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# Differential Cyclooxygenase-2 Enzyme Expression in Radiosensitive *versus* Radioresistant Glioblastoma Multiforme Cell Lines

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**Abstract.** *Background: Glioblastoma multiforme (GBM) is a high-grade primary brain tumor that is refractory to current forms of treatment. In cell studies, the growth rate of GBM cells correlates with the level of Cyclooxygenase-2 (COX-2) enzyme expression. COX-2 has been implicated in carcinogenesis of systemic cancers. Recently, COX-2 inhibition has been shown to increase the radiosensitivity of various tumors. We wished to assess whether the expression of COX-2 is greater in radioresistant versus radiosensitive forms of GBM. Materials and Methods: The radiosensitive (A172) and radioresistant (T98G) Glioblastoma multiforme cell lines were assayed for COX-2 expression using standard immunofluorescence histochemistry. Fluorescence readings were recorded per field. Western blot analysis was performed on both A172 and T98G GBM cell lines. The radioresistant cells were exposed to incremental doses of radiation in the presence and absence of a COX-2-selective inhibitor. Radioresistant cells were then exposed to incremental doses of COX-2-selective inhibitor at a constant dose of radiation. Results: The radioresistant cell line T98G had an approximate 1.7-fold greater expression of COX-2 than did the radiosensitive cell line A172, as per immunofluorescence histochemistry. Western blot analysis confirmed this finding. Statistical analysis (Bonferroni/Dunn) showed the results to be significant ($p<0.0001$). The wells containing radioresistant cells exposed to incremental doses of radiation and COX-2 inhibitors appeared to have higher cell kill when compared to radiation alone. Furthermore, increasing the COX-2 inhibitor concentration yielded higher cell kill. Conclusion: The results presented here show that the radioresistant GBM cell line, T98G, has a greater expression of COX-2 than does the radiosensitive GBM cell line, A172. These results suggest that: (i) COX-2 expression may serve as a marker for assessing radioresistance in GBM, (ii) COX-2 inhibition may lower the required doses of postoperative radiation, (iii) COX-2 inhibitors may have a role in radiosensitizing otherwise radioresistant forms of GBM.*

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Glioblastoma multiforme (GBM) is an incurable primary brain tumor with treatment limited to surgical resection and radiation. Chemotherapy, with the exception of temozolamide for GBM, is of equivocal benefit (1, 2). GBM shows variable response to radiation (3). In cell studies, the growth rate of GBM cells correlates with the level of Cyclooxygenase-2 (COX-2) enzyme expression and COX-2-derived prostaglandins are thought to mediate carcinogenesis (4). High COX-2 expression in tumor cells is associated with more aggressive gliomas and is a predictor of poor survival (5). Inhibition of COX-2 has been shown to increase the radiosensitivity of various tumors (6). The mechanism of radiosensitization is not clearly understood. However, for some systemic cancers, the radiosensitivity of the tumor directly correlates with degree of COX-2 expression (7). In this study, we analyzed the expression of COX-2 in two previously described GBM cell lines, one determined radiosensitive (A172) and the other radioresistant (T98G) by Yao *et al.* (8). Our results show that, among the two cell lines used in the study, A172 cells and T98G cells, there is a differential expression of COX-2 protein. Moreover, when COX-2 was inhibited in the high expression cells, there was a significant degree of radiosensitization conferred upon the cells.

## Materials and Methods

*Intracellular Cox-2 expression with Immunofluorescence*

*Cell culture.* T98G and A172 cell lines were purchased from the American Tissue Culture Consortium. Both cell lines were cultured in Dulbecco's modified minimal essential medium (DMEM-Cellgro, Inc) supplemented with 10% fetal bovine serum (Hyclone) at 37˚C with 5% $CO_2$. For experimental purposes, cells were released from the flasks using a tryspin/ETDA solution (Life Sciences) and equal numbers of viable cells were measured with

