

## ***In Vitro* Validation of Intratumoral Modulation Therapy for Glioblastoma**

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**Abstract.** *Background/Aim:* This proof-of-concept study evaluated the antitumor impact of a direct electrical stimulation technique, termed intratumoral modulation therapy (IMT) on glioblastoma (GBM) cells. *Materials and Methods:* An *in vitro* IMT model comprised of a calibrated electrode to deliver continuous, low-intensity stimulation within GBM preparations. Viability and apoptosis assays were performed in treated immortalized and patient-derived GBM cells, and post-mitotic neurons. IMT was delivered alone and with temozolomide, or gene silencing of the tumor-promoting chaperone, heat-shock protein 27 (HSP27). *Results:* GBM cells, but not neurons, exhibited >40% loss of viability, caspase-3 activation and apoptosis with IMT. Cell death was modest with temozolomide alone (30%) but increased significantly with concomitant IMT (70%). HSP27 silencing alone produced 30% viability loss, with significant enhancement of target knockdown and GBM cell death (65%), when combined with IMT. *Conclusion:* These findings warrant further evaluation of IMT as a potential novel therapeutic strategy for GBM.

Glioblastoma (GBM) is the most common primary brain tumor in adults, with highly invasive cells that infiltrate multiple cerebral lobes, deep nuclei and across midline commissures. Standard-of-care entails maximal safe resection followed by chemoradiation and affords a median survival of 12-18 months (1). Surgery may be limited or not safely feasible when eloquent brain regions are involved, thus further reducing the duration of tumor control and patient survival. There exists

rapidly emerging evidence that supports the use of electrotherapy to complement current options for GBM treatment. Although the vulnerability of tumor cells to electrochemical perturbations is known, direct neuromodulation techniques have not been described for glioma management (2, 3). If effective, such strategies may prompt development of implantable technology to deliver low-intensity stimulation within tumor-affected brain regions. We refer to this approach as intratumoral modulation therapy (IMT), that is conceptually supported by the clinical use of an external electrotherapy device for GBM (3) and intracerebral neuromodulation systems for non-oncological disorders (4). To date, however, the efficacy of IMT-type stimulation delivered directly to GBM cells has not been defined and remains an unanswered question fundamental to advancing this technology.

The impact of low-intensity current delivered to the brain is complex and present clinical applications, such as deep brain stimulation for movement disorders, typically produce no overt damage while altering the electrical activity of normal brain tissue in a titratable manner (4-6). GBM cells are susceptible to otherwise innocuous electrical stimulation owing to their high proliferative rate, metabolic dysfunction and genetic instability (2). The advantages of an IMT strategy, in the context of GBM treatment, are speculated to include direct tumor cytotoxicity and summative or potentiated antitumor benefits when combined with alternate treatment modalities. In particular, IMT may prove beneficial as a mediator of electrochemotherapy and electrogene therapy, wherein stimulation is used to facilitate uptake and function of pharmacological agents and therapeutic genetic material, respectively (7). The objective of the present study was to provide proof-of concept data supporting or refuting antitumor effects of low-intensity electrical current, using an *in vitro* IMT model, as a stand-alone treatment in GBM cell lines and in primary GBM cells isolated from operative patient specimens. In addition, the therapeutic impact of combined IMT with the standard chemotherapeutic agent, temozolomide, was assessed. Finally, IMT was evaluated as a facilitator of small interfering RNA (siRNA) therapy targeting

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HSP27, a heat-shock protein with known oncological function as an apoptosis inhibitor and efficacy as a therapeutic target in cancer cells (8).

