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Research Report

The Survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (–)-epigallocatechin-3-gallate

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ABSTRACT

Introduction: Glioblastoma multiforme's (GBM) aggressiveness is potentiated in radioresistant tumor cells. The combination of radiotherapy and chemotherapy has been envisioned as a therapeutic approach for GBM. The goal of this study is to determine if epigallocatechin-3-gallate (EGCg), a green tea-derived anti-cancer molecule, can modulate GBMs' response to ionizing radiation (IR) and whether this involves mediators of intracellular signaling and inhibitors of apoptosis proteins. *Material and methods:* U-87 human GBM cells were cultured and transfected with cDNAs encoding for Survivin, RhoA or Caveolin-1. Mock and transfected cells were irradiated at sublethal single doses. Cell proliferation was analyzed by nuclear cell counting. Apoptosis was detected using a fluorometric caspase-3 assay. Analysis of protein expression was accomplished by Western immunoblotting. *Results:* IR (10 Gy) reduced control U-87 cell proliferation by 40% through a caspase-independent mechanism. The overexpression of Survivin induced a cytoprotective effect against IR, while the overexpression of RhoA conferred a cytosensitizing effect upon IR. Control U-87 cells pretreated with EGCg exhibited a dose-dependent decrease in their proliferation rate. The growth inhibitory effect of EGCg was not antagonized by overexpressed Survivin. However, Survivin -transfected cells pretreated with EGCg became sensitive to IR, and their RhoA expression was downregulated. A potential therapeutic effect of EGCg targeting the prosurvival intracellular pathways of cancer cells is suggested to act synergistically with IR. *Conclusion:* The radioresistance of GBM is possibly mediated by a mechanism dependent on Survivin in conjunction with RhoA. The combination of natural anti-cancerous molecules such as EGCg with radiotherapy could improve the efficacy of IR treatments.

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1. Introduction

Glioblastoma multiforme (GBM) represents the most aggressive and invasive primary cerebral neoplasm in the adult population. Median length of survival without further therapy is usually less than 1 year from the time of diagnosis (Davis et al., 1999; Glioma Meta-analysis Trialists (GMT) Group, 2002). When surgical excision is considered, the goal should be gross total removal to prolong quality survival (Keles et al., 1999). However, the effect of surgical resection on the time to tumor progression and the median length of survival can only be optimized when combined with other therapies. For instance, conventional local field radiotherapy has been shown by itself to prolong median survival for 6–8 months (Fine et al., 1993). Unfortunately, 90% of patients receiving radiation therapy following GBM resection still develop tumor recurrence in the proximity of the primary site (Hess et al., 1994). Neither whole brain irradiation nor high focal radiation doses can decrease the incidence or change the location of recurrences (Sharma et al., 2003). Ionizing radiation (IR)-induced alterations of the malignant behavior are not unique to astrocytic tumors and are still poorly understood (Ohuchida et al., 2004). Recent studies have reported that GBMs' recurrence following IR is partially mediated by an enhanced invasive character of radioresistant tumor cells, which makes them more difficult to treat (Cordes et al., 2003; Wild-Bode et al., 2001).

Chemotherapy is thus used either as an adjuvant, concurrent or preirradiation treatment along with radiotherapy for malignant primary tumors. However, only modest benefits in survival have been reported (Fine et al., 1993; Lanzetta et al., 2003; Scott et al., 1999). More recently, the combination of chemotherapeutic drugs such as temozolomide with molecules exhibiting an anti-angiogenic activity has been shown to be safe and more effective with respect to survival than the administration of the chemotherapeutic agent alone (Baumann et al., 2004; Tuettenberg et al., 2005). Also attention has been focused on identifying naturally occurring substances capable of inhibiting, retarding or reversing the multistage carcinogenesis process. Recent reports have proposed that some phytochemicals can function as sensitizers, augmenting the effectiveness of conventional radiotherapy (Chendil et al., 2004; Fulda and Debatin, 2004). Epigallocatechin-3-gallate (EGCg), a major anti-oxidative green tea polyphenol, has been recognized for its anti-mutagenic and anti-carcinogenic properties (Demeule et al., 2002; Surh, 1999). More recently, we have shown that EGCg also possessed anti-angiogenic properties as it suppressed vascular endothelial growth factor receptors functions in endothelial cells (Lamy et al., 2002). We have also demonstrated that EGCg efficiently targeted endothelial cells that escaped IR-induced apoptosis (Annabi et al., 2003). Whether this natural polyphenol can be used to target prosurvival pathways involved in GBM radioresistant phenotype is unknown.

