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Research Article

Cyclin D3 and p53 mediate sulforaphane-induced cell cycle delay and apoptosis in non-transformed human T lymphocytes

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Abstract. Despite experimental evidence that sulforaphane can exert chemopreventive effects, whether these effects are specific for neoplastic cells is not known. Following our previous demonstration that sulforaphane induces cell cycle arrest and apoptosis in human T lymphoblastoid Jurkat leukemia cells and increases p53 and bax protein expression, we tested sulforaphane on non-transformed phytohemagglutinin-stimulated human lymphocytes. Here, we demonstrate that sulforaphane arrested cell cycle progression in G_1 phase, through a decrease in the protein expression of cyclin D3. Moreover, sulforaphane induced apoptosis (and also necrosis), mediated by an increase in the expression of p53. These findings suggest that sulforaphane is a growth modulator for T cells. Our in vitro evidence that sulforaphane is active and even cytotoxic in normal as well as transformed lymphocytes raises important questions regarding its suitability for cancer chemoprevention.

Key words. Sulforaphane; T lymphocyte; cell cycle; apoptosis; necrosis; cyclin D3; p53.

Glucosinolates are naturally occurring thioglucosides present in cruciferous vegetables, broccoli, cabbage, cauliflower, turnip, radish, and watercress [1]. The enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) – activated in damaged plant tissue and also present in the microflora of the human digestive tract [2] – converts these glucosinolates to a number of compounds including isothiocyanates, thiocyanates, nitriles, and epithiocyanoalkanes, depending on the substrate structure and the reaction conditions [3]. Most attention has focused on the cancer-preventive potential of isothiocyanates.

Isothiocyanates perturb several steps in the carcinogenic process. They block DNA damage by both inhibition of carcinogen activation through inhibition of phase 1 enzymes (mainly cytochrome P450) and detoxification of reactive carcinogens through induction of phase 2 enzymes (e.g., glutathione S-transferase) [4]. Moreover, isothiocyanates exert a pronounced antiproliferative effect on human leukemia cells in vitro [5].

One of the most commonly studied isothiocyanates is sulforaphane. Sulforaphane elicits high levels of mammalian phase 2 enzymes by antioxidant response element-mediated transcriptional activation [6, 7]. Moreover, in a rat model, sulforaphane reduced the incidence of breast cancer, and also delayed the appearance and reduced the size of the tumors [7]. It exerts cytostatic and cytotoxic effects on human colon cancer cells in vitro [8]. Moreover, it inhibits cell growth by cell cycle arrest and removes premalignant and malignant cells through activation of apoptosis in human colon and prostate cancer cells [9, 10].

We previously demonstrated that sulforaphane induces cell cycle arrest and apoptosis in human T leukemia cells and increases p53 and bax, but not bcl-2, protein expression [11]. However, whether the effects of sul-

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foraphane are specific for neoplastic cells has yet to be established. In the present work, we therefore tested the effects of sulforaphane on cell cycle progression and apoptosis on phytohemagglutinin (PHA)-stimulated, non-transformed T lymphocytes. We also assessed the molecular mechanisms involved in the apoptotic response and cell cycle delay by sulforaphane.

Materials and methods

Production of sulforaphane

To mimic dietary contact with sulforaphane following consumption of brassicas, sulforaphane was generated in situ by myrosinase-catalyzed hydrolysis of glucoraphanin. In our experimental cell culture conditions (pH 7.4, 37 °C), sulforaphane is the only enzymatic breakdown product. Since the formation of sulforaphane was quantitative, as confirmed by GC-MS techniques [12], we refer to sulforaphane in the text. Glucoraphanin was obtained by a recently developed procedure [13] whereby a quantitative yield is produced starting from glucoerucin. Glucoerucin was isolated with a high purity grade from the ripe seeds of rocket (*Eruca sativa* Miller), according to a well-defined protocol [14]. The transformation of glucoerucin into glucoraphanin is based on the oxidation reaction of sulfides into their corresponding sulfoxides. The semi-synthetic glucoraphanin produced was purified according to the method reported by Visentin et al. [14]. Purity was assessed by HPLC analysis of the desulfo-derivative according to the ISO 9167-1 method [15]. The myrosinase used in the present study was isolated from ripe seeds of white mustard (*Sinapis alba* L.), as reported by Pessina et al. [16]. The myrosinase stock solution had a specific activity of circa 60 units/mg of soluble protein. One myrosinase unit was defined as the amount of enzyme able to hydrolyze 1 µmol of sinigrin/min at pH 6.5 and 37 °C. The myrosinase solution was stored at 4 °C in sterile distilled water until use.

Cell culture

Human peripheral blood (20 ml) was obtained from normal healthy volunteers of AVIS (Italian Association of Voluntary Blood Donors); donors provide written, informed consent for study use of samples at the time of donation. Human mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). After depletion of adherent cells on plastic dishes, T lymphocytes were isolated by erythrocyte rosetting. The erythrocyte rosette positive fraction contained < 5% monocytes or B lymphocytes, as assessed by flow cytometric analysis [17]. T cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO), containing 10% (v/v) heat-inactivated fetal bovine serum (Sigma) and 30 µM bromodeoxyuridine (Sigma), and incubated at 37 °C.

