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RESEARCH ARTICLE

Repetitive Nicotine Exposure on Efficacy of Temozolomide and Radiotherapy on Cultured Glioblastoma Cell Lines

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ABSTRACT

Purpose: Of patients with glioblastoma who smoke tobacco, 16 to 28% will continue to smoke following diagnosis. Use of nicotine-containing products may enhance proliferation, migration and radioresistance and detrimentally affect treatment and prognosis of glioblastoma. The aim of this study is to identify effects of a period of nicotine exposure on efficacy of subsequent treatment with temozolomide and radiation therapy on 5 glioblastoma cell lines. We hypothesize that prior and continued nicotine exposure would reduce tumoricidal effects of temozolomide and radiation therapy.

Methods: After 5 glioblastoma cell cultures are exposed to nicotine prior to treatment with temozolomide and /or radiation, proliferation, migration, colony forming assays, and enzymatic expression of matrix metalloproteinases are assessed.

Results: Proliferation is not affected by exposure of physiologically relevant concentrations of nicotine prior to treatment with temozolomide or irradiation. Nicotine exposure has variable effects which include enhancement of migration rate, metalloproteinase expression, and colony formation for some glioblastoma cell lines subsequently treated with temozolomide and /or radiation therapy.

Conclusions: These findings suggest that continued smoking or use of other nicotine-containing products during treatment could result in increased aggressiveness and invasion of residual tumor cells causing a resistance to treatment in some glioblastoma.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and aggressive malignant brain tumor in adults. The fast-growing and infiltrative pattern of the tumor, along with its abundant vasculature makes complete resection difficult, leading to a poor prognosis.[1] While the etiology of glioblastoma is poorly understood, no association of tobacco smoking and the risk of development of GBM has been established. [3-5] Within the last decade the question of the use of tobacco products following the diagnosis and treatment of cancer, and the possible detrimental effects on treatment efficacy, prognosis and tumor spread in non-neural tumors has been raised.[6-9] Tobacco smoking is highly addictive and it has been estimated that approximately 56-64% of patients seeking cancer treatment will continue to smoke.[10, 11] Specifically, 16-28% of patients with GBM continue to smoke following their diagnosis and during treatment.[12, 13] Life-style modifications following a diagnosis of GBM may be explored by some patients in an effort to improve their quality of life or overall survival, while others have difficulties in accepting the restrictions necessitated by the diagnosis.[14, 15] Patients may be advised to switch to a cigarette replacement treatment such as a patch, chewing gum or e-vapor in an effort for smoking cessation, but these products still contain nicotine, one of the major compounds contained in tobacco associated with adverse effects. Data relating to the potential interaction of current smoking or nicotine-replacement products and chemoradiation therapy in glioblastoma patients and recommendations for cessation of smoking are lacking.

Nicotine, a major component of smoking tobacco, has been implicated in cancer spread and prognosis due to effects on apoptosis, tumor angiogenesis, invasion, and migration molecular pathways. [16, 17] Although nicotine's interactions with breast, lung, colon and pancreatic cancer cell lines have been investigated, studies on GBM are few. [18, 19] While nicotine's mechanisms of action are uncertain, nicotine as been shown to enhance glial tumor proliferation and invasion. Khali et al [18] postulated that nicotine leads to upregulation of the EGFR pathway, whereas Pucci et al[19] suggested that increased expression of α7- and α9- neuronal acetylcholine receptors (AChRs) are responsible for increased proliferation. Thompson *et al.*[20] recently highlighted the roles of increased activation of AChRs as a modulator of GBM behavior and had a direct correlation to a reduction in patient survival. Activation of AChRs, in addition to the EGFR pathway, is linked to increased expression and activity of matrix metalloproteases 2 and 9 (MMP-2 and MMP-9). These proteins play an important roles in cellular migration, angiogenesis, and invasion by basement membrane degradation.[1] Overexpression of both MMP-2 and MMP-9 have been associated with poorer survival in gliomas and are negative prognostic indicators for clinical progression of GBM.[21-23] Whether nicotine increases GBM cell proliferation, migration, and invasion by either, or both, of these mechanisms, the result may promote recurrence and resistance to chemotherapy.[21]

Optimal treatment for patients diagnosed with GBM includes maximal safe resection followed by a combination of the oral chemotherapy alkylating agent temozolomide (TMZ) and radiotherapy.[14] Acute nicotine exposure may enhance radioresistance of glioblastoma cells [18] but potential antagonist effects with TMZ have not been addressed, and neither has chronic exposure of these cells to nicotine, which is more indicative of a long-term smoker than acute exposure. The period of exposure of GMB cells to nicotine is critical as the action of nicotine is dependent on dose, frequency of exposure and cell type.[24] Chronic stimulation of neuronal cells to nicotine has been shown to upregulate AChRs but the increases differ in receptor subtypes and vary between brain regions.[25] Whether these upregulated nicotinic receptors are desensitized despite the increased in numbers of receptors is not certain.[25]

This study evaluates the effects of a period of nicotine exposure on proliferation and migration of four primary GBM cell lines and the commercially available U87-MG, a continuously growing GBM cell line. The effects of prior and concurrent nicotine exposure on efficacy of treatment of the cells with TMZ and/or radiation therapy is also assessed. Additionally, we assess MMP2 and MMP9 activity using gel zymography. We hypothesize that continued nicotine exposure will reduce therapeutic effects of TMZ and radiation therapy on GBM cell lines.

METHODS

Chemicals and Treatments

The reagents were purchased as follows: TrypLE™ Express (12604-013), Pen Strep (15141-122), DMEM (1X) (31053-028) and DMEM/F-12 (11039-021) from Gibco-Life Technologies, Grand Island, NY; fetal bovine serum FBS (F2442), dimethyl sulfoxide (DMSO) (D-2650), Thiazolyl Blue Tetrazolium Bromide (M5655) and (-)-Nicotine (N3876-25ml) from Sigma-Aldrich, St Louis, MO.

