemin are able to increase the activity of NF-kappaB in KAT5 cells, as the expression of NF-kappaB in the nuclei of cells treated with either cadmium or hemin was significantly higher than in untreated cell. Similarly, the increase in the nuclear level of NF-kappaB was markedly attenuated by SB203580 and PD09805, inhibitors of p38 MAPK and ERK, respectively. The p38 MAPK inhibitor was more

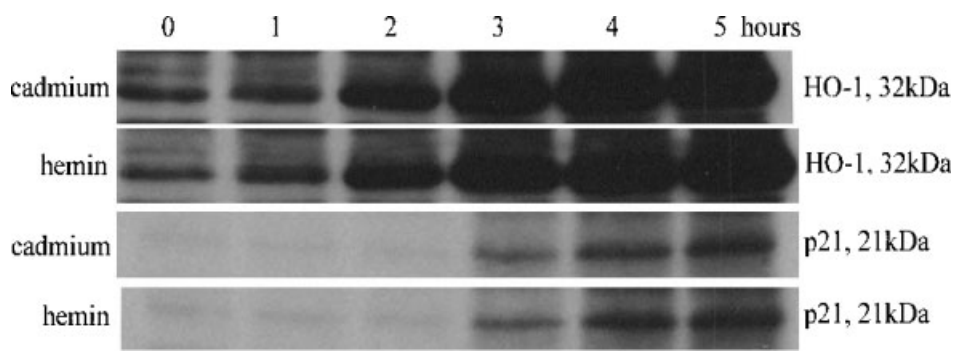


Fig. 2. The time-course of HO-1 and p21 expression in KAT5 cells. KAT5 cells were cultured in a complete medium for 48 h and subsequently in a serum-free medium for 24 h, and were then exposed to hemin and cadmium separately at a concentration of 10 μM for 1–5 h. The total protein was extracted and the expression of HO-1 and p21 was analyzed by Western blotting.

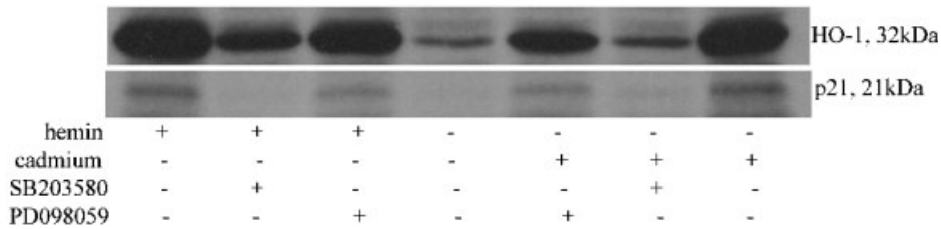


Fig. 3. The effect of kinase inhibitors SB203580 and PD098059 on the expression of HO-1 and p21. KAT5 cells were cultured in a complete medium for 48 h and subsequently in a serum-free medium for 24 h, and were then exposed to hemin and cadmium separately at a concentration of 10 μM for 4 h. The kinase inhibitors SB203580 and PD098059 were added at a concentration of 40 μM 1 h prior to the addition of hemin and cadmium. The total protein was extracted and the expression of HO-1 and p21 analyzed by Western blotting.

effective in reducing the levels of NF-kappaB than was the ERK inhibitor. To confirm that nuclear NF-kappaB plays a role in the regulation of HO-1 and p21 expression in KAT5 cells treated with HO-1 inducers, Ad5IkB was used to inhibit the translocation of NF-kappaB into the nucleus. Ad5IkB is a super-repressor of NF-kappaB activity and contains a mutated