The identification of the molecular mechanisms underlying GBM radioresistance thus becomes essential for the development of combination therapies against this lethal condition. Survivin, along with other markers, has been proposed as a major factor for radioresistance in glioblastomas (Chakravarti et al., 2004). Survivin, belonging to the family of inhibitor of apoptosis proteins, is involved in the modulation of apoptosis (Altieri, 2003; Conway et al., 2000; Li et al., 1998; Shin et al., 2001) in the regulation of cell growth (Altieri, 2003; Suzuki et al., 2000; Temme et al., 2005a), in the regulation of mitotic events such as chromosomal segregation and cytokinesis (Temme et al., 2003; Wheatley et al., 2001) and in the process of angiogenesis (Blanc-Brude et al., 2003; O'Connor et al., 2000b). Its expression has been associated with enhanced malignant potential of gliomas, increased cell viability after IR exposure and adverse clinical prognosis (Chakravarti et al., 2002; Kajiwara et al., 2003). Rho proteins, which belong to a family of small GTPases, are also involved in the control of key cellular processes such as modulation of the cytoskeleton, receptor internalization or cell adhesion (Aznar and Lacal, 2001; Etienne-Manneville and Hall, 2002; Wheeler and Ridley, 2004). More specifically, farnesylated RhoB pathway has been suggested as a key factor in glioblastoma resistance to IR. Indeed, overexpression of RhoB in radiosensitive cells increased cell survival after IR (Ader et al., 2002). Conversely, inhibition of RhoB led to the appearance of multinucleated cells and induced a post-mitotic cell death that led to decreased glioma cell survival (Ader et al., 2003). Interestingly, the same effect was observed in glioma cells transduced with a $p34^{cdc2}$ phosphorylation-defective SurvivinT34A, suggesting a link between Survivin and Rho proteins (Temme et al., 2005b). Although studies have shown that the expression of RhoA and RhoB was similar in brain tumors of grades II to IV (Forget et al., 2002), the specific role of RhoA, in conjunction with Survivin, in glioma radioresistance remains to be investigated. Caveolin-1, a protein associating RhoA to caveolae-enriched membrane domains (Gingras et al., 1998; Regina et al., 2004), is proposed to participate in cell survival and angiogenesis (Massimino et al., 2002; Razani et al., 2001). The molecular implication of Caveolin-1 in glioma radioresistance has also not yet been investigated. Hence, the goal of this study is to determine whether EGCg can sensitize GBMs' response to radiation and whether specific molecular markers are involved.

2. Results

2.1. *Sublethal, low, single dose IR partially inhibits U-87 and DAOY cell proliferation*

We examined the proliferation rates of several human high grade astrocytoma (U-138, U-118, U-87) and medulloblastoma (DAOY) cell lines in response to increasing doses of IR (Fig. 1). We observed a dose-dependent decrease of the cell proliferation rate at increasing IR doses, up to 30 Gy. DAOY was the most radiosensitive cell line, as the proliferation rate was decreased by 70% after 10 Gy and by 95% after 30 Gy exposures. U-87 cells were the most radioresistant cell line when compared to the other astrocytoma cell lines; their proliferation rate was decreased by only 40% after 10 Gy and by 50% after 30 Gy exposures.

2.2. *Sublethal, low, single dose IR inhibits U-87 cell proliferation by a caspase-independent mechanism*

In order to assess whether the decrease in cell proliferation was due to IR-induced caspase-mediated apoptosis,

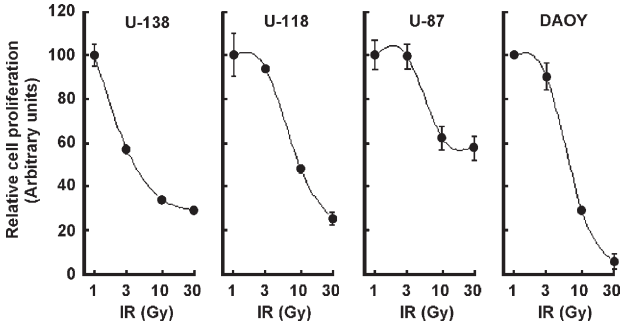

Fig. 1 – Effects of ionizing radiation on the proliferation rates of malignant glioma and medulloblastoma cell lines. Single dose ionizing radiation (IR) was applied to subconfluent cells, and cell proliferation rate was assessed 18 hour after IR using an automatic nuclear counter and Trypan blue staining. Cell proliferation is expressed as a percent of the non-irradiated (control) cell proliferation.

we measured caspase-3 activity. In all human astrocytoma cell lines analyzed, radiation exposure up to 10 Gy did not induce caspase-3 activity (Fig. 2). However, at 30 Gy, the caspase-3 activity was increased 2-fold in U-138 and U-118 cells, whereas in U-87 cells, no significant increase in caspase-3 activity was detectable. In contrast, IR induced significant caspase-3 activity in DAOY cells even at doses as low as 3 Gy. These results prompted us to further investigate the possible apoptosis-independent mechanisms underlying the observed radioresistance of U-87 cells.

