Cancer Chemother Pharmacol (1998) 41: 343–346

SHORT COMMUNICATION

Wai M. Liu · Clare Bamford · Maurice Slevin
Simon P. Joel

Effects of haemopoietic growth factors in combination with etoposide on sister chromatid exchange frequencies in peripheral blood mononuclear cells

Received: 12 May 1997 / Accepted: 29 August 1997

Abstract Prior to work on the influence of dosing and scheduling of the drug etoposide in bone marrow cells, the DNA-damaging effects of three haemopoietic growth factors, either alone or in combination with etoposide, were investigated. Sister chromatid exchange (SCE) frequencies in phytohaemagglutinin-stimulated mononuclear cells of six normal volunteers were used as an indicator of DNA damage. The effects of three growth factors on SCEs were investigated at concentrations ranging between 0 and 100 ng/ml and those of etoposide alone, at concentrations varying between 0 and 2 μM. The effect of combinations of growth factor (GF) and etoposide were assessed at a 40-ng/ml concentration of each cytokine and at 0.4 μM etoposide. Results showed not only a dose-dependent rise in SCE frequency in cells treated with etoposide but also a cytokine effect. Stem-cell factor did not cause a significant change in SCE numbers. However, cytokines with activity at the progenitor cell level induced small but significant increases in SCE numbers at concentrations of 50 and 100 ng/ml ($P < 0.001$). Results of combination studies indicated a significant 60% increase in SCE numbers in cells treated with GF and etoposide as compared with etoposide alone ($P < 0.00001$). This finding suggests a sensitivity of peripheral blood mononuclear cells to SCE induction by GFs given either as single agents or in combination with etoposide.

Key words Sister chromatid exchange · Etoposide · Stem-cell factor · Interleukin 3 · Granulocyte/macrophage colony-stimulating factor

This work was presented in part at the 1997 meeting of the American Association for Cancer Research

W.M. Liu (✉) · C. Bamford · M. Slevin · S.P. Joel
Barry Reed Oncology Laboratory,
St. Bartholomew's Hospital,
4th Floor, 38 Little Britain,
West Smithfield, London EC1A 7BE, UK
Tel.: +44 171 601 8927; Fax: +44 171 600 4265

Introduction

Many researchers have shown that a large variety of exogenous agents that are capable of inducing sister chromatid exchanges (SCEs) are also carcinogenic and that the concentration needed to cause SCE is significantly lower than that needed to cause an increase in chromosomal mutation [1]. It has also been reported that SCE numbers rises significantly after exposure to alkylating agents and chemotherapy drugs such as etoposide and cisplatin. The exact mechanism is unknown, but changes in the post-replication DNA repair mechanisms that remove illegal recombinations have been implicated [2]. As the presence of SCE reflects damage to DNA, increasing levels may indicate increasing carcinogenic potency.

Growth factors play an important role in chemotherapeutic regimens, many being given in conjunction with cytotoxic drugs to reduce neutropenic episodes. Currently, the most common dose-limiting toxicity for many cytotoxic drugs is myelosuppression, manifesting as severe leucopenia and/or thrombocytopenia and this prolonged immunosuppression is a cause of treatment-related death. This can be ameliorated by the prophylactic administration of extrinsic sources of growth factors responsible for leucopoiesis [3–5]. The clinical use of haemopoietic growth factors to minimise chemotherapy-induced myelosuppression may become more widespread with the increased use of high-dose chemotherapy.

There are few reports describing the effect of growth factors on SCE frequency or on the subsequently increased risk for long-term chromosomal changes. These, notably including that of Bussing et al. [6], concluded that administration of such cytokines did not influence SCE frequency in peripheral blood mononuclear cells (MNCs) and, consequently, would not produce severe mutagenic effects.

As part of on-going studies into the activity and schedule dependence of etoposide in bone-marrow

progenitor cells and cell lines, SCE frequency is being used as one of a number of measures of drug-induced DNA damage. These studies include the effect of etoposide in short-term bone marrow cultures that involve the use of granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3) and stem-cell factor (SCF). Consequently, the effect of these cytokines alone and in combination with etoposide on SCE frequency in MNCs was investigated.

