Eur J Cancer, Vol. 27, No. 11, pp. 1406–1411, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Studies of the Differentiation Properties of Camptothecin in the Human Leukaemic Cells K562

Paul M.J. McSheehy, Marco Gervasoni, Vito Lampasona, Eugenio Erba and Maurizio D'Incalci

Camptothecin, a specific inhibitor of topoisomerase I, caused erythroid differentiation of the human leukaemia cell-line K562, as assessed by benzidine staining at 70 h recovery following a 60 min treatment of the cells. Differentiation was confirmed by increased levels of ε-globin and γ-globin mRNA in the treated cells and was accompanied by down-regulation of c-*myb* mRNA. Synchronisation of K562 cells by non-cytotoxic doses of methotrexate increased the differentiation induced by camptothecin, without affecting the camptothecin-induced inhibition of cellular proliferation. Camptothecin induction of differentiation and inhibition of proliferation may occur by independent mechanisms.
Eur J Cancer, Vol. 27, No. 11, pp. 1406–1411, 1991.

INTRODUCTION

CAMPTOTHECIN is an alkaloid isolated from the Chinese plant *Camptotheca acuminata* and is an effective anticancer agent in several animal solid tumour models [1]. The cellular target for camptothecin is the nuclear enzyme DNA topoisomerase I. Camptothecin is specific for topoisomerase I since topoisomerase II is not affected by the drug [2] and camptothecin-resistant cells either possess a drug-resistant form of topoisomerase I [3, 4], or have significantly reduced levels of the enzyme [4, 5]. Camptothecin inhibits the normal activity of topoisomerase I and stabilises the intermediate enzyme-linked DNA breaks [2]. These single-strand DNA breaks (DNA ssB) are readily reversible [6], but induce events that culminate in cytotoxicity.

Topoisomerase I levels are unchanged during the cell cycle [7] and camptothecin-induced DNA ssB frequencies are comparable in S and G1 phase cells [8]. However, although this implies that camptothecin has no specificity for any particular phase of the cell cycle, experiments using mouse leukaemia cells [9, 10] and hamster fibroblasts [10] suggested that S-phase was most sensitive, possibly because of a lethal collision between DNA replication forks and the drug-induced DNA topoisomerase I complexes [11, 12]. Thus in principle, mechanisms designed to increase the number of cells actively synthesising DNA in S-phase [13, 14] should increase the cytotoxicity of camptothecin as has been reported recently for the topoisomerase II inhibitor etoposide [15, 16].

In addition to its cytotoxic effect camptothecin has been reported to induce myeloid differentiation of human leukaemic cell lines [17, 18]. In the present work we show that camptothecin can also induce erythroid differentiation of the human leukaemia cells K562 and we report some studies on the mechanisms possibly involved in this process.

Correspondence to P.M.J. McSheehy, CRC Biomedical Magnetic Resonance Research Group, Department of Cellular and Molecular Sciences, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.
The authors are at the Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milano, Italy.
Revised 17 June 1991; accepted 9 July 1991.

MATERIALS AND METHODS

Materials

The sodium salt of camptothecin (NSC 100880) and methotrexate were gifts from the National Cancer Institute, Bethesda, Maryland. Bromodeoxyuridine (BrdU) and anti-BrdU products were from Becton Dickinson. Propridium iodide (PI) and normal goat serum were purchased from Calbiochem and Dakopatts, Denmark, respectively. Benzidine reagent and hydrogen peroxide were obtained from Sigma. The reagents used for RNA isolation from cells and the northern blotting were from Bethesda Research Laboratories, London. The c-*myb* probe was a complete human cDNA probe kindly provided by Riccardo Dalla Favera, New York University, New York.

Cell culture and synchronisation

K562 cells were routinely propagated in suspension in RPMI 1640 tissue culture medium (Gibco) containing 10% heat-inactivated foetal bovine serum (Flow) at 37°C in an atmosphere of 95% O_2, 5% CO_2. The cells were maintained between 6×10^4 and 6×10^5/ml for exponential growth. For synchronisation, cells at a concentration of 2×10^5/ml were treated with 0.02 μmol/l methotrexate for 23 h. After treatment, the medium containing methotrexate was removed, the cells were washed twice with phosphate-buffered saline (PBS) at 37°C and incubated in fresh medium. Non-treated cells were diluted to the same concentration as methotrexate-treated cells and at different intervals synchrony was checked using flow cytometric methods.

