

CYTOKINE AND GROWTH FACTOR MODULATION OF CELL CYCLE EVENTS IN HUMAN MELANOMA CELL LINES

A.R. ANDALIB,* J. LAWRY,** B.J. BURNS,** A.K. MURRAY,**
M. HERLYN,*** AND R.C. REES**

*From * Esfahan Medical School, Esfahan, I.R. Iran,*

*** Sheffield University Medical School, U.K., and*

**** Wistar Institute, Philadelphia, USA.*

ABSTRACT

Cytokines influence cell cycle events, which in some but not all instances can be associated with melanoma progression. Analysis of the G0/G1 and S phase fractions of the cell cycle was used to assay the proliferative or inhibitory activity of cytokines against ten human melanoma cell lines, including pairs of cell lines derived from primary and metastatic tissue of individual patients. Cytokines and growth factors were generally capable of inducing either a proliferative or an inhibitory effect, depending upon the melanoma cell line tested.

Distinct differences in responsiveness between the primary and metastatic partners of related pairs of melanoma cell lines was observed for IL-1 β , IL-6, TNF α , TGF β 2, IFN α , IFN γ and IGF-2. This data is complementary to previous works^{1,2} showing IL-6 and TGF β 2 to be inhibitory to cell lines derived from early, but not advanced-stage melanomas. These alterations in cell cycle events were coincidental with a reduced expression of ICAM-1 and/or MHC antigen expression in response to some of these cytokines.

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INTRODUCTION

The development of primary melanoma from its precursor lesion, and further progression to a highly metastatic tumor is known to be associated with stepwise genotypic and phenotypic alterations,³⁻⁵ and a complex network of cytokines is involved in the regulation of melanoma cell growth.^{6,7} The ability of tumor cells to grow locally and metastasize can be affected by the presence of

adjacent normal tissues and cells, particularly mesenchymally-derived stromal cells such as fibroblasts.^{8,9} A large number of growth factors and cytokines function as potential stimulators or inhibitors of melanocyte or melanoma cell growth.¹⁰⁻¹² However, the requirement for exogenous growth factors, such as insulin-like growth factor-1 (IGF-1), α -melanocyte stimulating hormone, b-fibroblast growth factor (FGF) and phorbol esters is reduced as melanocytes acquire a malignant phenotype.^{10,13,14} Resistance to growth inhibitory actions of cytokines may occur with disease progression as has been shown for IL-

*Corresponding author.

6, TGF β and oncostatin M.^{1,2,8,15}

It has been suggested that an altered response to host tissue factors may confer a growth advantage upon a small number of metastatically competent cells, allowing them to escape local growth constraints both at the primary tumor site and at distant ectopic tissue sites.⁹ Lu¹⁵ indicated that rIL-6 was able to arrest cells derived from early stage melanoma lesions but not from advanced stage lesions in the G0/G1 phase of the cell cycle. In addition, it has been reported that human melanoma cells progressively lose sensitivity to endothelium-derived growth inhibitors (mostly cytokines and growth factors) during disease progression,¹⁶ inferring that the process of angiogenesis may contribute to the selection of more malignant 'cytokine-resistant' melanoma cell variants.^{1,11,16}

In this study cytokines and growth factors including IL-1 β , IL-4, IL-6, TNF α , TGF β 2, IFN γ , IFN α and IGF-2 were assessed for their ability to modulate proliferation in a series of ten human melanoma cell lines including three pairs of related cell lines derived from primary and metastatic lesions. Flow cytometry was used to assess the percentage of cells in each phase of the cell cycle. Both proliferative and anti-proliferative effects were observed in response to treatment with cytokines/growth factors for the panel of cell lines assayed; in addition, a diversity of response was noticed between cell lines derived from primary versus metastatic tissue from the same patient, which was accompanied by a simultaneous decrease in ICAM-1 and/or MHC antigen expression in response to the same cytokine.

