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Original Paper

High and Low Dose Rate Irradiation Have Opposing Effects on Cytokine Gene Expression in Human Glioblastoma Cell Lines

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Effects of radiation on five cytokine expressing human glioblastoma cell lines were studied. In comparison to unirradiated controls, IL-1β and IL-6 mRNAs were generally reduced after low (LDR, 1.0 cGy/min) and very low (VLDR, 0.35 cGy/min) dose rate irradiation. In contrast, high (HDR, 200 cGy/min) and intermediate (IDR, 4.1 cGy/min) dose rates increased steady-state levels of IL-1β and IL-6 mRNAs. The surviving fraction was generally inversely proportional to the dose rate; however, these glioma cells were unusually susceptible to LDR. In the two cell lines tested, IDR was less cytotoxic than either HDR or LDR irradiation. Although cytokine gene expression had no clear effect on radiation survival *in vitro*, autologous cytokines could be important to radiation response *in vivo* by affecting immune response, tumour stroma, vasculature or surrounding tissues. Adjusting dose rates to account for inverse dose rate effects and altered gene expression may be a useful strategy in optimising radiation therapy of glioblastomas. © **1997 Elsevier Science Ltd. All rights reserved.**

Key words: glioblastoma, radiation, cytokines

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INTRODUCTION

ASTROCYTOMAS REPRESENT the largest group of primary brain tumours. Radiation therapy with or without resection is the mainstay of management, and histological grade is the most significant clinical prognostic factor. *In vitro* studies have confirmed that cell lines derived from low-grade astrocytomas are more radiosensitive than those from high-grade tumours [1]. More research is needed to substantiate these observations, and to explore underlying mechanisms.

The role of cytokines in radiation response is largely undefined. A few studies have shown that exogenous cytokines can modulate radiation sensitivity, but no clear pattern has emerged [2]. For example, although IL-1 can protect mice from radiation-induced haematopoietic injury [3, 4] and dramatically increase radiation survival of human myeloid bone marrow progenitor cells *in vitro* [5], it can act as either a radiosensitiser or as a radioprotector in murine intestinal stem cells, depending on the timing of administration [6, 7]. *In vivo* tumour necrosis factor (TNF) can sensitise a number of cell types to radiation including human squamous cancer cells, but the mechanism of radiosensitisation remains unclear and has been difficult to duplicate *in vitro* [8]. IL-6 also appears to function as a radioprotector under certain circumstances [9], probably largely through interaction with other cytokines such as IL-1 and TNF. While the mechanisms underlying these observations are unknown, a recent report has implicated cytokines in regulation of apoptosis following irradiation [10].

Endogenous cytokine gene expression in human gliomas has received limited attention. In a study of 22 human glioma cell lines, along with tumours from 16 patients with malignant gliomas, significant expression of IL-1β and IL-6 was observed, but no attempt was made to correlate cytokine expression with radiation response or with tumour histology [11]. Other investigators reported that irradiation with 10–20 Gy resulted in increased IL-6 and IL-8 in culture supernatants from three glioma cell lines [12]. TGFβ has also been detected in glioma cell lines and in the CSF of patients with glial tumours [13]. To date, the role of en-

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dogenous cytokines in glioma pathophysiology and response to irradiation remains undefined. The five human glioblastoma cell lines studied here produce cytokines (IL-1β, IL-6, GM-CSF and TGFβ) that, in various tumour models, have been implicated in tumour growth, invasion and metastasis and immune interactions, and may modulate radiation resistance *in vivo*.

We have reported that high endogenous IL-1β expression has only minimal effect on *in vitro* radiation resistance in three of the cell lines studied here [14]. Cytokine gene expression could, however, indirectly influence tumour growth and sensitivity to treatment, for example, by modulating the immune response, tumour vasculature or stromal development [15–18]. New information is emerging both on the expression of cytokines by human gliomas and on the influence of cytokines on radiation response in a variety of cell lines and normal tissues. Because both fractionated high dose rate (HDR) external beam irradiation and continuous low dose rate (LDR) brachytherapy are employed in treatment of patients with glial tumours, we have studied high and low dose rate irradiation of glioma cell lines *in vitro*.

MATERIALS AND METHODS

Cell lines

Low-grade, non-tumorigenic (Hs683, U138) and high-grade, tumorienic (U373, U87) glioblastoma cell lines were obtained from the ATCC (American Type Culture Collection, Rockville, Maryland, U.S.A.). An additional grade IV glioblastoma cell line (ACBT) was a generous gift of G. Granger (UCI, Irvine, California, U.S.A.). Cells were cultured in DMEM (Fisher Scientific, Pittsburgh, Pennsylvania, U.S.A.) supplemented with 5% each of fetal and newborn calf serum (Irvine Scientific, Irvine, California, U.S.A.), and containing penicillin/streptomycin (100 U/0.1 mg/ml) (Fisher). Cultures were grown in humidified incubators at 37°C in 5% CO_2 and were passaged weekly.

