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In vitro folate deficiency induces apoptosis by a p53, Fas (Apo-1, CD95) independent, bcl-2 related mechanism in phytohaemagglutinin-stimulated human peripheral blood lymphocytes

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In vitro folate deficiency is associated with S phase accumulation and apoptosis in various cell types. To investigate the role of p53 and two apoptosis-related molecules, bcl-2 and Fas antigen (Apo-1, CD95), in the mechanism whereby folate-deficient lymphocytes accumulate and undergo apoptosis in the S phase, normal human peripheral blood lymphocytes were cultured for 3–9 d in control medium or in specially ordered and formulated HAM's F-10 medium lacking folic acid, thymidine and hypoxanthine. Cells were stimulated with phytohaemagglutinin for the final 72 h prior to harvesting. The results indicate that p53 expression was downregulated in folate-deficient lymphocytes when compared with the control lymphocytes during the relevant period of S phase accumulation and apoptosis. In addition, folate deficiency was also found to downregulate IL-2, Fas antigen and bcl-2 expression, in terms of either mRNA or protein levels. The downregulation of Fas antigen suggests that folate deficiency-induced apoptosis probably does not occur via the Fas pathway. As IL-2 is a known inducer of bcl-2, and the downregulation of bcl-2 induces apoptosis, the downregulation of IL-2 and bcl-2 is suggested to play an important role in apoptosis. The complete rescue of folate-deficient lymphocytes from apoptosis was achieved by folic acid, thymidine or hypoxanthine alone or thymidine and hypoxanthine in combination. These results suggest that IL-2 depletion by folate deficiency in lymphocytes reduces the bcl-2 level, thereby triggering deoxynucleoside triphosphate pool imbalance and p53-independent apoptosis.

Folate deficiency: Apoptosis: p53: bcl-2

Folate, in the form of tetrahydrofolate coenzymes, is an essential carrier of methyl groups within cells. These act as either donors or receivers of one-carbon moieties in a variety of reactions involved in the synthesis of thymidylate and purines, and in the methylation of cytosine in DNA (Shane & Stockstad, 1985). Thus, folate deficiency can lead to an alternation in DNA by limiting the number of intracellular thymidylate, purine deoxynucleotides or methyl groups available for cytosine methylation. Clinically, a deficiency of this vitamin is associated with an increased risk of certain types of cancer (Rosenberg & Mason, 1989; Freudenheim *et al.* 1991) and many other diseases, including megaloblastic anaemia, infant neural tube defects (Smithells *et al.* 1976; Fleming & Copp, 1998) and CHD (Bunout *et al.* 2000; Michalis *et al.* 2001).

Although the mechanisms by which folate deficiency causes these diseases remains unclear, it has been demonstrated that *in vitro* folate deficiency is associated with S phase accumulation in various cell types (Huang *et al.* 1999; Koury *et al.* 2000), gene instability (Duthie & Hawdon, 1998; Duthie *et al.* 2002) and DNA damage (James *et al.* 2003; Courtemanche *et al.* 2004). In addition to genetic damage, it is well established that folate deficiency induces programmed cell death, or apoptosis, a process marked by cell shrinkage, membrane blebbing, chromatin condensation and eventual internucleosome DNA cleavage (Huang *et al.* 1999). A critical regulator of the cellular response to DNA damage is the transcription factor encoded by the p53 tumour-suppressor gene (Donehower & Bradley, 1993; Zambetti & Levine, 1993). Normal cells express very low levels of p53 protein, but its level rises rapidly after irradiation or exposure to agents that damage DNA (Maltzman & Czyzyk, 1984; Kuerbitz *et al.* 1992; Lowe *et al.* 1993). These genotoxic insults are known to induce apoptotic cell death through p53-dependent pathways, yet Strasser *et al.* demonstrated that genotoxic insults could also induce apoptosis in activated T-cells obtained from $p53^{-/-}$ animals (Strasser *et al.* 1994). This indicates that, in certain settings, cell death induced by DNA damage can also be mediated by a p53-independent mechanism.

In spite of an earlier study showing that erythroblasts undergoing apoptosis due to intracellular folate deficiency had increased levels of p53 protein, Koury *et al.* showed, in a more recent study, that the S phase accumulation and increased rate of apoptosis during folate deficiency were p53-independent (Koury *et al.* 2000). The apoptosis of HepG2 cells during folate deficiency has also been shown to be independent of increased p53 expression (Huang *et al.* 1999).

Abbreviations: CM, control medium; FDM, folate-deficient medium; PBL, peripheral blood lymphocyte; PHA, phytohaemagglutinin; FCS, fetal calf serum.

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Dietary folate deficiency has been shown to induce DNA strand breaks within a highly conserved region of the p53 tumour-suppressor gene in rat liver (Pogribny *et al.* 1995) and a decreased steady state of p53 transcript in rat colon (Kim *et al.* 2000). However, the level of p53 transcript in folate-deficient human lymphocytes has not been reported. To obtain more information on folate deficiency-induced apoptosis, two apoptosis-related molecules, bcl-2 and Fas antigen (Apo-1, CD95) were studied.

