Effect of radiation on cytokine and cytokine receptor messenger-RNA profiles in p53 wild and mutated human glioblastoma cell lines

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Abstract

Objective: Glioblastoma cells produce cytokines with proinflammatory or immunosuppressive properties, or both, which, in addition to altered *p53* gene expression, have been shown to be associated with glioblastoma resistance to radiotherapy. The reported data concerning cytokines have been isolated and sometimes discordant, and a comprehensive profile analysis of cytokines and their corresponding receptors in irradiated glioblastomas has received limited attention. The object of this study was to test the hypothesis that radiation alone in clinically relevant doses would not significantly alter expression of endogenous cytokines and their receptors in human glioblastoma celll ines with wild-type and mutant *p53*.
Design and method: Culture specimens of 4 glioblastoma cell lines of different *p53* gene expression (U87, U118, U251, U373) were irradiated with cobalt 60 at a dose of 10 Gy. After 48 hours, radiosensitivity was defined through a colony formation assay, cell cycle distribution was analyzed by flow cytometry, and cytokine and cytokine receptor messenger-RNA (mRNA) profiles were defined with an RNase protection assay. Different single doses of radiation at varying time intervals after culture were applied also to wild-type *p53* cell lines.
Results: All cell lines were relatively radioresistant at lower doses of 1 and 2 Gy. Immunosuppressive cytokine and cytokine receptor mRNA of the Th2 (*IL-13Rα*, *IL-4*) and Th3 family (*TGF-β1*, *2* and *3*, *TGF-βRI* and *RII*) were expressed. In contrast, only 2 proinflammatory Th1 cytokine receptor genes (*IFN-γRa* and *IFN-γRβ*), but no significant Th1 cytokine gene expression, were detected. Even though the population examined included a large fraction of reproductively dead cells, cytokine and cytokine receptor mRNA profiles were not altered significantly by irradiation in all cell lines, regardless of the *p53* status.
Conclusion: These results suggest that cobalt irradiation alone at clinically relevant doses does not significantly alter the cytokine and cytokine receptor profiles in human glioblastoma cell lines.

Résumé

Objectif : Les cellules de glioblastome produisent des cytokines aux caractéristiques pro-inflammatoires ou immunosuppressives, ou les deux, et qui, sans compter

qu'elles modifient l'expression du gène *p53*, sont associées à la résistance du glioblastome à la radiothérapie. Les données sur les cytokines qui ont fait l'objet de rapports sont isolées et parfois divergentes et une analyse détaillée du profil des cytokines et de leur récepteurs correspondants dans des glioblastomes irradiés a attiré peu d'attention. La présente étude visait à vérifier l'hypothèse selon laquelle la radiothérapie administrée à des doses pertinentes sur le plan clinique ne modifierait pas de façon significative, à elle seule, l'expression des cytokines endogènes et de leurs récepteurs dans les lignées de cellules de glioblastome humain comportant un gène *p53* du type sauvage et mutant.
Conception et méthode : On a irradié avec du cobalt 60 à une dose de 10 Gy des spécimens de culture de quatre lignées de cellules de glioblastome présentant une expression différente du gène *p53* (U87, U118, U251, U373). Après 48 heures, on a défini la radiosensibilité au moyen d'une épreuve de formation de cellules souches, analysé la répartition du cycle cellulaire par cytométrie de flux et défini au moyen d'une épreuve de protection de la RNase les profils de l'ARN messager (ARNm) des cytokines et de leurs récepteurs. On a appliqué à des lignées de cellules *p53* du type sauvage des doses uniques différentes de rayonnement à intervalles différents après les cultures.
Résultats : Toutes les lignées de cellules étaient relativement radiorésistantes à des doses plus faibles de 1 et 2 Gy. Des cytokines immunosuppressives et l'ARNm de récepteurs de cytokines des familles Th2 (*IL-13Rα*, *IL-4*) et Th3 (*TGF-β1*, *2* et *3*, *TGF-βRI* et *RII*) ont été exprimés. Par ailleurs, on a détecté seulement deux gènes des récepteurs des cytokines Th1 pro-inflammatoires (*IFN-γRα* et *IFN-γRβ*), mais aucune expression importante des gènes des cytokines Th1. Même si la population analysée incluait une fraction importante de cellules mortes pour la reproduction, l'irradiation n'a pas modifié considérablement les profils de l'ARNm des cytokines et des récepteurs de cytokines dans toutes les lignées de cellules, sans égard à l'état du gène *p53*.
Conclusion : Ces résultats indiquent que l'irradiation au cobalt à des doses pertinentes sur le plan clinique ne modifie pas de façon significative, à elle seule, les profils des cytokines et des récepteurs des cytokines des lignées de cellules du glioblastome humain.