Treatment with camptothecin and detection of differentiation

Cells in exponential growth were treated for 60 min or 24 h with camptothecin, washed once in PBS, resuspended in fresh

medium and put into recovery. Erythroid differentiation of the cells was assessed by the benzidine staining method of Wanda *et al.* [19]. Briefly, at least 10^6 cells were washed twice in PBS, resuspended in 1 ml PBS and 100 μl of the suspension was added to 50 μl 0.2% benzidine HCl which had been freshly activated by the addition of 50 μl of hydrogen peroxide to 1 ml of benzidine HCl. The cells were moved to the dark for at least 20 min before examination under a light microscope at 200 × magnification for benzidine staining (Bz +). K562 cells are normally of diameter about 12 μm with less than 2% of the cells twice this diameter (large cells). In each experiment (except where shown) the different treatments were examined in triplicate and in each of the triplicates a minimum of 150 cells were counted with the number of Bz + cells recorded as a % of the total number of cells.

Flow cytometric analysis

Monoparametric conventional cell cycle analysis using PI [20] and biparameter analysis using the PI and BrdU indirect immunofluorescent method [21] were performed using a FACS Star Plus (Becton Dickinson) coupled with a Hewlett Packard Consort 30 computer system. To assess the cell cycle phase percentages, the method of Krishan and Frei [22] was used. Relative movement was calculated by the method of Begg *et al.* [23].

Isolation of RNA and northern blotting

RNA was isolated from cells using the thiocyanate–phenol–chloroform method [24] and 10 μg of each sample were electrophoresed on a 1% agarose–formaldehyde gel. RNA was then transferred to a nylon filter (Amersham Hybond-N +) and hybridisation was performed following the protocol of the producer. To ensure equal volumes of RNA were added to each lane samples were tested spectrophotometrically and then after electrophoresis by ethidium bromide staining. All the probes that were used were labelled by the random priming–labelling extension method.

Alkaline elution

DNA ssB were analysed by DNA-denaturing alkaline elution (pH 12.1) under deproteinising conditions [25]. Drug-induced ssB were compared with ssB induced by X-rays on ice.

Statistics

The statistics used were either a paired *t*-test or Gabriel's one-way analysis of variance.

RESULTS

A 60 min treatment of K562 cells with 1 μmol/l camptothecin caused near or almost complete cessation of cell growth and over 70 h led to a gradual increase in the percentage of cells staining positively for benzidine (Bz +) (Fig. 1). On average, by day 3 of recovery from a 1 μmol/l dose, 20% of the cells were Bz + whereas of untreated cells between 2 and 3% were positive; while for comparison, using Hemin (40 μmol/l) as a positive control, we found between 10 and 30% (95% confidence limits, $n = 3$) of the cells to be Bz + after 24 h continuous treatment. Differentiation by camptothecin was always accompanied by a block in the S-late, G2M cell cycle phase (SLG2M block) and a consequent increase in the average cell size. By increasing the camptothecin concentration and exposure time, a larger SLG2M block at 24 h recovery as well as an increased inhibition of cell growth and increased differentiation were evident (Table 1 and Fig. 2). However, the SLG2M block did not seem to be prerequisite for differentiation, since in all experiments the small cells (normal size) had a much greater percentage staining Bz + than the large cells (Table 2).

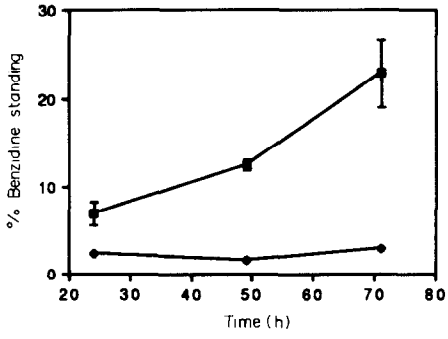

Fig. 1. Time-course of differentiation of K562 cells by CPT. Mean (S.E.) of three independent experiments. ◆ = Control and ■ = camptothecin treated cells.

Table 1. *Dose-response for differentiation and growth inhibition of K562 cells treated by camptothecin*

Treatment	% Bz +	Cell no. (% control)	*n*
5 μmol/l: 1 h	44.7	4.4	1
1 μmol/l: 1 h	20.9 (3.4)	13.4 (2.7)	5
0.25 μmol/l: 1 h	9.1 (1.0)	34.9 (4.7)	5
0.01 μmol/l: 1 h	2.0 (0.3)	95.2 (4.3)	2
0.01 μmol/l: 24 h	11.0 (0.6)	36.3 (3.3)	2

Differentiation (Bz + cells) and cell no./ml were determined after 70 h. Mean (S.E.) of *n* experiments when the % Bz + staining of untreated cells was from 1.9 to 3.3, 95% confidence limits ($n = 14$).