## Materials and Methods

**GBM tissue preparation and cell cultures.** This study was approved by the Research Ethics Board at the University of Western Ontario (Approval #17290). GBM specimens were obtained at the time of operative resection and placed immediately into phosphate-buffered saline (PBS) with 0.5% fetal bovine serum (FBS; Life Technologies, Burlington, ON, Canada). The tissue was washed, digested and filtered through a 100- $\mu$ m cell strainer. Samples were then centrifuged and resuspended in Dulbecco's modified Eagle's medium (DMEM; Wisent Bioproducts, St. Bruno, PQ, Canada) supplemented with 10% FBS, 1% non-essential amino acids and 1% penicillin/streptomycin (Life Technologies) before plating to a 35-mm dish for 30 min to allow blood cells to separate. The upper cell suspension was then transferred to two wells of a 24-well plate, freshly pre-coated with 10  $\mu$ g/ml poly-L-lysine (Trevigen Inc., Gaithersburg, MD, USA) and incubated at 37°C with 5% CO<sub>2</sub>. Cultures were passaged at approximately 80% confluence and split 1:2 using 0.25% trypsin with 0.53 mM ethylenediaminetetraacetic acid (EDTA; Wisent). The medium was changed twice per week. All assays were conducted using GBM cells from cultures at passages 4 through 12.

Human LN229 GBM cells (ATCC, Manassas, VA, USA) were maintained in DMEM supplemented with 10% FBS, 1% non-essential amino acids and 1% penicillin/streptomycin (Life Technologies) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were passaged every 2-3 days using 0.25% trypsin-EDTA (Wisent). At the exponential phase of growth, cells were seeded in 35 mm wells of a 6-well plate in maintenance medium for 24 h prior to treatments.

**Embryonic rat neuronal cultures.** This protocol met the standards of the Canadian Council on Animal Care and was approved by the University of Western Ontario Animal Use Subcommittee (Approval #2014-016). IMT was performed in primary neuronal cultures (N=3) to determine its effects on post-mitotic neural cells. As primary human neurons are not readily available, these studies were conducted in preparations isolated from embryonic rat brain. Pregnant female Wistar rats (Charles River, Montreal, PQ, Canada) were sacrificed by cervical dislocation for surgical removal of E18 embryos. Cortices from each embryo were extracted and placed in a 14 ml conical tube containing 1.8 ml of Hank's balanced salt solution (HBSS; Wisent) and centrifuged at 4000  $\times$  g for 1 min at room temperature. HBSS was aspirated and 1.8 ml of solution A containing 5 ml HBSS, 6  $\mu$ l MgSO<sub>4</sub> (1 M) and 2 ml trypsin (Sigma Aldrich, St. Louis, MO, USA) were added. The tube was mixed well, ensuring the neurons were free floating, and placed in an automated rotator at 37°C for 25 minutes. After rotation, 3.6 ml of solution B containing 7 ml HBSS, 8  $\mu$ l MgSO<sub>4</sub> (1 M), 175  $\mu$ l DNase1 (10 mg/ml) and 112  $\mu$ l trypsin inhibitor (100  $\mu$ g/ml; Roche Life Sciences, Indianapolis, IN, USA) was added to the conical tube and mixed for 2 minutes, centrifuged at 4000  $\times$  g for 5 min at room temperature, after which the HBSS was aspirated. Finally, 6 ml of a solution C containing 20 ml of HBSS, 48  $\mu$ l MgSO<sub>4</sub> (1 M), 1.3 ml DNase1 (10 mg/ml), and 1 ml trypsin inhibitor (100  $\mu$ g/ml) was

added to the resulting cell pellet (Roche). These cells were transferred to a 50 ml falcon tube and another 6 ml of solution C was added. The cells were titrated, centrifuged at 4000  $\times$  g for 5 minutes and the supernatant aspirated. The cell pellet was resuspended in 36 ml of neurobasal plating media containing 96% neural basal media (Wisent), 2% B27 supplement, 0.8% N2 Supplement, 0.5% penicillin/streptomycin, 0.25% Glutamax (Life Technologies), and 0.1% Amphotericin B solution (Sigma Aldrich). Cells were counted with a hemocytometer, plated in 35 mm wells coated with 7% poly-L-Ornithine (Sigma Aldrich) at density of 0.5 $\times$ 10<sup>6</sup> cells/well and kept in an incubator at 37°C with 5% CO<sub>2</sub>. The medium was changed on the third day of culture, then wells were fitted with the IMT apparatus (see below) for delivery of 72 h of sham or IMT conditions.