Introduction

Radiosensitivity of malignant glioma cells can be modulated by glioma-derived products such as cytokines. For example, tumour necrosis factor-a (TNF-a) has been shown to sensitize glioma cells to radiation1 and, in contrast, interleukin-6 (IL-6) appears to function as a radioprotector in other models.2 Recent in vitro studies suggested that malignant glioma cells express proinflammatory Th1 cytokine receptors for TNF-a and interferon-g (IFN-g) but not Th1 cytokines. Interestingly, these cells express immunosuppressive Th2 cytokines, such as IL-6, and Th3 cytokines, such as transforming growth factor-b (TGF-b), as well as all corresponding receptors (Hao C, Parney IF, Roa WH, Turner J, Petruk KC, Ramsey DA. Unpublished data). These unbalanced cytokine and cytokine receptor profiles support the formation of autocrine growth loops. Clinically, the lack of proinflammatory Th1 cytokine expression in malignant gliomas may be associated with the tumour's virulence, whereas constant production of immunosuppressive Th2 and Th3 cytokines may permit tumour growth after radiation treatment.

Expression of *p53* gene has been shown to be associated with glioblastoma suppression.[3] Genetic analysis indicates that alterations of the tumour suppressor gene are associated with the development of malignant gliomas as well as various cell cycle changes in response to genotoxic stress.[4] Although many studies have examined either tumour suppressor gene or cytokine expression in glioblastomas, few have examined the interactions between these potent tumour growth-promoting factors and irradiation. In this study, we hypothesize that irradiation alone in clinically relevant doses would not significantly alter the unbalanced cytokine profile regardless of the *p53* status. Such persistent cytokine dysregulation may have clinical implications and contribute to tumour virulence and regrowth.[5] To test this hypothesis, we examined Th1, Th2 and Th3 cytokine and cytokine receptor profiles in both *p53* wild-type and mutant human glioblastoma cell lines in regard to radiation effect.

Materials and methods

Malignant glioma cell lines

Human glioblastoma cell lines (U87, U118, U251, U373) were obtained through the American Type Culture Collection (ATCC) and were cultured according to ATCC protocols. The *p53* gene status has recently been established in these cell lines.[6] Specifically, only U118 and U251 were reported to have *p53* mutations. U118 and U251 have been reported to have *p53* and *PTEN* mutations and *p14ARF/P16* deletion. U87MG are wild-type *p53*, but have been reported to have *p14ARF/P16* deletion and *PTEN* mutation.

Irradiation

Cells were irradiated at room temperature with a cobalt 60 (^{60}Co) gamma source (5.97 Gy/min). Culture medium was changed 1 day before irradiation; on the day of irradiation, cell cultures were observed with light microscopy to rule out any significant cell replication. Cells were irradiated with 10 Gy while they were attached. Forty-eight hours after further culture, the cell lines were assayed for cell cycle distribution and cytokine and cytokine receptor genes. Additional single doses (1 Gy, 5 Gy) and time intervals after culture (3 hours, 24 hours) were applied also to wild-type and mutated *p53* cell lines so as to delineate other possible effect.

Colony formation assay

Cells were counted and plated to form 50 to 200 colonies per 60-mm dish and were incubated for 24 hours before irradiation. After irradiation, the cells were incubated for 14 days, fixed with 5% formalin and rendered visible with crystal violet staining. Colonies containing more than 50 cells were counted under a microscope. The surviving fraction at each dose point was calculated as the ratio of the plating efficiency at that dose point to that of non-irradiated controls. Experiments at each dose point were repeated in triplicate for each cell line.