Northern blots from RNA extracts of cells treated for 60 min by 0.25 μmol/l or 1.0 μmol/l camptothecin showed increases in ϵ-globin mRNA content at 24 h recovery (60–90%) and in γ-globin mRNA content at 70 h recovery (70–190%) (Fig. 3). This confirmed that a significant number of cells had been induced towards partial or complete erythroid maturation. Accompanying this differentiation was a reduction in the mRNA levels of c-*myb* mRNA at 70 h recovery while actin mRNA levels were not significantly changed (Fig. 4).

The doubling time of the K562 cells during exponential growth was between 20 and 22 h and treatment during exponential growth for 23 h by a low dose of methotrexate (0.02 μmol/l) blocked a large number of cells at the G1/S border of the cell cycle (Fig. 5d). Release of this block by washing the

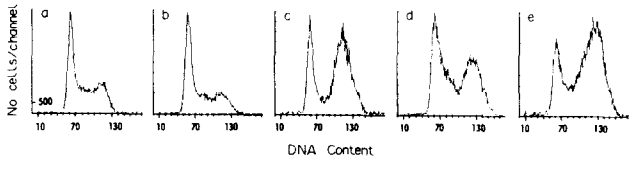

Fig. 2. Flow-cytometric analysis of cell cycle phase distribution evaluated at 24 h recovery. K562 cells in logarithmic growth were given (a) no treatment, (b) 60 min treatment, (c) 24 h treatment with 0.01 μmol/l camptothecin or (d) 0.25 μmol/l and (e) 1.0 μmol/l camptothecin-treatment for 60 min.

Table 2. Camptothecin-induced differentiation in normal-sized compared to large cells

Cell population	Recovery time (h) 0	20	45	70
Bz +				
All cells	2.7, 3.9	3.4, 3.9	9.3, 9.9	25.8, 29.3
Normal size	2.7, 3.9	21.1, 23.1	29.2, 44.4	54.8, 58.1
Large size		1.8, 1.9	4.9, 6.3	18.6, 21.9

Duplicate readings (as a %) from a 60 min treatment by camptothecin (1 μmol/l). Non-treated cells act as 0 h recovery and have < 2% large cells. Mean percentage of large cells in the treated population was 91% at 20 h, 87% at 45 h and 80% at 70 h.

cells caused a wave of synchronisation which led to a doubling of the number of cells in S-middle (SM) in the methotrexate-treated cells compared to control cells at 6 h recovery. After 12 h recovery, the methotrexate-treated cells had a normal cell cycle phase distribution (Fig. 5). The incorporation of BrdUrd (a thymidine analogue) following a 20 min pulse of 10 μmol/l BrdUrd was similar in both synchronised and asynchronised cells and the exponentially growing and synchronised BrdU-positive cells proceeded through S to G2M at the same rate (Fig. 5a and c, respectively). The methotrexate treatment was considered non-cytotoxic, since there was no change in the rate of cell proliferation or ^{3}H-Thd incorporation during 70 h recovery compared to synchronous cells (results not shown).

The methotrexate treatment did, however, consistently cause a small but significant increase ($P < 0.02$ in a paired t-test) in the number of cells staining Bz + (Table 3). Treatment of these methotrexate-synchronised cells at 6 h post-methotrexate recovery (maximum increase in SM) with camptothecin (0.25 μmol/l) for 60 min caused a 2.5-fold increase in the percentage of cells staining Bz + at 70 h recovery compared to camptothecin-treatment of asynchronous cells. This effect was synergistic when compared to the differentiation caused by the single treatments of methotrexate and camptothecin (Table 3). Alkaline elution analysis of DNA ssB immediately following the camptothecin-treatment indicated a small but non-significant increase of these DNA ssB in the synchronised cells compared to the non-synchronised cells (Fig. 6), a result consistent with observations in Hela cells that camptothecin-induced DNA ssB frequencies are comparable in both S and G phases. Non-camptothecin-treated methotrexate-synchronised cells showed the same very low break frequencies as synchronous cells (controls) (Fig. 6).

Although the methotrexate-synchronised cells were more sensitive to the differentiation induced by camptothecin, the same dose (0.25 μmol/l, 60 min) did not cause an increased inhibition of cellular proliferation at any of the recovery time-points (Table 4), or in the degree of the SLG2M-block.