Cell treatment

A 4 mM stock solution of glucoraphanin was prepared in 0.9% NaCl and stored at 4 °C. 0.23 units of myrosinase were directly added to 5 ml of the complete cell culture medium containing increasing concentrations of glucoraphanin (3, 10 and 30 µM), prepared by diluting the stock solution with 0.9% NaCl. The activity of native glucoraphanin or myrosinase was tested by treating the cell cultures with 30 µM glucoraphanin in the absence of the enzyme or with myrosinase in the absence of glucoraphanin, respectively. 0.9% NaCl was used as control. Lymphocytes at a concentration of 3×10^5 cells/ml were added with 5 µg/ml PHA (Sigma). For each experiment, treatments were done in duplicate and separate cultures were set up for each treatment.

Cell viability

Cell viability was assayed by trypan blue exclusion, which distinguished viable and non-viable cells. Cells were diluted with an equal volume of 0.5% (w/v) trypan blue in phosphate-buffered saline (PBS: NaCl 145 mM in phosphate buffer 150 mM) and counted. To separate clumps of adherent cells adequately, EDTA was added to a final concentration of 0.2 mg/ml, 20 min prior to counting test samples, without loss of viability. Cell viability was expressed as a percentage calculated as follows: (number of live cells/total number of live and dead cells) × 100. This percentage was then normalized to the percent viability of the control cells that were given a value of 100%. The control cells were the lymphocytes stimulated with mitogen alone. ID_{50}, the drug concentration causing 50% cell toxicity following a 48-h exposure, was calculated by interpolation from dose-response curves.

Flow cytometry

Flow cytometry was performed using a FACStar$^+$ flow cytometer (Becton Dickinson, Sunnyvale, Calif.) equipped with an argon laser (Innova 90; Coherent Radiation, Palo Alto, Calif.) operating at 488 nm (500 mW) for excitation of the ethidium bromide, propidium iodide, and fluorescein isothiocyanate, and a second argon laser (Innova 100; Coherent Radiation) adjusted to the UV multilines (351.1–363.8 nm, 300 mW) for excitation of Hoechst.

Flow cytometric analysis of the cell cycle

The preparation of lymphocyte samples for measurements of cell proliferation by the bromodeoxyuridine/Hoechst/ethidium bromide quenching technique and the cell cycle distribution of nuclei by DNA content was performed according to a two-step method reported elsewhere [18]. Briefly, cultures were treated with glucoraphanin plus myrosinase, centrifuged for 5 min at 800 g, resuspended in 0.5 ml of solution A (100 mM Tris pH 7.4, 154 mM

$CaCl_2$, 0.5 mM $MgCl_2$, 0.1% Nonidet P40, 0.2% bovine serum albumin, 1.2 µg/ml Hoechst), and stored for 30 min at 4 °C. After adding 1.5 µg/ml ethidium bromide, flow cytometric measurements were performed. Pulse height (trigger) and the areas of ethidium bromide fluorescence (measured using a combination of KV550 and OG590 longpass filters) and of Hoechst fluorescence [measured by a 424DF20 bandpass filter (Becton Dickinson)] were recorded for cell nuclei in list mode. For each sample, 10,000 events were registered. The fluorescence from the bis-benzimidazole Hoechst bound to bromodeoxyuridine-substituted DNA is decreased in cells after the first cell cycle (quenching technique). The extent of Hoechst fluorescence quenching increases with increasing bromodeoxyuridine substitution, thus allowing discrimination between cells in the first and the second cell cycle. The fraction of cells in the different compartments of the cell cycle and in the second cell cycle was calculated as described by Schreiber et al. [19].

Analysis of cell cycle proteins

Detection by flow cytometry of different proteins involved in cell cycle regulation and in the apoptotic process offers rapid and objective quantification of protein levels [20–23]. Under the same exposure conditions described above, 1×10^6 cells were fixed and permeabilized by a commercially available kit, namely Leucoperm solution A and B (Serotec, Oxford, UK). They were then incubated with 20 µl of each antibody, i.e., fluorescein isothiocyanate cyclin D2 (1 mg/ml; Serotec), fluorescein isothiocyanate cyclin D3 (BD PharMingen, San Diego, Calif.), cyclin-dependent kinase (CDK) 4 (BD PharMingen), CDK6 (1 mg/ml; Serotec), or isotype-matched negative control (Serotec). The cells (except those stained with cyclin D2 and D3) were washed and incubated with 20 µl of fluorescein isothiocyanate-labeled secondary antibody (5 µg/ml; Serotec). Finally, the cells were washed and resuspended in 0.2 ml of 1% paraformaldehyde. The cells were then analyzed to quantitate fluorescein isothiocyanate binding by flow cytometry. From each sample, 10,000 cells were analyzed and non-specific binding was excluded by gating around those cells which were labeled by the fluorescein isothiocyanate-conjugate isotype control.