Phosphate-buffered saline (PBS) 1X without Ca/Mg (14190144) and Phosphate-buffered saline 1X with Ca/Mg (14040133) were obtained from Thermo-Fisher, Waltham, MA. Temozolomide (S1237) from Selleckchem (Houston, TX) was diluted in DMSO according to manufacturer's instructions to prepare a 100 µM stock solution. Corresponding control cultures received equal volumes of solvent (final concentrations in culture was always less than 0.07%). The dose of TMZ used was based on highest plasma levels in clinical studies converted to µM which fell within or below concentrations provided by Selleckchem in published studies for proliferation and growth in vitro cell cultures for U87 cells, as well as the range provided by Chung and Khali. [26-28]

Cell culture

U87-MG cells, obtained from the American Tissue Culture Collection (HTB-14™) (ATCC, Rockville, MD), were seeded in Corning tissue culture flasks (mfr #430639) (ThermoFisher, Rochester, NY) in Dulbecco Modified Eagle Medium supplemented with 10% Fetal Bovine Serum and 1 % penicillin-streptomycin in a humidified incubator with 5% CO_2 at 37° C. Human GBM primary cell lines, designated as MU-1400, MU-1431, MU-1454 and MU-1458, were harvested at surgical resection of tumors of four patients of the senior author (NSL) following the ethical procedures approved by University of Missouri Institutional Review Board (#1044138) with initial blinding of the patients' smoking status. The tumors from which these cultures were derived were diagnosed based on histopathological criteria as glioblastomas; these cultures were initially harvested prior to the era of routine determination of molecular genetic characteristics of high-grade gliomas; in particular, neither the MGMT promoter methylation status nor the IDH1/IDH2 mutation status was known for two of the tumors from which the cell lines were derived. Clinical characteristics of the source patients are shown in Table I. The primary cells were cultured with Dulbecco Modified Eagle Medium/F-12 (containing no Phenol Red), Fetal Bovine Serum (20%) and Penicillin-Streptomycin (1%) as described by others [18, 29-31] in a humidified incubator with 5% CO_2 at 37° C and were passaged by TrypLE™ every 3-4 days at confluence (2 to 8 passages). Acute nicotine exposure consisted of treatment of the naïve cells for the first time prior to each of the experimental protocols. Long-term nicotine repetitive exposure consisted of a minimum of seven days of daily exposure of 0.1 µM or 0.5 µM nicotine (henceforth referred to as 0.1 LTN or 0.5 LTN), replacing the culture media every 48 hours, prior to initiating other conditions. Control un-exposed cells were also cultured.

Table 1. Patient Demographics

Cell ID	Molecular Characterization	Smoking History	Demographics	Tumor Size (mm)	Location	Imaging Characteristics
MU-1400	Not performed	Non-smoker	47-year-old; female	45 x 54 x 56	Frontal, left	Heterogenous Enhancement
MU-1431	Not performed	38 pack-years	61-year-old; female	56 x 75 x 60	Temporal, right	Heterogenous enhancement
MU-1454	IDH – wild; 1p19q – wild	45 pack-years	56-year-old; male	41 x 54 x 49	Temporo-occipital, right	Heterogenous enhancement
MU-1458	IDH – wild; p53 – wild; MGMT – indeterminant; ATRX – wild	90 pack-years	77-year-old; female	35 x 61 x 47	Frontal, right	Heterogenous enhancement

Radiation Treatment

GBM cells at 70–80% confluence were irradiated with a XRAD 320 Biological Irradiator (Precision X-ray, North Branford, CT, USA) at 320 Kev, 12.5 mA, and 50 cm FSD with filter 1 (280 cGy/min). Irradiation curves were completed using 0, 2 Gy (43 sec), 5 Gy (107 sec), 10 Gy (214 sec) and 15 Gy (320 sec). For combined irradiation and TMZ studies, GBM cells were treated with TMZ at 73µM for 1-2 hours, followed by 5 Gy irradiation or mock treatment per the methods of Pasi et al.[32] Cells were irradiated at room temperature in multi-well plates and cultured for 24 hours for migration experiments, 48 hours for gel zymograms and Western blots, and 72 hours for proliferation assays.

MTT Assay for viability and proliferation

Initially, effects of nicotine on viability of U87-MG and MU-1454 were measured to define appropriate dilutions for further studies. Criteria included dilutions that resulted in 30-40 % change in viability/proliferation as follows. Cells were plated when confluency reached 95% or greater at density of 3000 cells per well into Falcon 96-multiwell plates (Cat no. 353072, Becton Dickinson Labware, Franklin Lakes, NJ) for 24 hours. U87 and MU1454 cells were cultured using 100 µM stock nicotine diluted in PBS and added at various concentrations (0.03, 0.1, 0.5 and 1 µM) to determine nicotine toxicity and define experimental dosages of nicotine. Additionally, a pilot study was conducted on two nicotine- naive cell lines (U87 and MU1454) over a four-day treatment period in which cells were pre-treated with nicotine (negative controls were included) and a subsequent three-day treatment of either TMZ alone, or nicotine before and/or after TMZ treatment. Overall, six arms of cells were set up accordingly. The dosage of nicotine was 0.1 µM, 0.3 µM and 0.5µM as determined from published LC50 and working dosages in the literature that could approximate or mimic natural physiologic levels expected in human serum, light, moderate and heavy smokers. [24, 33] Cells were incubated for 24, 48 and 72 hours at standard conditions (5% CO_2 at 37°C) and were then removed from medium. The different dosages were evaluated by a colorimetric assay using reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to measure cellular metabolic activity as previously described.[4] Briefly, cells were cultured in complete media in 96-well plates for defined time period. After media removal 200 µL fresh media without FBS was added and incubated with 10 µl of MTT (5 mg/mL) for 4 hours. Subsequently media/MTT was removed, cells were lysed by adding 100 µl DMSO solution to each well, and they were returned to the CO_2 incubator for 15 minutes. Optical density at 570 nm was then measured using a microplate reader (ELx-800, BioTek, Winooski, VT). MTT assays were performed on fifteen wells per dosage per cell line; the experiment was repeated in triplicate.