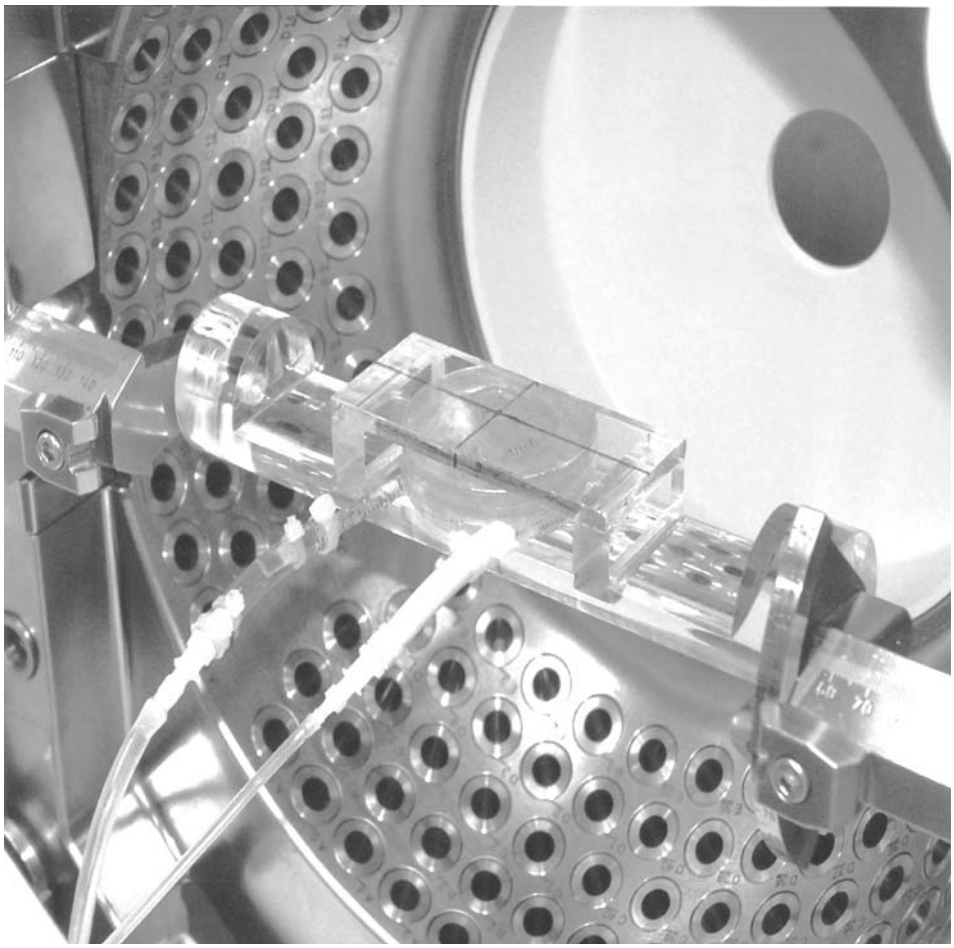

Figure 1. *Leksell Gamma Knife® model "B" (Elekta Instruments, Atlanta, GA USA) with attached "cell irradiator" device.*

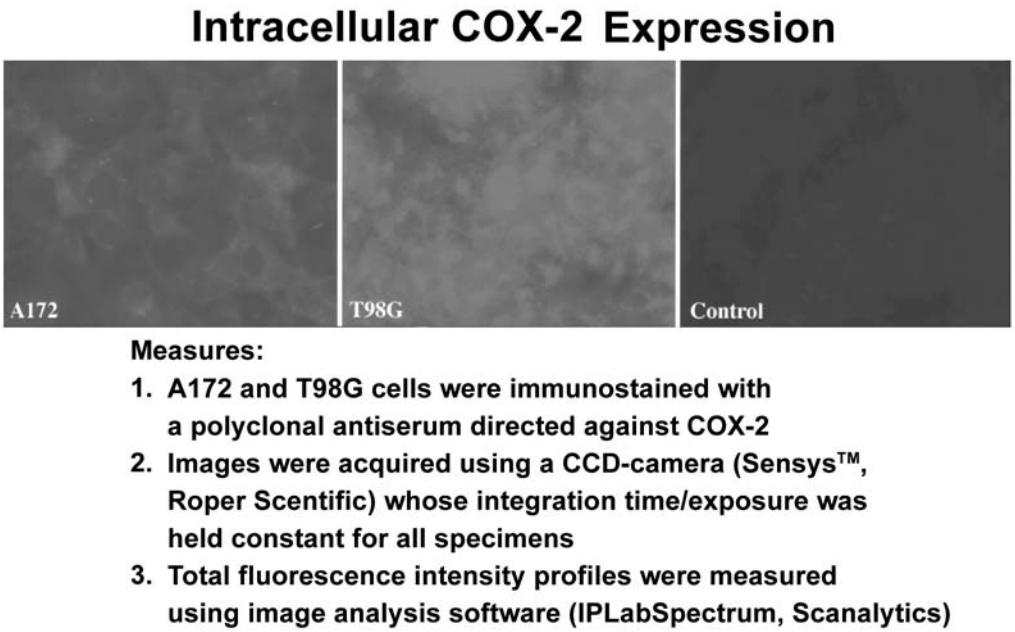


Figure 2. *Immunofluorescence staining of cell lines A172 and T98G with COX-2 antibody.*