2.3. *Low dose IR induces the expression of prosurvival proteins in U-87 cells*

We investigated the protein expression of Survivin, RhoA and Caveolin-1 in irradiated U-87 cells. We selected 10 Gy as the delivered IR dose since it was associated with a significant decrease in cell proliferation and absence of caspase-3 activity (Figs. 1 and 2). After exposure to IR, Survivin expression in U-87 control cells increased twofold over basal expression, while that of RhoA increased fourfold (Figs. 3A and B). Interestingly, the expression of Caveolin-1, a protein regulating RhoA functions, also increased by 4.5-fold in irradiated U-87 cells. β-actin and total Erk protein levels were not affected by IR (Fig. 3A).

2.4. *Overexpression of Survivin and RhoA exhibit antagonistic effects on the radioresistant phenotype of U-87 cells*

U-87 cells were cultured to 60% confluence and transfected with cDNA constructs encoding Survivin, RhoA or Caveolin-1 proteins. Transfection efficiencies were assessed by the presence of GFP positive cells in Survivin and Caveolin-1 transfectants (Fig. 4A) and by immunodetection for ectopic RhoA protein in RhoA-transfected cells (Fig. 4B). Surprisingly, ectopic expression of Survivin induced RhoA, whereas recombinant Caveolin-1 did not (Fig. 4B). Mock-transfected controls and cells either expressing Survivin, RhoA or Caveolin-1 were then exposed to increasing doses of IR. In mock-transfected U-87 cells, we observed a dose-dependent decrease in the cell proliferation rate (open circles) when cells were irradiated with doses up to 30 Gy. Interestingly, the proliferation rate of U-87 cells overexpressing Survivin (closed circles) did not decrease upon IR treatment and maintained a proliferation rate similar to non-irradiated cells (Fig. 4C). This clearly demonstrates that Survivin confers resistance to irradiation. Strikingly, cells overexpressing RhoA (closed squares) exhibited a significantly decreased cell proliferation rate in comparison to mock U-87 cells (Fig. 4C). Overexpression of RhoA thus seems to render U-87 cells more sensitive to IR resulting in a decreased proliferation capacity. Overexpression of Caveolin-1 (open squares) had no influence on the U-87 cell proliferation rate (Fig. 4C).

2.5. *Survivin overexpression does not antagonize EGCg's anti-proliferative effect*

Recent studies have demonstrated that EGCg, a green tea-derived polyphenol, inhibits the growth of various human cancer cell lines (Baatout et al., 2004; Rosengren, 2003; Takada et al., 2002), particularly human glioblastoma cells (Yokoyama et al., 2001). We further investigated the effect of EGCg on U-87 glioma cell proliferation. We show that EGCg indeed inhibited cell proliferation in a dose-dependent manner with an optimal inhibitory effect at 25 μM of more than 70% (Fig. 5A). Caspase-3 activity was measured in parallel and found not to be significantly induced by EGCg (Fig. 5A), suggesting that the inhibition in cell proliferation was not due to apoptosis. Cells were cultured to 60% confluence and transfected with cDNAs encoding for Survivin, RhoA or Caveolin-1, treated with EGCg and left to recuperate overnight. Maximal inhibition of proliferation (35–40% of untreated cells) was achieved with 25 μM EGCg (Fig. 5B). The growth of U-87 cells overexpressing RhoA, Survivin and Caveolin-1

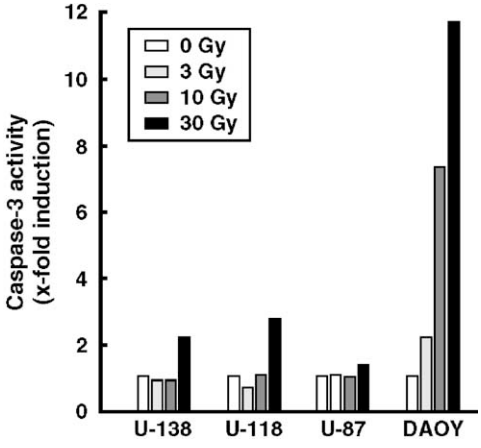

Fig. 2 – Ionizing radiation effects on caspase-3 activity in malignant glioma and medulloblastoma cell lines. Cells were grown to 60% confluence and were exposed to a single dose of IR. A representative caspase-3 activity profile for each cell line tested is presented and was assessed as described in the Experimental procedures section.