MATERIALS AND METHODS

Tumor cell lines

The WM melanoma cell lines were established by Dr. M. Herlyn.¹³ Two pairs of human melanoma cell lines derived from primary (P) and metastatic (M) tumors from the same patients were chosen in order to compare differences in response to cytokine stimulation: WM983A (P), WM983B (M); WM1361A (P), WM1361C (M). A third pair of cell lines was established from a primary melanoma (WM793) whilst its metastatic partner was generated following growth in nude mice (WM1205Lu). The WM278 (P) and WM1617 (M) were also derived from primary and metastatic lesions but were not related. HLA typing and karyotyping confirming the relationship between these cell lines are shown elsewhere.¹⁷

The A375 melanoma cell line, and the early stage primary cell line WM35 were also included in this study. The cell lines were cultured in Dulbecco's medium (Life Technologies Ltd; Paisly, Scotland) plus 10% fetal calf serum (Northumbria Biologicals Ltd., UK) at 37°C in a 5% CO₂ humidified incubator. All experiments were performed using cells cultured within 10 passages of the stock cultures.

Cell culture conditions and cytokines

The following cytokines were used in the study: rhIL-1 β 1000 U/mL (Immunex Corporation), rhIL-45 1000 U/mL (Sterling Pharmaceuticals), rhIL-6 1000 U/mL (gift from ICI Pharmaceuticals), rhTNF α 1000 U/mL and IFN γ 500 U/mL (Boehringer Ingelheim), TGF β 2 5ng/mL (Sandoz Ltd), IFN α 500 U/mL (Wellcome Diagnostics), and IGF-2 60 ng/mL (Pepro Tech Inc.). Cytokine concentrations were

Table I. Examples of cell cycle analysis of human melanoma cell lines following culture with cytokines and growth factors. The proportion of cells in each cell cycle phase was assessed after incubation with cytokine or growth factor.

The percentage of cells in cell cycle phases following incubation with (+) or without (-) cytokine/growth factor									
Exp. no.	Cytokine	Cell line	G0/G1-phase %		S-Phase %		G2/M-phase %		n
			-	+	-	+	-	+	
1	IL-6	A375	61.2 \pm 6.6	*73.2 \pm 8.9	24.4 \pm 9	*11.4 \pm 6.1	14.2 \pm 11	15.3 \pm 11.4	5
2	IL-1 β	WM983B (M)	50.6 \pm 5.1	57.0 \pm 3.9	46.2 \pm 7.2	37.3 \pm 4.7	3.10 \pm 2.0	5.7 \pm 0.8	3
3	IL-4	WM983A (P)	70.2 \pm 0.2	68.4 \pm 1.4	20.6 \pm 0.6	22.8 \pm 0.4	9.2 \pm 0.8	8.6 \pm 1.0	3
4	TNF α	WM35 (P)	73.7 \pm 2.0	*67.5 \pm 3.1	15.5 \pm 2.7	19.2 \pm 3.8	10.6 \pm 3.0	13.2 \pm 1.0	3
5	TNF α	WM793 (P)	61.6 \pm 4.4	66.0 \pm 4.7	25.6 \pm 7.5	21.4 \pm 4.6	14 \pm 2.8	12.5 \pm 3.5	4
6	TGF β ₂	WM278 (P)	74.2 \pm 6.3	*82.4 \pm 1.5	17.1 \pm 5.4	*8.2 \pm 0.9	8.6 \pm 0.8	9.3 \pm 0.6	4
7	TGF β ₂	WM1617 (M)	51.5 \pm 6.6	*60.0 \pm 8.4	38.9 \pm 5	*31.7 \pm 8.0	9.5 \pm 2.2	8.2 \pm 1.1	4
8	IFN γ	WM1361A (P)	67.8 \pm 4.3	*72.0 \pm 5.2	21.2 \pm 5.0	16.9 \pm 4.7	10.9 \pm 1.1	11 \pm 3.1	4
9	IFN γ	WM1361C (M)	69.2 \pm 2.1	*65.0 \pm 0.7	16.5 \pm 2.5	22.9 \pm 7.5	17.8 \pm 1.6	21.6 \pm 8.6	3
10	IFN α	WM1205 (M)	77.1 \pm 2.5	*58.6 \pm 4.2	15.7 \pm 3.3	*34.6 \pm 9.8	7 \pm 1.9	6.7 \pm 5.7	4
11	IGF-2	A375	70.4 \pm 6.3	*62.5 \pm 6.2	12.3 \pm 6.5	10.7 \pm 2.9	26.9 \pm 3.6	*17.3 \pm 0.6	4

* = Significant alteration ($p \leq 0.05$) using paired t-test for analysis between sets of treated and untreated cell lines.

n=number of experiments performed, (P)= primary and (M)= metastatic melanoma cell line.