Materials and methods

Peripheral blood was taken from six healthy and haematologically normal volunteers without recent viral infections. The same six subjects were used for all of the experiments. In each case, 0.4 ml of whole blood was maintained in 10 ml of RPMI 1640 supplemented with 10% fetal calf serum, 1% glutamine, and 1% penicillin (10 MU/ml) and streptomycin (10 mg/ml) solution. Cell mitogenic activity was stimulated with phytohaemagglutinin-M (PHA-M) at 20 μg/ml and was maintained in darkness at 37 °C. Bromodeoxyuridine (BrdU) at a concentration of 10 μ*M* was added at 24 h of incubation. Following a further incubation period of 24 h, one of three methods was employed, according to the assay involved: (1) growth factor alone – 10-, 50- and 100-ng/ml concentrations of either SCF (Amgen Ltd, Cambridge, UK), GM-CSF or IL-3 (both Sandoz Pharmaceuticals, Frimley, UK) were added; (2) etoposide alone – serial concentrations ranging between 0 and 2 μ*M* etoposide (Sigma, Poole, UK) were added; (3) combination – 0.4 μ*M* etoposide and a 40-ng/ml concentration of one of the three cytokines were added. After a third period of 24-h incubation, colcemid at 0.1 μg/ml was added at 1 h prior to lymphocyte harvest by centrifugation. Chromosome spreads were obtained using hypotonic treatment with 75 m*M* potassium chloride for 10 min. Cells were washed in three successive fixations in freshly prepared methanol:glacial acetic acid (3:1). Slides were prepared and stained according to the method of Perry and Wolff [7]. Next, 15 well-spread metaphases with a full complement of 46 properly stained chromosomes were analysed for SCE status in each side. All slides were analysed at random to reduce any possibility of experimental bias in the scoring procedure.

Table 1 Effect of growth factors on SCE frequency. Figures are expressed as median numbers of SCEs per cell (range); $n = 90$ in all cases – 15 metaphases in each of the 6 subjects

Growth factor	Concentration (ng/ml)			
	0	10	50	100
SCF	5 (0–12)	5 (0–13)	5 (1–10)	5 (1–11)
GM-CSF	4 (0–10)	5 (0–16)	5 (0–11)	6 (1–14)*
IL-3	5 (0–11)	5 (0–9)	7 (2–11)*	7 (2–10)*

*$P < 0.001$ versus control cultures with no added cytokine

Results

SCEs were observed in all cell preparations, including those without added cytokines. The frequency of SCE at each concentration studied was variable and was not normally distributed. For instance, in the absence of cytokines, control incubations typically showed a median frequency of 5 SCEs/cell, which for 15 metaphases in the 6 subjects ranged from 0 to 11 SCEs/cell.

Table 1 shows the median number of SCEs determined for each of the growth factors studied at each of three concentrations. Non-parametric analysis of variance (Friedman's ANOVA) of all the data for each cytokine, controlling for subject and concentration, indicated differences in SCE numbers with concentration in both GM-CSF-treated ($P < 0.05$) and IL-3-treated ($P < 0.001$) MNCs but not in SCF-treated cells. Subsequent paired analysis (Wilcoxon signed-rank matched-pairs test) of the number of SCEs determined at specific concentrations against incubations with no added cytokine indicated significant increases in SCE frequency for IL-3 at 50 and 100 ng/ml and for GM-CSF at 100 ng/ml ($P < 0.001$ in all cases).

Figure 1 shows the dose-dependent increase in SCE frequency obtained with etoposide. A rise in etoposide