Measurement of apoptosis

Loss of phospholipid asymmetry of the plasma membrane is an early event of apoptosis. The annexin V binds to negatively charged phospholipids, such as phosphatidylserine. During apoptosis, the cells react to annexin V once chromatin condenses but before the plasma membrane loses its ability to exclude propidium iodide. Hence, by staining cells with a combination of fluoresceinated annexin V and propidium iodide, non-apoptotic live cells, early apoptotic cells and late apoptotic or necrotic cells can be detected [24]. Cells from exponentially growing cultures were collected at different times (24, 30, 48, and 72 h). Aliquots of 0.5×10^6 cells were centrifuged (100 g) for 5 min and washed with PBS. The cell pellet was resuspended in 100 µl of labeling solution (ANNEXIN-V-FLUOS; Boehringer Mannheim, Mannheim, Germany) containing 2 µl annexin V labeling reagent and 0.1 µg propidium iodide (Sigma) and incubated for 10–15 min, following the manufacturer's instructions. Immediately after adding 0.1 ml of incubation buffer (10 mM HEPES/NaOH, 140 mM NaCl, 5 mM $CaCl_2$), green (annexin V-fluorescein isothiocyanate) and red (propidium iodide uptake) fluorescence of individual cells were measured with a FACStar$^+$ flow cytometer (Becton Dickinson), using 488-nm excitation and a 530-nm bandpass filter for fluorescein isothiocyanate detection and a filter >590 nm for propidium iodide detection. For each sample, 10,000 events were registered. Electronic compensation was required to exclude overlapping of the two emission spectra. Moreover, during the cell cycle analysis described above, cells were considered to be in apoptosis if they exhibited sub-G_1 DNA fluorescence and had a forward angle light scatter the same as or slightly lower than that of cells in G_1 [24].

Evaluation of p53, bcl-2, and bax proteins

Under the same exposure conditions described above, 1×10^6 cells were fixed and permeabilized with Leucoperm solution A and B (Serotec). They were then incubated with 10 µl of each antibody, i.e., fluorescein isothiocyanate p53 (35 µg/ml; Novocastra, Newcastle, UK), fluorescein isothiocyanate bcl-2 (200 µg/ml; Serotec), and bax (200 µg/ml; Santa Cruz Biotechnology, Santa Cruz, Calif.), or isotype-matched negative control (Serotec). The cells were washed and incubated with 10 µl of fluorescein isothiocyanate-labeled secondary antibody (5 µg/ml; Serotec) for the bax-stained cells. Finally, the cells were washed and resuspended in 0.2 ml of 1% paraformaldehyde. The cells were then analyzed to quantitate fluorescein isothiocyanate binding by flow cytometry. From each sample, 10,000 cells were analyzed and non-specific binding was excluded by gating around those cells which were labeled by the isotype negative control antibodies.

Statistical analysis

All data are the mean ± SE of at least three experiments. Fisher's exact test was adopted for statistical evaluation of the results. T test was used to analyze protein level measurements. All p values are two-sided. All statistical analyses were performed in GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, Calif. If the standard error bars of the determinations were less than 10% of the mean value, they were not included in the figures.

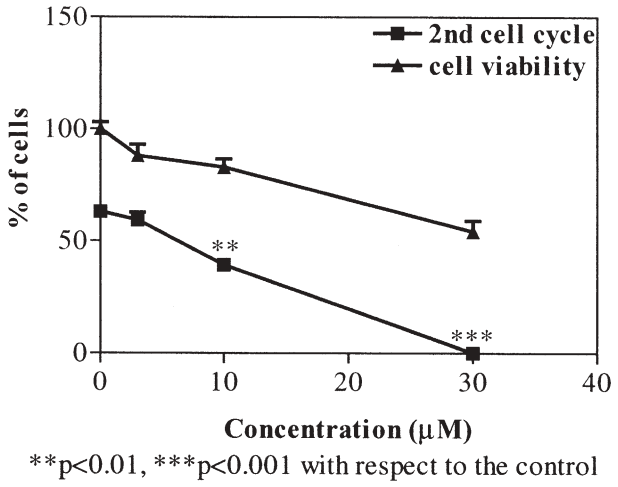

Figure 1. The effect of sulforaphane on T cell viability (Trypan blue exclusion) and proliferation (FACStar+ flow cytometry). The glucoraphanin-myrosinase mixture was added at the concentrations indicated to exponentially growing T cell cultures. Aliquots were removed from drug-treated and untreated control cultures after 48 h. Data are means ± SE (deviation bars, except when smaller than the symbol size) of three independent experiments.