**In vitro IMT model.** GBM cells (2 $\times$ 10<sup>5</sup> cells in 2 ml maintenance DMEM) were transferred to 35 mm wells in standard 6-well plates and allowed to grow to ~70% confluence before treatment. The IMT model was created by fashioning each well with a clinical grade, platinum-based reference strip electrode (AD-Tech, Racine, WI, USA) around the periphery and a stimulating electrode (Medtronic Ltd., Brampton, ON, Canada) in the centre of the well. The electrodes were connected to a waveform generator set to produce monophasic, square-wave pulses of 4 volts, with pulse width of 90  $\mu$ sec and frequency of 130 Hz. This setting is in the range of that commonly used in clinical neuromodulation treatment for symptoms of movement disorders, such as Parkinson's disease (4-6). Control wells (*i.e.* sham-treated) were fitted with electrodes but no current was delivered. Treatment durations between 24-96 h were used to allow adequate time for antitumor effect while avoiding the need for medium change once IMT was initiated. Thus, all intact GBM cells, adherent and floating, contributed to the viability measures described below. GBM cells treated with chemotherapy were plated with DMEM containing temozolomide (50  $\mu$ M; Sigma Aldrich) in 35 mm wells fitted with the IMT apparatus and received 72 h of concomitant IMT or sham conditions. The 50  $\mu$ M temozolomide concentration reflects clinically relevant levels corresponding to the *in vivo* plasma concentration of 150 mg/m<sup>2</sup> in the adjuvant phase of GBM treatment (9).

**Concomitant IMT and HSP27 knockdown.** Primary patient GBM cells (1 $\times$ 10<sup>5</sup> cells in 2 ml maintenance DMEM) were seeded into one 35 mm well equipped with the IMT system and allowed to grow to ~70% confluence. Cells were transfected with siRNA targeting human HSP27 mRNA (50 nM) or an equivalent concentration of non-specific control siRNA (siRNA Universal Negative Control, Sigma Aldrich) using jetPRIME™ transfection reagent (Polyplus Transfection, New York, NY, USA) (10). The culture medium was replaced with 210  $\mu$ l of jetPRIME-siRNA complex in 2 ml DMEM with 10% FBS. The transfected cells were incubated for 48 h at 37°C with 5% CO<sub>2</sub>. In the IMT-siRNA conditions, IMT was initiated at the time of transfection and maintained for the entire 48 h, after which the extent of target knockdown and GBM cell viability were assessed.

**Cell viability assays.** Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) spectral analysis (Sigma Aldrich). This colorimetric assay measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase to an insoluble, dark purple Formosan product. Immediately

following the GBM cell treatments described above, MTT (80  $\mu$ l at 5 mg/ml) was added to the 35 mm wells and incubated for 3 hours at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The cells were then lysed to release the purple Formosan product by the addition of 600  $\mu$ l dimethyl sulfoxide for 15 min at room temperature. Absorbance was measured using an enzyme-linked immunosorbent assay plate reader (Fisher Scientific, Nepean, ON, Canada). Cell viability was estimated using optical density values at 570 nm with references at 655 nm detected in each well.

Trypan blue exclusion was used as a confirmatory, qualitative measure of cell viability. Briefly, 0.1 ml of a 0.4% trypan blue solution (Lonza, Walkersville, MD, USA) was added for every 1 ml culture media and the cells then incubated for 2 min at room temperature. Brightfield images of cells were obtained using a Motic AE31 inverted microscope fitted with an Infinity1-3 scientific complementary metal-oxide semiconductor camera (Lumenera Corp., Ottawa, ON, Canada).