To examine the effects of nicotine with TMZ and/or irradiation on proliferation, nicotine-exposed cells (0.1 LTN or 0.5 LTN) were seeded at 3000 cells/well in 96-well culture plates for MTT assay overnight for full attachment. When cells were at least 80% confluent, media were removed and cells were treated with complete media without or with 73 µM TMZ in addition to nicotine. Where indicated, cells were incubated with TMZ for 2 hours prior to irradiation (0 or 5 Gy) as described by Bobola et al[34] Cells were incubated for 72 hours at standard conditions (5% CO_2 at 37°C) after which plates were evaluated by MTT assay as described above. MTT assays were performed on 10 wells per dosage per cell line and repeated in triplicates. Cells not treated with nicotine with or without TMZ and/or irradiation were included as controls.

Colony forming assay

To test the effects of nicotine on radioresistance, colony-forming assays were performed. Cells seeded at a density of 250 cells/mL in a 6-well CytoOne ™culture plate (cat # CC7682-7506, USA Scientific Inc, Ocala, FL) were allowed to attach. Twenty-four hours later they were treated with 5 Gy irradiation. Two wells per treatment (including the controls) were used per replicate. Plates were checked at 14 days for colony formation and fixed with 100% ethanol for 20 minutes and then stained with 0.5 % crystal violet, air-dried and counted. Colonies consisted of ≥ 50 cells. A proliferation-based assay was used for U87-MG cells, which do not form colonies.[35] Briefly, cells were seeded at a density of 500 cells well in a 96-well Falcon plate (cat. # 353072, Corning Incorporated, Tewksbury, MA). Cells were allowed to attach and 24 hours later were treated with a single dose of 5 Gy irradiation. Control wells without radiation were included. A minimum of 9 wells per treatment group (including the controls) were used per replicate. The plates were incubated for 10 days and MTT assay performed as described above. The experiments were repeated in triplicates. The data was recorded as a percentage of the non-irradiated (control) cells.

Migration Scratch Assay

The combined effects of long-term nicotine exposure followed by TMZ and/or irradiation on migration was examined. Briefly, cells were seeded into 6-well culture plates in complete medium until 80 % confluent. Confluent cell monolayers were incubated in serum-free media for 8 hours overnight to stop cell proliferation. Monolayers were carefully scratched using a 1000 µL sterile pipette tip forming approximately a 600-micron wound, and dislodged cells were removed by washing with serum-free media. Wounded monolayers were incubated in 1% FBS with or without TMZ and/or irradiation for 14 and 24 hours. Cell migration across the wound was imaged using an inverted Nikon TMZ phase microscope equipped with an EP50 digital camera (Olympus

Life Science, Waltham, MA). Gap width in each image was measured at 150 points along the length of the wound using Image J. Each scratch was repeated at a minimum in triplicate. Photographs of the wounds were taken at time 0, 14 and 24 hours with measurements recorded at 14 hours. Due to the variation in the initial wound between wells within an experimental period, as well as between replicates, wound closure was expressed as distance traveled by the cells in microns per hour rather than area and percent of wound closure to eliminate incorrect interpretation of the data.

Gelatinase Zymography

Cells ($1x10^6$) were seeded into 6-well plates in complete media. The next day the media was removed, wells were rinsed with PBS (with Ca/Mg), and 2 mL FBS-free media containing specific treatment was added. After 24-hour incubation, cell culture media was collected and stored at -80 °C until used. Gelatinase zymography was performed using hand-casted 7.5% Polyacrylamide gel and 0.1 w/v % gelatin (1mg/ml) in non-reducing conditions. Culture media (20 µl) were mixed with zymogram sample buffer (BioRad, Hercules, CA) and loaded for SDS-PAGE with Tris-glycine SDS buffer (1610732, BioRad, Hercules, CA). Following electrophoresis, gels were washed three times in 1X BioRad Renaturation Buffer (1610765, 10x Zymogram Renaturation Buffer, Bio Rad, Hercules, CA) for 15 minutes at room temperature on a shaker to remove SDS. Gels were then incubated at 37° C overnight in substrate buffer containing 1X Zymogram Development Buffer (1610766, 10x Zymogram Development Buffer, Bio Rad, Hercules, CA). Finally, gels were stained with 0.5% Coomassie Blue R250 in 40% methanol and 10% glacial acetic acid for 30 minutes and then destained in 40% methanol and 10% glacial acetic acid solution to reveal zones of gelatin lysis. The zymograms were scanned using Amersham™ Imager 600. Band intensity examined using ImageJ 1.52a (NIH) with U87 control (non-treated) supernatant used as a housekeeping control in each gel (band not shown in results).

Statistical Analysis

Experimental data was analyzed for statistical significance using the student-t test with $p < 0.05$ considered significant (GraphPad QuickCalcs , San Diego California, at Website: https://www.graphpad.com/quickcalcs/ttest1.cfm [accessed Jan-Apr 2021]).