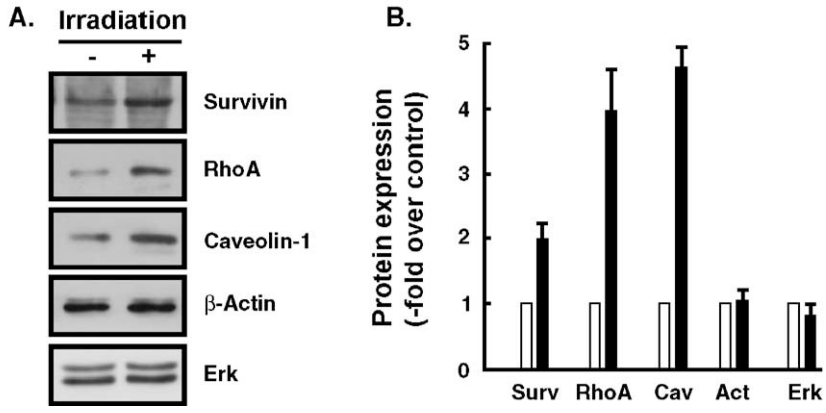

Fig. 3 – Expression of prosurvival proteins in malignant glioma cells exposed to ionizing radiation. U-87 cells were cultured and exposed to 10 Gy IR. Cell lysates isolated from control and irradiated conditions were electrophoresed on SDS-gels, and transferred to PVDF membranes. (A) Immunodetection of the specific proteins was carried out as described in the Experimental procedures section. (B) Quantification of protein expression was performed by scanning densitometry of control, non-irradiated U-87 cells (white bars) and of irradiated cells (black bars).

was also decreased by EGCg, similarly to the mock U-87 cells. We conclude that neither RhoA, Survivin, nor Caveolin-1 was able to fully reverse EGCg's anti-proliferative effect. Individual, maximally inhibitory treatments with EGCg (25 μM) and IR (10 Gy) were also compared to show that only Survivin was able to reverse the inhibitory effect that IR has on cell proliferation.

2.6. *Effect of combined EGCg and low dose IR on U-87 glioma cells proliferation*

We studied the effect of a combined approach consisting of pretreating U-87 cells with EGCg followed by exposure to sublethal single IR doses on the protein expression levels of Survivin, RhoA and Caveolin-1. EGCg did not affect the steady-state protein levels of Survivin, RhoA and Caveolin-1 in non-irradiated cells (Fig. 6A). Interestingly, while IR induced the expression of Survivin, RhoA and Caveolin-1, EGCg pretreated cells showed a dose-dependent decrease in the IR-induced protein expression of Survivin and of RhoA (Fig. 6A). The protein expression levels of Caveolin-1 were increased in cells treated with both EGCg and irradiation. The effect of this combined treatment was further investigated with respect to RhoA expression. U-87 cells were transfected with the Survivin cDNA, which induced RhoA expression (Figs. 4B and 6B). Interestingly, this effect was reversed when Survivin-transfected cells were treated with EGCg but not when they were exposed to IR (Fig. 6B). Consequently, the cytoprotective effect that Survivin has towards IR may, in part, be caused by RhoA signaling, which is significantly abrogated when cells are treated with EGCg. Most importantly, cell proliferation assays showed that the combined EGCg/IR treatment was able to significantly decrease cell proliferation when compared to individual EGCg or IR treatments (Fig. 6C, left panel). Furthermore, the combined EGCg/IR treatment in U-87 cells overexpressing recombinant Survivin was able to reverse the cytoprotective effect that Survivin had towards IR (Fig. 6C, right panel). Prosurvival intracellular pathways might thus be targeted by EGCg, a phytochemical that could be efficiently used as an adjunct to radiotherapy.