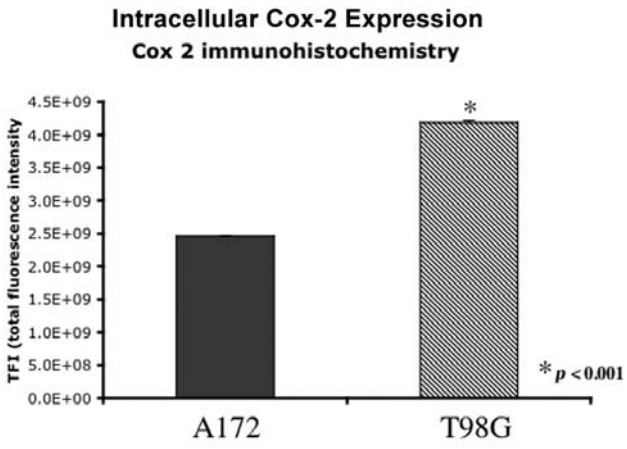


Figure 3. *Bar graph showing COX-2 expression in A172 and T98G cells from Figure 1. Statistical analysis (Bonferroni/Dunn) showed the results to be significant (p<0.0001).*

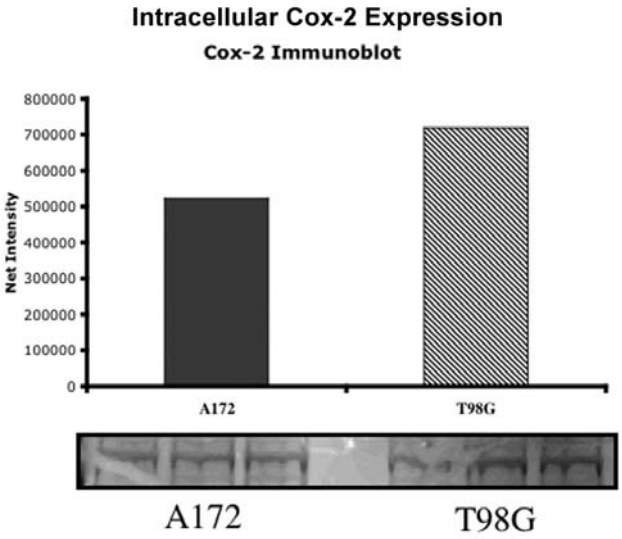


Figure 4. *Western blot analysis for COX-2 expression in A172 and T98G cell lines using COX-2 antibody. Bar graph shows relative expression of COX-2 in A172 and T98G cells as per Western blot analysis.*

an automated cell counter (Beckman Instruments) and seeded into 50-mm cell culture dishes.

*Gamma knife protocol.* Cells are seeded to both 12-mm (Fisher; Pittsburgh, PA, USA) and 18-mm circular cover slips (VWR; Media, PA, USA). The cultures are used at 1 week in culture (approximately 3 days after culture confluency is established). These are then fixed onto an ingeniously designed "cell irradiator" device (Figure 1). The cell cultures in the irradiator are then exposed to single session gamma ray irradiation using the Leksell Gamma Knife® model "B" (Elekta Instruments, Atlanta, GA, USA). Maximal doses 10, 20 and 30 Gy were administered using the 18-mm collimator. This was previously described by Sharp *et al.* (9).

*Immunohistochemistry.* Cells from each cell line were immunostained with a polyclonal antiserum directed against COX-2 and localization of primary antibody binding done using a Cy3 conjugated donkey anti-rabbit IgG (Jackson Immunoresearch). The specimens were imaged using an Olympus IX-70 inverted microscope equipped for epifluorescent illumination interfaced to a Sensys ™(Roper Instruments) cooled CCD camera. The signal from the camera was ported to a Macintosh G4 PowerPC hosting the imaging software IPLabSpectrum (Scanalytics). Total fluorescence intensity profiles were measured using a subroutine present in the IPLab image analysis software.