DISCUSSION

We have demonstrated that brief exposure of K562 cells to camptothecin causes a significant percentage of cells to become differentiated after three days as expressed by increased Bz + staining and increased cellular content of ε and γ-globin mRNA. In common with other inducers of differentiation of K562 cells such as cytarabin [26], cisplatin [27] or methylating agents [28] we found the commitment to differentiation was accompanied

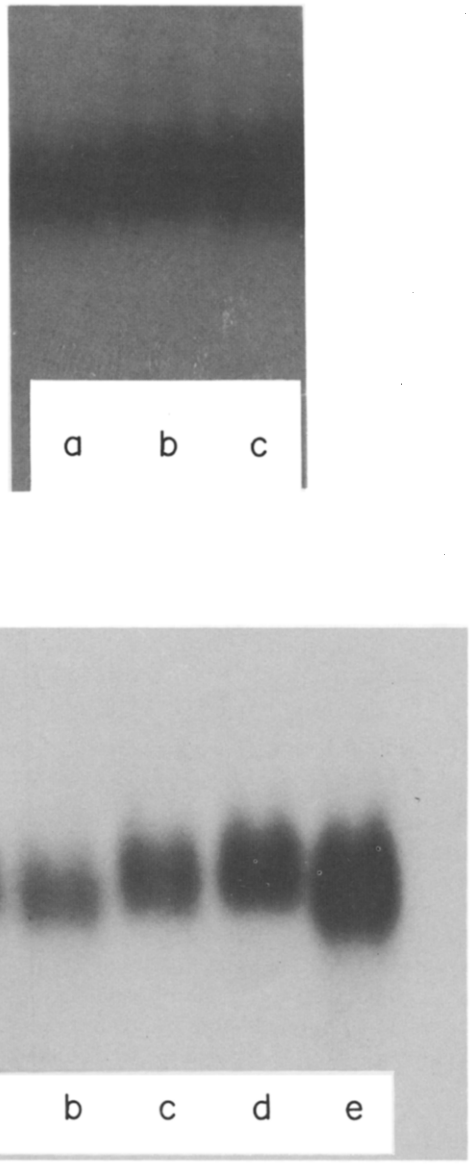

Fig. 3. Increase in the (upper) ε-globin and (lower) γ-globin mRNA content of K562 cells. Cells were treated with camptothecin for 60 min and put into recovery. At 24 h (ε-globin) and 24 and 70 h (γ-globin) RNA was extracted and northern blotted. (a) control, (b) 0.25 μmol/l camptothecin, (c) 1.0 μmol/l camptothecin 24 h, (d) 0.25 μmol/l camptothecin and (e) 1.0 μmol/l camptothecin 70 h. Gelblots intensity, normalised to controls: (upper) (a) 1.0, (b) 1.6, (c) 1.9; (lower) (a) 1.0, (b) 0.9, (c) 0.7, (d) 1.7, (e) 2.9.

by an increase in average cell size due to an accumulation of cells in the G2M phase. However, the differentiation caused by camptothecin was not necessarily related to the G2M block and to the increase in cell size, since the normal-sized cells were always more Bz + than the larger cells.

Camptothecin is an inhibitor of topoisomerase I and although levels of this enzyme do not change during the cell cycle [7], camptothecin is thought to be most cytotoxic against S-phase cells [10]. We were able to synchronise reproducibly K562 cells in SM by using low-levels of methotrexate (0.02 μmol/l). This concentration of methotrexate was non-cytotoxic since following removal of the drug the synchronised cells behaved identically to synchronous cells in rate of growth, movement through S-phase and frequency of DNA ssB. The methotrexate-treatment did, however, cause a small but significant increase in differen-

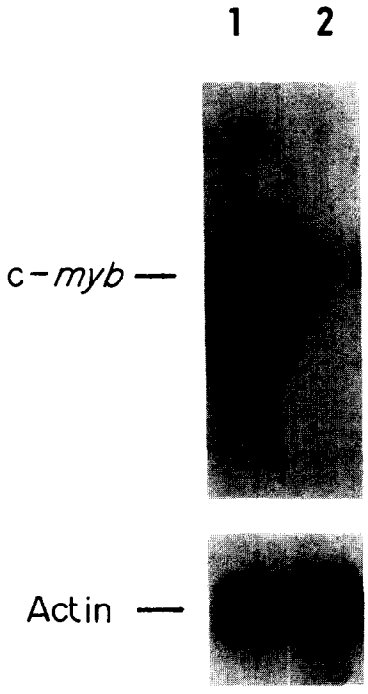

Fig. 4. The effect of camptothecin on mRNA content of c-*myb* in K562 cells. Lane 1: control, lane 2: 1.0 μmol/l camptothecin 70 h.