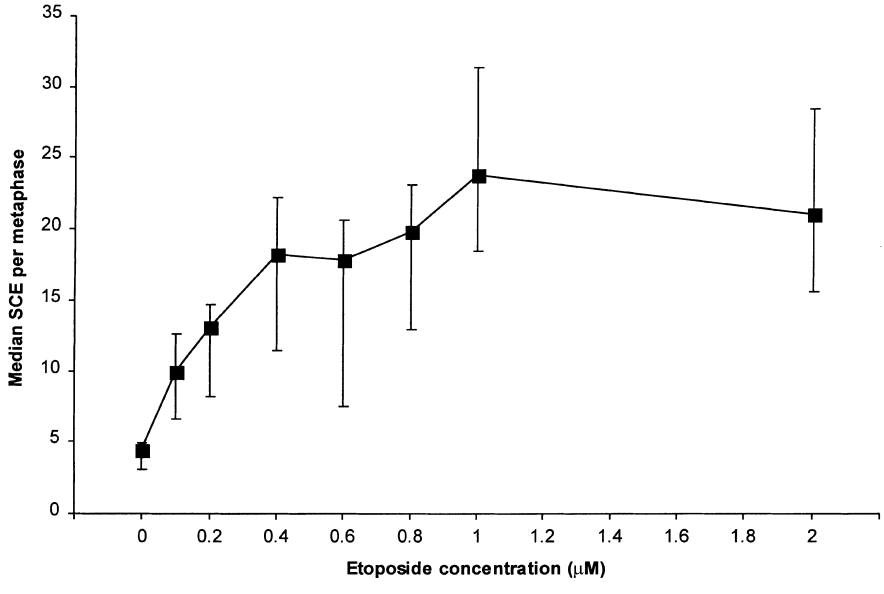

Fig. 1 Effect of etoposide on SCE numbers. MNCs were cultured in serial concentrations of drug. *Points* represent the median values and the minimal maximal range of SCE numbers per metaphase spread of the 6 subjects

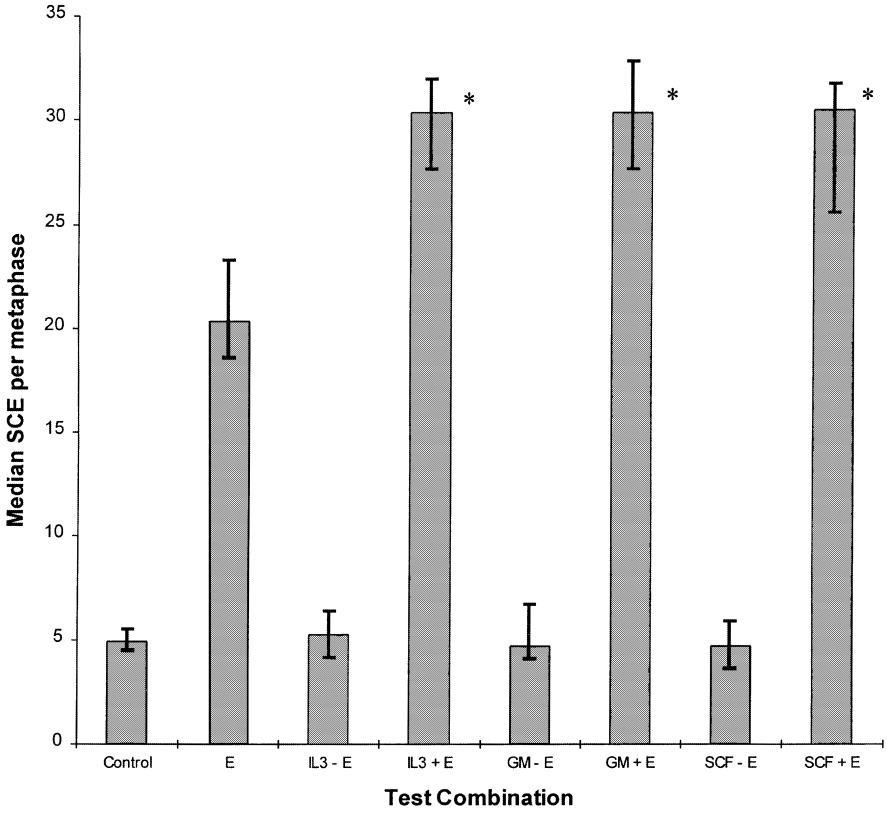

Fig. 2 Effect of growth factor and etoposide on SCE numbers. MNCs were treated with sub-optimal concentrations of 0.4 μ*M* VP-16 and 40 ng GF/ml. Each *bar* represents the median number of SCEs detected per metaphase after culture with IL-3 ± etoposide (*E*), GM-CSF (*GM*) ± E, SCF ± E or E alone ($n = 90$). *$P < 0.00001$ versus control cultures incubated with 0.4 μ*M* etoposide but no added cytokine

concentration resulted in an increase in SCEs to a plateau of around 20/cell from the 0.4 μ*M* dose.