3. Discussion

Glioblastomas multiforme (GBM) are highly invasive primary tumors of the adult central nervous system. Although radiotherapy is routinely prescribed in the management of high grade gliomas, its efficacy remains limited because of tumor cell radioresistance, enhanced invasive character following radiation and resultant tumor recurrence (Cordes et al., 2003; Wild-Bode et al., 2001). In our study, we first analyzed the sensitivity of various human high-grade astrocytoma- and medulloblastoma-derived cell lines to ionizing radiation (IR). U-87 glioma cells were found to be the most radioresistant cell line, while DAOY medulloblastoma cells were the most radiosensitive cell line tested. This is in accordance with the reported radioresistance of glioblastoma cells (Chakravarti et al., 2004; Cordes et al., 2003) and radiosensibility of medulloblastoma cells (Leith et al., 1994). Studies have indeed shown that p53-mediated apoptosis is an important response of medulloblastomas to radiotherapy (Dee et al., 1995). In contrast, p53 mutations are present in as much as 40–60% of GBMs (Wu et al., 1993), suggesting that either prosurvival mechanisms or p53-independent events may regulate their therapeutic response to IR (Haas-Kogan et al., 1996).

In light of the emergence of several markers of glioma malignant progression, the molecular characterization of GBMs' radioresistance was next investigated. Among these markers, Survivin has been recently related to the malignant phenotype of gliomas as its expression is associated with glioma progression from low- to high-grade (Chakravarti et al., 2002; Kajiwara et al., 2003). Furthermore, Survivin expression is well recognized as a predictor of adverse clinical prognosis for patients with gliomas (Chakravarti et al., 2002), as it increases survival of primary GBM cell lines through its capacity to suppress caspase-mediated apoptosis (Chakravarti et al., 2004) by directly binding to caspase-9 or by interacting

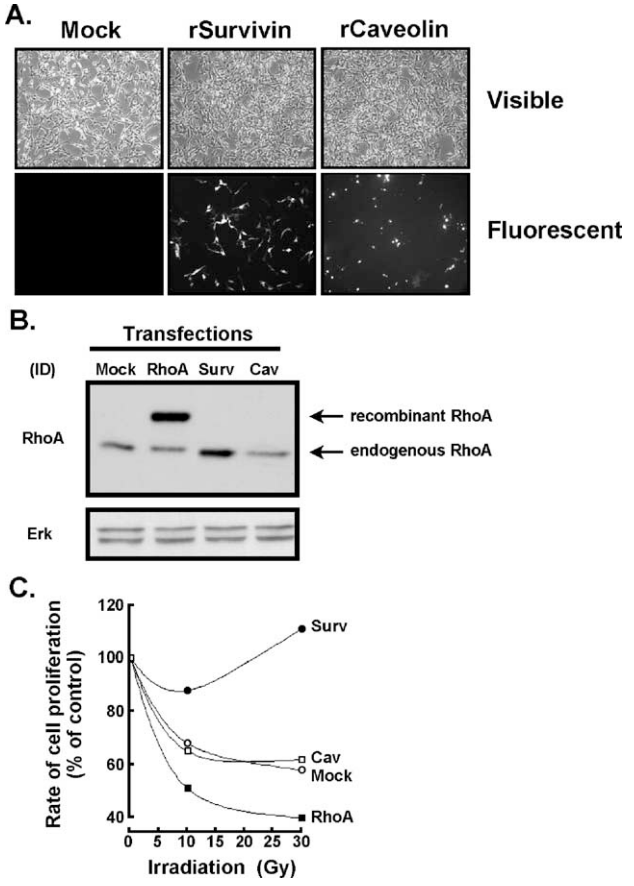

Fig. 4 – Effect of IR exposure on the proliferation rates of malignant glioma cells transfected with proteins involved in radioresistance. U-87 cells were transfected with cDNA constructs encoding for recombinant Survivin, Caveolin-1 or RhoA. (A) Transfection efficiency was confirmed by fluorescent microscopy in cells transfected with Survivin and Caveolin-1 and (B) by Western blotting in cells transfected with RhoA. (C) A dose-dependent decrease in the mock U-87 (open circles) proliferation rate is observed as cells are irradiated with increasing sublethal doses. The proliferation rate of U-87 cells transfected with Survivin (closed circles), RhoA (closed squares) and Caveolin-1 (open squares) is also shown.

with smac-DIABLO (O'Connor et al., 2000a; Song et al., 2003). The effects of Survivin in our study support the lack of IR-induced growth inhibition that we observed in GBM. Furthermore, Survivin is also implicated in the regulation of cell division (Altieri, 2003; Suzuki et al., 2000) as it is localized in multiple components of the mitotic apparatus and centrosomes and participates in cell cytokinesis (Temme et al., 2003; Wheatley et al., 2001), a process that involves RhoA (Amano et al., 1996). Novel functions of Survivin have been proposed to emerge following IR such as double-strand DNA break repair and enhancement of tumor cell metabolism potentially mediating radioresistance (Chakravarti et al., 2004). In addition, some studies have suggested that overexpression of Survivin may facilitate evasion from checkpoint mechanisms of growth arrest and promote resistance to chemotherapeutic regiments targeting the mitotic spindle (Giodini et al., 2002). Whether these mechanisms also regulate the radioresistant phenotype of GBM remains to be confirmed.