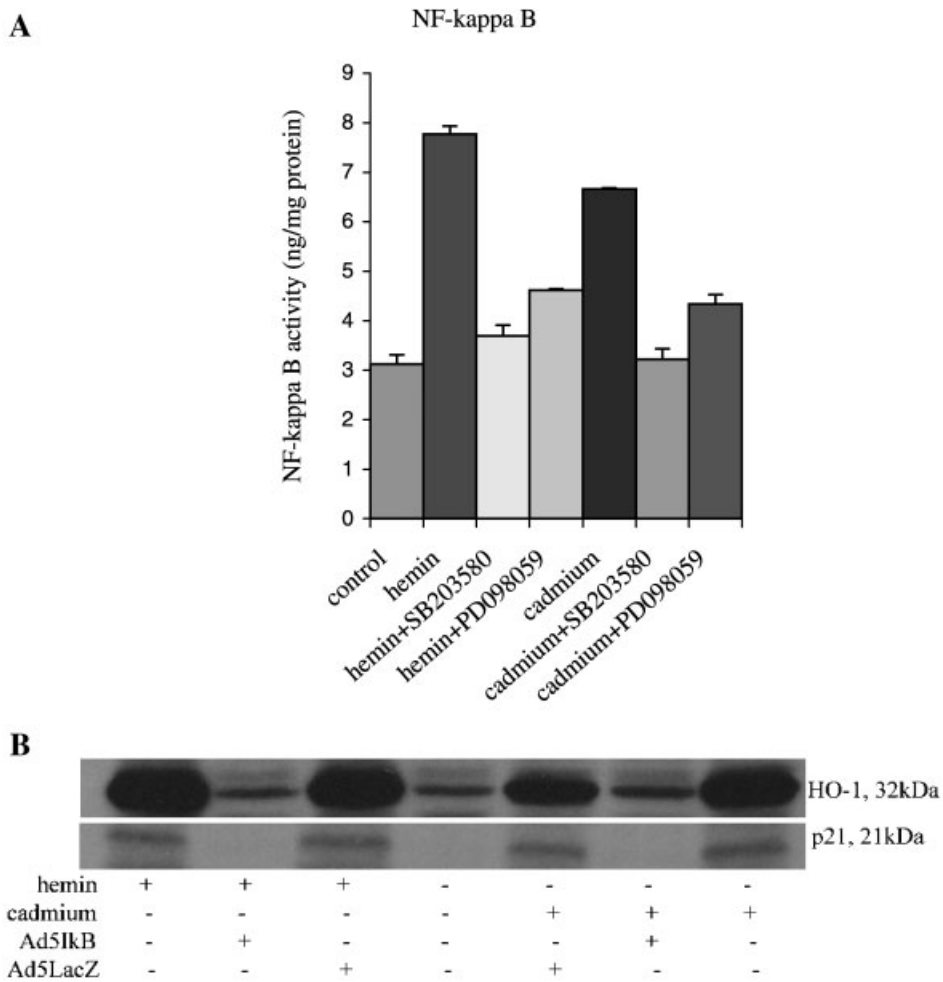


Fig. 4. Nuclear NF-kappaB is involved in the expression of HO-1 and p21. KAT5 cells were cultured as described in Figure 3. At the end of the culture, the nuclear protein was extracted. The concentration of nuclear NF-kappaB was determined using a chemiluminescence based sandwich ELISA kit. The result was expressed as ng of NF-kappaB in mg of nuclear protein. The data represents a mean of three independent experiments (**A**). For the NF-kappaB inhibition experiment, the cells were infected with either Ad5IkB or Ad5LacZ and then treated with hemin and cadmium separately for 4 h. The expression of HO-1 and p21 was determined by Western blotting. A typical result of Western blotting is shown (**B**). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control; $^{++}P < 0.01$, compared with cells treated with hemin; $^{\#\#}P < 0.01$, compared with cells treated with cadmium.