In the combination assays, all three cytokines were used at sub-optimal concentrations of 40 ng/ml and etoposide was applied at 0.4 μ*M*. Figure 2 shows the number of SCEs determined for each of the etoposide and growth factor combinations studied. In cells incubated with etoposide alone the median SCE value was 19 (range 13–34) versus 30.5 (range 13–46) for IL-3, 30.5 (range 13–46) for GM-CSF and 29.5 (range 19–35) for SCF (all $P < 0.00001$).

Discussion

Prior to the investigation of factors that influence the activity of etoposide in bone marrow and tumour cells, involving short-term bone marrow cultures, the intrinsic SCE-inducing effect of growth factors, used as a necessary agent in successful colony growth, was tested. The data presented in this report describe the effect of these growth factors on SCE frequency in MNCs.

Growth factors were used at optimal (100 ng/ml) and sub-optimal (10 and 50 ng/ml) concentrations with regard to their proliferative and differentiating activity in normal donor marrow-colony assays (unpublished data). This range of concentration was in line with documented in vivo plasma concentrations of the growth factors studied [8, 9]. There is no published information on plasma levels of SCF.

The differences in the SCE numbers seen with these growth factors were related to the maturity of the cells. Peripheral blood contains a high proportion of cells that are well differentiated down the lymphoid lineage. SCF had no effect on the SCE frequency, as *c-kit* expression is reduced [10]. In contrast, both GM-CSF and IL-3, showing activity in cells at a later stage of maturation, induced dose-dependent increases in the frequency of SCEs in PHA-stimulated MNCs.

There are few reports describing the effect of commonly used growth factors on SCE numbers. Most of these have concentrated on G-CSF, a cytokine with differentiative effects similar to those of GM-CSF, and have reported no increased induction of SCEs at concentrations of up to 3 μg/ml [6]. However, we demonstrated a small but significant increase in SCE frequency in MNCs using GM-CSF at 100 ng/ml and IL-3 at 50 and 100 ng/ml.

Following this preliminary work on growth factor alone, a combination of cytokine and etoposide was used in cultures to study any synergistic effect that might influence the activity of etoposide in this short-term culture system. The subjects tested were the same six used in our preliminary study. Our data indicate a positive effect of growth factor combined with etoposide. As compared with control cultures, etoposide alone caused an increase in SCE numbers by 280% (19 versus 5), whereas a combination of etoposide with any of the cytokines studied resulted in an increase of 500% (30 versus 5). This represents an increase in SCE frequency

of 60% in cultures containing both etoposide and cytokine as compared with etoposide alone.

Stimulated MNCs have a large range of cycling times and were not synchronised in this experiment, as only a single time point of 24 h was studied. Cells cycling at more than 24 h would be excluded from SCE scoring by the resultant incorporation of BrdU to both chromatids, causing both to be stained with Hoechst 33258 such that SCEs would not be visible. Conversely, cells cycling at fewer than 24 h would not have sufficient intercalation of BrdU into chromatid strands for SCE analysis. Only correctly stained metaphases with the full complement of 46 chromosomes and clearly distinguishable sister chromatids were counted.

SCE frequency is used as an indicator of damage to chromosomes in prediction of tumour sensitivity in vivo [11], and correlations between SCE induction and the induction of carcinogenesis have been established [12]. Although a number of models have been proposed, the observation that exchange of material between chromatids involves double-stranded DNA breaks implicates topoisomerase II (topo II) as suggested by Pommier et al. [13]. The enzyme normally causes transient double-stranded DNA breaks during replication, but these breaks become stabilised in the presence of etoposide. The proximity of DNA breaks on homologous DNA strands may result in incorrect rejoining, causing an SCE.

Topo II catalytic activity is dependent on the growth state of cells, with topo II protein expression increasing during the S and G_2 phases and peaking at G_2/M [14]. Thus, in the presence of GF the number of cycling cells would be expected to increase, but an increase in SCE frequency would result only from an increase in topo II during the S phase relative to control cultures or from changes to a post-replication repair system. Such an increase in topo II after exposure to GF has been reported [15] and would result in a greater number of cleavable complexes, which in turn may give rise to an increase in SCE frequency as seen in our own studies.