Our study shows that IR exposure increases the basal expression of Survivin in U-87 glioma cells, possibly through RhoA-mediated intracellular signaling, and that this overexpression subsequently confers radioprotection against IR. Rho GTPases function as molecular switches that modulate the activation of several enzymes involved in different biological processes related to tumor progression, such as cell growth, transcriptional regulation and apoptosis (Aznar and Lacal, 2001; Etienne-Manneville and Hall, 2002; Wheeler and Ridley, 2004). Interestingly, RhoA-induced apoptosis is independent of p53 but dependent on modulation of anti-apoptotic proteins (Esteve et al., 1998). In our study, whether RhoA expression was induced by IR or through cDNA cell transfection, a cytosensitizing effect was observed in U-87 cells which resulted in a significant decrease in cell proliferation following IR. On the other hand, although the overexpression of recombinant Survivin also induced RhoA expression, the resultant was rather a cytoprotective effect on cell proliferation following IR. This paradoxical effect might be explained by post-transcriptional modifications

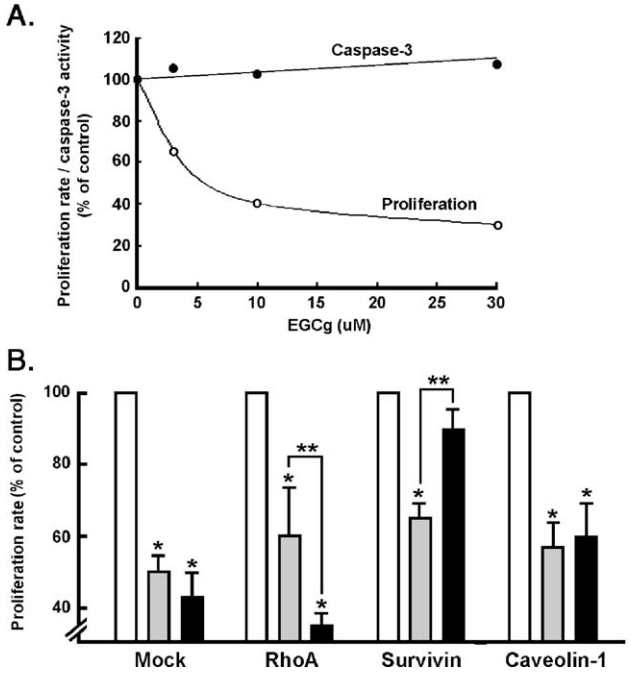

Fig. 5 – Survivin overexpression does not antagonize EGCg's anti-proliferative effect. Subconfluent cells were exposed for 6 h to increasing concentrations of EGCg (0–30 μM) and cell proliferation rate (white circle) and caspase-3 activity (black circle) were assessed following EGCg treatment as described in the Experimental procedures section. Results are expressed as a percent of the control untreated Mock cells (A). The proliferation rate was also assessed in U-87 cells transfected with Survivin, RhoA or Caveolin-1 cDNAs and further exposed to IR (black bars) or EGCg treatment (grey bars). Cell proliferation rate is expressed as a ratio of control untreated Mock cells (white bars).