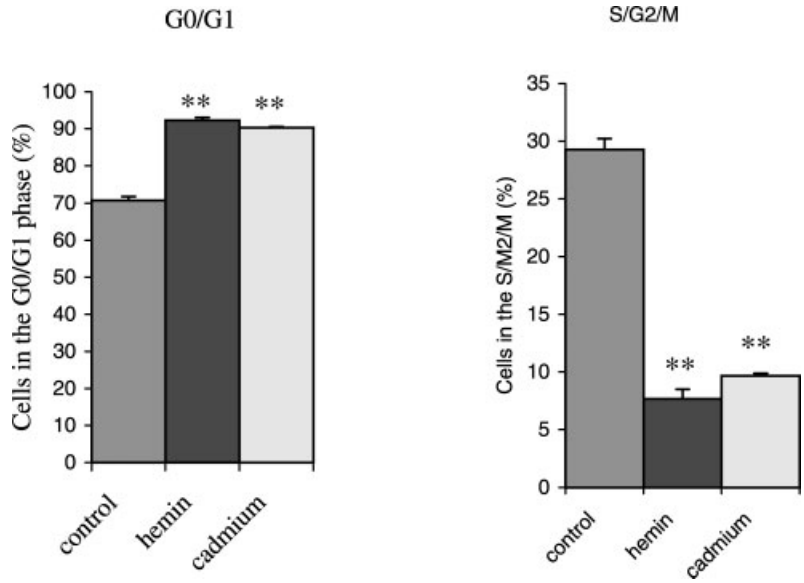


Fig. 5. The effect of hemin and cadmium on the cell-cycle. KAT5 cells were treated with either 10 μM hemin or 10 μM cadmium for 4 h. After treatment, the cells were stained with propidium iodide. The cell-cycle was analyzed by fluorescence activated cell sorter analysis (**A**). Data is expressed as the percentage of cells in the G_0 and G_1 phase (G_0/G_1) of the cell-cycle versus the percentage of cells in the S, G_2 and M phase of the cell-cycle ($S/G_2/M$). The data represents a mean of three independent experiments. $^{**}P < 0.01$, compared with control.

non-degradable IkBα that is resistant to phosphorylation and degradation. Ad5IkB significantly inhibited the levels of HO-1 and p21 expression in KAT5 cells treated with either hemin or cadmium when compared to cells not treated with Ad5IkB and to controls treated with Ad5LacZ (Fig. 4B).

HO-1 and p21 Arrest Cell Growth in the G_0/G_1 Phase of the Cell-Cycle

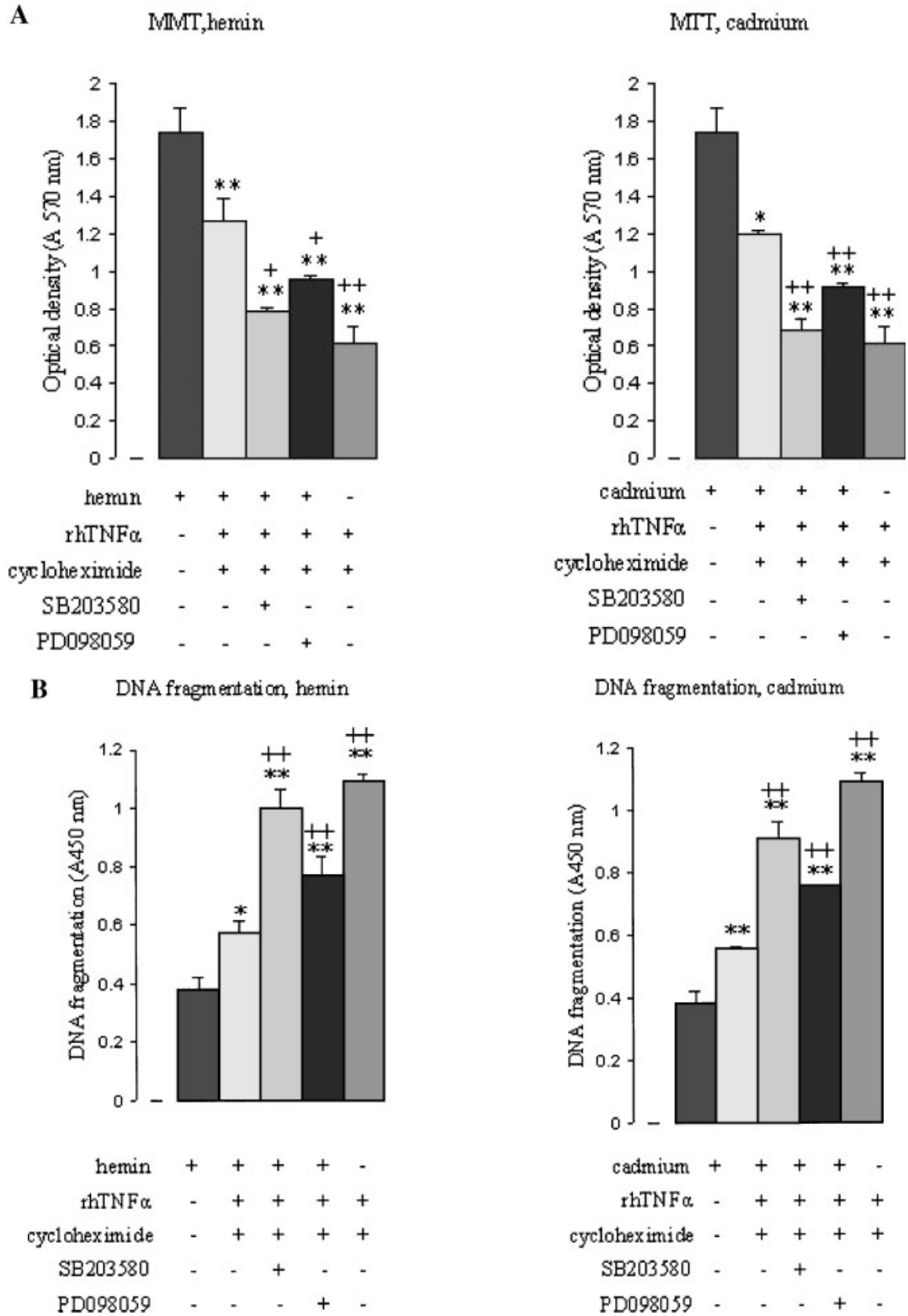
The cell-cycle was analyzed using FACS after KAT5 cells were treated with hemin or cadmium. The percentage of KAT5 cells in the G_0/G1 phase of the cell-cycle was significantly higher after treatment with hemin or cadmium than in untreated (control) cells (Fig. 5). A marked decrease in the $S/G_2/M$ phase was also recorded in treated cells. These results suggest that the up-regulation of HO-1 and p21 by hemin or cadmium is associated with the arrest of cell growth in the G_0/G_1 phase of the cell-cycle.