Irradiation

Adherent cells were allowed to grow to near confluence in T-25 flasks (3–5 days post plating) and were irradiated with gamma rays from a self-shielded ^{137}Cs irradiator at dose rates of 0.35, 1.0, 4.1 and 200 cGy/min. Different dose rates were achieved by appropriate use of lead shielding and were confirmed by both Fricke's and Victoreen dosimetry. For the highest dose rate of 200 cGy/min, flasks were irradiated in the upright position at room temperature for not longer than 5 min. For the remaining dose rates, cells were irradiated in sealed flasks filled with media equilibrated with 5% CO_2 in air in a 37°C water bath with continuous mixing to maintain even temperature. No pH changes were observed during or after irradiation.

Assessment of radiation sensitivity

A cell proliferation assay was employed since most of these cell lines form very loose colonies when plated at low cell density making colony forming assays extremely difficult to score accurately. Cells were irradiated as subconfluent monolayers and plated into triplicate T-25 flasks (1×10^5 cells per flask) after holding at 37°C for 16 h post-irradiation to allow for potentially lethal damage repair (PLDR). Cells were counted by Coulter counter (Coulter Electronics, Hialeah, Florida, U.S.A.) at various times up to 10 days post-irradiation. The data were analysed by plotting growth curves and measuring the delay to grow to 2×10^5 cells/flask as a function of radiation dose. Radiation sensitivity was assessed by determining the dose required for an 8 day growth delay.

Cell cycle distribution

At least 1×10^6 cells per condition were harvested, fixed with ethanol and stained with propidium iodide by standard methods. Analysis was performed on a Becton-Dickinson flow cytometer and DNA profiles were analysed using CELLFIT version 2.01.2.

Analysis of mRNA

Cytoplasmic RNA was obtained from adherent cells by detergent lysis and phenol–chloroform extraction as described previously [19]. Each experiment was performed at least twice and representative Northern blots are shown. Northern blots of 20 μg/lane were sequentially hybridised as described previously, washed to a stringency of 0.1 × SSC at 65°C and exposed to film for 1–4 days at −80°C [19]. Blots were stripped in 0.1 × SSC, 0.1% SDS at 80°C between hybridisations. β-actin was used as a measure of radiation effect on housekeeping genes and rpL-32, which has been reported to be unaffected by irradiation, was used as a loading control. Autoradiographs were quantitated by scanning densitometer and analysed using the Molecular Analyst program (BioRad Laboratories, Hercules, California, U.S.A.).

DNA probes

GM-CSF cDNA probe (EcoR1, 800 bp) from pCSF1 and IL-6 cDNA probe (XhoI, 1.2 K bp) from pXM309 were generous gifts of S. Clark (Genetics Institute, Cambridge, Massachusetts, U.S.A.). IL-1β cDNA probe (PstI, 900 bp) was from pA-26 [20]. β-actin probe was from pHFA3'UT (EcoRI/BamHI, 0.7 bk) [21]. $TGF\beta_1$ probe was from pλBC1 (EcoRI, 1.0 kb) [22]. rpL32 was a generous gift of A. Maity [23]. Probes were labelled with [^{32}P]dCTP by random priming.

RESULTS

Effects of high dose rate irradiation on expression of IL-1β and other cytokines

Cultures were irradiated with 5 and 10 Gy at 2 Gy/min as described in the Materials and Methods section. Preliminary experiments were carried out to determine the time-course of changes in cytokine mRNA expression (not shown). Increased IL-1β, IL-6, GM-CSF and TGFβ mRNAs were detected within 1 h after irradiation returning to or below basal expression by 4 h. By 24 h post-irradiation, long lasting changes in cytokine gene expression began to occur, reached a maximum at around 36 h, were maintained for at least 72 h and resulted in altered cytokine levels in the culture supernatants. It was felt that the earliest changes in cytokine mRNA levels probably represent a non-specific response to injury while the changes at 24 h and beyond are of more interest in that they most likely represent specific responses to irradiation. We therefore chose to examine stable changes in cytokine gene expression 48–72 h after irradiation.

HDR irradiation induced expression of IL-1β in Hs683 and U138 in a dose-dependent manner with up to a 4-fold