RESULTS

Nicotine has no consistent effect on GBM cellular proliferation

Initial cell proliferation studies to determine acute nicotine dose response on naïve cell lines was noted in MU1454 at 0.1 µM and 0.5 µM, and at 24 hours at dosing concentration of 1.0 µM on the U87 cell line. Experimental dosing of nicotine at 1 µM was considered out of the range of an acceptable rate based on physiological plasma levels in smokers, so nicotine dosages under 1 µM was selected for further pilot studies. [33] The initial 6-leg study on the acute effects of nicotine without or with TMZ on two cell lines did not show significant differences between the different levels of acute dosing of nicotine in the two cell lines. Acute exposure of nicotine on the remaining cell lines exposed to nicotine prior to harvest showed variability and lack of significant effects of an acute exposure on naïve cells, so the effects of repetitive, or chronic, dosing of the cells with 0.1 and 0.5 µM concentration, mimicking the real-life situation of a light and heavy chronic smoker pre-morbid to glioblastoma diagnosis, was subsequently performed. As noted in Table II and Figure 1, proliferation of U87-MG 0.1 LTN followed by TMZ ($p < 0.01$), TMZ + 5 Gy ($p < 0.05$), and U87-MG 0.5 LTN and TMZ + 5 Gy ($p < 0.05$) was diminished. Long-term exposure to nicotine had little to no effect on therapeutic treatment when compared to the controls for any of the four primary GBM cell lines (Figure 1). Long-term -exposure nicotine had no effect on cell proliferation at increasing dose of irradiation.

TABLE II. Summary of Cell Lines and Assay Results

	1400	*p* value	**1431**	*p* value	**1454**	*p* value	**1458**	*p* value	**U87**	*p* va
MTT										
CR vs 0.1R	NS		NS		NS		NS		NS	
CR vs 0.5R	NS		NS		NS		NS		NS	
CT vs 0.1T	NS		NS		NS		NS		NS	0.07
CT vs 0.5T	NS		NS		NS		NS		↓	0.04
CTR vs 0.1TR	NS		NS		NS		NS		↓↓	0.0(
CTR vs 0.5TR	NS		NS		NS		NS		↓	0.02
IR Curve	NS		NS		NS		NS		NS	
CFA										
C vs 0.1	NS		NS	0.088	NS		NS		NS	0.14
C vs 0.5	NS		↑	0.017	NS		NS		NS	0.07
Zymography										
MMP2										
C vs 0.1	NS		NS		NS		↓	0.0438	NS	
C vs 0.5	↑	0.0403	NS		NS		NS		NS	
C vs 0.1R	NS		NS		NS		↓	0.0468	↑	0.02
C vs 0.5R	NS	0.099	NS		NS		NS		↑	0.04
C vs 0.1T	↑↑	0.01	NS		NS		↑↑↑	0.0008	NS	
C vs 0.5T	↑	0.038	NS		NS		↑↑	0.003542	NS	
C vs 0.1TR	↑↑	0.008	NS		NS		NS		NS	
C vs 0.5TR	NS		↑	0.023	NS		NS		NS	
Migration										
C vs 0.1	NS		↑↑	0.004	↑	0.019	NS		NS	
C vs 0.5	NS		NS		NS		NS		↑↑	0.0(
C vs 0.1R	NS		↑↑	0.0035	↑	0.025	NS		NS	
C vs 0.5R	NS		NS		↑	0.035	NS		↑↑	0.0(
C vs 0.1T	NS		↑	0.011	NS		NS		NS	
C vs 0.5T	↑↑	0.001	↑↑	0.001	↑↑↑	0.001	NS		↑	0.03
C vs 0.1TR	NS		↑↑	0.008	NS		NS		↑	0.01
C vs 0.5TR	↓	0.023	↑↑	0.001	↑↑↑	0.001	↑	0.0465	NS	

T, Temozolomide; R, irradiation; TR, Temozolomide and irradiation; NS, not significant; ↑ or ↓, $p > 0.5$; ↑↑ or ↓↓, $p > 0.01$; ↑↑↑ or ↓↓↓, $p > 0.001$.

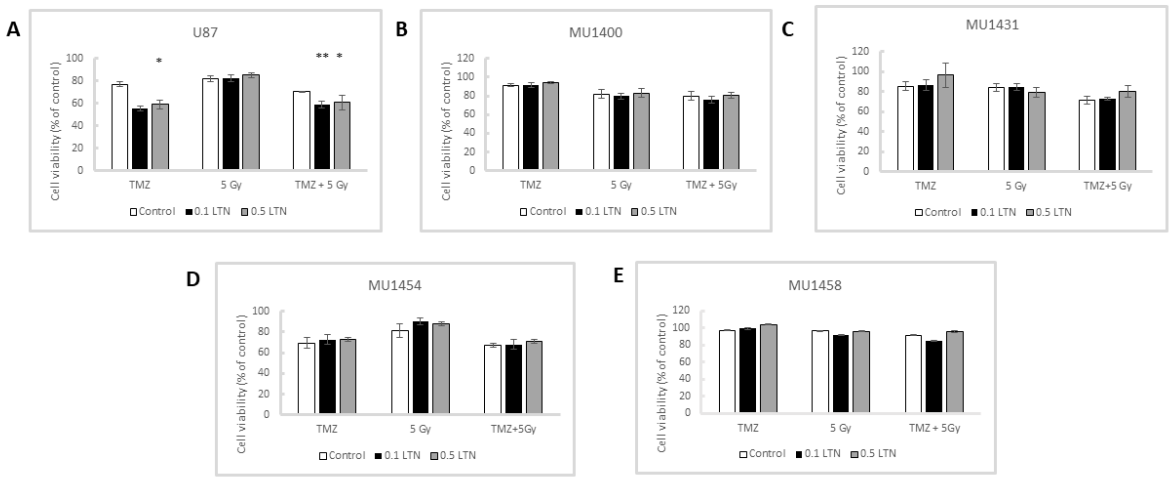

Figure 1. The effect of pre-exposure to 0.1 μM, 0.5 μM nicotine on proliferative response to Temozolomide and 5 Gy irradiation. Data shown as mean +/- SEM. Significance denoted as *p < 0.05, **p < 0.01, ***p < 0.001. The size of the error bars are too small to be seen in some graphs. Control = no nicotine; 0.1LTN = long-term pre-exposure 0.1 μM nicotine; 0.5LTN = long-term pre-exposure 0.5 μM nicotine; Gy = Gray

Nicotine increased colony formation in a dose-dependent manner in some cell lines

Colony forming assays were performed on all cell lines treated with exposure to nicotine either alone or followed by 5 Gy irradiation (Figure 2). Controls with no treatment were included. Colony formation increased when compared to controls as nicotine dose increased with U87-MG and MU-1431 (p = 0.0165). No significant change with colony formation occurred with MU-1400, MU-1454 and MU-1458 primary cell lines compared with control cells, although increased colonies were visible with increasing nicotine concentration in MU-1454.