HO-1 and p21 Increase Cell Resistance to Apoptosis Induced by rhTNF-α/Cycloheximide

To determine whether the up-regulation of HO-1 and p21 has an effect on the vulnerability of cells to apoptosis, we evaluated apoptosis induced by rhTNF-α/cycloheximide in KAT5 cells treated with hemin, cadmium, SB203580, and PD098059. Figure 6 shows the results of this study. In the experiment, cell death/apoptosis was assessed by MTT (Fig. 6A) and cellular DNA fragmentation ELISA (Fig. 6B). Hemin- or cadmium-treated KAT5 cells were resistant to apoptosis induced by rhTNF-α/cycloheximide. Both kinase inhibitors, SB203580 and PD098059, attenuated cellular resistance to apoptosis. However, the inhibition to apoptosis by SB203580 was stronger than that by PD098059. We also found that hemin and cadmium treatment changed the levels of caspase-6 activity (Fig. 6C). The alteration of caspase-6 activity by hemin, cadmium, and the kinase inhibitors paralleled the change in cell death determined by MTT and cellular DNA fragmentation ELISA.

DISCUSSION

Tissue homeostasis is maintained by factors that govern the balance between cell proliferation and cell death or apoptosis. Apoptosis is a biological phenomenon that is critically important for the regulation of cell populations in physiological conditions, and in pathological conditions such as cancer. Recent studies have demonstrated that human thyroid cancer cells show an increase in pro-apoptotic molecules such as Fas and Fas ligand (FasL) [Arscott et al.,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MMT,hemin
MTT, cadmium
Optical density (A 570 nm)
hemin
cadmium
rhTNFα
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Fig. 6.