The present *in vitro* study of folate deficiency in normal human peripheral blood lymphocytes (PBL) was used to address the following questions: (1) Is the apoptosis related to folate deficiency p53 dependent? (2) Can the addition of folate, hypoxanthine and thymidine rescue folate-deficient lymphocytes from apoptosis? (3) Are the apoptosis-related molecules bcl-2 and Fas expressed in folate-deficient lymphocytes?

Methods

Materials

Folate (pteroylmonoglutamic acid), amino acids, nucleosides, nucleotides and other chemical compounds were purchased from Sigma Chemical (St. Louis, MO, USA). Modified standard HAM's F-10 medium with L-glutamine but without ribosides, ribotides, deoxyribosides, deoxyribotides, glycine or folic acid was specially ordered from and formulated by Biological Industries (Beit Haemek, Israel). Fetal calf serum (FCS) also came from Biological Industries. Penicillin and streptomycin were purchased from GIBCO Laboratories (Grand Island, NY, USA).

Cell preparation

Approval for the study was obtained from Kaoshiung Medical University Human Ethics Committee. Human peripheral blood mononuclear cells were isolated from healthy volunteers by density gradient centrifugation using a Ficoll–Paque cushion (Amersham Biosciences, Uppsala, Sweden). These cells were plated on Petri dishes at 37°C in a 5 % CO_2 and 99 % humidity incubator for 2 h; non-adherent cells were collected as PBL, washed and then resuspended in control (CM) or folate-deficient medium (FDM) at a concentration of 1×10^6/ml. Viability was assessed by trypan blue exclusion. This viability was over 90 % in CM or FDM. For stimulation, 10 ng/l phytohaemagglutinin (PHA) was added.

Culture medium and cell culture

Standard HAM's F-10 medium contains folic acid (0·88 μg/ml), thymidine (8·7 μg/ml) and hypoxanthine (4·9 μg/ml). The medium was specially ordered and formulated without folic acid, thymidine or hypoxanthine in order to stress the availability of both precursors for the *de novo* pathway of nucleotide synthesis (folate derivatives) and precursors for the salvage pathway biosynthesis (thymidine, hypoxanthine). To minimise any exogenous folate source, FCS was dialysed at 4°C for 16 h against 6×10 volumes of sterile PBS. Using this procedure, serum complement contributed virtually no folic acid (Huang *et al.* 1999) and less than 1 μmol/l hypoxanthine and thymidine.

FDM was designated as folate-deficient HAM's F-10 medium supplemented with 10 % dialysed FCS. This FDM contained less than 1 ng/ml folate as contributed by FCS. CM was standard F-10 medium supplemented with 10 % dialysed FCS. Lymphocytes cultured in FDM and stimulated with PHA were designated as folate-deficient cells. The culture in this condition was designated the folate-deficient culture. Cells cultured in CM and stimulated with PHA were referred to as control cells. The culture under this condition was designated the control culture. For the culture, the PBL were allowed to grow in forty-eight- or ninety-six-well flat-bottom microtiter plates in triplicate at a concentration of 1×10^6/ml CM or FDM for 3–9 d. Cells were stimulated by PHA for the final 48–72 h prior to harvesting. The harvested cells were used for following experiments.

Determination of intracellular folate concentration

Intracellular folate levels were determined by a folic acid determination kit and device (IMMULITE 2000; Diagnostic Products Corporation, Los Angeles, CA, USA). This procedure is a solid-phase, competitive-binding chemiluminescent immunoassay. Cells were lysed by brief sonication in a lysis buffer containing 20 g/l sodium ascorbate, 50 mM-HEPES, 50 mM-2-(N-cyclohexylamino)ethanesulfonic acid and 14 μg/ml L-2-mercaptoethanol. The supernatant was collected by centrifugation and incubated first with ligand-labelled folic acid and 2 mM-dithiothretol at 37°C for 30 min, and then with NaCl/KCN. The treated sample was transferred to a fresh tube containing folate-binding protein and folate-binding protein antibody-coated polystyrene beads, and was then incubated at 37°C for another 30 min. The beads were washed to remove free folic acid and alkaline phosphatase-labelled anti-ligand, which specifically binds to the ligand-labelled folate that was added to the reaction.

After removal from the unbound alkaline phosphatase conjugate by centrifugation, the sample was incubated with the chemiluminescent substrate (a phosphate ester of adamantyl dioxetane) at 37°C for 5 min, during which the substrate underwent hydrolysis to yield an unstable intermediate that emitted light. The amount of light, which is inversely proportional to the concentration of folic acid in the sample, was measured using IMMULITE 2000, and the concentration of folic acid was calculated using a standard curve.

Analysis of cell cycle phase by flow cytometry

Harvested cells were fixed in ice-cold 70 % ethanol at 4°C for 30 min. After centrifugation, the cell pellets were washed and resuspended in PBS. Cells were then treated with ribonuclease A (25 mg/l) and 0·5 % Triton X-100 at 37°C for 60 min. Cellular DNA was stained with propidium iodide (50 mg/l) for 30 min. After centrifugation, the pellets were resuspended in PBS. The cellular DNA in 10^4 cells was analysed in an Elite-ESP flow cytometer (Beckman Coulter, Miami, FL, USA) and fixed with an argon laser set at 488 nm. Cell debris and doublets were excluded. The percentage of three phases in the cell cycle was determined by the Multicycle DNA analysis software program (Beckman Coulter, Miami, FL, USA).