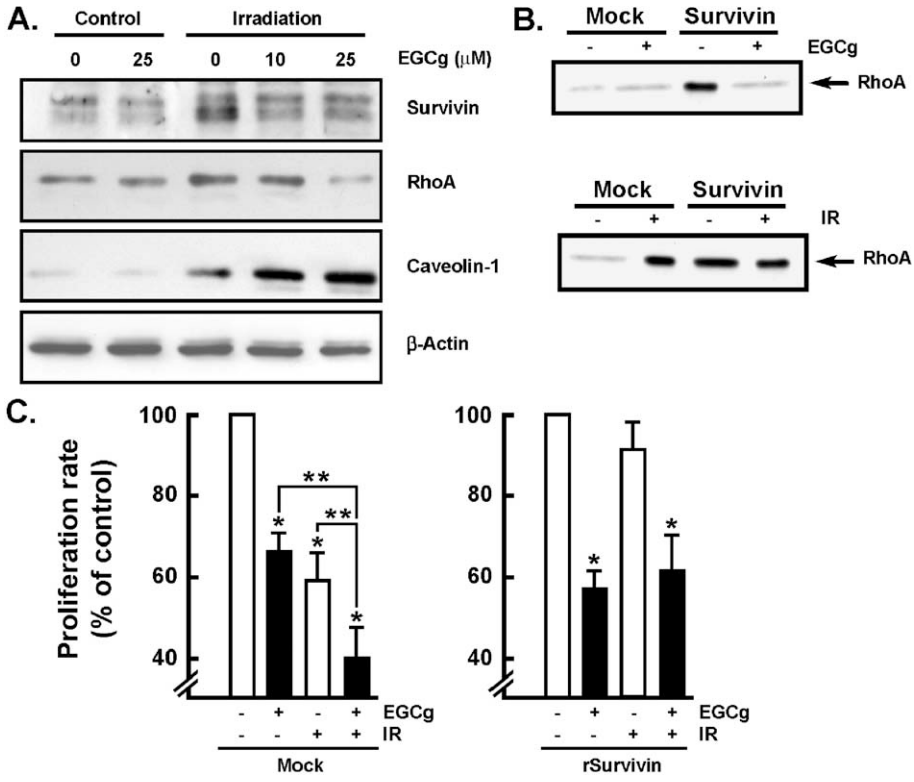

Fig. 6 – Effect of combined EGCg and low dose IR on U-87 glioma cell proliferation. U-87 cells were cultured and treated with EGCg (0–25 μM) for 6 h before IR (10 Gy) and left to recuperate overnight at 37 °C. (A) Cell lysates of each condition were electrophoresed on SDS-gels, and immunodetection was carried out as described in the Experimental procedures section. (B) Mock or Survivin-transfected U-87 cells were also treated with either EGCg (upper panel) or IR (lower panel) and RhoA immunodetection performed in cell lysates. (C) Cell proliferation was performed in Mock and Survivin-transfected cells that were either treated with EGCg, irradiated (IR) or submitted to combined EGCg/IR treatment.

that would be induced by IR and that may alter RhoA's function or cellular distribution to specialized membrane domains. We previously reported that RhoA is associated with caveolae-enriched membrane domains, possibly through physical interaction with Caveolin-1 (Gingras et al., 1998). Although caveolae regulate cell survival processes (Massimino et al., 2002), we show that overexpression of Caveolin-1 induced either by IR or cDNA cell transfection did not influence cell proliferation but may potentially have regulated RhoA's functions or localization. The interaction between RhoA and Caveolin-1 in the context of IR is currently under investigation.

One of the major features characterizing high-grade radioresistant gliomas is their infiltrating character. Recently, we showed that this phenotype might be linked to RhoA/Rok-mediated CD44 cell surface shedding which could be targeted and inhibited by EGCg, a green tea-derived polyphenol with anti-cancerous properties (Annabi et al., 2005). In light of this evidence and of recent studies which have shown that EGCg could serve as an IR enhancer on cancer cell lines (Baatout et al., 2004), we investigated whether EGCg pretreatment prior to IR could reverse the cytoprotective effect of Survivin. We observed that the combined EGCg/IR treatment was able to significantly decrease cell proliferation when compared to individual EGCg or IR treatments. Furthermore, the combined EGCg/IR treatment in U-87 cells overexpressing recombinant Survivin was able to downregulate RhoA expression and reverse the cytoprotective effect that Survivin exhibited towards IR. Although the exact molecular mechanism of EGCg is not completely understood, recent studies have shown that the inhibitory effect of EGCg on tumor cell proliferation might be transduced through its binding to the 67-kDa laminin receptor (67LR) (Tachibana et al., 2004), a protein whose expression is strongly correlated with tumor aggressiveness (Berno et al., 2005). Interestingly, studies have shown that through its binding to the 67LR, EGCg may also reduce the phosphorylation of myosin II regulatory light chain, resulting in an increase in cells in the G2/M phase of cell cycle and ultimately in an inhibition of cell growth (Umeda et al., 2005) possibly through increased cell radiosensitivity (Bernand and Bourhis, 2001).