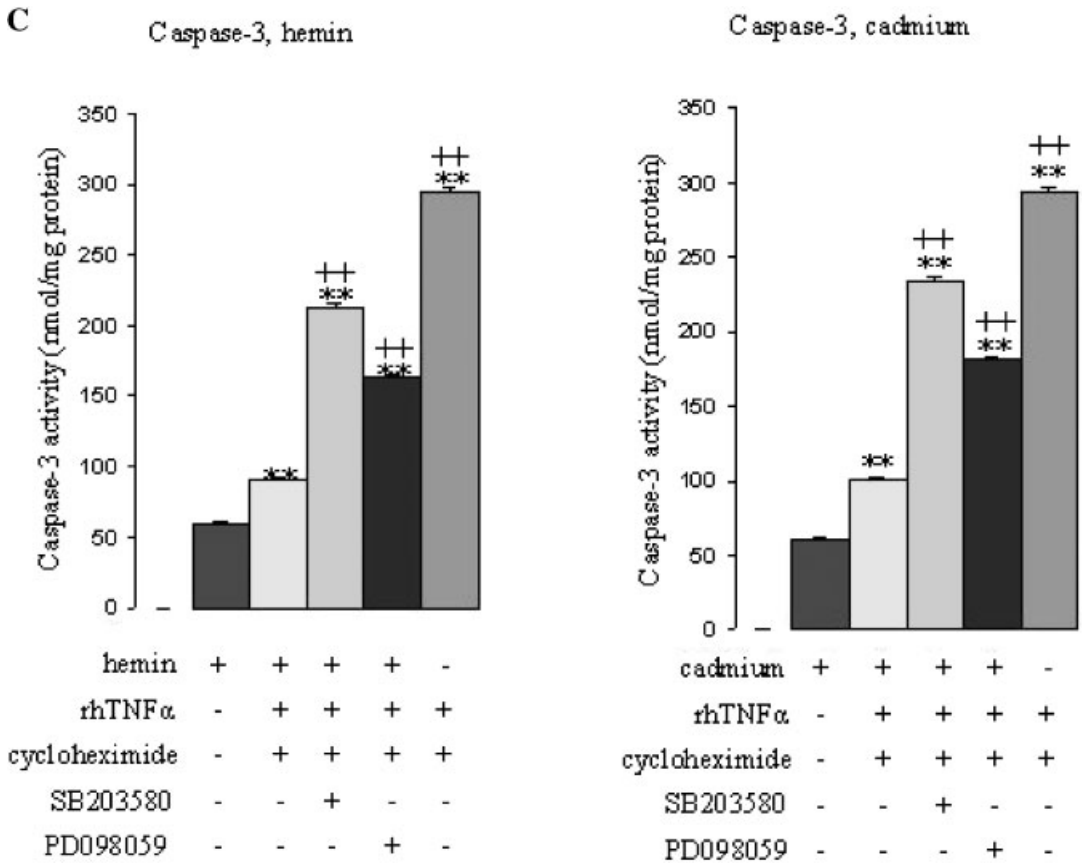

Fig. 6. (*Continued*)

1999; Mitsiades et al., 2003]. It remains unclear as to why cancer cells continue to proliferate rather than undergo apoptosis. In this study, we have shown that human papillary thyroid carcinoma cells that have an increased level of HO-1 and p21 are resistant to apoptosis induced by TNF-α and cycloheximide.

HO-1 is known to participate in the development of some malignant tumors such as prostate cancer and renal adenocarcinoma [Maines and Abrahamsson, 1996; Goodman et al., 1997]. Although the pathway by which HO-1 contributes to the development of cancer is unknown, a number of publications show that HO-1 acts as an anti-apoptotic agent in several types of cells [Chen et al., 2000; Petrache et al., 2000; Inguaggiato et al., 2001; Liu et al., 2002]. Epithelial cells, fibroblasts, neurons, and vascular smooth muscle cells that over-express HO-1 have been shown to be resistant to apoptosis induced by chemicals, stress, and cytokines. On the other hand, these cells are particularly sensitive to apoptotic stimuli when HO-1 is inhibited or when there is a defect in the expression of HO-1 [Chen et al., 2000; Petrache et al., 2000; Inguaggiato et al., 2001; Liu et al.,

Fig. 6. HO-1 inducers protect the cell from death. KAT5 cells were cultured as described in Figure 3. Kinase inhibitors SB203580 and PD098059 were added in a concentration of 40 μM 1 h prior to the addition of either hemin or cadmium. The cells were then treated with rhTNF-α (5 ng/ml) in conjunction with cycloheximide (10 μg) for 3 h. Cell death was assessed by MTT assay (**A**) and cellular DNA fragmentation ELISA (**B**). The result of MTT assay was expressed as an optical density reading at 570 nm; the lower the reading the higher the cell death. The result of the cellular DNA fragmentation ELISA was expressed as a reading at 450 nm; the higher the reading the greater the DNA fragmentation. Caspase-3 activity was measured at 405 nm with correction at 570 nm and expressed in Ac-pNA cleavage or released absorbance (**C**). One unit is the amount of enzyme that will cleave 1.0 nmol of the colorimetric substrate Ac-DEVD-pNA per hour at 37°C under saturated substrate concentrations. The data represents a mean of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with cells treated with hemin or cadmium alone; $^{+}P < 0.05$, $^{++}P < 0.01$, compared with cells treated with hemin plus rhTNF-α/cycloheximide or cadmium plus rhTNF-α/cycloheximide.