Table 3. Camptothecin-induction of differentiation (Bz +) *in methotrexate-synchronised or unsynchronised* K562 *cells*

Treatment	% Bz + at 70 h recovery
Methotrexate	1.9 (0.2)
Camptothecin	2.7 (0.2)
Methotrexate plus camptothecin	6.6 (1.1)*

Normalised mean (S.E.) of four independent experiments where control (no treatment) = 1.0.
* Significant difference ($P < 0.002$) from single treatments of methotrexate or camptothecin alone (Gabriel's one-way analysis of variance).

tiation (Bz +). Treatment of the synchronised cells with camptothecin at the maximum increase in SM caused a much larger increase in differentiation compared to treatment of asynchronous cells, but did not alter the amount of inhibition of cell proliferation or indeed the degree of the SLG2M block. This appeared to confirm that the cell cycle block was not related to differentiation and suggested camptothecin-induced differentiation and cytotoxicity occurred by independent mechanisms.

Others have demonstrated that different phases of the cell cycle have differential sensitivity to induction of differentiation. Cytarabine was most effective at the G1/S phase in K562 cells [26] and DMSO at S phase in mouse erythroleukaemia cells [29]. Neither of these agents, or others that induce differentiation

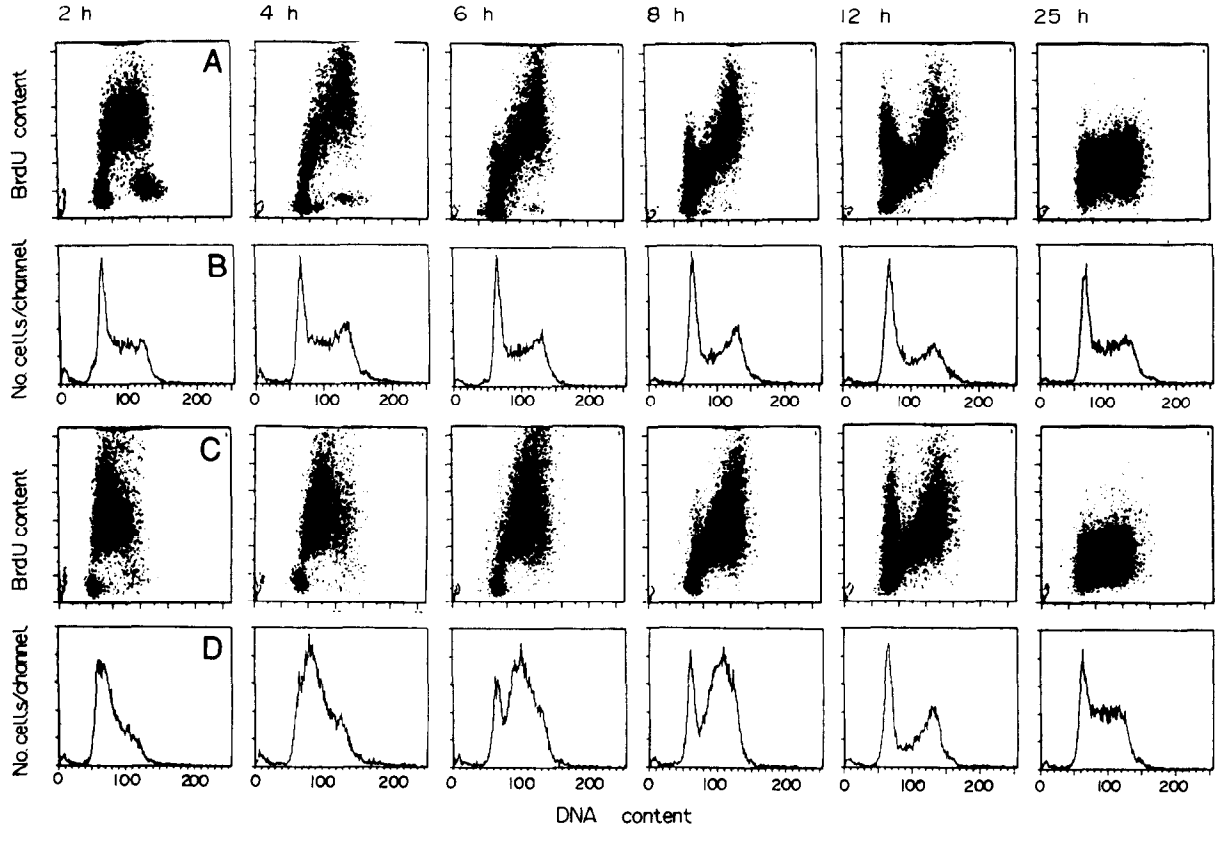

Fig. 5. Biparametric BrdU/PI analysis of control cells (a) and (b) and methotrexate-treated (c) and (d) cells.