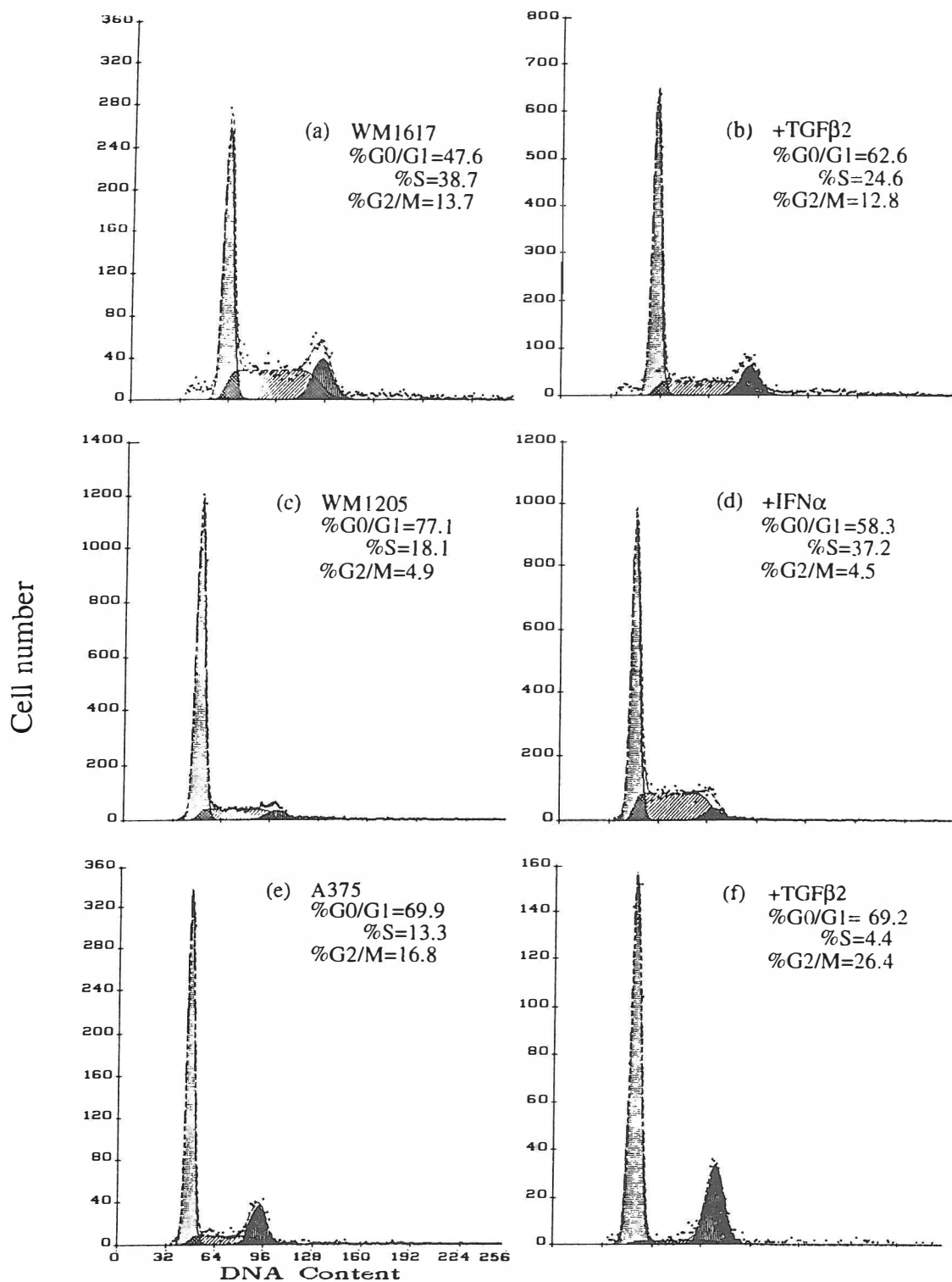


Fig. 1. Cell cycle analysis-representative results of flow cytometric profiles of three different melanoma cell lines following cytokine treatment. The fluorescence intensity is plotted on the X-axis against the relative cell number on the Y-axis. Shaded areas indicate the G0/G1, S and G2/M population within each histogram. Untreated (a) and TGFβ2 treated (b) WM1617(M) cells: results show a reduction of cells in S-phase from 38.7% to 24.6% (c.v. G1 5.4 and 4.8, respectively). WM1205 (M) cells show an accumulation of cells in S-phase from 18.1% control (c) to 37.2% IFNα treated (d) (c.v. G1 6.0 and 5.9, respectively). A375 cells accumulation in G2/M-phase measured as 16.8% for control cells (e) and 26.4% for TGFβ2 treated cells (f) (c.v. G1 5.2 and 6.2, respectively).