2002]. Our present study agrees with the concept in the literature that HO-1 functions as an anti-apoptotic molecule in human papillary thyroid carcinoma cells. The possible involvement of HO-1 in the development of thyroid cancer is also supported by several observations. An increase in HO-1 activity can result from high levels of iodine [Delaney et al., 1988]. A high iodine intake may be a key environmental factor that can lead to the development of thyroid cancer [Glattre et al., 1993; Galanti et al., 1997; Boltze et al., 2002]. Treatment with iodine has resulted in an increase in epidermal hyperplasia [Nyska et al., 2001]. Therefore, one of the mechanisms by which a high level of iodine causes thyroid cancer appears to be associated with an induced elevated level of HO-1. Follicular thyroid cells that have an increased expression of HO-1 grow at a pathological rate due to an alteration in the balance between cell proliferation and apoptosis.

Our study detailed the downstream pathway of HO-1. We observed that p21, increased by HO-1 inducers, is a downstream molecule in the HO-1 pathway of papillary thyroid carcinoma cells, as the time course study showed that the appearance of HO-1 predated the expression of p21. This finding agrees with a previous study in which the over-expression of HO-1 resulted in a significant increase in the levels of p21, and in which the inhibition of HO-1 activity led to the down-regulation of p21 [Inguaggiato et al., 2001]. Our study indicated that the HO-1-mediated anti-apoptotic pathway is related to p38 MARK, as SB203580, a potent inhibitor of p38 MARK, inhibits the expression of HO-1 induced by hemin or cadmium. It is likely that the level of p21 is not directly regulated by p38 MAPK, but is regulated as a result of HO-1 expression, as the increased expression of HO-1 occurs before a change in the levels of p21. We showed that in addition to p38 MAPK, ERK is also involved in the anti-apoptotic pathway of HO-1. The inhibition of ERK significantly attenuated the levels of HO-1 and p21 in the papillary thyroid carcinoma cells. Similar to the mechanism by which p38 MAPK works, the effect of ERK on p21 may be through its association with HO-1. This assumption is supported by the fact that the activation of HO-1 promoter requires both p38 MAPK and ERK activity [Elbirt et al., 1998]. As in human thyroid cancer cells, the expression of HO-1 in human cervix carcinoma cells is known to be regulated by both p38 MAPK and ERK [Chen and Maines, 2000]. In our experiment, the levels of HO-1 and p21 were inhibited more profoundly by SB203580, a p38 MAPK inhibitor, than by PD098059, an ERK inhibitor. However, neither of them completely blocked the expression of HO-1 and p21, suggesting that the expression of HO-1 and p21 in papillary thyroid carcinoma cells is complex and that molecules other than p38 MAPK and ERK are involved.

Both p38 MAPK and ERK are closely associated with NF-kappaB, an anti-apoptotic transcription factor [Chen et al., 2002]. ERK enhances the transcription activity of NF-kappaB while p38 MAPK is necessary for NF-kappaB-dependent gene expression [Carter et al., 1999; Tuyt et al., 1999]. Inhibition of apoptosis by NF-kappaB has been reported to be in a p38 MAPK-dependent manner [Zechner et al., 1998]. Our present study demonstrated that the up-regulation of HO-1 and p21 was associated with an increase in nuclear NF-kappaB activity. In the inactivated condition, NF-kappaB is located in the cytosol as a three-subunit complex consisting of two prototypical subunits, p50 and p65, and an inhibitory subunit called IkB [Gilmore, 1999]. When suitably stimulated, the inhibitory subunit IkB will dissociate from the two prototypical subunits and enable the p50-p65 dimmer to translocate to the nucleus, where it binds to its regulatory element and activates relevant genes. Using this concept, our finding of an increase in nuclear NF-kappaB activity is an indicator that NF-kappaB has been functionally activated. Our results further show that when the cells were treated with a potent NF-kappaB inhibitor, the expression of induced HO-1 and p21 is reduced to the levels found in untreated controls. This experiment strongly indicates that NF-kappaB plays a critical role in the expression of HO-1 and p21 in human papillary thyroid carcinoma cells. Our results suggest that NF-kappaB is a signaling molecule that is associated with p38 MAPK, ERK, HO-1, and p21. However, the induction of HO-1 may not only be regulated by NF-kappaB, as the inhibition of nuclear NF-kappaB affects the expression of p21 more than the expression of HO-1. Nevertheless, our data suggests that the inhibition of NF-kappaB may provide a potential strategy in the control of thyroid cancer by promoting apoptosis.