Results

The effect of sulforaphane on T cell viability

The effects of sulforaphane on cell viability of PHA-stimulated lymphocytes measured at 48 h are shown in fig. 1. When the harvested cells were counted, the numbers of treated cells decreased in a dose-dependent manner with respect to the control. The maximum effect was registered at a concentration of 30 μM sulforaphane, with a 46% reduction in the number of viable cells with respect to the control (fig. 1). These changes appeared to be irreversible, as they persisted when the treated cells were recultured in sulforaphane-free medium (data not shown). Only a slight decrease in the number of viable cells in cultures treated with myrosinase or glucoraphanin 30 μM was observed (data not shown). The ID_{50}, as measured by the number of viable cells in cultures after the addition of sulforaphane, was seen at a concentration of about 33 μM.

Effects of sulforaphane on T cell proliferation

As shown in fig. 1, treatment with PHA for 48 h stimulated cell proliferation, as indicated by the percentage of cells that entered the second cycle. Sulforaphane significantly suppressed the progression of cells into the cell cycle and in a concentration-dependent manner. At 3.0 μM, sulforaphane suppressed PHA-treated cell proliferation by 4% ($p = n.s.$), and at 10.0 μM by 24% ($p < 0.001$ vs controls). At 30.0 μM, cell cycle progression was completely suppressed and the cells were in the first cycle. T cell viability decreased after treatment with 30 μM sulforaphane for 48 h, indicating that the inhibitory effect of sulforaphane was also related to direct cytotoxicity.

Effects of sulforaphane on the PHA-stimulated T cell cycle

After stimulation with PHA, T cells enter G_1 phase in 2–4 h, S phase after approximately 18–24 h, and reach the G_2/M phase by 36–48 h [25]. At baseline, all cells were at G_0/G_1 in both control and treated samples (fig. 2). Twenty-four hours after sulforaphane treatment, fluorescence intensity increased from that of the G_0/G_1 phase to the S phase and G_2/M phase.

A DAS V4.37 data analysis system was then used to determine the percentage of T cells in the G_0/G_1, S, and G_2/M phases. At all concentrations, addition of sulforaphane caused a dose-related accumulation of cells in the G_0/G_1 phase (fig. 2). In particular, after 24 h of treatment at 3 μM, 79% of cells were in the G_0/G_1 phase. This fraction increased to 92% at 10 μM and to 95% at 30 μM. At 10 and 30 μM, there was also a decrease of cells in S phase with respect to the control (6% and 3%, respectively vs 30% in the control; $p < 0.001$). Interestingly, after 48 and 72 h of treatment, only the highest doses of sulforaphane significantly affected the fraction of cells either in the G_0/G_1 or S phase (fig. 2). For example, after 72 h of treatment at 30 μM, the fraction of cells in the G_0/G_1 phase increased to 94%, while that in the S phase decreased to 4%. Remarkably, unlike in Jurkat leukemia cells [11], the fraction of normal T cells in the G_2/M phase was not affected by sulforaphane at any of the concentrations and time points evaluated.

Expression of cyclin D2, cyclin D3, CDK4, and CDK6 in T cells treated with sulforaphane

Because the cell cycle is controlled by expression and activation of several cyclins and CDKs, we asked whether their expression levels changed after cell exposure to sulforaphane. We therefore analyzed the expression of cyclin D2, cyclin D3, CDK4, and CDK6, the major D cyclins and associated kinases in human peripheral blood T lymphocytes [26], after T cell activation. The analyses were performed at the same time points used for the cell cycle analysis. We found that sulforaphane treatment caused a marked decrease in cyclin D3 (fig. 3), whereas the expression of cyclin D2, CDK4, and CDK6 was more mildly attenuated (data not shown). Expression of cyclin D3 in non-stimulated cells (0 h) cannot be detected in either untreated or sulforaphane-treated cells. Their stimulation by PHA led to a rapid induction of cyclin D3, whose expression declined especially on the second day of stimulation. Treatment with sulforaphane greatly decreased the expression of cyclin D3 at 24, 48, and 72 h. The maximum effect was registered at 24 h, where the expression of cyclin D3 was decreased by 60 (890.0 vs 356.0; $p < 0.001$).