Table II. Altered responsiveness to cytokine modulation between primary and metastatic pairs of WM melanoma cell lines, as measured by simultaneous changes in DNA content (represented by % G0/G1 phase cells) and phenotypic marker expression (MHC class I, II and ICAM-1).

	G0/G1 phase		Class I		Class II		ICAM-1	
	(P)	(M)	(P)	(M)	(P)	(M)	(P)	(M)
WM1361A/C								
IFN γ	↓	↑	1	0.5	7.5	3	5	1
TNF α	↓	↑	1.5	0	0	0	10	1
WM983A/B								
IFN γ	↓↓	↓	0	0.5	7.5	3.5	1.5	1.5
IL-6	↓↓	↓	0	0	1.5	0.5	1	1
WM793/1205								
IFN γ	↓	↑↑	0.5	0	10	5	0.5	0.5
IFN α	↑↑	↑↑↑↑	0.5	0	10	1.5	0.5	0.5

↑ = 3 - 5% G0/G1 phase decrease (proliferation effect).

↓ = 3 - 5% G0/G1 phase increase (inhibition effect).

Numbers represent fold increases (from constitutive levels of expression of untreated cells) in antigen expression, using the median channel value.

0 = no response to cytokine modulation from constitutive level of antigen expression.

(P) = primary, (M) = metastasis melanoma cell line.

selected on the basis of published data and from the results of other investigations performed in this lab (data not shown). Only IGF-2 was used at a range of concentrations (30, 60, 120 ng/mL) for cell cycle studies but only data obtained from 60 ng/mL is included in the present study. Similarly, the period of incubation with cytokine was standardized at 48 hours based on our (and other) previous findings with the exception of IGF-2. Time course studies with IGF-2 indicated that G1 accumulation was optimal at 24 hours. All melanoma cell lines were cultured at 37°C in a humidified 5% CO₂, 95% air atmosphere, with or without the addition of cytokines. Briefly, 3-5×10⁵ cells in 2 mL of medium containing 10% FCS were seeded into 6 well plates (Costar) (in 2 mL/well) and incubated overnight at 37°C. The medium was then removed and replaced with serum free medium and incubated at 37°C for one hour. The medium was removed once again, the cells washed with serum free medium and incubated with or without cytokine for 48 hours in a further aliquot of serum free medium, or 24 hours in the case of IGF-2.

Flow cytometric analysis

Medium was removed from cultured cells (usually 48 hours after exposure to cytokines or controls) and the cultures washed twice using PBS; 0.5 mL trypsin 0.25% v/v in PBS (Life Technologies Ltd) was added, and the cultures incubated at 37°C for 5 min or until the cells detached from the plastic. The cells were then washed ×2 in PBS. Cytokine-treated and control (untreated) cells were pelleted and assayed using a Cycle TEST DNA analysis kit (Becton Dickinson) in which the nuclei are extracted and

stained with propidium iodide in a stabilizing buffer containing RNase. Using flow cytometry approximately 10,000 nuclei were analyzed and the percentages of cells in each sample with diploid (2n) DNA (those in G0/G1 phase); 2n to 4n DNA (those in S phase); and 4n DNA (those in G2/M phase) subsequently determined by using the Multicycle DNA analysis software (Phoenix Flow Systems, USA) (Fig. 1). Only those cell cycle histograms in which the coefficient of variation (c.v.) of the G0/G1 peak was less than 8.0 were accepted in the present study. Typically the c.v. was 5.0, range 3.8 to 6.5.

Experiments were repeated 3-5 times and untreated cell samples were included as controls for each cell line. Statistical analysis was performed using the paired t-test for analysis using the SPSS software package. Result sets from treated samples were compared to result sets from control (untreated) samples, and a P value calculated. These results were used to calculate the significance of a cytokine's ability to induce growth inhibition (G0/G1 phase accumulation) or proliferation (G0/G1 phase reduction, increase S+G2/M) as indicated when $p \leq 0.05$.