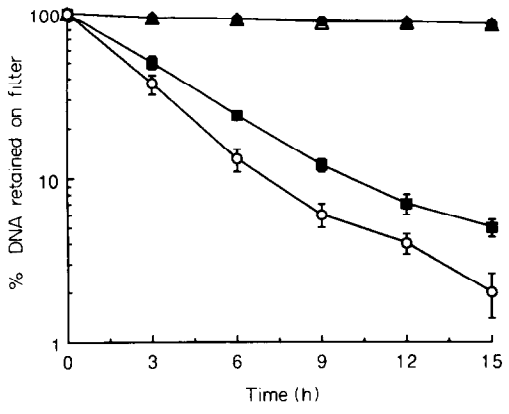

Fig. 6. Alkaline elution profile of asynchronised and methotrexate synchronised K562 cells treated with camptothecin. K562 cells were treated for 60 min by 0.25 μmol/l CPT, chilled in ice and without washing were immediately used for alkaline-elution as described in methods. Mean (S.D.) of triplicate determinations. △ = Control, ● = methotrexate, ■ = camptothecin and ○ = methotrexate plus camptothecin.

Table 4. Camptothecin-induction of the inhibition of K562 cell proliferation in methotrexate-synchronised and asynchronous cells

	Recovery time (h)		
Treatment	24	48	70
Asynchronous	65.1 (2.2)	48.0 (1.6)	39.5 (4.6)
Synchronised	62.7 (4.6)	46.0 (9.2)	39.4 (8.8)

Cell number was determined at the recovery times shown. % of the non camptothecin-treated cells; mean (S.E.) of three independent experiments.

in K562 cells [27, 28, 30, 31] and HL60 cells [32, 33], are specific inhibitors of topoisomerase I and it may be the differentiation induced by camptothecin is independent of its effects on this enzyme. Indeed, a recent report demonstrated topoisomerase I activity was actually increased following the induction of differentiation in HL60 cells by phorbol myristate acetate [34]. Finally, we noticed no significant difference in the number of camptothecin-induced DNA ssB in methotrexate-synchronised and asynchronous cells, although this may be as expected since topoisomerase I levels are reported to be cell cycle independent [7].

What does appear to be common for these different drugs and cellular systems is a downregulation in expression of the oncogenes c-*myc* or c-*myb* [30–33, 35, 36] as we also observed at 70 h recovery from camptothecin treatment. While a down regulation of c-*myb* and/or c-*myc* may be critical events in differentiation, the primary event is unknown and may indeed vary from one cell line or drug to another. However, our results suggest that further studies of the phase-specificity of the process of differentiation would be useful in developing drug regimes for this approach to the chemotherapy of leukaemia.