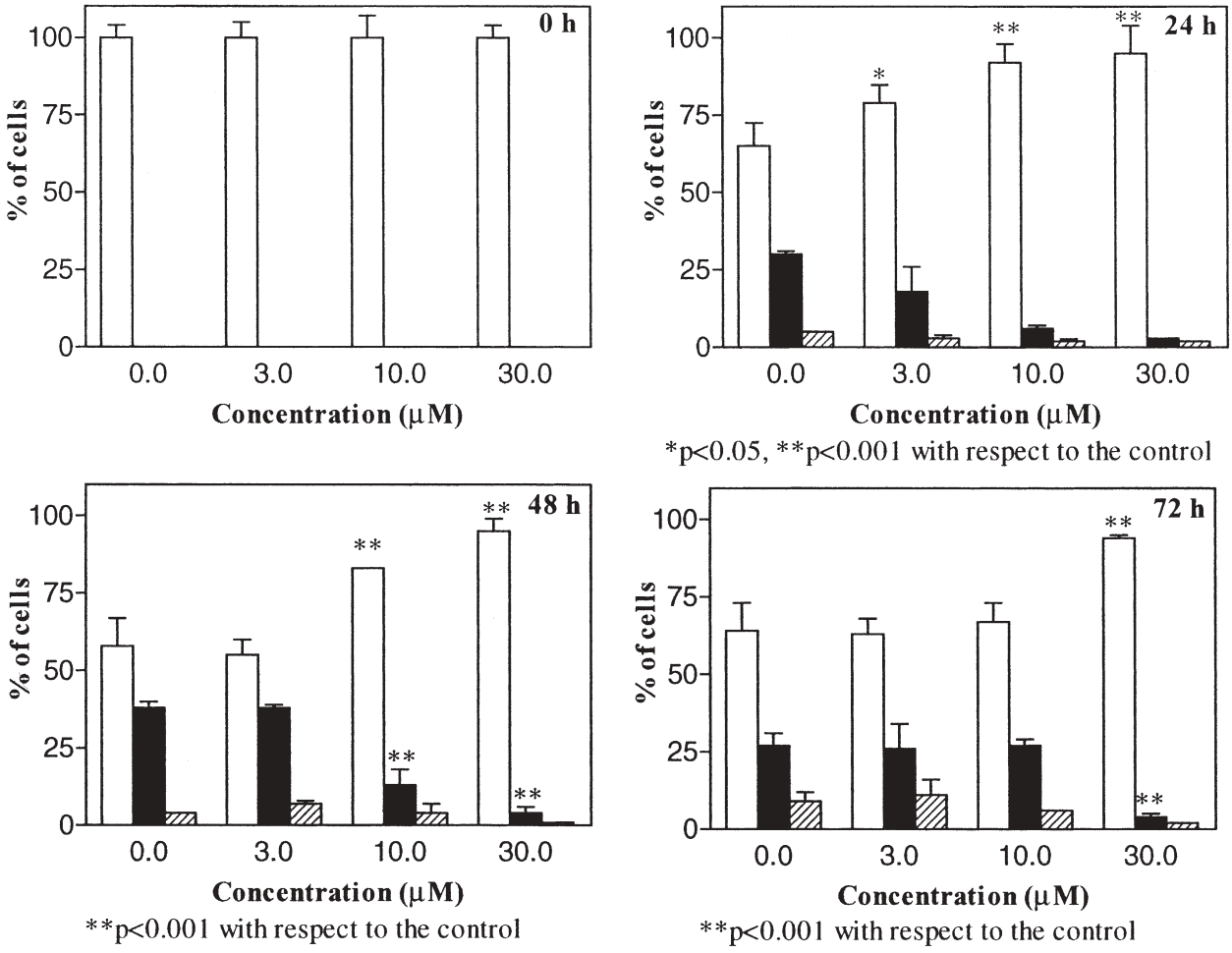

Figure 2. Effects of sulforaphane on T cell cycle with respect to controls. At the indicated time, the distribution of the cells in G_0/G_1, S, and G_2/M phase was analyzed by FACStar$^+$ flow cytometry. Results are expressed as percentages of total cell counts. Data are means ± SE (deviation bars, except when smaller than the symbol size) of three independent experiments.

Effects of sulforaphane on T cell apoptosis and necrosis

At 24 h, only a small proportion (about 8%) of both untreated and treated T cells displayed features of apoptosis (i.e., annexin V^{positive} and propidium iodide^{negative} cells) or necrosis/degeneration (i.e., annexin V^{positive} and propidium iodide^{positive} cells) (fig. 4). At 30 h, only non-significant increases in apoptosis or necrosis were recorded (at all sulforaphane concentrations) with respect to controls. At 48 and 72 h, these differences became significant at concentrations of 10 μM (for apoptosis) and 30 μM (both apoptosis and necrosis). For example, after 72 h of treatment at 30 μM, only 16% of the lymphocytes were viable and the number of apoptotic and necrotic cells increased to 50% ($p < 0.001$) and 34% ($p < 0.001$), respectively. Similar effects were observed from the analysis of cells with decreased DNA content (i.e., 'sub-G_1-cells' with DNA content distribution values below those of G_1 cells). For example, after treatment with 30 μM sulforaphane, the

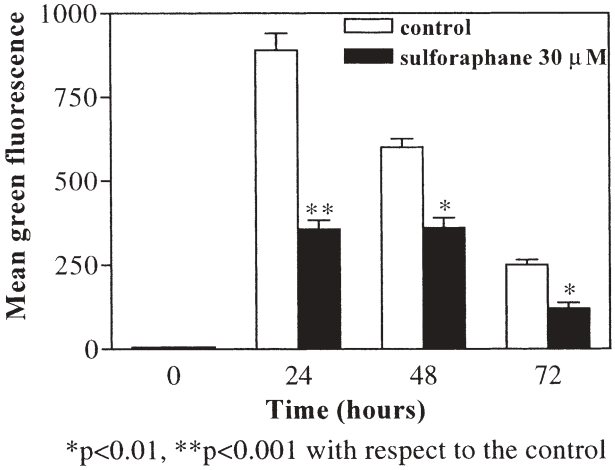

Figure 3. Representative histograms showing cyclin D3 levels following 0, 24, 48, and 72 h culture in the absence or presence, respectively, of sulforaphane (30 μM). Each bar represents means ± SE (deviation bars, except when smaller than the symbol size) of three independent experiments performed by FACStar$^+$ flow cytometry.