The increase in the number of SCEs found in the presence of cytokines alone, although numerically small, was statistically significant, and when cytokines were used in combination with etoposide they increased SCE frequency by 60% over etoposide alone. Investigators should therefore be aware that the necessary use of growth factors in bone marrow cultures may influence the activity of DNA-damaging agents, possibly through up-regulation of topo II activity. Clinically, the possibility that the application of growth factors may influence the activity of these agents as seen in the present in vitro studies needs to be investigated further.

Acknowledgements This work was supported by the Frances & Augustus Newman Foundation, UK. We are grateful to both Amgen Ltd. (SCF) and Sandoz Pharmaceuticals (GM-CSF, IL-3) for their supplies of recombinant human growth factors for this study.

References

1. Tice RR, Lambert B, Morimento K, Hollaender A (1984) A review of the international symposium on sister chromatid exchanges: twenty-five years of experimental research. Environ Mutagen 6: 737–752
2. Dillehay LE, Jacobson-Kram D, Williams JR (1989) DNA topoisomerases and models of sister-chromatid exchange. Mutat Res 215: 15–53
3. Orazi A, Cattoretti G, Schiro R, Siena S, Bregni M, Di Nicola M, Gianni AM (1992) Recombinant human interleukin-3 and recombinant human granulocyte-macrophage colony stimulating factor administered in vivo after high-dose cyclophosphamide cancer chemotherapy: effect on haematopoiesis and microenvironment in human bone marrow. Blood 79: 2610–2619
4. Laughlin MJ, Kirkpatrick G, Sabiston N, Peters W, Kurtzberg J (1993) Haematopoietic recovery following high-dose combined alkylating-agent chemotherapy and autologous bone marrow support in patients in phase-I clinical trials of colony-stimulating factors: G-CSF, GM-CSF, IL-1, IL-2, M-CSF. Anal Haematol 67: 267–276
5. Crawford J, George M (1995) The role of haematopoietic growth factors in support of ifosfamide/carboplatin/etoposide chemotherapy (review). Semin Oncol 22 [Suppl 7]: 18–22
6. Bussing A, Azhari T, Mertens R, Schweizer K (1995) Recombinant human granulocyte colony-stimulating factor does not influence distribution of sister chromatid exchange frequency of proliferating mononuclear cells of the peripheral blood. Oncology 52: 215–218
7. Perry P, Wolff S (1974) New Giemsa method for differential staining of sister chromatids. Nature 25: 156–158
8. Kurzrock R, Talpaz M, Estrov Z, Rosenblum MG, Gutterman JU (1991) Phase I study of recombinant interleukin-3 in patients with bone marrow failure. J Clin Oncol 9: 1241–1250
9. Steward WP (1993) Granulocyte and granulocyte-macrophage colony stimulating factors. Lancet 342: 153–157
10. McGuckin CP, Uhr MR, Gordon-Smith EC (1995b) C-kit protein (stem cell factor receptor) expression on cells with erythroid characteristics and on normal human bone marrow without the use of monoclonal antibodies. Exp Haematol 23: 14–20
11. Latt SA, Allen J, Bloom SE, Carrano A, Flake D, Wolff S (1981) Sister chromatid exchanges: a report of the Gene Tox program. Mutat Res 98: 17–62
12. Carrano AV, Thompson LH, Lindl PA, Minkler JL (1978) Sister chromatid exchange as an indicator of mutagenesis. Nature 271: 551–553
13. Pommier Y, Zwelling LA, Kao-Shang CS, Whang-Peng J, Bradley MO (1985) Correlations between intercalator-induced DNA strand breaks and sister chromatid exchanges, mutations and cytotoxicity in Chinese hamster cells. Cancer Res 45: 3143–3149
14. Hsiang YH, Wu HY, Liu LF (1988) Proliferation-dependant regulation of DNA topoisomerase II in cultured human cells. Cancer Res 19: 3230–3235
15. Kreuser ED, Wadler S, Thiel E (1995) Biochemical modulation of cytotoxic drugs by cytokines: molecular mechanisms in experimental oncology (review). Recent Results Cancer Res 139: 371–382