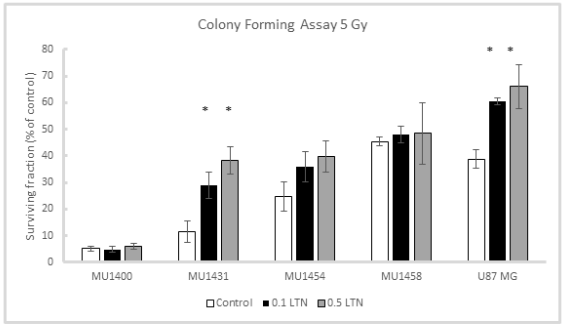

Figure 2. Colony formation in cells pre-exposed to 0.1 μM and 0.5 μM nicotine followed by irradiation at 5 Gy. Significance denoted as *$p < 0.05$. Data shown as mean +/- SEM. Control = no nicotine; 0.1LTN = long-term pre-exposure 0.1 μM nicotine; 0.5LTN = long-term pre-exposure 0.5 μM nicotine; Gy = Gray; TMZ = Temozolomide

Long-term nicotine exposure increased cell migration in some cell lines

The migration scratch assay was performed to discover if nicotine had any effect on cell migration before and after treatment with TMZ and 5 Gy irradiation (Figure 3). Long-term exposure to nicotine had varying effects on cell line responses after addition of TMZ or 5 Gy irradiation. Migration rates following 0.1 LTN exposure were increased without other treatments on two cell lines,

MU-1431 (p = 0.004) and MU-1454 (p = 0.019). U87-MG was the only cell line to demonstrate increased migration at 0.5 LTN (p = 0.0089).

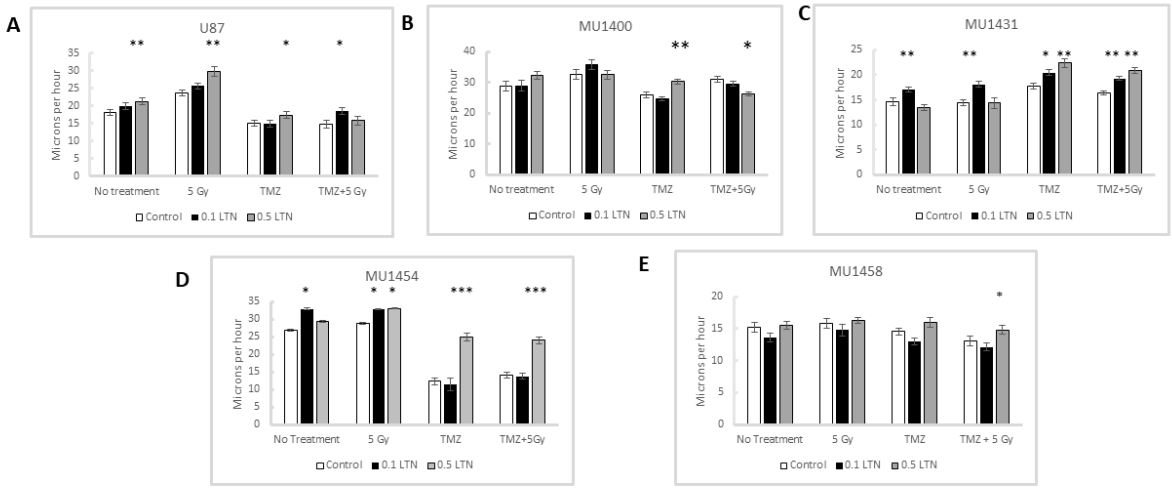

Figure 3. Quantitative analysis of scratch migration assays. Significance denoted as * $p < 0.05$, ** $p<0.01$, *** $p < 0.001$. Data is shown as mean +/- SEM. Size of the error bars are too small to be seen in some graphs. Control = no nicotine; 0.1LTN = long-term pre-exposure 0.1 μM nicotine; 0.5LTN = long-term pre-exposure 0.5 μM nicotine; Gy = Gray; TMZ = Temozolomide

For MU-1431, both 0.1 LTN and MU-1431 0.5 LTN demonstrated enhanced migration rate despite the addition of TMZ (p = 0.011 and p = 0.001 respectively). While MU-1458 0.1 LTN and MU-1458 0.5 LTN did not show a significant response, U87-MG 0.5 LTN (p = 0.023), MU-1400 0.5 LTN (p = 0.001), and MU-1454 0.5 LTN (p = 0.0001) had enhanced migration rates despite TMZ treatment.

Migration following irradiation was also enhanced if cells were pre-exposed to nicotine as seen with MU-1454 0.1 LTN (p = 0.024), MU-1431 0.1 LTN (p = 0.003), U87-MG 0.5 LTN (p = 0.0099) and MU-1454 0.5 LTN (p = 0.35). The greatest migration response for four of the cell lines occurred after 0.5 LTN pre-exposure and treatment with both TMZ and 5 Gy irradiation. (MU-1431 0.5LTN p = 0.0010, MU-1454 0.5 LTN p = 0.0001, MU-1458 0.5 LTN p = 0.46). Conversely, long-term nicotine exposure had a synergistic effect on the efficacy of radiation and TMZ (p = 0.023) for MU-1400 0.5 LTN where migration was significantly slowed compared to the control wells. Both MU-1431 0.1 LTN and U87-MG 0.1 LTN treated with TMZ + 5 Gy irradiation also exhibited increased migration (p = 0.00766, p = 0.012).