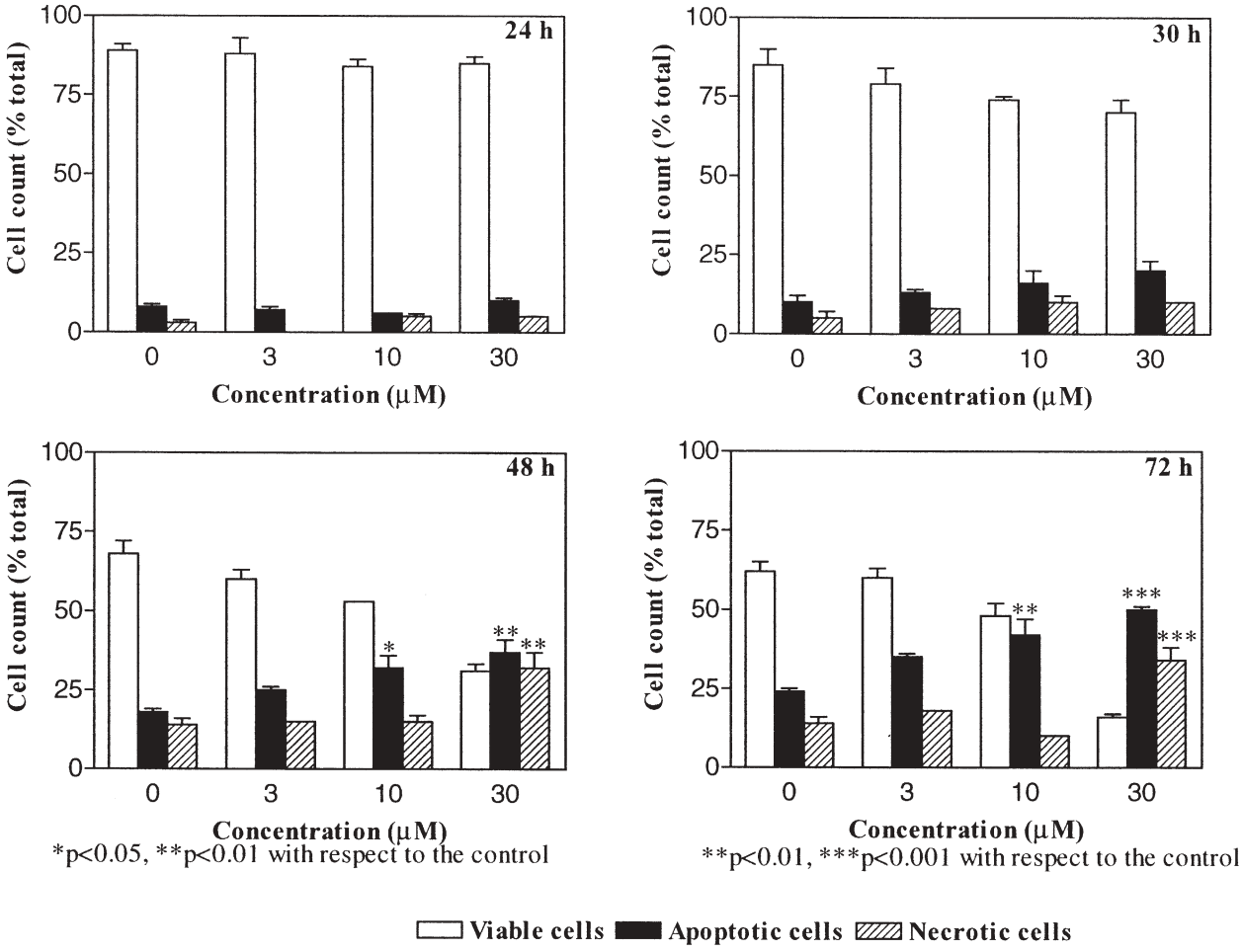

Figure 4. Fraction of viable, apoptotic, and necrotic cells, as detected at different time points in T cells treated with sulforaphane at the indicated doses. Aliquots were removed from treated and untreated cultures at the various time points, stained with annexin V-fluorescein isothiocyanate and propidium iodide, and analyzed by FACStar+ flow cytometry. Each bar represents the means ± SE (deviation bars, except when smaller than the symbol size) of three independent experiments.

fraction of apoptotic cells increased from 5% at 24 h to 15% at 30 h and 28% at 48 h ($p < 0.05$ and $p < 0.001$, respectively).