**Flow cytometry.** An Annexin V Apoptosis Detection Kit with propidium iodide (PI; BioLegend, San Diego, CA, USA) was used for identification of apoptotic and dead cells, as per the manufacturer's instructions. Cell fractions were analyzed using a Becton Dickinson LSR II SORP flow cytometer running FACSDiva software (BD Biosciences, Mississauga, ON, Canada). Cells were first gated on forward scatter (FSC-) *versus* side scatter (SSC-) characteristics before excluding doublets using consecutive gating FSC-Area *versus* FSC-Width and SSC-Area *versus* SSC-Width plots. The populations of annexin V+/PI-, annexin V+/PI+, annexin V-/PI+ and annexin V-/PI- were then calculated with quadrant gates. Approximately 30,000 single cells were acquired per sample at a maximum event rate of 5,000 events per second. Data were analyzed using FlowJo v 9.6.3 (TreeStar, Inc., Ashland, OR, USA).

**Western blot analysis.** Cells were collected in lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P40, pH 7.4) supplemented with SIGMAFAST™ Protease Inhibitor cocktail (1:10), incubated on ice for 15 min then sonicated (Sigma Aldrich). The cell lysates were centrifuged and the protein supernatant collected. Twenty micrograms of each protein extract were separated on a 10% sodium dodecyl sulphate polyacrylamide gel and transferred electrophoretically to Immobilon-Blot® membranes (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The membranes were blocked, then incubated overnight at 4°C with primary antibodies to HSP27 (1:1000), HSP90 (1:800), or activated caspase-3 (1:500; EMD Millipore Corp., Billerica, MA., USA). Membranes were washed then incubated with a horseradish peroxidase-conjugated secondary antibody (1:3,000; Bio-Rad) for 1 hour at room temperature. Peroxidase activity was visualized using an enhanced chemiluminescence and detection system imager (GE Healthcare Biosciences, Piscataway, NJ, USA). Membranes were then stripped, blocked and re-probed with an anti- $\beta$ -actin antibody (1:5,000; Abcam Inc, Toronto, ON, Canada) to assess protein loading.

**Immunofluorescence labeling of activated caspase-3 and confocal microscopy.** GBM cells were plated on 12 mm round cover slips (VWR International, Mississauga, ON, Canada) and collected 24 h after treatment. Cells were washed, fixed in 4% paraformaldehyde and permeabilized prior to blocking with 1% bovine serum albumin (EMD Millipore Corp.) and incubation with a primary rabbit antibody to activated caspase-3 (1:100, EMD Millipore Corp.)

overnight at 4°C. Cells were then washed and incubated with Alexa Fluor® 546 goat anti-rabbit IgG secondary antibody (1:200; Life Technologies) for 1 h at room temperature and counter-stained with 4'-6-diamidino-2-phenylindole (DAPI; Life Technologies) for nuclear visualization. Control cover slips were processed in parallel without primary antibody. Cells were imaged using a Zeiss LSM-510 META laser-scanning microscope with a Zeiss 63x NA 1.4 oil immersion lens, appropriate filters and AIM software (Carl Zeiss GmbH, Jena, Germany, EU).

**Statistical analysis.** Paired and multiple comparisons were made with Student's *t*-test or one-way analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* analysis, respectively (SigmaStat, Systat Software Inc., San Jose, CA, USA). All data are presented as the mean $\pm$ standard deviation and comparisons were considered significant at  $p < 0.05$ .

## Results

**IMT produces tumor-selective death of immortalized and primary patient GBM cells.** The effects of IMT (4V, 90  $\mu$ s, 130 Hz) were evaluated in LN229 GBM cells and GBM cells derived from three patient primary tumors. IMT, but not sham-treated GBM cells became pyknotic with disruption of membrane integrity and uptake of trypan blue. The MTT assay revealed a significant (*i.e.* 48-56%) loss of metabolic viability in patient GBM cells treated with IMT at both the 24-hour (relative MTT values: sham=0.33 $\pm$ 0.09; IMT=0.17 $\pm$ 0.03;  $p=0.043$ ) and 96-hour (relative MTT values: sham=0.45 $\pm$ 0.07; IMT=0.20 $\pm$ 0.03;  $p=0.005$ ) time points. The sham-treated cultures continued to expand between 24 and 96 hours of therapy (Figure 1). In contrast to the impact on GBM cells, IMT did not produce overt alterations in morphology or viability of rat post-mitotic neurons (relative MTT values: sham=0.63 $\pm$ 0.00; IMT=0.64 $\pm$ 0.02;  $p=0.36$ ; Figure 2).