Nicotine exposure has variable and inconsistent effects on MMP2 and MMP9 activity

Standard gelatinase zymography (Figures 4A-E) was used to examine expression of MMP activity in response to nicotine exposure and subsequent treatment with TMZ and 5 Gy irradiation. All five cell lines expressed a strong band corresponding to pro-MMP-2 (72 kDa) at baseline. MU-1454 expressed a slight band at 92 kDa indicative of pro- MMP-9 gelatinase activity. No differences in density for the MMP-9 band between treatment groups in MU-1454 was detected although the density of this band for 0.5 μM was more strongly expressed within the treatment groups (data not graphed).

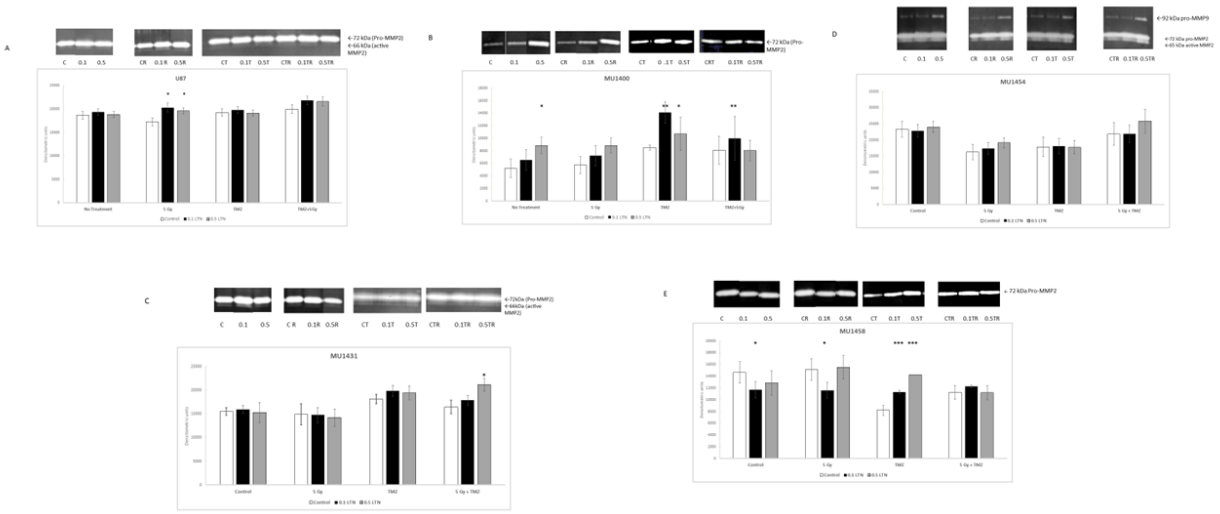

Figure 4. Representatives of gel zymograms and densitometric analysis. for matrix metalloproteinases-2. A. U87 B. MU1400 C. MU1431 D. MU1454 (and metalloproteinases -9) E. MU1458. Significance denoted as * p< 0.05, ** p<0.01, *** p < 0.001. C = control; 0.1 = long-term pre-exposure 0.1 μM nicotine; 0.5 = long-term pre-exposure 0.5 μM nicotine; LTN = long-term pre-exposure; R = irradiation 5 Gy; T = Temozolomide; TR = Temozolomide and irradiation 5 Gy

Efficacy of treatment with TMZ and/or irradiation after exposure to nicotine showed a varied and limited response among the five cell lines. MU-1400 was the only cell line to show significance in pro-MMP2 enzymatic activity in the absence of treatment and only at the highest concentration of nicotine (significance at 0.5 LTN vs control p = 0.0403). MU-1400 0.1 LTN and MU-1400 0.5 LTN MMP2 band density were the strongest when treated with TMZ alone (p = 0.0098 and p = 0.038 respectively) as compared to control, as well as when TMZ treatment was followed by 5Gy irradiation (MU-1400 0.1 LTN p = 0.008). Differences for U87 0.1 LTN and U87 0.5 LTN were only detected in irradiated cells versus matching controls (p = 0.028, p = 0.040 respectively). MU-1454 had a consistent linear nicotine-dose-dependent increased density for all treatments; a visible MMP-9 band density was also present. MU-1458 0.1 LTN showed decreased MMP-2 enzymatic activity at baseline with no additional treatments (p = 0.044). This decreased activity was also evident when MU-1458 0.1 LTN was subjected to irradiation (p = 0.047). MU-1458 0.1 LTN and MU-1458 0.5 LTN exhibited a linear response to treatment with TMZ (p = 0.025, p = 0.021). The combination of TMZ and irradiation provoked an increase in activity only for MU-1458 0.1 LTN, but not statistically significant compared to control. MU-1431 showed few differences between the three groups when challenged with the different therapeutic provocations with one exception - TMZ + 5 Gy treatment of MU-1431 0.5 LTN had enhanced MMP2 enzymatic activity (p = 0.023). In summary, exposure to nicotine at 0.1 LTN and/or 0.5 LTN prior to treatment with either TMZ and/or 5 Gy resulted in increased activity of MMP-2 in a few of the cell lines, however the increased activity was rarely linear with nicotine concentration and was variable among the cell lines.