In conclusion, we demonstrate that the growth inhibitory effect of EGCg pretreatment efficiently antagonized both the IR-induced expression of Survivin, as well as that of RhoA induced by overexpression of recombinant Survivin. A potential therapeutic impact of EGCg in targeting prosurvival and RhoA-mediated intracellular pathways in cancer cells is suggested to act synergistically with IR. We propose that EGCg might potentiate IR's inhibitory effect on tumor cell proliferation by increasing the proportion of cells in a radiosensitive state. This natural phytochemical might thus be an important

molecule to consider in combined adjuvant chemotherapy and radiotherapy.

4. Experimetal procedures

4.1. Materials

Agarose, (–)-epigallocatechin-3-gallate (EGCg), sodium dodecylsulfate (SDS), gelatin and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). FUGENE-6 transfection reagent was from Roche Diagnostics Canada (Laval, QC). Mouse anti-Survivin monoclonal antibody was from Cell Signaling Technology (Beverly, MA), mouse anti-RhoA monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-Caveolin-1 monoclonal antibody was from BD Pharmingen (Mississauga, ON), and mouse β-actin monoclonal antibody was from Sigma. Horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). BCA protein assay kit was purchased from Pierce (Rockford, IL) and enhanced chemiluminescence (ECL) Western blot kit from Chemicon International (Temecula, CA). Products for electrophoresis were bought from Bio-Rad (Mississauga, ON) and polyvinylidene difluoride (PVDF) membranes from Boehringer Mannheim. Trypsin was from INVITROGEN (Burlington, ON).

4.2. Cell culture and cDNA transfection

The U-118, U-138 and U-87 human glioblastoma cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and were cultured at 37 °C under a humidified atmosphere containing 5% CO_2. U-87 cells were transiently transfected with cDNA constructs using the non-liposomal FUGENE-6 transfection reagent. The EGFP-tagged WT-Survivin cDNA construct has been described previously (Temme et al., 2003). The Myc-tagged WT-RhoA cDNA construct was provided by Dr. Allan Hall (London, UK). GFP-tagged WT-Caveolin-1 was provided by Dr. Sung-Soo Yoon (Sung Kyon Kwan University, Korea). Transfection efficiency was analyzed by Western blotting and fluorescent microscopy. All experiments involving these cells were performed 36 h following transfection. Mock transfection of U-87 cultures with the empty pcDNA (3.1+) expression vector was used as controls.

4.3. EGCg and irradiation treatment

Cells were treated in serum-free MEM supplemented (or not) with EGCg (3–30 μM) for 6 h and were overlaid with sufficient medium to subsequently provide efficient build up doses. Cells were irradiated with a 6 MV photon beam from an Elekta SL75 linear accelerator. The delivered radiation doses were measured using a thermoluminescence dosimetry (TLD) system with an accuracy of 7%. After irradiation, MEM containing 20% FBS was added, and cultures were left to recuperate for 18 h. Non-irradiated control cells were handled similarly to the cells which were subjected to IR.

4.4. Cell proliferation assay

The extent of cell proliferation was assessed 18 h after irradiation. Cells were collected by gentle scraping and were resuspended in the overlaying medium. From each probe, 150 μl homogenate was saved for nuclear cell counting using an automatic nuclear counter and its specific reagents (New Brunswick Scientific Co., Edison, NJ) and for cell number determination using Trypan blue for exclusion of dead cells.

4.5. Fluorimetric caspase-3 activity assay

U-87 cells were grown to about 60% confluence and treated with EGGg or IR doses. Cells were collected and washed in ice-cold PBS pH 7.0. Cells were subsequently lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for 1 h at 4 °C, and the lysates were clarified by centrifugation at 16,000 *g* for 20 min. Caspase-3 activity was determined by incubation with 50 μM of the caspase-3-specific fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) in assay buffer [50 mM HEPES–NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0,1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 5 mM DTT and 1 mM EDTA] in 96-well plates. The release of AFC was monitored for at least 30 min at 37 °C on a fluorescence plate reader (Molecular Dynamics) (λ_{ex} = 400 nm, λ_{em} = 505 nm).

4.6. Immunoblotting procedures

Total protein lysates from control cells and treated cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4 °C with 5% non-fat dry milk in Tris-buffered saline (150 mM Tris, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1-h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000 dilution) in TBST containing 5% non-fat dry milk. The secondary antibodies were visualized by enhanced chemiluminescence and quantified by densitometry.

4.7. Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t* test and was used to compare the relative proliferation rates. Probability values of less than 0.05 were considered significant. In each figure, statistically significant differences are identified by an asterisk (*) for ECGg or IR treatment compared to control, while double asterisk (**) shows significance between combined EGCg/IR treatment and either EGCg or IR treatment alone.

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