No effect on apoptotic event induction was detected in cell cultures treated with glucoraphanin or myrosinase (data not shown).

Expression of p53, bax, and bcl-2 in sulforaphane-induced T cell apoptosis

To examine the effect of sulforaphane on levels of p53, bcl-2, and bax, cells were treated for 72 h with sulforaphane at 30 μM. Apoptotic cells were gated, based on their modified forward and side-angle light scatter distributions, and p53, bcl-2, and bax proteins were then quantified. Figure 5 shows histograms of the p53, bcl-2, and bax protein level following incubation with and without sulforaphane. Treated cells showed raised p53 expression (292.5 vs 151.5 in controls, $p < 0.001$), which, however, did not reach the seven-fold increase

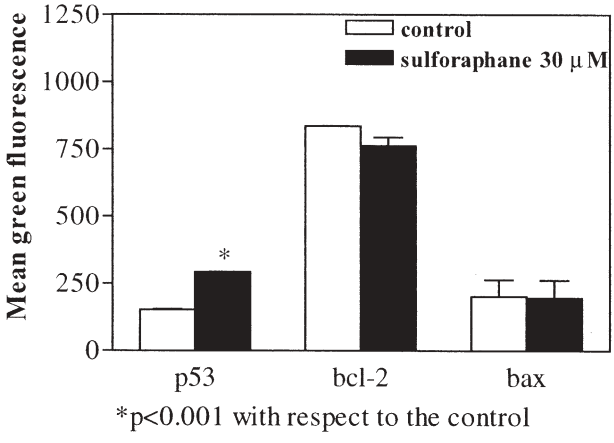

Figure 5. Representative histograms showing p53, bcl-2, and bax protein levels following 72 h culture in the absence or presence of sulforaphane (30 mM). Each bar represents the means ± SE (deviation bars, except when smaller than the symbol size) of three independent experiments performed by FACStar+ flow cytometry.

we previously recorded in Jurkat leukemia cells [11]. As in Jurkat cells, bcl-2 levels showed a slight, non-significant decrease (835.0 vs 761.5 in controls). On the other hand, bax levels rose only slightly (202.0 vs 196.5, p = n.s.) compared with the highly significant three fold increase previously found in Jurkat cells.

Discussion

Selective targeting and low toxicity for normal host tissues are fundamental requisites for proposed chemopreventive agents such as sulforaphane. A non-selective mechanism of action may lead to potentially severe toxicity: in neoplastic or preneoplastic cells, inhibition of proliferation and induction of apoptosis can be regarded as a therapeutic function aimed at eliminating damaged cells, but in normal cells they can be related to DNA damage. We previously reported [11] that sulforaphane, formed in vivo from the glucosinolate glucoraphanin (as occurs during mastication of brassica vegetables), induced growth arrest and apoptosis in T lymphoblastoid Jurkat leukemia cells in vitro. In the present study, we investigated whether sulforaphane selectively affects cancer cells. To do this, we employed the same methods used in our previous study to analyze the effects of sulforaphane on normal T cell cultures. Our data show that sulforaphane is also active, and even cytotoxic, in normal lymphocytes, raising important questions regarding its suitability for cancer chemoprevention.

We found that the most marked effect of sulforaphane is the suppression of PHA-driven proliferation of normal T cells. In particular, cell cycle analysis revealed that sulforaphane arrested the PHA-stimulated normal T cells in the G_1 phase of the cell cycle. In eukaryotes, passage through the cell cycle is governed by the function of a family of protein kinase complexes [27–30]. Each complex is composed minimally of a catalytic subunit, the CDK, and its essential activating partner, the cyclin [27–30]. Under normal conditions, these complexes are activated at specific intervals and through a series of events. This results in the progression of cells through different phases of the cell cycle, thereby ensuring normal cell growth. Any defect in this machinery alters cell cycle regulation. Because our study demonstrated that sulforaphane treatment of T cells resulted in a G_1 phase delay of the cell cycle, we examined the effect of sulforaphane on cell cycle regulatory molecules operative in the G_1 phase of the cell cycle, specifically, cyclins D2 and D3, and CDK4 and CDK6. Sulforaphane treatment of the cells resulted in a significant down-modulation of cyclin D3, whereas the expression of cyclin D2, CDK4, and CDK6 was more mildly attenuated. Our data therefore indicate that sulforaphane has specific mechanisms for inducing cell cycle delay. Cyclins D2 and D3 are well established as two members of the cyclin D family that play distinct and non-redundant roles in regulating the proliferation of normal lymphocytes. After recruitment of normal quiescent T lymphocytes to the cell cycle, the gene for cyclin D2 is activated several hours earlier than that for cyclin D3. This differential regulation suggested that 'G_1 cyclins' may actually participate in more than one checkpoint in G_1, perhaps an early checkpoint near G_0/G_1 (cyclin D2), and a later event near the advancement from G_1 into the S phase (cyclin D3) [31].