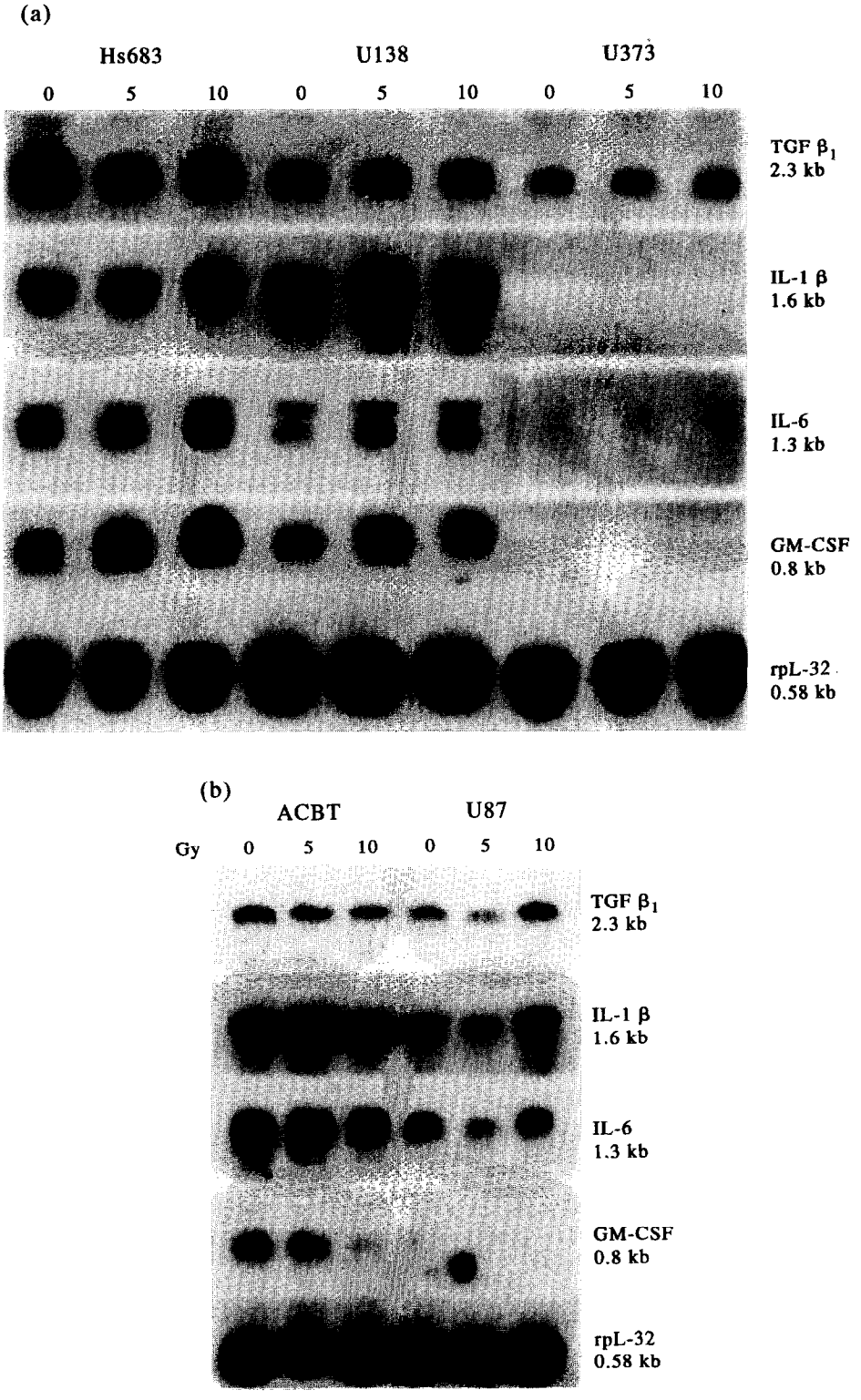

Figure 1. Response of cytokine genes to HDR irradiation. Subconfluent cell cultures were irradiated with 0, 5 and 10 Gy at 2 Gy/min. Northern blots prepared from Hs683, U138, U373 (a); ACBT and U87 (b) RNA extracted 72 h after irradiation were probed sequentially for the mRNAs shown. Autoradiographs were exposed for 1–4 days except for IL-6 in U373 which required an 8-day exposure. Signals were quantitated densitometrically as described in Materials and Methods.

increase seen 72 h after treatment with 10 Gy (Figure 1a). Similar inductions were seen for GM-CSF (6-fold) and IL-6 (4-fold). In contrast, TGFβ was slightly (<2-fold) suppressed in Hs683 and slightly increased in U138. U373 expressed only TGFβ which was minimally affected by HDR and IL-6 which was increased at least 4-fold after irradiation. It should be noted that U373 expresses very low amounts of IL-6 RNA compared to the other cell lines necessitating a longer exposure of the autoradiograph as shown.

Figure 1b shows that 10 Gy at HDR resulted in increased IL-1β (4-fold), IL-6 (2-fold) and TGFβ (2-fold) mRNAs in U87 cells. Conversely, IL-1β, IL-6, GM-CSF and TGFβ mRNAs were suppressed by 10 Gy in ACBT (<2-fold change in TGFβ, IL-1β and IL-6, but 3-fold decrease in GM-CSF). In contrast to the cell lines shown in Figure 1a, ACBT and U87 cells responded differently to 5 Gy than to 10 Gy. For ACBT IL-1β, IL-6 and GM-CSF, RNAs were slightly increased after 5 Gy but suppressed by 10 Gy. The opposite effect was seen in U87.