By analyzing the cell-cycle, our study showed a correlation between an increase in G_0/G_1 phase arrest and the induction of HO-1 and p21. HO-1 inducers hemin and cadmium significantly stimulate HO-1 and p21 expression in papillary thyroid carcinoma cells. Those cells expressing high levels of HO-1 and p21 showed a significant increase in an arrested G_0/G_1 phase of the cell-cycle and a decrease in the S and G_2/M phases of the cell-cycle. In parallel with an accumulation of cells in the G_0/G_1 phase, papillary thyroid carcinoma cells with high HO-1 and p21 levels displayed a significant resistance to apoptosis. Defects in cell-cycle checkpoints can result in the replication of damaged DNA, gene mutation, genetic instability and aneuploidy, all of which are known to contribute to tumorigenesis. It is well known that p21, a cyclin-dependent kinase inhibitor, causes cell-cycle arrest in the G_1 phase in response to DNA damage [Deng et al., 1995; Waldman et al., 1995]. Therefore, the increased level of p21 following the expression of HO-1 seen in the present study is a factor that may be responsible for the G_0/G_1 phase arrest. However, the pathological significance of the arrest of the cell-cycle in the G_0/G_1 phase in papillary thyroid carcinoma cells with elevated levels of HO-1 and p21 is uncertain at present.

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REFERENCES

Arscott PL, Stokes T, Myc A, Giordano NW, Thompson NW, Baker JR, Jr. 1999. Fas (CD95) expression is upregulated on papillary thyroid carcinoma. J Clin Endocrinol Metab 84:4246–4252.

Bacher-Stier C, Riccabona G, Totsch M, Kemmler G, Oberaigner W, Moncayo R. 1997. Incidence and clinical characteristics of thyroid carcinoma after iodine prophylaxis in an endemic goiter country. Thyroid 7: 733–741.

Boltze C, Brabant G, Dralle H, Gerlach R, Roessner A, Hoang-Vu C. 2002. Radiation-induced thyroid carcinogenesis as a function of time and dietary iodine supply: An in vivo model of tumorigenesis in the rat. Endocrinology 143:2584–2592.

Busnardo B, De Vido D. 2000. The epidemiology and etiology of differentiated thyroid carcinoma. Biomed & Pharmacother 54:322–326.

Carter AB, Knudtson KL, Monick MM, Hunninghake GW. 1999. The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP). J Biol Chem 274:30858–30863.

Chen K, Maines MD. 2000. Nitric oxide induces heme oxygenase-1 via mitogen-activated protein kinases ERK and p38. Cell Mol Biol 46:609–617.

Chen K, Gunter K, Maines MD. 2000. Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. J Neurochem 75:304–313.

Chen GG, Lee JF, Wang SH, Chan UP, Ip PC, Lau WY. 2002. Apoptosis induced by activation of peroxisome-proliferator activated receptor-gamma is associated with Bcl-2 and NF-kappaB in human colon cancer. Life Sci 70:2631–2646.

Davis JN, Kucuk O, Djuric Z, Sarkar FH. 2001. Soy isoflavone supplementation in healthy men prevents NF-kappaB activation by TNF-alpha in blood lymphocytes. Free Rad Biol Med 30:1293–1302.

Delaney JK, Mauzerall D, Drummond GS, Kappas A. 1988. Photophysical properties of Sn-porphyrins: Potential clinical implications. Pediatrics 81:498–504.

Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. 1995. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell 82:675–684.

Elbirt KK, Whitmarsh AJ, Davis RJ, Bonkovsky HL. 1998. Mechanism of sodium arsenite-mediated induction of heme oxygenase-1 in hepatoma cells. Role of mitogen-activated protein kinases. J Biol Chem 273:8922–8931.

Elledge RM, Clark GM, Fuqua SA, Yu YY, Allred DC. 1994. p53 protein accumulation detected by five different antibodies: Relationship to prognosis and heat shock protein 70 in breast cancer. Cancer Res 54:3752–3757.

Galanti MR, Hansson L, Bergstrom R, Wolk A, Hjartaker A, Lund E, Grimelius L, Ekbom A. 1997. Diet and the risk of papillary and follicular thyroid carcinoma: A population-based case-control study in Sweden and Norway. Cancer Causes Control 8:205–214.