RNase protection assay

Total RNA from irradiated cells and controls was harvested using the TRIZOL method according to the manufacturer's (Canada Life Technologies, Burlington, Ont.) protocol. Quantification of mRNA encoding for cytokines and cytokine receptors was performed with the RiboQuant Multi-Probe RNase Protection Assay (RPA) System (PharMingen, San Diego). In brief, antisense RNA probes labelled with phosphorus 32 were formed from human cytokine and cytokine receptor templates using T7 RNA polymerase; 10 mg of RNA was hybridized overnight at 56 °C with a ^{32}P-labelled RNA probe set. RNase was used to digest free probe and other single-stranded RNA. Protected mRNAs were purified and resolved on denatured 5% polyacrylamide gels and then visualized by autoradiography for 15 hours at –80 °C. Specific cytokine transcripts were identified by the length of the respective fragments. Bands on gels were then quantified by densitometry on PhosphorImage analysis (Fujix, Tokyo). Relative cytokine and receptor levels were calculated by normalizing the specific cytokine band to the control ribosomal RNA L32 band included in the panel of probes with the commercially available kits. Experiments for each cell line were repeated in triplicate. Statistical comparison of the normalized bands from each gel was performed with Student's *t*-test for independent samples. Any statistically significant difference of 10% or more from baseline would be scored as a positive change in this study.

Flow cytometric analysis of cell cycle

Cells were prepared for flow cytometric DNA analysis after irradiation according to the technique outlined by Vindeløv and colleagues.[7] In brief, the cells were stained by sequential treatment with 0.003% trypsin, 0.05% trypsin inhibitor, 0.01% RNase buffer, followed by 0.0416% propidium iodide. Each treatment was performed for 10 minutes with continuous shaking at room temperature. Cell cycle analysis was performed within 2 hours of staining on a Becton Dickinson FACScan flow cytometer (Beckton Dickinson, San Jose, Calif.) equipped with the Doublet Discrimination Module (Becton Dickinson) and CellQuest software (Beckton Dickinson). Cell cycle distributions were analyzed with Modfit version 2.0 software (Beckton Dickinson).

Results

Radiosensitivity of cell lines

Radiation survival curves derived from colony formation assays of all cell lines are shown in Fig. 1. The general pattern of this collection of curves demonstrates the limited cell kill at lower radiation doses and increased cell kill at higher doses. Survival fraction at 2 Gy (SF2) ranged from 0.41 to 0.72. This wide range follows the pattern of reported in vitro radiosensitivities of human glioblastoma cell lines.[8]

Effect of irradiation on cell cycle distribution

Dose-dependent levels of cell cycle arrest were indicated in all cell lines after irradiation.

Cytokine and cytokine receptor profiles

Tables 1, 2 and 3 demonstrate the profiles of all cell lines, displaying Th1, Th2 and Th3 cytokine dysregulation. Cytokine and cytokine receptor genes belonging to the Th3 family (*TGF-β1*, *2* and *3* and *TGF-βRI* and *RII*) and the Th2 family (*IL-13*, *IL-13Rα*, IL-4 and *IL-4R*) were detected. Two Th1 cytokine receptor genes were detected in all cell lines (IFN-γRα and *γRβ*), although only 1 Th1 cytokine (*IFN-γ*) was barely detected.

Effect of irradiation on cytokine and cytokine receptor profiles

In all cell lines, levels of cytokine and cytokine receptor gene expression did not change (less than

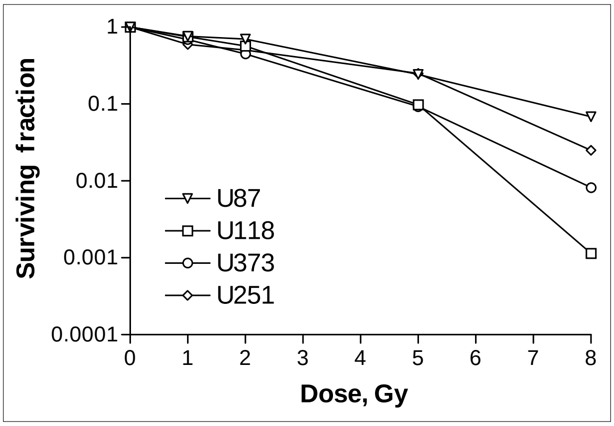

Fig. 1: Radiation survival curves of cell lines tested. All cell lines, independent of their *p53* status, display relative radioresistance at lower single doses of radiation at 1 and 2 Gy.

10% from baseline) 48 hours after 10 Gy irradiation with ^{60}Co (Table 1). Levels of cytokine and cytokine receptor gene expression did not change after 1 Gy, 5 Gy and 10 Gy irradiation with ^{60}Co in the *p53* wild-type cell line U87 or *p53* mutant cell line U373 (Table 2). Moreover, levels of cytokine and cytokine receptor gene expression did not change in the same cell lines at 3, 24 and 48 hours after 10 Gy irradiation with ^{60}Co (Table 3).