In all five cell lines, HDR resulted in acutely increased cytokine mRNAs (4 h) that we have interpreted as a response to injury. Cytokine gene expression returned to or below baseline by 24 h in four of the five cell lines, followed by a second increase resulting in the elevated mRNA levels found at 36–72 h. In ACBT, however, IL-1β, IL-6 and GM-CSF mRNA levels were persistently increased 24 h after 10 Gy (not shown), but were suppressed by 48 h. The anomalous findings in ACBT may indicate a different mechanism of alteration of cytokine gene expression in these cells, or a delayed time-course of response, perhaps due to some alteration in signal transduction. Further experiments would be needed to define this phenomenon better.

Effects of low dose rate irradiation on cytokine gene expression

Cells were irradiated at 37°C with up to 10 Gy at 1.0 cGy/min. In all cell lines except U87, IL-1β and IL-6 mRNA expression was decreased at least 5-fold 48 h after completion of irradiation (Figure 2). This decrease did not represent a general inhibition of gene expression in response to LDR irradiation, since β-actin mRNA was increased in all the cell lines except U373. In U87, IL-1β mRNA was increased 4-fold and IL-6 mRNA was increased 2-fold after LDR irradiation.

Effects of very low dose rate irradiation on cytokine gene expression

To explore further the unexpected suppression of cytokine mRNA levels by low dose rate irradiation, three cell lines were irradiated with 2.5 Gy at 0.35 cGy/min (VLDR, Figure 3). For the most part, changes in cytokine gene expression were of similar character and magnitude as for LDR (Figure 2). One exception was lack of suppression of IL-6 mRNA levels in the high-grade U373 cell line. It should also be noted that while β-actin was markedly suppressed by LDR in U373, no suppression was seen after VLDR. This difference may relate either to dose rate or to lower total dose although 5 Gy at LDR also decreased β-actin mRNA (not shown). In the low dose rate set-up used, it was impractical to maintain the cells in the irradiator for longer than 1000 min, hence the lower total dose at VLDR.

Effects of intermediate dose rate irradiation

To ascertain whether a threshold dose rate existed at which modulation of cytokine gene expression changed from suppression (low dose rates) to induction (high dose rates), we examined a low-grade cytokine expressing cell line (Hs683) and a high-grade, low cytokine expressing cell

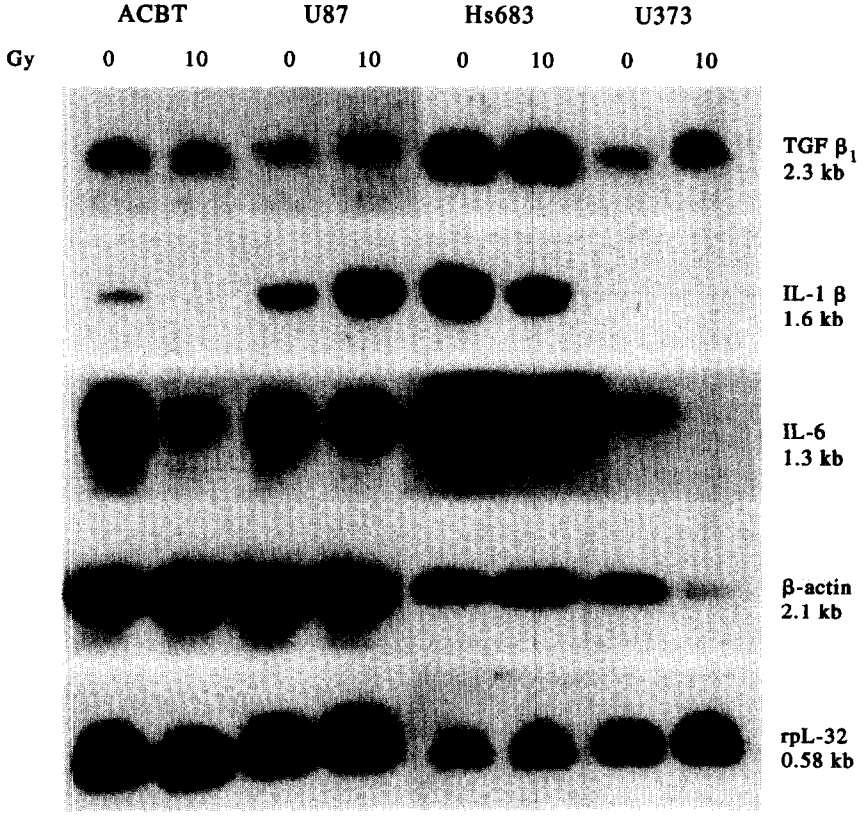

Figure 2. Response of cytokine genes to LDR irradiation. Subconfluent cell cultures were irradiated with 0 and 10 Gy at 1 cGy/min. Northern blots prepared from RNA extracted 48 h after irradiation were probed sequentially for the mRNAs shown. Autoradiographs were exposed for 1–4 days.