RESULTS

Changes in cell cycle progression, occurring as a result of the presence of exogenous cytokines, was assessed in a series of melanoma cell lines derived from early, intermediate and advanced tumors. This series included cell lines established from primary and metastatic melanoma tissue derived from the same patient. Flow cytometric

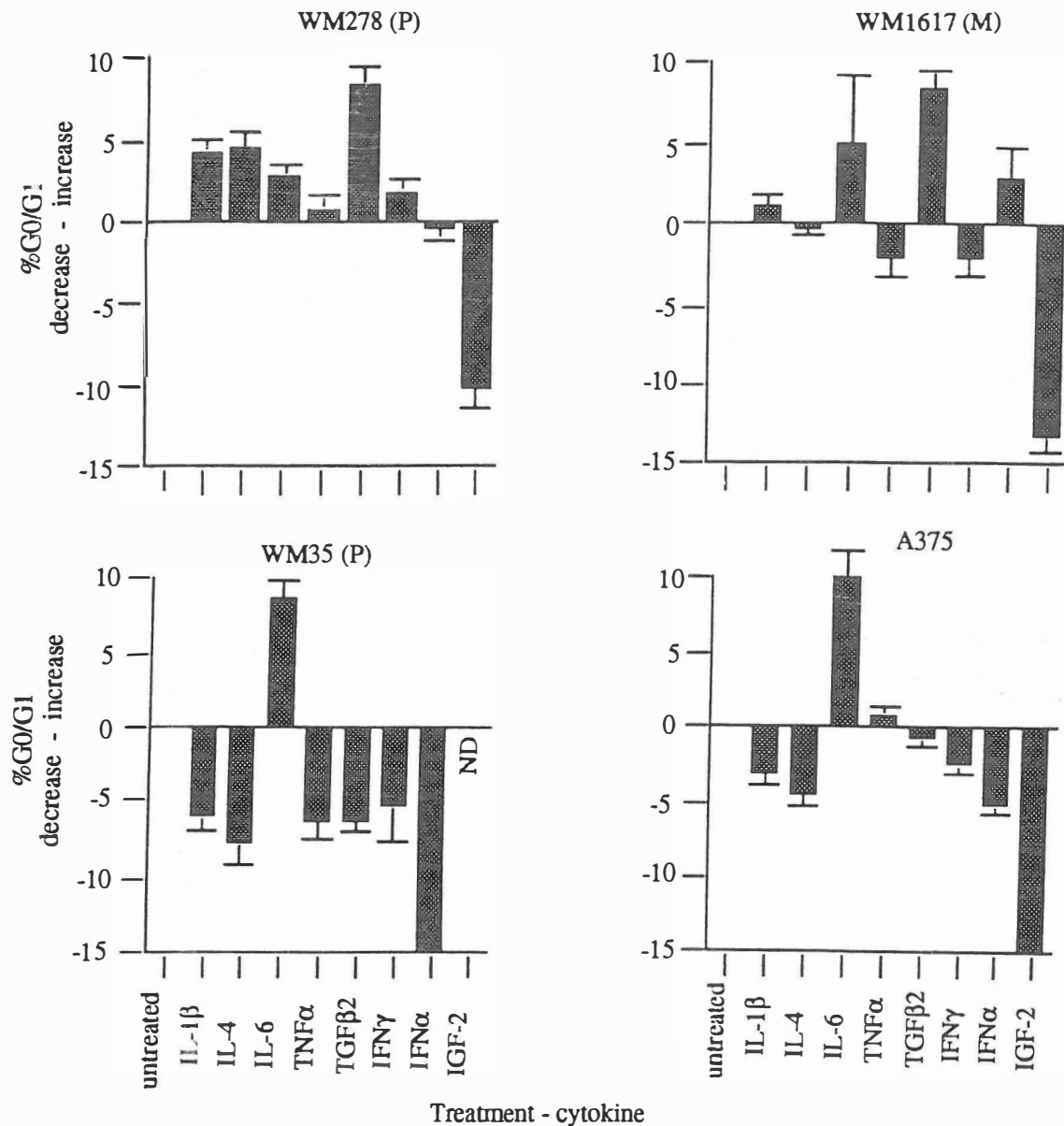


Fig. 2. Analysis of the percentage changes for G0/G1 phase of the cell cycle of the unrelated melanoma cell lines following cytokine and growth factor treatment. Mean and S.E. for differences between the two calculated sets of treated versus untreated cells are shown. 0=level of G0/G1 phase in untreated (control) cells, positive values (+) = accumulation of cells, and negative values (-) = decrease of cell numbers in G0/G1 phase. Alteration in G0/G1 less than 2.5% has been assumed to be equal to normal experimental variation for the control. Changes in G0/G1 were graded as follows; <5% = weak effect, 5-10% = moderate effect and >10% = strong effect. ND = not done.