*Western blot immunoassay.* Fifty thousand cells from both cell lines were extracted with Tris-HCl 50 mM (pH 7.0) , 0.1% Triton X-100, EDTA 10mM, E-aminocaproic acid 100mm, benzamidine 5mm and PMSF 0.2mM. Fifty microliter aliquots from both cell extracts were used for SDS-PAGE. The resultant gel was electrophoretically transferred to nitrocellulose for immunoassay. A polyclonal antibody directed against COX-2 was used to probe the immunoblot and localization was detected using a species appropriate alkaline phosphatase- conjugated secondary antibody (Jackson Immunoresearch). Color development of the blot was done using NBT/BCIP (BioRad) and densitometry of COX-2 positive bands was performed using a Kodak ISO2000 gel documentation system. Radiosensitization of GBM cell line T98G was achieved with COX-2 inhibitor Cay10404 (Cayman Chemical Co) with a selective index of >500, 000. Twenty-four hours prior to irradiation, the medium was exchanged for serum-free DMEM and the appropriate amount of COX-2 inhibitor was added to the cultures. Two types of radiation studies were done: 1). Protocol A-The radiation was kept constant at 10G and the dose of the COX-2 inhibitor varied at 100nM and 1000nM; 2.) Protocol B-the COX-2 inhibitor was kept constant at 100nM and the radiation dose varied at 0, 5, and 10G. After 5 days, the cells were imaged live using Hoescht 3342 (Molecular Probes) staining for nuclear imaging and Cell tracker dye (CMFDA, Molecular Probes). Using the double label protocol as such we were able to identify live cells at the time of measurement because only live cells are able to hydrolyze the ester bond in CMFDA to render it fluorescent, and relate that to specific changes in nuclear morphology. Also, the protocol allowed for the rapid measure of cell area/dish as a relative indicator of cell density.

## Results

The results in Figure 2 show the immunofluorescence analysis of A172 and T98G cells with respect to COX-2 expression. In both cell populations, COX-2 immunostaining appeared to be evenly distributed within

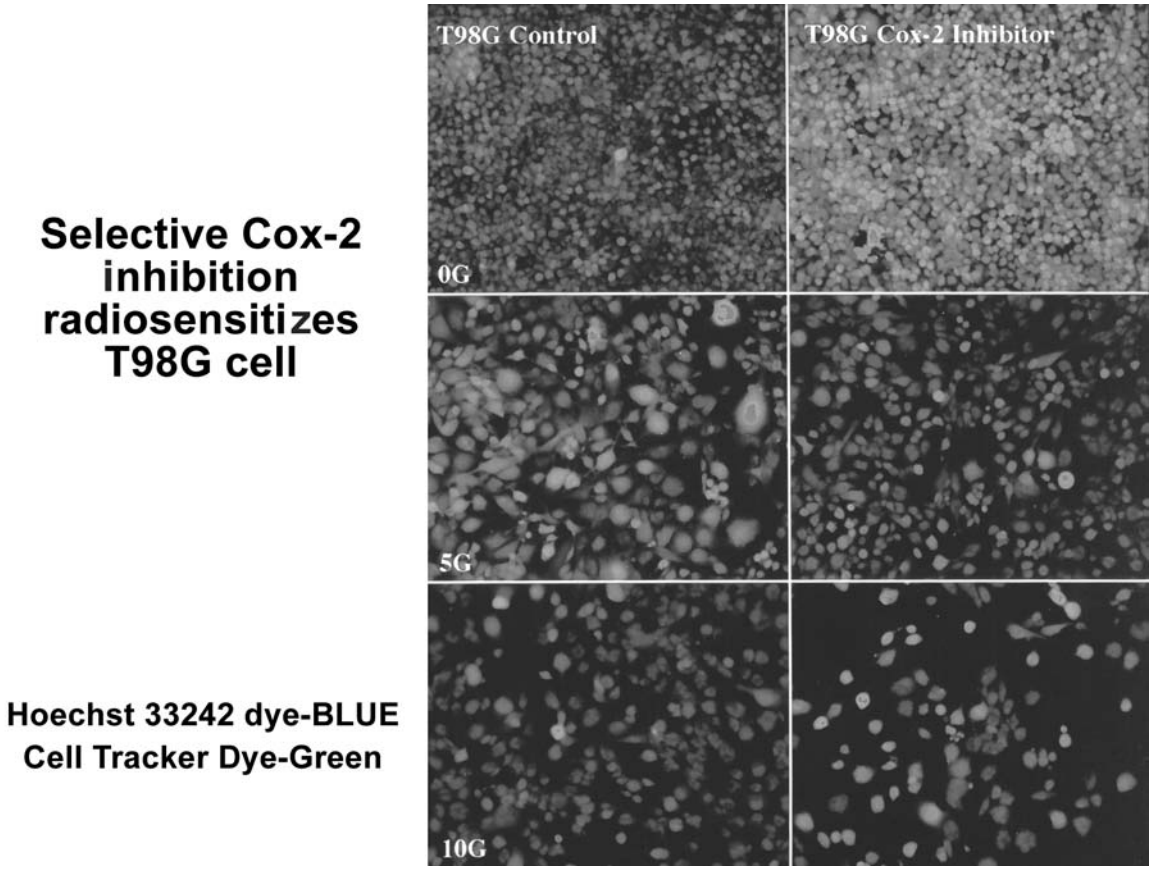


Figure 5. *Analysis of T98G cell line at 0 G, 5 G and 10G radiation in the absence and presence of the COX-2 inhibitor, Cay10404, at a dose of 100nM.*