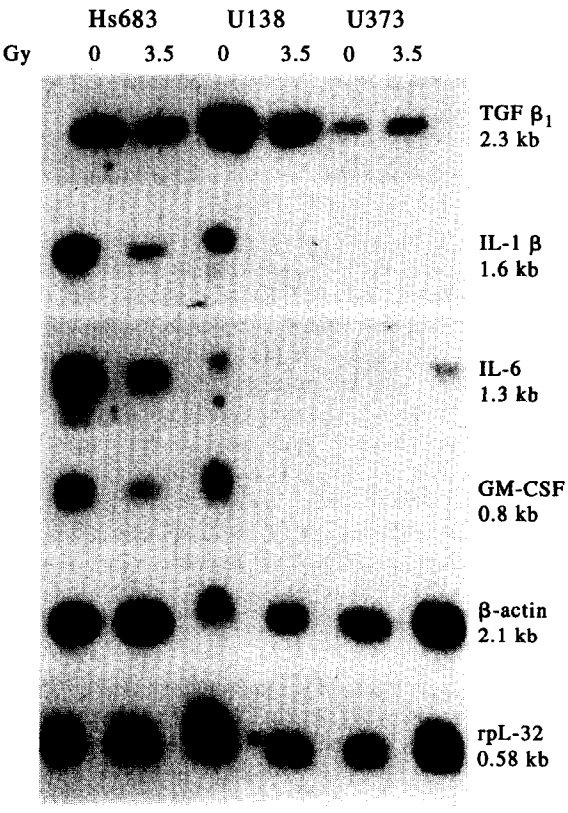

Figure 3. Response of cytokine genes to VLDR irradiation. Subconfluent cell cultures were irradiated with 0 and 3.5 Gy at 0.35 cGy/min. Northern blots prepared from RNA extracted 48 h after irradiation were probed sequentially for the mRNAs shown. Autoradiographs were exposed for 1–4 days.

line (U373) after intermediate dose rate (IDR) irradiation (10 Gy at 4.1 cGy/min). RNA was extracted after 36 h PLDR. Figure 4 shows that IL-1β, IL-6 and GM-CSF mRNA levels were increased 4-fold in response to IDR in Hs683 cells. IL-6 mRNA was also increased in U373 cells. β-actin mRNA levels were increased by IDR but to a lesser extent than the cytokine genes.

Effects of irradiation on cytokine protein levels

With the few exceptions noted above, high and intermediate dose rate irradiation tended to increase cytokine mRNA levels while low and very low dose rates tended to reduce them. ELISA (R&D Systems) confirmed that changes in cytokine mRNA are reflected in protein levels for all the cytokines tested. Data for IL-1β in one cell line are presented. For example, Hs683 produced 6.4 pg IL-1β per 10^6 cells in control flasks (4 ml media per flask), 32.6 pg/10^6 cells 72 h after 10 Gy HDR and 28.8 pg/10^6 cells 72 h after 10 Gy IDR. Levels were below the limit of detection (0.3 pg/ml) after LDR irradiation.

Growth delay after HDR and LDR irradiation

Because of the effect of dose rate on cytokine gene response to radiation, we were interested in possible differential effects on cell survival. Since most of these cell lines exhibited very poor colony formation, assessment of colony number was difficult necessitating a different approach to assess radiosensitivity, so it was decided to utilise a proliferation-based assay and measure growth delay as a function of dose (see Materials and Methods).

Figure 5 illustrates the growth curves obtained for one cell line irradiated with HDR, IDR and LDR. From plots such as this, the growth delay to reach a cell number of 2×10^5 was measured. Figure 6 shows these growth delays as a function of radiation dose for HDR and LDR irradiation for each cell line. Table 1 shows the dose required to obtain an 8 day delay in growth for both HDR and LDR irradiation, as well as relative sensitivity to LDR versus HDR for each cell line.

Only Hs683 showed the expected reduction in radiosensitivity typically associated with LDR radiation [24]. U87, U373 and U138 had, within experimental error, essentially equal sensitivities to LDR and HDR, while ACBT was considerably more sensitive (factor of 1.6) to LDR. Thus, four of the five cell lines can be stated to show an inverse dose rate effect at the low dose rate of 1 cGy/min. An inverse dose rate is thought to be a result of alterations in cell cycle distribution during irradiation such that a larger fraction of cells blocked in the radiosensitive G2 phase of the cell cycle would be exposed at the critical low dose rate [24]. However, the results reported here are somewhat unusual in