Analysis of apoptosis with propidium iodide staining by flow cytometry

Harvested cells were stained with hypotonic fluorochrome solution containing 25 mg/l propidium iodide, 0·1 % Triton X-100 and 0·1 % sodium citrate in PBS for 30 min, washed in PBS and fixed with 4 % paraformaldehyde for 30 min. After centrifugation, the pellet was resuspended in PBS. The cellular DNA in 10^4 cells was analysed by Elite-ESP flow cytometry. The percentage of apoptosis was determined by E4win-ESP analysis software program.

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Rescue from apoptosis by Annexin V assay

At 0 h of culture, folic acid, thymidine and/or hypoxanthine alone, or a combination of thymidine and hypoxanthine, was added to the medium. Cells were harvested at days 0, 4, 5 and 6, washed in PBS and incubated in a binding buffer containing Annexin V–fluorescein and propidium iodide for 15 min at room temperature. After centrifugation, the pellets were resuspended in PBS. A total of 10^4 cells were analysed by Elite-ESP flow cytometry. The percentage of apoptosis was determined by E4win-ESP analysis software program.

RT-PCR for mRNA expression of bcl-2 Fas/Apo-1/CD95 and IL-2

Total RNA was extracted from 5×10^6 cells incubated and stimulated with PHA in CM or FDM for 3 d using REzol C & T (Promega, Madison, WI, USA) according to the manufacturer's instructions. Reverse transcription was performed with 1 μg RNA, 20 μl reaction reagent containing 5 mM-$MgCl_2$, 1 mmol/l dNTP mixture, 62·5 U RNase inhibitor, 0·5 μg oligo$(dT)_{15}$ primer and 15 U avian myeloblastosis virus RT in reverse transcription buffer (10 mmol/l Tris-HCl (pH 9·0 at 25°C), 50 mmol/l KCl, 0·1 % Triton X-100). The mixture was incubated at 42°C for 15 min, followed by 99°C for 5 min and 4°C for 5 min. All reagents used in the cDNA synthesis were included from the manufacturer's instructions (Promega).

After reverse transcription, 0·625 U Taq DNA polymerase, 1·5 mmol/l $MgCl_2$, 0·8 mmol/l dNTP and 50 pmol/l of each primer in PCR buffer were added to the cDNA mixture, the final volume being 50 μl. After denaturation at 95°C for 5 min, 30 cycles of β-actin amplification were performed at 94°C for 45 sec, 60°C for 45 sec and 72°C for 1·5 min/cycle; bcl-2 amplification at 94°C for 45 sec, 52°C for 45 sec and 72°C for 1·5 min/cycle; and Fas and IL-2 amplification at 94°C for 45 sec, 55°C for 45 sec and 72°C for 1·5 min/cycle.

The primers for β-actin message amplification were 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ (sense) and 5′-CGTCATACTCCTGCTTGCTGATCCACATATGC-3′ (antisense). The primers for bcl-2 message amplification were 5′-GGTGCCACCYGYGGTCCACCTG -3′ (sense) and 5′-CACTTGTGGCTCAGATAGG-3′ (antisense). The primers for Fas message amplification were 5′-TTATCGTCCAAAAGTGTTA -3′ (sense) and 5′-TTCTGTTCTGCTGTGTCTTG -3′. The primers for IL-2 message amplification were 5′-ATGTACAGGATGCAACTCCTGTCTT-3′ (sense) and 5′-CTCAGT GTTGAGATGATGCTTTGAC-3′ (antisense). The message amplifications of β-actin, bcl-2, Fas and IL-2 were electrophoresed on a 2 % agarose minigel at 100 V for 30 min and visualised with ethidium bromide staining under UV illumination. Multimers of 100 bp DNA ladder plus were used as markers (MBI Fermentas, Hanover, MD, US).

ELISA for measurment of IL-2

Cells (2×10^5) were incubated in CM or FDM and stimulated with PHA for 3 d. The supernatants were harvested and assayed for IL-2 production using an ELISA kit (Pierce Endogen, Rockford, IL, US) according to the manufacturer's instructions. Absorbance was measured at 450 nm by a MRX model ELISA reader (Dynatech Laboratories, Billinghurst, West Sussex, UK).

Immunofluorescence staining and flow cytometric analysis of bcl-2 and Fas/Apo-1/CD95

Harvested cells were incubated with fluorescein isothiocyanate-conjugated monoclonal antibody to human bcl-2 and Fas/Apo-1/CD95 (Ancell Bayport, MN, USA) for 30 min on ice and then washed three times; the cells were first fixed in 4 % paraformaldehyde in PBS for 30 min on ice, followed by washing and resuspension in PBS. Samples were analysed by flow cytometry (Coulter Epics, Beckman Coulter, Miami, FL, USA).

Western blot analysis

Polyclonal antibodies against human p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used in this study. Total cellular protein isolation and protein blotting were performed as previously described with slight modifications (Yu *et al.* 2002; Liao *et al.* 2004). Total cellular protein 30 μg was electrophoresed in 10 % SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was hydrated with p53 antibody. Protein blots were visualised by using an enhanced chemiluminescence substrate kit (Pierce, Rockford, IL, USA).

Statistics

Data were analysed to determine the statistical significance of differences between the control and folate-deficient groups by Student's paired *t* test. Values $P < 0{\cdot}05$ were considered significant.