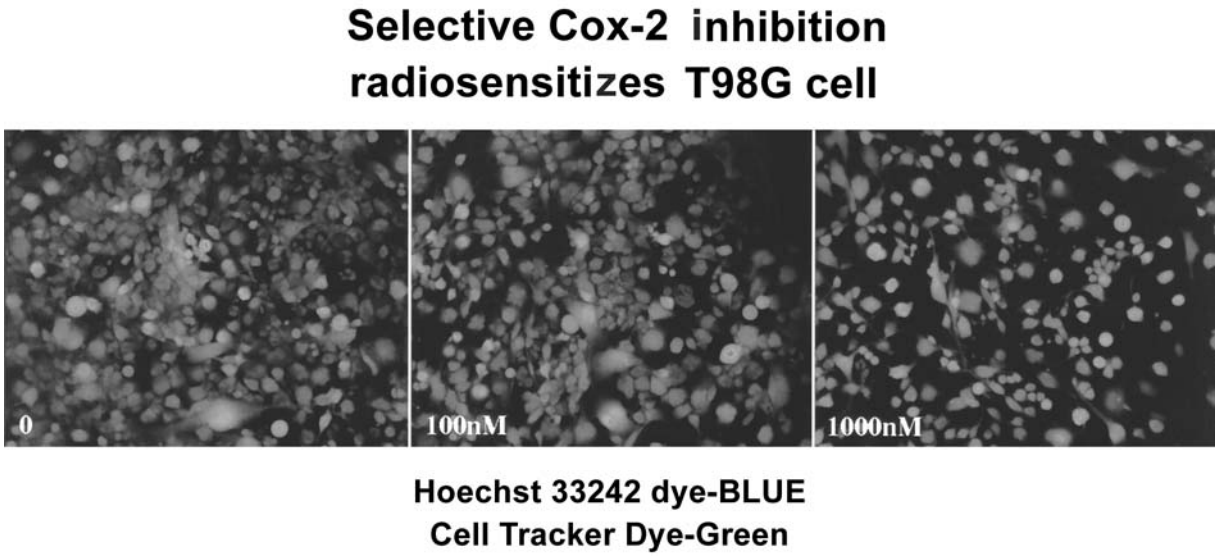


Figure 6. *Analysis of T98G cells in the presence of a constant dose of radiation (10G) with a progressive increase in the dose of the COX-2 inhibitor Cay10404 (0nM, 100nM and 1000nM).*

the cytoplasm of the cells. However, the A172 cells showed a significantly lower level (1.7-fold) of COX-2 expression ($p<0.001$) than that seen in the T98G cells (Figure 3). To validate the immunofluorescence data, extracts of both cell lines were analyzed by SDS-PAGE and Western blot immunoassay. The immunoassay confirmed the higher expression of COX-2 in T98G (Figure 4).

Treating the A172 cells with COX-2 inhibitor in either protocol prior to irradiation had no effect on the survival of the cells (data not shown). On the other hand, T98 G cells exposed to the same radiation/COX-2 treatment protocols (Figures 5 and 6) showed enhanced cell death.

## Discussion

Glioblastoma multiforme remains a challenge to surgeons and oncologists alike. Although surgical debulking remains a major arm for treatment, most patients receive postoperative radiation (10). Interestingly, GBM responds variably to radiation with some tumors more responsive than others (3). Yao *et al.* have shown in cell studies that the human GBM cell line A172 is more radiosensitive than the human GBM cell line, T98G (8). Cyclooxygenase expression has been determined to have a role in conferring radioresistance; although, the exact mechanism for radioresistance remains unclear (11). We determined if the radioresistant cell line, T98G, can be radiosensitized with exposure to a COX-2 inhibitor.

The data presented in this paper shows that the radioresistant cell line, T98G, has a greater expression of COX-2 than does the radiosensitive cell line, A172. Furthermore, the combination of radiation and COX-2 inhibitor led to a higher cell kill than did either radiation or COX-2 inhibitor alone. Progressive increase in either radiation or COX-2 inhibitor led to incremental cell kill. These results suggest that: (i) the degree of COX-2 expression may serve as a marker for assessing radioresistance in GBM and (ii) COX-2 inhibitors may have a role in radiosensitizing otherwise radioresistant forms of GBM.

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