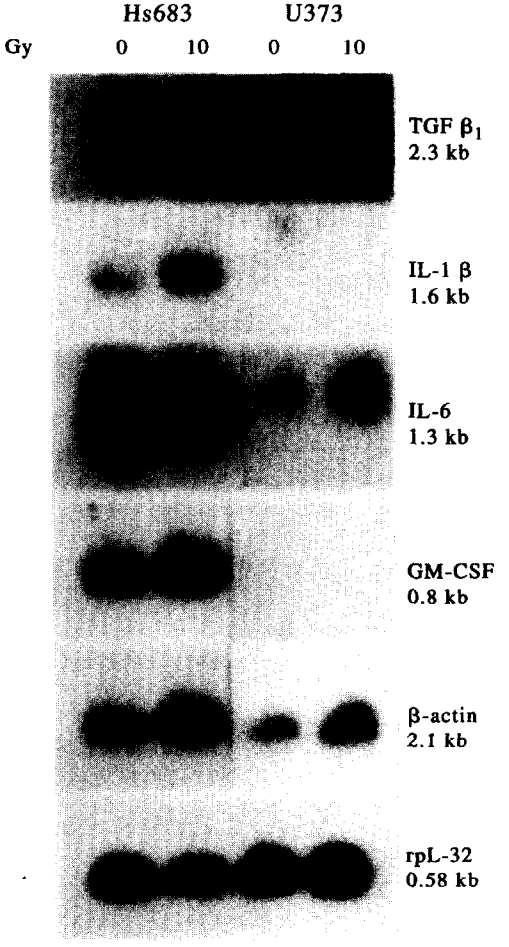

Figure 4. Response of cytokine genes to IDR irradiation. Subconfluent cell cultures were irradiated with 0 and 10 Gy at 4.1 cGy/min. Northern blots prepared from RNA extracted 36 h after irradiation were probed sequentially for the mRNAs shown. Autoradiographs were exposed for 1–4 days.

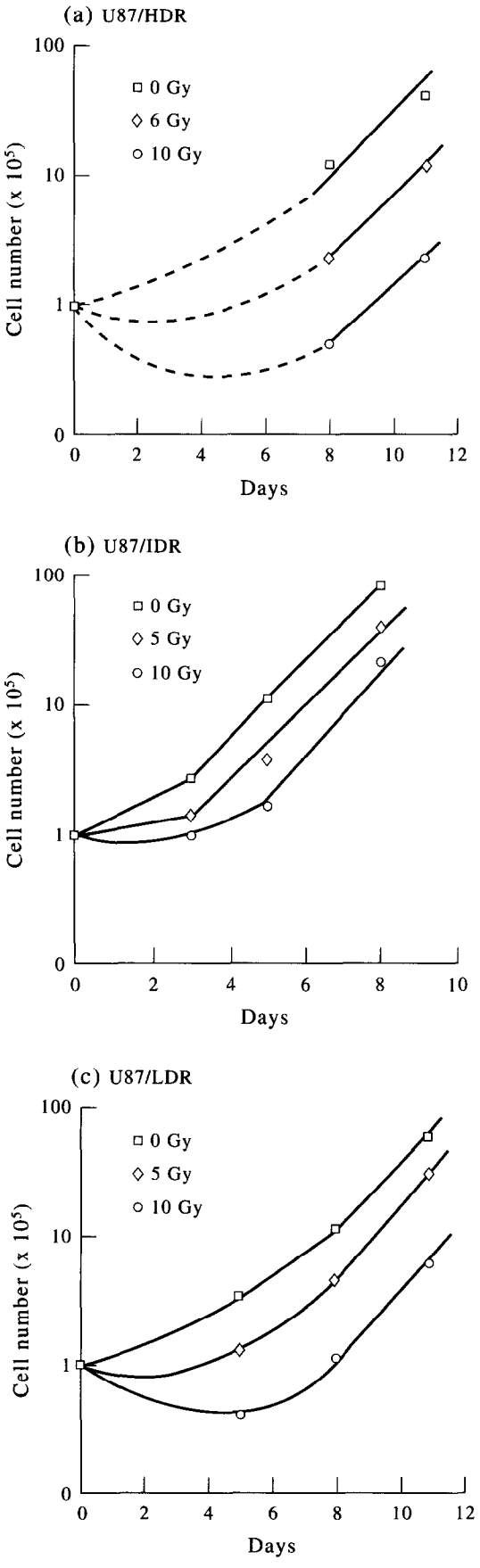

Figure 5. Growth delay after HDR, IDR and LDR irradiation. Subconfluent U87 cells were irradiated with 0, 6 and 10 Gy (HDR) or 0, 5 and 10 Gy (IDR, LDR), allowed to undergo PLDR, and plated in triplicate at 1×10^5 cells/T-25 flask. Cells were counted on the days shown. Viability of counted cells was >95% by Trypan blue exclusion. Standard deviations for the raw cell counts were less than 5%.