Discussion

In this study, we have shown that whereas single-fraction irradiation at clinically relevant doses is capable of inducing reproductive death in a significant proportion of human glioma cells in culture, the expression and character of the cytokine and cytokine receptor gene profile of the studied cells are not altered significantly. Nevertheless, alteration of cytokine profiles may still be induced by different radiation dose rates and total doses owing to different biologic processes.[9,10] Reported significant changes in TGF-b secretion in glioma cell lines after irradiation at doses higher than the ones used in this study (e.g., 50 Gy) may reflect a death process that is expected at such high doses.[5]

Single fractions of radiation were used in this study, as its purpose was to examine the early effects of radiation on the expression of cytokine and cytokine receptor genes. The use of fractionated radiation treatments may have allowed for the accumulation of delayed effects of radiation over a protracted course of radiation treatments, which could potentially obscure observations of the early effects of the treatments. In addition, a protracted course of radiation could possibly induce different repair mechanisms over time. If these concerns can be adequately understood and controlled for, incorporating fractionated radiation treatments will be our next step toward a more accurate model of clinical practice.

The human glioblastoma cell lines used in this study have a predominant immunosuppressive (Th2 and Th3) cytokine and cytokine receptor gene profile. The strongest genes detected in all cell lines are in the TGF-β family, which others have also detected in glioma cell lines.[11–13] Cytokines in the TGF-β family are known to play a prominent role in glioblastoma pathophysiology. TGF-β2 has significant immunosuppressive effects[14] and may contribute to impaired host immune response to gliomas and neovascularization of tumour tissue.[15] The confirmed presence of genes for the TGF-β family of cytokines and their receptors in all cell lines studied in this paper suggests that the TGF-β cytokines may exert effects on glioma cells by way of autocrine loops. The paucity of proinflammatory Th1 cytokine and cytokine receptor gene expression among all studied cell lines supports the speculation that Th1 cytokines may act to adversely affect the growth of glioblastoma. The ability of TNF-α, a Th1 cytokine, to sensitize prostate cancer cells to radiation-induced apoptosis has been reported.[16]

The RNase protection assay used in this study detected only genes for cytokine and cytokine receptors and not the active proteins. This is a potential limitation of the study, as changes in the level of expression of these genes or lack thereof may not accurately reflect what changes, if any, are occurring at the level of actual proteins. For example, radiation doses well below 1 Gy have been shown to activate a latent form of TGF-β. Assays for radiation-induced changes at the levels of the actual proteins would be a valid avenue of investigation in the future.

Classic radiobiology suggests that mitotic cell death is a major contributor to the decrease in clonogens after irradiation, and this phenomenon is often accompanied by cell cycle arrest and redistribution of cells throughout the cell cycle. Despite observations that *p53* status can be an important factor in radiation-induced apoptosis in response to radiotherapy,[17,18] there is evidence that *p53*-independent pathways determining radioresponsiveness exist[19] and

Table 2: Effect of various doses of cobalt-60 irradiation on levels of cytokine and cytokine receptor messenger-RNA profiles*

	Cell lines					
	U373			U87		
Profile	1 Gy	5 Gy	10 Gy	1 Gy	5 Gy	10 Gy
IFN-γ	–	–	–	–	–	-
IFN-γRα	++	++	++	+	+	++
IFN-γRβ	+++	+++	+++	++	++	+++
IL-13	–	–	–	–	–	–
IL-13Rα	+	+	++	+	+	+
IL-4	–	–	–	–	–	–
IL-4R	+	+	++	+	+	++
TGF-β1	++++	++++	++++	++++	++++	++++
TGF-β2	++++	++++	++++	++	++	++
TGF-β3	+	+	+	+	+	+
TGF-βRI	+++	+++	+++	+++	++++	++++
TGF-βRII	+++	++++	+++	++++	++++	+++
M-CSF	++++	+++	+++	+	+	++

*Levels of expression are indicated relative to the control L32 ribosomal RNA band.
– = <1%, + = 1% to 5%, ++ = 6% to 10%, +++ = 11% to 20%, ++++ = >20%