Results

Kinetics of intracellular folate concentration

Using chemiluminescent assay, the intracellular folate concentration in five normal human PBL in the resting phase (day 0) was 2·24 ng/10^6 cells. When cells were cultured in FDM with PHA stimulation, the intracellular folate concentration dropped from 2·24 (SD 0·43) to 1 (SD 0·67) ng/10^6 cells on day 3, and to 2·1 (SD 0·56) ng/10^6 cells on day 9. In contrast, when PBL were cultured in CM, the intracellular folate concentration increased from 2·24 (SD 0·43) to 6·31 (SD 2·74) ng/10^6 cells on day 3, and to 40·94 (SD 13·07) ng/10^6 cells on day 9 (Table 1). The folate content of PHA-activated lymphocytes cultured in FDM did not respond to the same degree as that of the cells cultured in CM; in contrast, it remained nearly as low as the level in the resting cells. The difference in folate level between the cells cultured in FDM and in CM reflected the relative folate deficiency in the cells cultured in FDM.

Folate deficiency induces an accumulation of cells in S phase

The distribution of cell cycle phases for cells from control or folate-deficient cultures is shown in Fig. 1. A pronounced change in cell-cycling was observed in the folate-deficient culture, with an accumulation of cells in the S phase accompanied by a decrease in the proportion of cells in G_0/G_1 phase beginning from day 4 (Fig. 2). The percentage of cells entering S phase was 1·9–2·2-fold higher in the folate-deficient culture than in the control culture on days 7–9. These data indicate that when human lymphocytes are cultured in FDM for at least 4 d and stimulated

Table 1. Intracellular folate concentrations of human peripheral blood lymphocytes incubated with phytohaemagglutinin stimulation for the final 3 d

	Folate concentration (ng/10^6 cells)			
	Control culture (*n* 5)		Folate-deficient culture (*n* 5)	
Culture days	Mean	SD	Mean	SD
0	2·24	0·43	2·24	0·43
3	6·31	2·74	1·00	0·67
4	16·45	3·15	1·63	0·85
5	17·22	2·39	1·63	0·48
6	26·64	6·43	1·55	0·53
7	43·32	19·17	2·06	1·09
8	47·22	19·46	1·93	0·36
9	40·94	13·07	2·10	0·56

by PHA for at least 72 h in the final stage of culture, there is an associated accumulation of a large proportion of cells in S phase.

Apoptosis in folate-deficient lymphocytes

As shown in Fig. 1, the percentage of apoptosis was significantly increased in folate-deficient cells compared with controls. The difference began from day 4 and values remained significantly higher at all subsequent sampling intervals ($P<0·05$; Fig. 3).

Folate deficiency in lymphocytes downregulates IL-2, bcl-2 and Fas/Apo-1/CD95

As shown in Figs 4 and 5, IL-2, bcl-2 and Fas mRNA were down-regulated in PHA-stimulated lymphocytes cultured in FDM compared with those in CM on day 3. bcl-2 and Fas protein