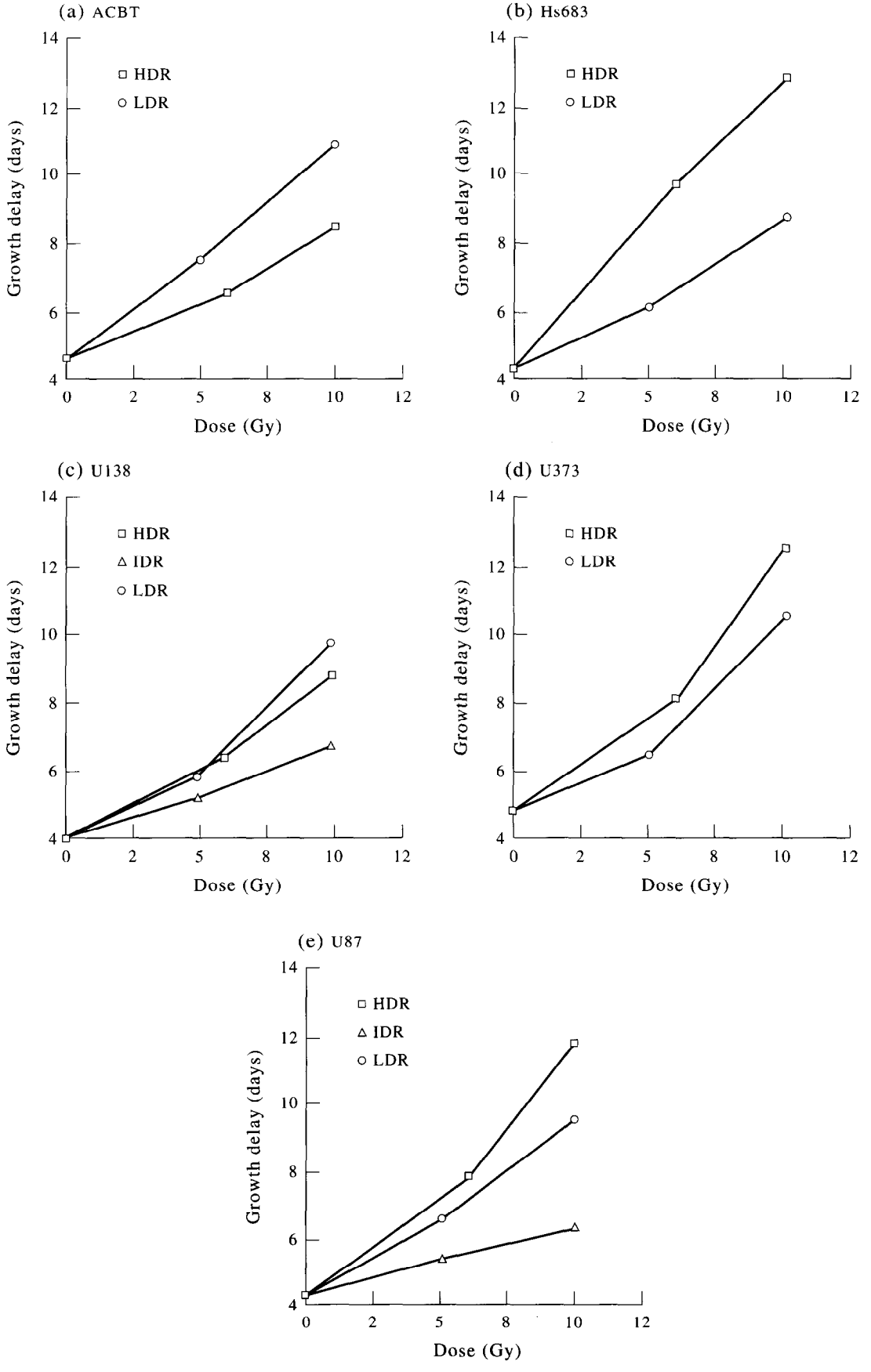

Figure 6. Growth delay as a function of radiation dose. Data from graphs, such as that shown in Figure 5, were plotted to show growth delay as a function of dose for HDR and LDR for each cell line. Data for IDR are included for U87 and U138.

Table 1. Dose for 8-day growth delay

Cell line	Dose for 8-day growth delay (Gy)		
	HDR	LDR	DMF (HDR/LDR)
Hs683	4.0	8.4	0.47
U87	6.0	7.4	0.81
U373	6.0	7.0	0.86
ACBT	9.0	5.5	1.63
U138	9.0	8.0	1.12

HDR, high dose rate; LDR, low dose rate; DMF, dose modifying factor.

Table 3. Relative change in cytokine mRNA level during low dose rate irradiation

	ACBT			Hs683		
	0 h	12 h	17 h	0 h	12 h	17 h
IL-β	1.0	2.0	4.0	1.0	0.7	1.0
IL-6	1.0	1.0	1.0	1.0	0.8	1.1

Values are derived from densitometric analysis of Northern blots. Ratio of baseline expression of IL-1β (0 time) is 5:1 for ACBT:Hs683. Ratio of baseline expression of IL-6 is 1:10 for ACBT:Hs683.

that the inverse dose rate effect has brought the sensitivity at LDR back to or even beyond that at HDR. For this reason, we chose to examine the sensitivity of two of the cell lines to IDR (4.1 cGy/min) where we rationalised that the typical reduction in effectiveness should be seen. The results of this study are shown in Figure 6 (c) and (e) where it can indeed be seen that IDR is less effective than either HDR or LDR.