DISCUSSION

We evaluated the effects of exposure of five GBM cell lines to low- and high- dose nicotine alone, and then with treatment with 73 μM TMZ and/or 5 Gy irradiation. We found variable effects of enhanced colony formation, enhanced migration and enhanced MMP2 expression in some cell lines. While initial pilot data utilized acute treatment of nicotine on naïve cell lines, the subsequent study design was intended to mimic the situation of long-term smokers prior to, and during, chemo-radiotherapy, recognizing that rarely will a cancer patient begin using nicotine-containing products following a cancer diagnosis. Rather, cancer patients struggle with abstaining from smoking post-diagnosis.[14] Only two other studies have specifically challenged GBM cells with nicotine, and those were acute exposure studies.[18, 35] Cultured cells were exposed to nicotine daily for 7 to 14 days prior to subsequent experiments. Concentration of the liquid nicotine solution used was based on the steady-state range of serum concentrations in smokers between 0.1– 0.5 μM[24] and nicotine replacement product users between 0.035-0.2 μM.[33] Exposure to nicotine prior to and during treatment with TMZ and/or radiation could potentially have a negative effect on treatment

results. The varied responses seen in this preliminary study suggest that cell types and molecular determinants (IDH mutation status; MGMT promoter methylation status) likely play roles in their sensitivity to nicotine.[24] While proliferation is not affected by the inclusion of nicotine exposure prior to treatment with TMZ or irradiation, migration rate, MMP expression, and colony formation are enhanced in some of the cell lines. These findings that nicotine could enhance colony formation following irradiation agree with Khalil, *et al.*[18]

Nicotine is highly addictive. Many patients who smoke when diagnosed with GBM continue to use nicotine-containing products during treatment despite counseling against such use. Studies examining nicotine effects on GBM are limited. Khalil, *et al.*[18] showed that acute treatment of cultured cells with nicotine at the time of assay enhanced proliferation, migration, radio-resistance and invasion of GBM cells, and concluded that nicotine increased radio-resistance of GBM cells as demonstrated by enhanced colony formation. Nicotine exposure of cultured GBM cells can upregulate functional nAChR receptors, thereby increasing cell proliferation.[19] Upregulation in nAChR expression has been shown to play a role in GBM tumor cell proliferation [19] and invasion primarily due to increased activity of MMPs, especially MMP-9, via downstream activation of the AKT pathway.[20] Khali *et al* [18] propose that nicotine exerts *in vitro* effects on malignant behavior of GBM via activation of P13K/AKT and MEK/ERK pathways through increased expression of EGFR. Increases in MMP-2 and MMP-9 have been implicated in worsened prognosis for GBM patients.[21-23, 36] On the other hand, radiation therapy can cause an increase in MMP-2 expression in GBM via the EGFR pathway.[29] Thus, one could reasonably consider that nicotine could cause a synergistic or additive effect if being used during radiation treatment. Our results do not support such an effect. Future studies utilizing PCR and Western blot techniques to identify expression of P13K, AKT and EGFR status are necessary to address this unexpected lack of responsiveness of the cells and to identify the pathways affected by the long-term exposure of the cells to nicotine in the media, as well as to determine if nicotine might have different effects in high grade diffuse gliomas depending on their IDH mutational status.

The effect of nicotine on TMZ efficacy has not been well described. U87-MG was reported to have enhanced growth when treated with 100 nM TMZ with acute exposure to nicotine.[28] By contrast, TMZ at physiological doses of 25-100 μM has no effect on U87-MG growth in this same laboratory; correlation of TMZ and nicotine effects is difficult to ascertain as TMZ concentrations in this instance are significantly below a feasible effective dose. The primary pathway for intrinsic resistant of GBM to TMZ has been attributed to increased expression of the P13K/AKT pathway, but not the MEK/ERK pathway, which leads to desensitization of tumor cells to TMZ, However the MEK/ERK pathway has been incriminated in the resistance of GBM cells to TMZ when in the presence of increased MGMT methylation.[37,38] Nicotine increases autophosphorylation of EGFR and subsequent cellular proliferation.[29] Phosphorylated EGFR and increased EGFR expression promote activation of the p13K/AKT and MEK/ERK pathways which suggests that nicotine could desensitize GBM cells to TMZ.[18] In our study, cellular proliferation in the presence of 73 μM TMZ for the five cell lines was unaffected in the setting of long-term nicotine exposure. However, migration activity increased in three cell lines (two of these lines at both concentrations of nicotine) either after TMZ alone or in combination of TMZ + 5 Gy, whereas an increase in MMP2 expression was noted in all five cell lines when cells are treated with TMZ following longer-term exposure to nicotine, especially at 0.5 LTN. As increased migration can occur through the EGFR-activated MEK/ERK pathway [18], nicotine probably does not directly affect TMZ efficacy. Additional studies, including immunohistochemistry on archival matched surgical tissues, or assaying signal transduction of the activated EGFR receptors by Western Blot techniques would be useful for the determination of the EGFR status of the primary cell lines at collection. Identification of primary cell lines over-expressing EGFR would potentially allow these cells to be more susceptible to nicotine challenges and may explain the varied responses seen in this study.

Previous published studies have shown that U87-MG cells and two primary glioblastoma cell lines, of unknown smoking status (one TMZ-resistant and the other over-expressing EGFR), are responsive to nicotine exposure with increased cellular proliferation.[18, 19] Our observations are in conflict with those results. One explanation for the difference is that the other studies exposed naive cells acutely to nicotine only at the time of assay. Nicotine can interact with the cell membrane through activation on nAChR, Beta Adrenergic Receptors (BAR) and EGFR.[1] Pucci *et al* [19] demonstrated that challenging naïve GBM cells with nicotine increased expression of several nAChR,

specifically α7-and α9-nAChR subtypes, activating downstream pathways leading to cellular proliferation. Chronic nicotine exposure of neural cells *in vivo* was associated with increased density of α7-nAChR as well as desensitization to additional nicotine, requiring higher doses of nicotine for same effect as non-sensitized cells.[25,41] Possibly, the physiological dose of nicotine used in this study was not sufficient to activate the pathway through nAChRs to lead to an increased cellular proliferation. Another explanation is that different experimental systems for measuring cell viability were used. While one study used manual cell counting following trypsinization to measure proliferation,[19] the other used serum starvation (0.5% FBS vs 20%).[18] Serum deprivation or starvation in cell viability/proliferation assays can create an environment in which the effects of nicotine on proliferation are more pronounced.[33] The ability to identify the density of α7-nAChR in the various cell lines before and after nicotine exposure by cytospin immunocytochemistry and Western Blotting could lend some insight into the effects of nicotine on these cell lines.