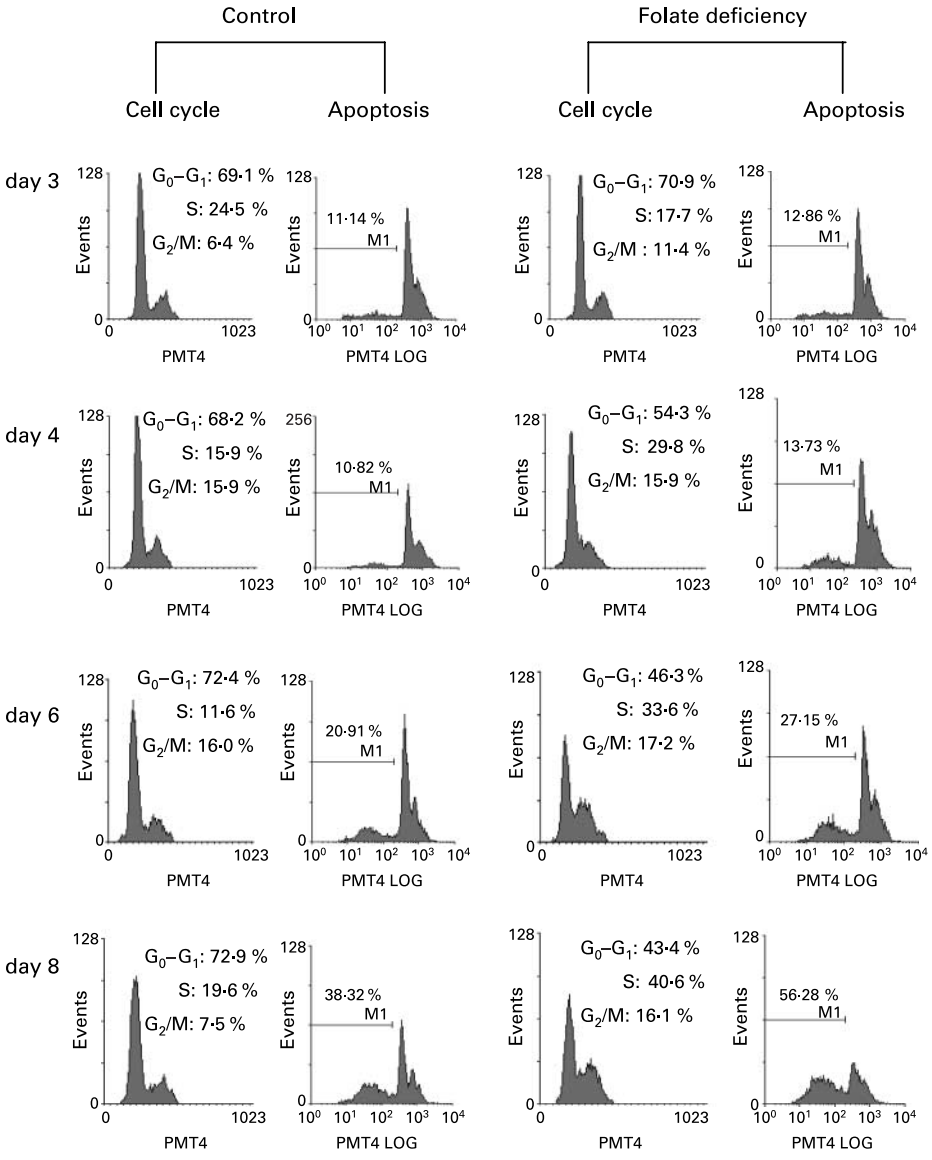

Fig. 1. Histogram of the cell cycle and apoptosis of phytohaemagglutinin-activated human lymphocytes. Cells were incubated in control medium or folate-deficient medium and stimulated by phytohaemagglutinin for the final 72 h prior to harvesting. Cells were harvested each day and then stained with propidium iodide for the cell cycle, and hypotonic fluorochrome propidium iodide containing solution for apoptosis, for 30 min. Cellular DNA per 1×10^4 cells was analysed by flow cytometry. Fig. 1 is representative of eight similar experiments.

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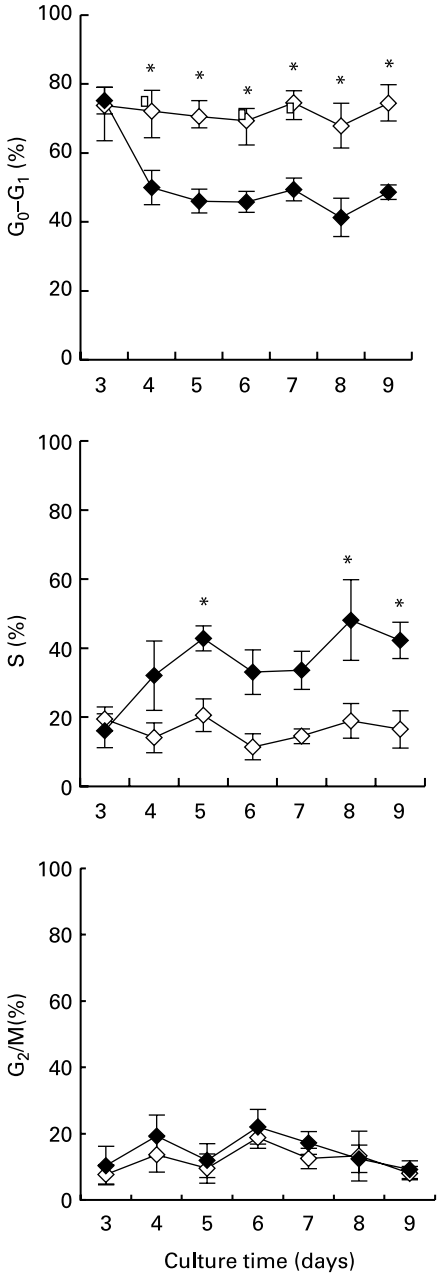

Fig. 2. Cell cycle analysis of phytohaemagglutinin-activated human peripheral blood lymphocytes. Cells were incubated in control medium (◇) or folate-deficient medium (◆) and stimulated by phytohaemagglutinin for the final 72 h before harvest. Cells were harvested each day and then stained with propidium iodide for 30 min. Cellular DNA per 1×10^4 cells was analysed by flow cytometry. Data are means and standard deviations, *n* 8. **P*<0·05 relative to control as evaluated by Student's *t* test.

production was significantly lower in the folate-deficient culture relative to the control culture on day 6, as shown in Fig. 6.

p53 protein expression during folate-deficient derived apoptosis

As shown in Fig. 7, p53 protein expression was downregulated in PHA-stimulated lymphocytes cultured in FDM compared with CM on day 7. These data indicate that the apoptosis of folate-deficient human PBLs occurred independent of increased p53 expression.

Effects of folate, thymidine or hypoxanthine alone or combination of thymidine and hypoxanthine on apoptosis

Using the Annexin V assay, supplementation of the FDM with folic acid, thymidine or hypoxanthine alone, or a combination of thymidine and hypoxanthine, rescued folate-deficient lymphocytes from apoptosis on days 5 and 6 (Fig. 8). This indicated that a full rescue of the folate-deficient lymphocytes from apoptosis was achieved by folic acid at a concentration of 2 μM.