Since the presence of an inverse dose rate effect has been hypothesised to be due to a significant change in cell cycle distribution during irradiation, we thought it pertinent to examine such changes in these cells during LDR irradiation.

Effects of irradiation on cell cycle distribution

Single-parameter flow cytometric analysis revealed induction of a significant G2 block in all of the cell lines during LDR irradiation. Table 2 indicates the percentage of cells in G2/M during the final 4.5 h of a 16.5-h dose delivery period. It can be seen that the percentage of cells in G2/M (36–65%) during this substantial fraction (25%) of the radiation dose delivery period is two to three times that for cells during the brief HDR exposure time. As no obvious correlation can be seen between the percentage of cells in G2/M and either the cell cycle time or the radiation response, some other reason must underlie the observed dose rate dependencies.

Cytokine gene expression during cell cycle delay after LDR irradiation

The effects of cytokine gene expression on the cell cycle in irradiated cells is unknown. Similarly, cell-cycle dependent cytokine gene expression has received little attention. Table 3 shows cytokine mRNA levels during the radiation-induced G2 block for Hs683 (the least sensitive to LDR) and for ACBT (the most sensitive to LDR). RNA was extracted at 0, 12 and 17 hours of irradiation (0, 7.2, 10.2 Gy) to correspond to the times at which cell cycle distribution was determined (Table 2). IL-1β and IL-6 mRNAs were reduced in both ACBT and Hs683 cells 48 h after LDR (Figure 2); however, ACBT cells exhibited significant induction of IL-1β mRNA during irradiation whereas there was no increase in IL-1β mRNA expression at these time-points in Hs683.

Table 2. Percentage of cells blocked in G2/M during low dose rate irradiation

Cell line	Tc (h)	Per cent G2/M (12–16.5 h)
ACBT	33	37–39
U87	29	36–46
U138	47	38–54
Hs683	38	44–52
U373	30	58–65

Tc, cell cycle time.

DISCUSSION

The data presented illustrate, for the first time, that the pattern of cytokine gene response to radiation can be dose rate dependent, at least in human glioma cell lines. Although a few exceptions have been noted, the general trend of our observations is that HDR irradiation induces an increase in the expression of certain cytokine mRNAs while LDR results in reduced expression. ELISA has confirmed these changes at the protein level.

The opposing effects of high and low dose rate irradiation on cytokine gene expression could not be correlated with radiation survival in our experiments indicating that differences in cytokine expression are unlikely to modulate *in vitro* radiosensitivity to any significant degree in glioma cell lines. Some authors have suggested that radioprotection could require cytokine interactions (e.g. between TNF and IL-1) [4]. Gliomas do not express TNF *in vitro*, but it is possible that TNF derived from endothelium or infiltrating haematopoietic cells could interact with tumour-derived IL-1 to influence *in vivo* radiosensitivity. Addition of TNF to our cultured tumour cells was cytotoxic, and TNF did not radiosensitise after either HDR or LDR irradiation (data not shown). This is not necessarily surprising, since most studies of cytokine modulation of radiosensitivity have been carried out *in vivo* and the synergistic interaction seen in whole animals is often no more than additive *in vitro* [2].

An inverse dose rate effect was seen at LDR (1 cGy/min) in four of the five cell lines. Only a few authors have addressed the inverse dose rate effect in glioma cells. Schultz and Geard reported a modest inverse dose rate effect in grade I astrocytoma cell lines, but no such effect was observed for higher grade tumours [1]. Marin and colleagues have also noted a mild inverse dose rate effect in a glioma cell line [25]. Considering whether these two observations relating to radiation dose rate—changes in the pattern of cytokine gene expression and the inverse dose rate effect—are causally linked, the results of the IDR studies showing attenuation of radiation toxicity in the face of similar effects on cytokine gene expression compared to HDR would suggest that, at least *in vitro*, it is not the case. However, the presence of an inverse dose rate effect indicates that optimising dose rates may be a useful strategy in maximising the efficacy of irradiation in patients with glioblastoma.

While a direct effect of endogenous cytokine gene expression on radiation survival *in vitro* is not apparent, it is likely that tumour-derived cytokines might influence *in vivo* radiation response. Possible areas of interaction might involve the tumour stroma or vasculature, host–tumour immune interactions or radiation damage to surrounding normal tissues. IL-1 and TGF-β have been reported to play a role in neovascularisation of tumours and could contribute to maintenance of the blood supply post-irradiation [26, 27]. All the cytokines studied here and many others can contribute to host immunosuppression and modulation of tumour–host interactions [28, 29]. A recent report has implicated tumour-associated cytokines in delayed cerebral radiation injury [18]. While this phenomenon probably would not influence tumour growth *per se*, it may be relevant to therapeutic design (i.e. low versus high dose rate irradiation).

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