analysis was performed on cells incubated in the presence of a single cytokine, and the response was depicted as the change in percent cells in any single phase of the cell cycle. Results are expressed as mean values \pm standard deviation which have been calculated from repeat experiments, in which cell lines were either treated with a cytokine, or left untreated. Data has all been taken from the percentage G0/G1, S and G2/M calculations generated by the cell cycle software (Table I). A statistically significant difference

between these sets is indicated by an asterisk (*). The specific changes in %G0/G1 phase, induced by cytokine, are illustrated in Figures 2 and 3 where they are expressed as mean \pm S.E. for all cell lines, and all cytokines/growth factors tested. Both growth inhibitory and stimulatory effects were observed and were seen to be dependent on the specific cytokine or growth factor used, as well as the target cell line.

Table I shows selected data taken from a total of 8

cytokines and growth factors which were assayed against ten cell lines. A range of selected results from experiments where cytokines caused a major effect on cell proliferation are shown. For instance, the accumulation of A375 cells in G0/G1 (growth inhibition) following incubation with IL-6 (Table I, exp. 1) which resulted in a concomitant decrease in the percentage of cells in S phase, is shown by comparing untreated (-) and treated (+) values. Similar modulatory effects are shown for IL-1 β , IL-4, TNF α , TGF β 2, IFN γ , IFN α and IGF-2 (Table I, exp. 2-11).

The results of individual experiments are illustrated in Figure 1, showing the flow cytometric calculation of cell cycle events using the Multicycle software package. WM-1617 (M) cells treated with TGF β 2 show a reduction in the percentage of cells progressing into S phase (a+b), IFN α increased the percentage of WM1205 cells entering S phase (c+d) and TGF β 2 increased the percentage of A375 cells accumulating in the G2/M phase of the cell cycle (e+f). Figure 2 shows the results obtained with the four unrelated melanoma cell lines where positive values above the zero line indicate an accumulation of cells in G0/G1 (growth inhibition), together with a decrease in the percent of cells in S or G2/M phases; whilst negative values shown below the zero line (growth progression) are indicated by reduction of cells in G0/G1 and a corresponding increase in the percentage of cells in S or G2/M phase. For example, IL-6 inhibited cell cycle progression of all four cell lines and IGF-2 was growth stimulatory for 3 out of 3 cell lines tested. The effect of other cytokines was variable, depending on the cell lines tested.

Further studies were performed on three pairs of related cell lines (see *Material and Methods*). Comparisons between paired cell lines indicated that the metastatic partner displayed an altered growth response profile compared to the parental line established from primary melanoma tissue. These responses are shown in Figure 3, in which (*) denotes differences which exceed 5% when measured between cell line pairs tested with the same cytokine. The metastatic partner line generally demonstrated a reduced growth inhibitory response and in several instances a loss of growth inhibition altogether and enhanced progression of cells into S phase (growth stimulation) was observed, in comparison with the effect seen in the primary cell line. These effects were shown for IL-6 (WM983A/B; WM-1361A/C), TNF α (WM1361A/C), TGF β 2 (WM793/WM1205; WM983A/B), IFN γ (WM793/WM1205; WM-1361A/C) and IGF-2 (WM983A/B). However, IFN α induced significant growth stimulation in the metastatic cell lines WM1361C and WM1205, whilst being less effective on the primary line WM793, and having no effect on WM1361A.

Table II summarizes the data produced by the three related pairs of WM melanoma cell lines shown in Figure 3. The table focuses specifically on those cytokines which

generated differential effects between the primary and metastatic partner, for both measurements on cell cycle phases, and also cell surface protein expression, the results of which have been previously presented in full.¹⁷ The simultaneous changes seen in response to cytokines were found for IFN γ for all three pairs. Moreover, the alterations in response of primary and metastatic cell lines for both DNA content and cell surface phenotype were also observed for TNF α : between WM1361A (P) and WM1361C (M), for IL-6: between WM983A and WM983B and for IFN α : between WM793 and WM1205.