Discussion

The results of the present study demonstrate that folate-deficient lymphocytes accumulate in S phase rather than progressing through the cell cycle to G_0/G_1 phase, and that accumulation in S phase is clearly associated with the increased apoptosis of folate-deficient lymphocytes. Folate deficiency-induced apoptosis and S phase accumulation have also been reported in erythroblasts, various cell lines (Benito *et al.* 1996; Ingram *et al.* 1997; Huang *et al.* 1999) and PHA-stimulated primary human lymphocytes (Courtemanche *et al.* 2004). The mechanism whereby folate-deficient lymphocytes accumulate and undergo apoptosis in S phase is, however, unknown.

Although p53 proteins are thought to increase in response to diverse cellular stress and to sense genotoxic stress or DNA damage, we demonstrated in the present study that p53 protein is markedly downregulated rather than upregulated in the folate-deficient cells compared with the control cells on day 7. These results are consistent with independence from p53 for either the S phase accumulation or the apoptosis of folate-deficient erythroblasts from $p53^{-/-}$ mice (Koury *et al.* 2000) and cell lines after treatment with specific inhibitors (Linke *et al.* 1996). p53 independence for either the S phase accumulation or apoptosis of folate-deficient, PHA-stimulated PBLs has not, however, been proposed.

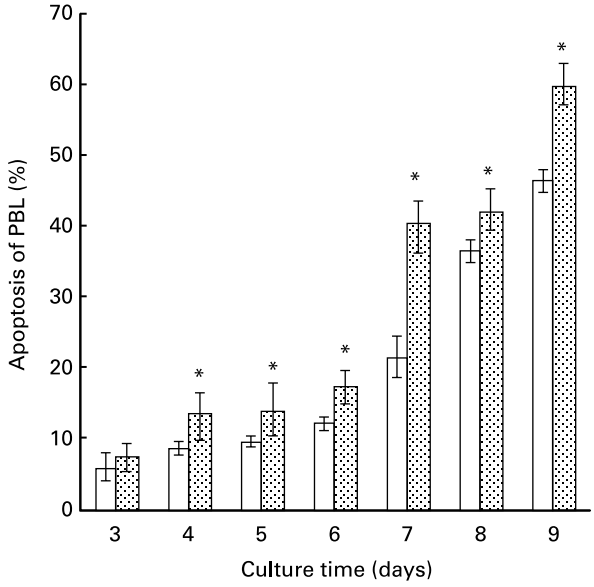

Fig. 3. Apoptosis of phytohaemagglutinin-activated human peripheral blood lymphocytes (PBL). Cells were incubated in control medium (□) or folate-deficient medium (▦) for 3–9 d. Cells were stimulated by phytohaemagglutinin for the final 72 h prior to harvest and stained with propidium iodide. Data were analysed by flow cytometry. Values were the percentage of apoptotic cells (subdiploid propidium iodide staining). Data are means and standard deviations, *n* 6. **P*<0·05 relative to control as evaluated by Student's *t* test.

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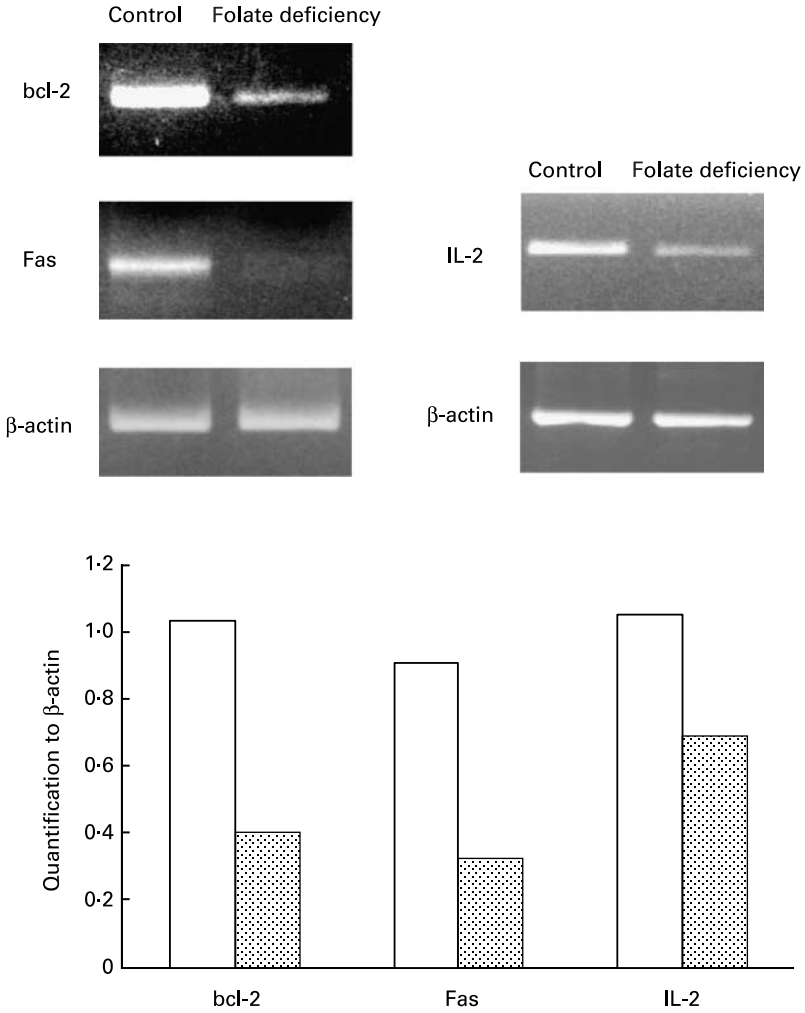