Table 1: Effects of 10 Gy of cobalt-60 (^{60}Co) irradiation on cytokine and cytokine receptor messenger-RNA profiles in 4 human glioblastoma cell lines*

	Cell lines							
	U251		U373		U118		U87	
Gene profile	0	^{60}Co	0	^{60}Co	0	^{60}Co	0	^{60}Co
IFN-γ	–	–	–	–	–	ND	–	–
IFN-γRα	+	+	++	++	++	+	+	++
IFN-γRβ	++	++	+++	+++	++	++	++	++
IL-13	–	–	–	–	–	–	–	–
IL-13Rα	+	+	+	++	ND	ND	+	+
IL-4	–	–	–	–	–	–	–	–
IL-4R	+	+	+	++	ND	ND	++	++
TGF-β1	++	+++	+++	++++	++++	++++	+++	++++
TGF-β2	++	+++	+++	++++	+++	++	+	++
TGF-β3	+	+	+	+	++	+	+	+
TGF-βRI	+++	+++	+++	+++	++++	++++	++++	++++
TGF-βRII	+++	+++	+++	+++	++++	++++	++++	++++
M-CSF	+	+	+++	+++	++	++	+	++

*Levels of expression are indicated relative to the control L32 ribosomal RNA band.
– = <1%, + = 1% to 5%, ++ = 6% to 10%, +++ = 11% to 20%, ++++ = >20%.

that, clinically, *p53* may not be the most important overall prognostic factor determining radioresponsivness.[20] Radiation-induced apoptosis in human glioblastoma cells that is independent of *p53* status has also been demonstrated,[21] and one of the *p53*-independent pathways determining radiation-induced apoptosis and radiosensitivity may involve glioma-derived Th1 cytokines.[22] Although the population examined for cytokines and cytokine receptors in this study included a large fraction of reproductively dead cells, there was no change in the cytokine expression profile. Because of the limited sample size used in this study, caution should be used in making sweeping generalizations about the importance of *p53* status in determining changes in the expression of the genes assayed for in this study.

Taken together, the observed reproductive cell death may play a pivotal role in the survival of clonogens in the studied cell lines after irradiation. The lack of changes seen in the cells' cytokine and cytokine receptor mRNA profiles after irradiation strongly suggests that these quiescent cells retain the ability to secrete dysregulated cytokine and cytokine receptor profiles. Upon re-entry into the cell cycle, cells that survived the various radiation-induced cytotoxic mechanisms will be fed again by autocrine loops. The predominantly immunosuppressive Th2 and Th3 cytokine profile may act after irradiation to promote tumour growth directly and suppress any effective immune response against the tumour. This can ultimately account for the clinical recurrences after irradiation that are characteristic of the natural history of human glioblastomas.

Modulation of cytokine-mediated immunosuppression, tumour radioresponsiveness and tumour regrowth after irradiation may provide promising therapeutic avenues in the future management of human glioblastomas. Studies involving rat gliomas treated with antisense therapy targeted against TGF-β and retroviral suppression of TGF-β1 secretion in human cell lines have reported promising results.[23–25] The translational hypothesis that locally infused Th1 cytokines would sensitize human glioblastomas used in clinical practice is yet to be confirmed. Further investigations on the effects of modulating tumour-derived cytokines in our laboratory and clinic will be pursued.

Table 3: Changes in cytokine and cytokine receptor messenger-RNA profiles over time after irradiation with 10 Gy of cobalt-60*

	Cell lines					
	U373			U87		
Profile	3h	24 h	48 h	3h	24 h	48 h
IFN-γ	–	–	–	–	–	-
IFN-γRα	+	++	++	+	+	++
IFN-γRβ	++	+++	+++	++	++	+++
IL-13	–	–	–	–	–	–
IL-13Rα	+	++	++	+	+	+
IL-4	–	–	–	–	–	–
IL-4R	+	+	++	+	+	++
TGF-β1	++++	++++	++++	++++	++++	++++
TGF-β2	+++	+++	++++	+	+	++
TGF-β3	+	+	+	+	+	+
TGF-βRI	+++	++++	+++	++++	++++	++++
TGF-βRII	++++	++++	+++	++++	++++	++++
M-CSF	+++	+++	+++	+	++	++

*Levels of expression are indicated relative to the control L32 ribosomal RNA band.
– = <1%, + = 1% to 5%, ++ = 6% to 10%, +++ = 11% to 20%, ++++ = >20%

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