1. Wall ME, Wani MC. Camptothecin. In: Cassadry JM, Duros JD, eds. *Anticancer Agents Based on Natural Product Models*. New York, Academic Press, 1980, 417–437.
2. Hsiang YH, Hertzberg R, Hecht S, Liu L. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* 1985, **260**, 14773–14878.
3. Gupta RS, Gupta R, Eng B, *et al.* Camptothecin-resistant mutants of Chinese-Hamster Ovary cells containing a resistant form of topoisomerase I. *Cancer Res* 1988, **48**, 6404–6410.
4. Andoh T, Ishii K, Suzuki Y, *et al.* Characterisation of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. *Proc Natl Acad Sci USA* 1987, **84**, 5565–5569.
5. Thrash C, Voelkel KA, DiNardo S, Sternglanz R. Identification of saccharomyces cerevisiae mutants deficient in DNA topoisomerase I activity. *J Biol Chem* 1984, **259**, 1375–1377.
6. Covey JM, Jaxel C, Kohn KW, Pommier Y. Protein-linked DNA strand breaks induced in mammalian cells by camptothecin, an inhibitor of topoisomerase I. *Cancer Res* 1989, **49**, 5016–5022.
7. Hsiang YH, Wu HY, Liu LF. Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. *Cancer Res* 1988, **48**, 3230–3235.
8. Horwitz SB, Horwitz MS. Effects of camptothecin on the breakage and repair of DNA during the cell cycle. *Cancer Res* 1973, **33**, 2834–2836.
9. Kessel D, Bosmann HB, Lohr K. Camptothecin effects on DNA synthesis in murine leukaemia cells. *Biochim Biophys Acta* 1972, **269**, 210–216.
10. Li LH, Fraser TJ, Olin EJ, Bhuyan BK. Action of camptothecin on mammalian cells in culture. *Cancer Res* 1972, **32**, 2643–2650.
11. Hsiang YH, Lichou MG, Liu LF. Arrest of replication forks by drug-stabilised topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res* 1989, **49**, 5077–5082.
12. Holm C, Covey JM, Kerrigan D, Pommier Y. Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerases I and II inhibitors in CH DC3F cells. *Cancer Res* 1989, **49**, 6365–6368.
13. Tobey RA, Valdez JG, Crissman HA. Synchronisation of human diploid fibroblasts at multiple stages of the cell cycle. *Exp Cell Res* 1988, **179**, 400–416.
14. Sen S, Erba E, D'Incalci M. Synchronisation of cancer cell lines of human origin using methotrexate: characterisation of the system. *Cytometry* 1990, **11**, 595–602.
15. Lorico A, Boiocchi M, Rappa G, Sen S, Erba E, D'Incalci M. Increase in topoisomerase II-mediated DNA breaks and cytotoxicity of VP16 in human U937 lymphoma cells pretreated with low doses of methotrexate. *Cancer Res* 1990, **48**, 2036–2041.
16. D'Incalci M, Erba E, Sen S. Induction of partial synchronisation of leukaemia cells by continuous infusion of low-dose methotrexate followed by citrovorum factor. *J Natl Cancer Inst* 1989, **81**, 1509–1510.
17. Ling YH, Nelson JA, Tseng MT. The DNA topoisomerase I inhibitor 10-hydroxycamptothecin induces differentiation of HL-60 cells. *Proc Am Assoc Cancer Res* 1989, **30**, 620.
18. Chou S, Kaneko M, Nakaya K, Nakamura Y. Induction of differentiation of human and mouse myeloid leukaemia cells by camptothecin. *Biochem Biophys Res Commun* 1990, **166**, 160–167.
19. Wanda PE, Lee LT, Howe CA. A spectrophotometric method for measuring haemoglobin in erythroleukaemic cells (K562). *J Histochem Cytochem* **29**, 1442–1444.
20. Erba E, Ubezio P, Colombo T, *et al.* Flow-cytometric analysis of DNA distribution after VP16-213 treatment of Lewis lung carcinoma. *Cancer Chemother Pharmacol* 1983, **10**, 208–211.
21. Dolbeare F, Beisker W, Pallavicini MG, Vanderlaan M, Gray JW. Cytochemistry for bromodeoxyuridine/DNA analysis. *Cytometry* 1985, **6**, 521–530.
22. Krishan A, Frei E. Effect of adriamycin on the cell cycle transverse and kinetics of cultured human lymphoblasts. *Cancer Res* 1976, **36**, 143–150.
23. Begg AC, McNally NJ, Shrieve DG, Karcher H. A method to measure the duration of DNA synthesis and the potential doubling time from a single sample. *Cytometry* 1985, **6**, 620–626.
24. Chromczynski IP, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, **162**, 156–159.
25. Kohn KW, Ewig RAG, Erickson LC, Zwelling LA. Measurements of strand-breaks and cross-links by alkaline elution. In: Friedberg EC, Hanawalt PC, eds. *DNA Repair: A Laboratory Manual of Research Techniques*. New York, Marcell Dekker, 1981, 379–401.
26. Wanda PE, Walker MM. Haemoglobin induction by Ara-C in