Gilmore TD. 1999. The Rel/NF-kappaB signal transduction pathway: Introduction. Oncogene 18:6842–6844.

Glattre E, Haldorsen T, Berg JP, Stensvold I, Solvoll K. 1993. Norwegian case-control study testing the hypothesis that seafood increases the risk of thyroid cancer. Cancer Causes Control 4:11–16.

Goodman AI, Choudhury M, da Silva JL, Schwartzman ML, Abraham NG. 1997. Overexpression of the heme oxygenase gene in renal cell carcinoma. Proc Soc Exp Biol Med 214:54–61.

Harach HR, Escalante DA, Day ES. 2002. Thyroid cancer and thyroiditis in Salta, Argentina: A 40-yr study in relation to iodine prophylaxis. Endocr Pathol 13:175–181.

Inguaggiato P, Gonzalez-Michaca L, Croatt AJ, Haggard JJ, Alam J, Nath KA. 2001. Cellular overexpression of heme oxygenase-1 up-regulates p21 and confers resistance to apoptosis. Kidney Int 60:2181–2191.

Liu XM, Chapman GB, Peyton KJ, Schafer AI, Durante W. 2002. Carbon monoxide inhibits apoptosis in vascular smooth muscle cells. Cardiovasc Res 55:396–405.

Maines MD, Abrahamsson PA. 1996. Expression of heme oxygenase-1 (HSP32) in human prostate: normal, hyperplastic, and tumor tissue distribution. Urology 47:727–733.

McCoubrey WK Jr, Huang TJ, Maines MD. 1997. Isolation and characterization of a cDNA from the rat brain that

encodes hemoprotein heme oxygenase-3. Eur J Biochem 247:725–732.

Mitsiades CS, Poulaki V, Mitsiades N. 2003. The role of apoptosis-inducing receptors of the tumor necrosis factor family in thyroid cancer. J Endocrinol 178:205–216.

Murphy BJ, Laderoute KR, Vreman HJ, Grant TD, Gill NS, Stevenson DK, Sutherland RM. 1993. Enhancement of heme oxygenase expression and activity in A431 squamous carcinoma multicellular tumor spheroids. Cancer Res 53:2700–2703.

Nyska A, Lomnitski L, Maronpot R, Moomaw C, Brodsky B, Sintov A, Wormser U. 2001. Effects of iodine on inducible nitric oxide synthase and cyclooxygenase-2 expression in sulfur mustard-induced skin. Arch Toxicol 74:768–774.

Petrache I, Otterbein LE, Alam J, Wiegand GW, Choi AM. 2000. Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. Am J Physiol Lung Cell Mol Physiol 278:L312–L319.

Takahashi S, Mikami T, Watanabe Y, Okazaki M, Okazaki Y, Okazaki A, Sato T, Asaishi K, Hirata K, Narimatsu E, et al. 1994. Correlation of heat shock protein 70 expression with estrogen receptor levels in invasive human breast cancer. Am J Clin Pathol 101:519–525.

Tuyt LM, Dokter WH, Birkenkamp K, Koopmans SB, Lummen C, Kruijer W, Vellenga E. 1999. Extracellular-regulated kinase 1/2, Jun N-terminal kinase, and c-Jun are involved in NF-kappa B-dependent IL-6 expression in human monocytes. J Immunol 162:4893–4902.

Waldman T, Kinzler KW, Vogelstein B. 1995. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res 55:5187–5190.

Wang SH, Phelps E, Utsugi S, Baker JR Jr. 2001. Susceptibility of thyroid cancer cells to 7-hydroxystaurosporine-induced apoptosis correlates with Bcl-2 protein level. Thyroid 11:725–731.

Williams JL, Nath N, Chen J, Hundley TR, Gao J, Kopelovich L, Kashfi K, Rigas B. 2003. Growth inhibition of human colon cancer cells by nitric oxide (NO)-donating aspirin is associated with cyclooxygenase-2 induction and beta-catenin/T-cell factor signaling, nuclear factor-kappaB, and NO synthase 2 inhibition: Implications for chemoprevention. Cancer Res 63:7613–7618.

Zechner D, Craig R, Hanford DS, McDonough PM, Sabbadini RA, Glembotski CC. 1998. MKK6 activates myocardial cell NF-kappaB and inhibits apoptosis in a p38 mitogen-activated protein kinase-dependent manner. J Biol Chem 273:8232–8239.