This study explicitly examines the effect of nicotine on MMP enzymatic activity in relation to migration rates of glioblastoma cells. The levels of expression of MMP-2 did not correlate with expected increases in migration rates. MU-1431 has little to no discernible changes in MMP expression following treatment except for MU-1431 0.5 LTN + TMZ + 5 Gy; however, this cell line is most sensitive to the increased migration in the setting of chronic nicotine exposure MU-1400 had increased migration only when MU-1400 0.5 LTN is followed by TMZ. MMP-2 activity was increased in MU-1400 at both pre-exposure nicotine concentrations for control and irradiated groups, as well as MU-1400 0.1 LTN + TMZ + 5 Gy and to a lesser extent with MU-1400 0.5 LTN +TMZ. TMZ does not affect the transcription, expression, or activity of MMP-2 or MMP-9.[40] The increased migration may be due to another pathway, perhaps HIF1α [41, 42] which is also activated through the AKT or MEK/ERK pathway[44, 45] and may have prognostication potential for GBM.[42] The lack of identifiable MMP-9 in most samples is not surprising as MMP-9 requires specific growth factors to be inducible in cultured cells whereas MMP-2 is often constitutively expressed.[46] Additionally, the migration assay in this study may not be as dependent on MMP as other assays.

The effect of prior smoking history of patients (Table I) on cellular responses is worth reflecting upon. One consideration is that primary low passage cells from smokers could be desensitized to experimental nicotine exposure or that baseline MMP activity could be high and not as responsive for the demonstration of significant changes. However, initially it would appear that neither mechanism appears present, as MU-1431, MU-1454 and MU-1458 obtained from patients with greater than 38 -, 45- and 90- pack-years smoking history respectively, responded differently; MU1431 ("light to moderate" smoker) had limited responses of increased MMP2 at high dose of nicotine combined with TMZ and irradiation, MU-1454 ("moderate" smoker) has no increased MMP2, whereas MU-1458 ("heavy smoker") had increased MMP2 enzymatic activity when nicotine was followed by TMZ. This could suggest that the level of smoking plays a role in the response of the cells to nicotine and TMZ and irradiation in expression of MMP2 activity, however it should be cautioned that cultured GBM cells may not retain their MMP expression pattern and the expression is altered as early as the first passage, especially with MMP-9, as compared to original tumor tissue.[47] Of further note, differences were not detected between cells propagated from smokers versus non-smokers (MU-1400) which weaken the hypothesis that pre-smoking history influenced the responses of the cultured cells. Following cessation of nicotine-containing products in laboratory animals, normalization of nicotinic receptor binding sites in neural tissues occurs within 6 days, matching those of the control animals not receiving nicotine-containing products.[48] During the initial propagation of these cells, nicotine was not present in the media, and therefore reversion to normal could have occurred regarding the density of nAChR. Also notable is that migration was affected by continuous use of nicotine in the cells obtained from the "light to moderate" (MU-1431) and "moderate" (MU-1454) smokers and not the "heavy" smoker (MU-1458), again suggesting that the level of smoking may be a determinant in the response of the cells to TMZ and irradiation, however the underlying cellular pathway is difficult to ascertain in this study. Other factors that differed between the smokers were their molecular determinants and profiles. Both cell lines are IDH wild-type as seen in Table I, but each has other molecular determinants. Whether these molecular differences can influence the tumor cells response to nicotine remains to be determined. As mentioned previously, future studies with these or other cell lines need to include determination of the expression of EGFR and the relationship to migration and MMP2 activity would be useful. Furthermore, the question of

desensitization also needs to be more rigorously considered.

The commercially available U87-MG cell line has the most variable and conflicting response after exposure to nicotine, especially following radiation. U87-MG is the only cell line with increased proliferation in response to nicotine, but nicotine appears to sensitize the U87-MG cells to TMZ alone or in combination with 5 Gy. The higher dose of nicotine enhance migration with all treatment combinations. This finding further exemplifies that tumor cells possess different intrinsic responses to experimental therapies. Additional studies are needed to identify the molecular pathways involved.

Limitations in this study include the lack of information related to the intrinsic pathways to explain the differences seen between the cell lines. Future studies to be included would be Western Blot techniques and qPCR to further identify gene and molecular expression of phosphorylation of $EGFR^{tyr992}$, AKT^{ser473}, and ERK, as well as density of nAChR, especially in the initial cell passage of the cell lines to obtain a base-line value. This information would also supplement investigations as to how previous smoking histories of patients from whom primary cells are obtained express these proteins, and how stable is this expression in multiple passages when using them in nicotine-exposure studies. Lastly, this study only assessed 5 cell lines, so generalization of these results to all GBM, a very heterogenous tumor within and between individuals, will require additional study on other cell lines and in *in vivo* models.

Recognition that nicotine-containing products may have a detrimental influence on efficacy of TMZ and irradiation in GBM is important. Continued use of nicotine during treatment could potentially result in increased aggressiveness and invasion of residual tumor cells, causing a resistance to treatment. The difficulty of abstention from nicotine-containing products could prove challenging as the low dose of nicotine used in this study mimics blood levels expected from using nicotine-replacement products.

CONCLUSION

At physiologically relevant concentration, exposure of nicotine variably enhances migration and/or MMP-2 expression in some GBM cell lines, even despite TMZ and/or irradiation. Additional studies confirming the role of molecular determinants may help identify those patients more at risk to the negative effects of nicotine.

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