**Apoptosis and enhanced chemotherapeutic effect in GBM cells treated with IMT.** The mechanism of IMT-mediated GBM cell death was evaluated by immunolabeling of activated caspase-3, a marker of apoptosis, and flow cytometric detection of the apoptosis and cell death markers, annexin and PI, respectively. IMT reliably and robustly increased the cellular level of activated caspase-3 in immortalized and primary patient GBM cells, consistent with the pyknotic morphology of IMT-treated GBM cells and indicative of an apoptotic death (Figure 3). Flow cytometry was performed in triplicate on primary GBM cells from three patient specimens (~30,000 cells per treatment condition for each patient specimen) to detect the apoptotic marker, annexin, and uptake of the membrane impermeant dye, PI (Figure 4 and 5A). The combined fraction of apoptotic (annexin<sup>+</sup>) and dead (annexin<sup>+</sup> and PI<sup>+</sup>) GBM cells rose dramatically from untreated (5.7 $\pm$ 2.5%) and sham conditions (5.9 $\pm$ 2.8%) to single-modality temozolomide (16.9 $\pm$ 7.4%) or

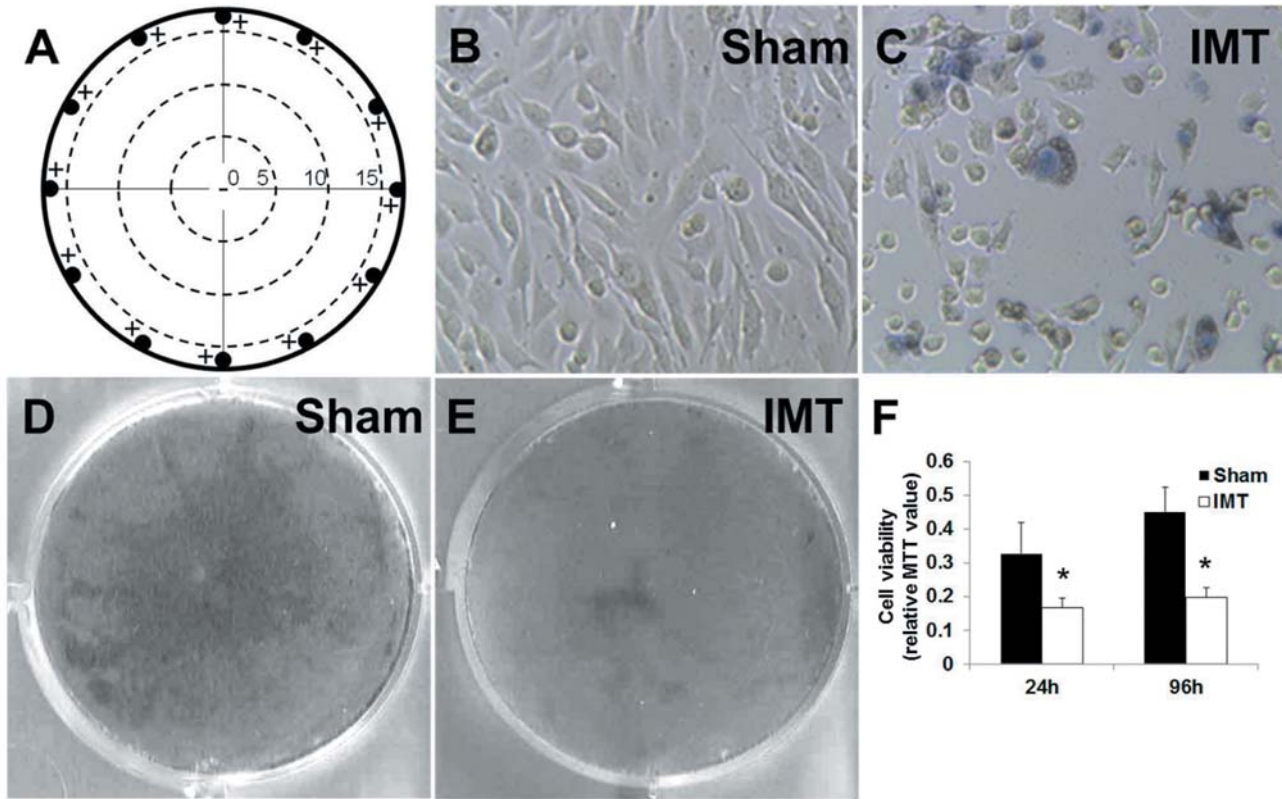


Figure 1. Intratumoral modulation therapy (IMT) induces glioblastoma (GBM) cell death. A: Schematic representation of the *in vitro* IMT model. Calibrated 35 mm wells were fitted with central stimulating (–) and peripheral reference (+; black circles) electrodes to deliver monophasic electric pulses (4V, 90  $\mu$ s, 130 Hz) into GBM cell preparations. The concentric, numbered circles indicate radial