DISCUSSION

Cytokines represent a broad class of agents that may affect tumor or normal cells in a variety of ways,^{18,19} which act alone or in concert to influence the clinical course of malignant disease.²⁰⁻²⁴ In addition, cytokines induce alterations in the shape and morphology of human melanoma cells.²⁵ Human tumors can constitutively express cytokines and growth factors, but in many cases, the mechanism of action of cytokines on cell growth remains unknown, and there is relatively little information available on cytokine receptor expression on tumor cells.^{26,27} A complex network of cytokines may regulate melanoma cell growth and further insight into these mechanisms may contribute to the development of new strategies for therapy.⁷

The response of a tumor cell to its environment may result in malignant progression, and cytokines produced by adjacent or infiltrating host leukocytes; or even the tumor cell itself has the potential to affect the biological response, growth potential and tumor phenotype.²⁸⁻³⁰ Human cancer cell lines provide useful tools to study further the mechanisms of cytokine action on cell growth. Furthermore, comparisons made between cell lines derived from different stages of the disease provides a unique opportunity to assess their response to cytokines relative to tumor progression.

The results of the present study demonstrate both proliferative and inhibitory effects of cytokines on melanoma cell growth *in vitro*, which appear to depend upon the origin of each cell line, and may indicate how tumor progression can be influenced by the presence of exogenous cytokines. We have previously reported the effects of cytokines on melanoma phenotype, and in particular the regulation of immune response genes, MHC class I and II and intracellular adhesion molecule-1 (ICAM-1).¹⁷ This study demonstrated differences in antigen expression between related melanoma cell lines derived from primary and metastatic tissue from the same patient. The present study further shows that a variety of cytokines and growth factors influence cell cycle progression, i.e. G0/G1 to S phase. Overall there was no consistent pattern