Fig. 4. The mRNA of IL-2, bcl-2 and Fas/Apo-1/CD95 expression in phytohaemagglutinin-activated human peripheral blood lymphocytes. Phytohaemagglutinin-stimulated lymphocytes were incubated in control medium □ or folate-deficient medium ▦ for 3 d. Total RNA was extracted from 5×10^6 cells. RT-PCR was performed for mRNA of IL-2, bcl-2 and Fas/Apo-1/CD95. The message of amplification of IL-2, bcl-2, Fas/Apo-1/CD95 and β-actin was electrophoresed on agarose minigels and visualised with ethidium bromide staining. Densitometric quantification of these blots with normalisation to β-actin is shown in the graph.

It has been demonstrated that folate deficiency induces severe DNA damage, including massive uracil incorporation and chromosome breakage based on decreased thymidylate synthesis (Duthie & Hawdon, 1998). Since intracellular 5,10-methylene tetrahydrofolate is the primary methyl donor for the *de novo* biosynthesis of dTMP from dUMP, folate deficiency results in an increased cellular dUMP:dTMP ratio. This increased ratio leads to an increase in the dUTP:dTTP ratio and finally increases the misincorporation of uracil into DNA (Wickramasinghe & Fida, 1994; Benjamin *et al.* 1997; James *et al.* 2003). Furthermore, folate-derived one-carbon groups are essential for the *de novo* synthesis of purines and pyrimidine, and dNTP are substrates for the DNA polymerase involved in DNA synthesis and repair. The fidelity of DNA synthesis is critically dependent on the current balance and availability of dNTP (Das *et al.* 1985).

Imbalances in the dNTP pool were noted in a variety of folate-deficient cells (van der Weyden *et al.* 1991; James *et al.* 1994; Oliver *et al.* 1997). Yoshioka and colleagues (1987) suggested that this dNTP pool imbalance could result in activation of the gene coding for an endonuclease, causing DNA double-strand breaks and cell death.

The relevance of dNTP pool alteration in cell death processes and the regulatory role of the salvage pathway of dNTP synthesis have been demonstrated (Lagergren & Reichard, 1987). A similar role of the salvage pathway has been demonstrated in a variety of cells in which the addition of thymidine or a combination of two or three dNTP was sufficient to protect the cells from apoptosis. The results were, however, rather controversial (Oliver *et al.* 1997; Koury *et al.* 2000; Mashiyama *et al.* 2004). The results of the present study show that either folate, thymidine or hypoxanthine alone, or a combination of thymidine and hypoxanthine, could completely rescue cells from apoptosis. The cause of this discrepancy in results is unknown.

In addition to the downregulation of p53, folate deficiency was also found to downregulate IL-2, Fas and bcl-2 expression in terms of either mRNA or protein level. Fas is a proapoptotic molecule; its downregulation in the present study suggests that folate deficiency-induced apoptosis was not likely to occur via the Fas pathway. It has been shown that overexpression of the antiapoptotic gene bcl-2 protects cells from apoptosis induced by inhibitors of *de novo* dNTP synthesis (Miyashita & Reed, 1992; Oliver *et al.* 1993), and that downregulation of bcl-2 induces

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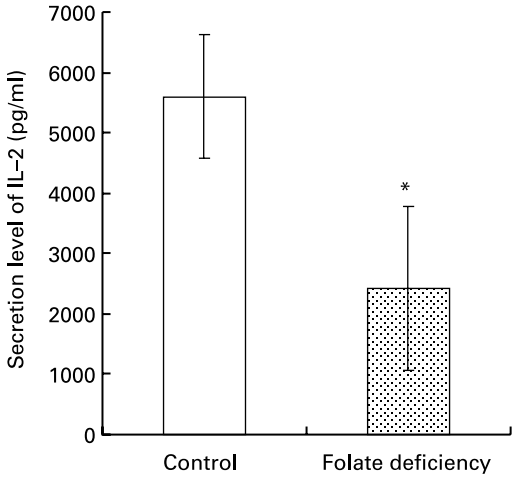

Fig. 5. Secretion of IL-2 by phytohaemagglutinin-activated human peripheral blood lymphocytes. Cells were incubated in control medium or folate-deficient medium and stimulated with phytohaemagglutinin. IL-2 secretion was analysed in the cell–free supernatant using ELISA and expressed as pg/ml. Mean values of IL-2 secretion in the supernatant of folate-deficient or control cells were 5599 (SD 1031) and 2418 (SD 1366) pg/ml, respectively (*n* 7). **P*<0·05 relative to control as evaluated by Student's *t* test.