The weak effect of sulforaphane on the expression of CDK4 and CDK6 is not surprising, in the light of our results indicating a delay of cell cycle progression. Down-modulation of CDK4 and CDK6 has been demonstrated to be involved in blocking, rather than delaying, the G1 phase [31].

Cell viability experiments revealed significantly increased levels of cell death in T cell cultures after 72 h of treatment with sulforaphane. In particular, we detected a progressive dose-related increase in the fraction of apoptotic cells. Interestingly, we also found that a prolonged exposure (72 h) to the highest dose of sulforaphane (30 μM) increased the incidence of necrotic cells. This suggests that sulforaphane exerts toxic effects on normal human T cells after prolonged exposure to relatively high concentrations.

To help elucidate the molecular mechanism involved in these events, as in our previous study on Jurkat cells, we analyzed p53, bcl-2, and bax protein expression levels. Interactions between *p53*, *bax*, *bcl-2* and their associated proteins are thought to form one pathway to apoptosis [32]. Moreover, bcl-2 expression, which is high in mature peripheral cells in the spleen, lymph nodes, and blood [33] and remains unchanged upon activation [34], is important in the maintenance of a mature population [35]. We found that sulforaphane significantly increased p53 protein levels, whereas it exerted little effect on bcl-2 and bax levels. These observations suggest that induction of *p53* expression might be an important step during sulforaphane-induced apoptosis.

It is interesting to compare these findings with the data from our previous parallel study (using identical methods) obtained on Jurkat cells [11]. Sulforaphane affected proliferation, cell cycle progression, and apoptosis in both the neoplastic and normal in vitro settings, but to different extents. For example, the dose required to reach ID_{50} in normal T cells was about double that recorded for Jurkat cells (33 vs 15 μM), indicating that sulforaphane is probably less cytotoxic in normal lymphocytes. Another important difference regards the phase in which the cell cycle block occurred. In Jurkat cells, we observed a complete derangement of cell cycle progression with a marked increase in the percentage of cells in the G_2/M cell cycle phase. By contrast, the normal lymphocytes employed in

the present study showed a block in the G_1 phase of the cell cycle, while the fraction of cells in the G_2/M phase remained substantially unchanged. Finally, the fraction of apoptosis induced in Jurkat cells (a seven-fold increase with respect to controls) was more than three times greater than that recorded in normal lymphocytes (two-fold with respect to controls).

The differences between normal and transformed T cells could be imputable to a different uptake of isothiocyanates, occurring mainly in the form of glutathione-conjugated dithiocarbamates, with glutathione S-transferases catalyzing the conjugation reaction. The uptake of isothiocyanates is related to intracellular glutathione levels [36], which are known to be rather similar in normal human lymphocytes and Jurkat cells (29.7 and 33.2 nmol/mg whole cell lysate, respectively) [37]. The differences in the activity of sulforaphane in normal cells are therefore too large to depend on minor differences in intracellular uptake.

Our gene expression data suggest that the block of cell proliferation and the induction of apoptosis could be p53 dependent in both normal T lymphocytes and Jurkat cells. However, sulforaphane could also target other important molecular events, at least partially explaining its differential effects in normal and transformed lymphocytes. One possibility involves Fas expression. Sen et al. [38] reported that whereas normal human peripheral blood lymphocytes are only 35% positive for the Fas receptor, Jurkat cells are 100% positive and express a ten-fold greater density of Fas receptors. We are currently investigating the role of Fas expression in sulforaphane activity on normal and transformed cells.

In conclusion, the results of the present study indicate that sulforaphane may be a growth modulator for normal T cells. Although in vitro studies do not necessarily predict in vivo outcomes, our findings indicate that sulforaphane is active not only in transformed lymphocytes but also in their normal counterpart. However, some questions may be raised about the concentrations and the exposure times used in in vitro experiments, which may not be representative of an in vivo situation. Ye et al. [39] reported that the cumulative 8-h urinary excretion of dithiocarbamates in humans who received 200 µmol of broccoli sprout isothiocyanates averaged 58.3 ± 2.8% of the dose. However, if sulforaphane is to be applied in clinical settings as a suitable cancer chemopreventive agent, the compound should be administered in such a way as to maintain a steady state of drug in the body. With regard to the doses, quantities of isothiocyanates up to 100 mg daily and even greater quantities of their glucosinolate precursors are widely consumed by humans [40]; 100 mg of isothiocyanates may contain 77.2% sulforaphane [39], corresponding to 435 µmol of isothiocyanates, comparable to the doses used in this study. Thus, any chemopreventive use of sulforaphane would have to be very carefully examined, because dietary supplementation with single, putative anticarcinogenic compounds is not warranted without extensive investigation of their possible harmful effects.

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