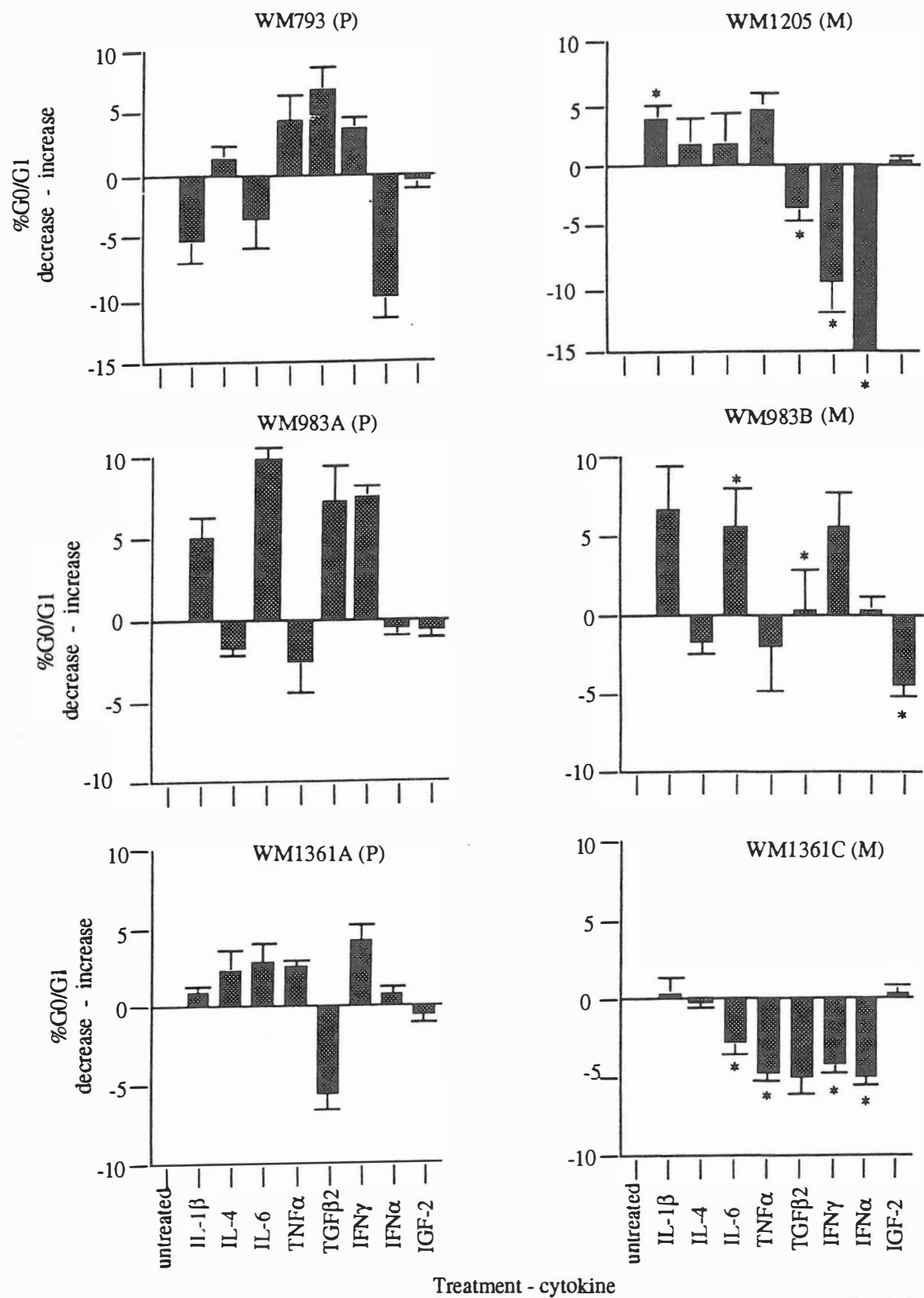


Fig. 3. Analysis of the percentage changes for G0/G1 phase of the cell cycle of paired (related) melanoma cell lines following cytokine and growth factor treatment. Mean and S.E. for differences between the two calculated sets of treated versus untreated cells are shown. * = >5% difference in response to cytokine treatment, between the metastatic cell line and its primary counterpart.

for any single cytokine, and in most instances the same cytokine caused both growth inhibition and cell cycle progression depending on the individual cell line studied. However, cell lines derived from metastatic tissue demonstrated an altered pattern in response to number of cytokines compared with their related partner line derived from primary melanoma tissue from the same patient (Fig. 3).

The analysis of cytokine effects on tumor growth has been assessed previously by measuring the uptake of radio-labelled nucleotides,^{8,31} whilst few reports have included an analysis of individual compartments of the cell cycle.²⁹ Using flow cytometry, it was possible to determine the effects of exogenous cytokines on melanoma cell progression and accumulation in each phase of the cell cycle, i.e. G0/G1, S phase and G2/M. Several cytokines have previously been shown to be capable of inhibiting and stimulating melanoma cell growth *in vitro*^{7,12,32-38} and *in vivo*,^{39,40} and certain combinations of cytokines, e.g. TNF α and IFN γ , may act synergistically *in vivo* to induce regression of human tumor xenografts in nude mice.^{20,22,34,41}

It has been suggested that some of the multifunctional effects of cytokines and growth factors can be related to tumor progression. For example, whereas non-metastatic mouse fibrosarcoma/melanoma or mouse mammary carcinoma cells were resistant, or growth inhibited, upon exposure to TGF β , their metastatic counterparts were growth stimulated.^{2,42} Likewise well-differentiated, non-aggressive human colorectal carcinoma cells may be growth inhibited by TGF β while their more aggressive anaplastic counterparts are stimulated into cell division when treated with TGF β under the same experimental conditions. A similar transition has been noted during progression of low-grade to high grade human gliomas,⁴³ and it has been demonstrated that rIL-6 inhibited early but not advanced stage melanoma cell growth, and in many cases multi-cytokine resistance occurs.^{1,2,15,44,45} In addition, it has been documented that growth factor independence in advanced (metastatic) stages of tumor progression,⁴⁶ as well as resistance to antiproliferative effects of cytokines, correlates with immunophenotype changes in human melanoma cells.³⁷

The results presented here allow us to conclude that cell lines derived from metastatic tissue appear to show a reduction in their response to cytokine-induced growth inhibition compared with the parental cell line. This complements the phenotypic traits previously reported for cell lines derived from advanced disease.^{1,2,15,47} Moreover, it has been shown that cytokine use in cancer patients often fails to achieve the desired antiproliferative effect.²⁵ We consider that our findings may help us to understand differences in the basic responsiveness of tumor cells to cytokines, which could influence tumor cell behaviour *in vivo*.

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