human erythroleukaemia cells (K562) is cell-cycle dependent. *Leukemia Res* 1989, **13**, 683–688.
27. Tonini GP, Parodi MT, Bologna R, Persici P, Cornaglia-Ferraris P. Cisplatin induces modulation of transferrin receptor during cellular differentiation *in vitro*. *Cancer Chem Pharmacol* 1986, **18**, 92.
28. Tisdale MJ. Induction of haemoglobin synthesis in the human leukaemia cell line K562 by monomethyltriazenes and imidazotetrazinones. *Biochem Pharmacol* 1985, **34**, 2077–2082.
29. Levy J, Terada M, Rifkind R, Marks P. Induction of erythroid differentiation by dimethylsulphoxide in cells infected with Friendly virus: relationship to cell cycle. *Proc Natl Acad Sci USA* 1984, **81**, 7461–7465.
30. Bianchi-Scarra GL, Romani M, Coviello DM, *et al.* Terminal erythroid differentiation in the K562 cell line by Ara-C: accompanied by c-*myc* messenger RNA decrease. *Cancer Res* **46**, 6327–6332.
31. Tonini GP, Radzioch D, Cronberg A, *et al.* Erythroid differentiation and modulation of c-*myc* expression induced by antineoplastic drugs in K562. *Cancer Res* 1987, **47**, 4544–4547.
32. Gailoni D, Cadwell FJ, O'Donnel PS, Hromas RA, Macfarlane DE. Absence of phorbol ester-induced down-regulation of *myc* protein in the phorbol-ester tolerant mutant of HL-60 promyelocytes. *Cancer Res* 1989, **49**, 5329–5333.
33. Reitsma PH, Rothberg PG, Astrin SM, *et al.* Regulation of *myc* gene expression in HL-60 leukaemia cells by a Vitamin D metabolite. *Nature* 1983, **306**, 492–494.
34. Gorsky LD, Cross SM, Morin MJ. Rapid increase in the activity of topoisomerase I, but not topoisomerase II, in HL60 promyelocytic leukaemia cells treated with a phorbol diester. *Cancer Commun* 1989, **1**, 83–92.
35. Craig RW, Bloch A. Early decline in c-*myb* oncogene expression in the differentiation of human myeloblastic leukaemia (ML-1) cells induced with 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res* 1984, **44**, 442–446.
36. Ramsay RG, Ikeda K, Rifkind RA, Marks PA. Changes in gene expression associated with induced differentiation of erythroleukaemia: protooncogenes, globin genes and cell division. *Proc Natl Acad Sci USA* 1986, **83**, 6849–6853.

Acknowledgements—The funding for this work by the European Commission is gratefully acknowledged. Statistical advice was given by Dr. M. Bland of St. George's Hospital Medical School.

Eur J Cancer, Vol. 27, No. 11, pp. 1411–1416, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Dysplasia and the Natural History of Cervical Cancer: Early Results of the Toronto Cohort Study

Steven A. Narod, D.W. Thompson, M. Jain, Claus Wall, Lois M. Green and Anthony B. Miller

A sample of 176808 Pap smears, taken from 70236 women, was constructed from the records of a large cytopathology laboratory between 1962 and 1981. The prevalence of cervical dysplasia, based on the distribution of initial smear results, rose from 42.7 to 94.9 per 1000 during the study period. The relative risks (RR) for the manifestation of a malignancy (carcinoma *in situ* or worse) in a subsequent cervical smear were 1.48, 3.42, 20.9 and 71.5 for women with minimal, mild, moderate and severe dysplasia, respectively, compared with the entire cohort. The initial degree of dysplasia for women developing a malignancy was much more likely to be interpreted as moderate (RR = 5.0) or severe (RR = 42.3) than were those for controls. These results are strongly supportive of the hypothesis that the degree of dysplasia is related to the risk of development of cancer of the cervix.
Eur J Cancer, Vol. 27, No. 11, pp. 1411–1416, 1991.

INTRODUCTION

SCREENING FOR cancer of the cervix is based on the premise that there is a natural progression from a normal cervical epithelium through dysplasia to carcinoma *in situ* to invasive cancer of the cervix, and that if individuals could be identified and treated early in this process, progression to invasive disease could be prevented. A successful community-based screening program should ultimately lead to a decrease in the incidence of cervical cancer and reduced mortality from the disease. The Canadian Task Force on Cervical Cancer Screening [1] supported cytological screening programs for women who have had sexual intercourse, and recommended that annual screens be performed between the ages of 18 and 35 years, and every 5 years thereafter until age 60, providing the results of satisfactory smears are negative. Consensus regarding the ideal screening interval has not been achieved, possibly because the natural history of carcinoma of the cervix at different ages is not entirely clarified.

Several studies have demonstrated that the cumulative incidence of preneoplastic lesions exceeds the expected incidence of invasive disease [2–5], and spontaneous regression of some early lesions has been demonstrated [5]. The optimum screening interval would be ideally estimated from data generated by a controlled trial, but as such a study is unlikely to occur, results from non-experimental designs must suffice.

An unusual opportunity for review of preneoplastic abnormalities of cervical epithelium was available through the experience of a large laboratory in Toronto during the period 1962 to 1981. Pap smears had been performed on a large part of the