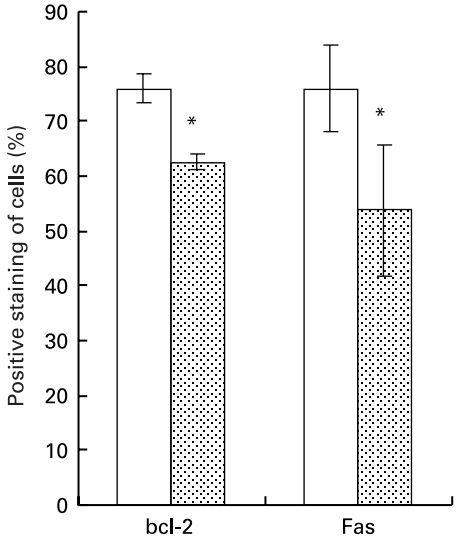

Fig. 6. Bcl-2 and Fas/Apo-1/CD95 expression in phytohaemagglutinin-activated human peripheral blood lymphocytes. Cells were incubated in control medium (□) or folate-deficient medium (▦) for 6 d and stimulated by phytohaemagglutinin for 48 h before harvesting. Cells were harvested and incubated with fluorescein isothiocyanate conjugated monoclonal antibody to human bcl-2 and Fas/ Apo-1/CD95 for 30 min. 1×10^4 cells was analysed by flow cytometry. Data are means and standard deviations, *n* 6. **P*<0·05 relative to control as evaluated by Student's *t* test.

apoptosis (Fleischer *et al.* 2002). Although Oliver and collaborators described changes in dNTP metabolism that preceded DNA fragmentation in a model of apoptosis driven by deprivation of the cytokine IL-3 (Collins *et al.* 1992; Oliver *et al.* 1996, 1997), recent studies have shown that IL-2 deprivation also induces apoptosis through different mechanisms (Duke & Cohen, 1986; Hildeman *et al.* 2002; Devireddy & Green, 2003). As IL-2 is a known inducer of bcl-2 (Miyawaki *et al.* 1992), a downregulation of bcl-2, as in the present study, could be

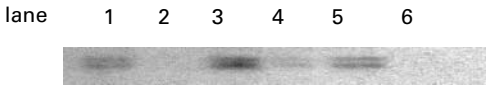

Fig. 7. The expression of p53 protein in phytohaemagglutinin-activated human peripheral blood lymphocytes. Phytohaemagglutinin-stimulated lymphocytes were incubated in control medium or folate-deficient medium for 7 d and stimulated by phytohaemagglutinin for 48 h before harvest. Total protein was extracted from 5×10^6 cells. The protein was electrophoresed in a 10 % SDS-PAGE and transferred to nitrocellulose membrane. The membrane was hydrated with p53 antibody. Lanes 1, 3 and 5 are from control cells. Lanes 2, 4, and 6 are from folate-deficient cells.

expected. These results suggest that IL-2 depletion by folate deficiency in lymphocytes reduces bcl-2 protein expression, thereby facilitating a dNTP pool imbalance and p53-independent triggered apoptosis.

These folate-deficient cells induced cell cycle arrest in S phase, cell apoptosis and reduced cell proliferation (data not shown). This change is probably also present *in vivo*. When folate deficiency is apparent in the peripheral blood, as in some populations in the world, the critical concentration of folate in resting PBL is able to meet the needs of the resting state, but once a critical situation occurs, for example a crisis of infection, it cannot meet the host's emerging needs. These data suggest that folate deficiency may affect the immune system by reducing the capacity of lymphocytes to proliferate and then differentiate into the effector cells that will remove the pathogen from the host in response to antigenic stimulation.

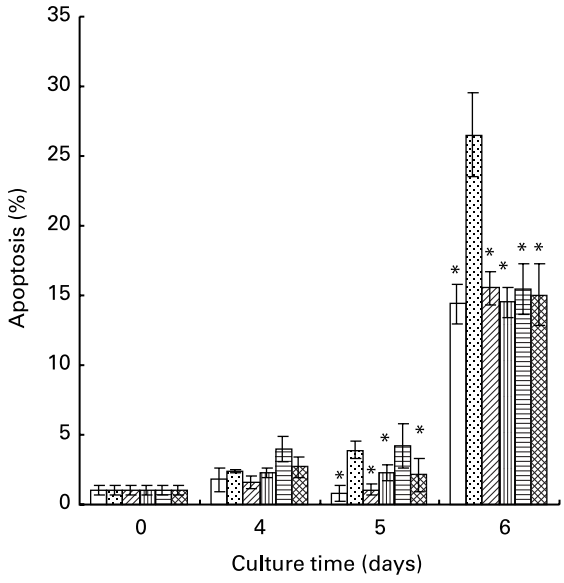

Fig. 8. Rescue of folate-deficient peripheral blood lymphocytes by folate, thymidine or hypoxanthine alone, or a combination of thymidine and hypoxanthine. Peripheral blood lymphocytes were cultured in folate-deficient medium (D) or control medium (C) stimulated by phytohaemagglutinin for the final 72 h prior to harvesting. At 0 h of culture, folic acid (F) (0·88 μg/ml), thymidine (T) (8·7 μg/ml) or hypoxanthine (H) (4·9 μg/ml) alone, or a combination of thymidine and hypoxanthine (H + T), was added to the medium. At the indicated times, the cells were harvested. Apoptosis was determined by Annexin V assay. Values were the percentage of apoptotic cells (subdiploid fluorescein isothiocyanate-conjugated staining). Data are mean and standard deviations, *n* 5. **P*<0·05 relative to folate deficiency as evaluated by Student's test. □, C; ▨, D; ▨, F; ▥, H; ▤, T; ▩, H + T.

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