distances (mm) from the central electrode. In these studies, IMT was delivered for between 24-96 h. Brightfield microscopy ( $\times 20$ ) of primary patient GBM cells treated with 96 h of sham conditions (B) or IMT (C) and stained with the membrane-impermeant dye, trypan blue. Note the reduced cell density, extensive pyknosis and trypan blue uptake in the IMT-treated preparations. Cell viability was also evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) spectral analysis in sham- (D) and IMT-treated (E) cells, shown here in representative culture wells prior to cell lysis and spectrophotometric analysis. The sham cultures stained purple with MTT and extended across most of the culture well. In contrast, the IMT-treated preparations exhibited markedly diminished, patchy staining, consistent with extensive GBM cell death. F: Histogram showing the mean cell viability in three primary patient GBM cell preparations treated with sham conditions or IMT for 24 or 96 h (mean $\pm$ standard deviation). Note the significant loss of viability with IMT at both time points (\* $p < 0.05$ ).

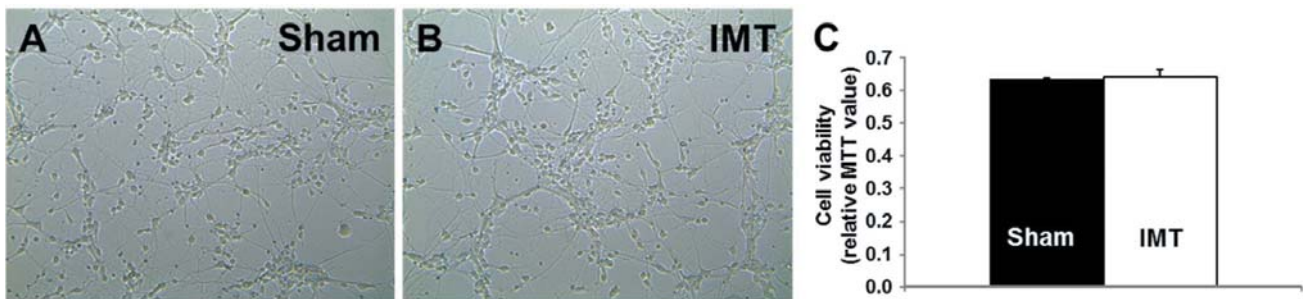


Figure 2. Intratumoral modulation therapy (IMT) produces no significant cytotoxicity in post-mitotic neurons. A, B: Embryonic rat neuronal cultures treated for 3 days with sham conditions or IMT are shown imaged with brightfield microscopy ( $\times 20$ ) after exposure to trypan blue viability dye. No significant labeling or morphological changes were identified after IMT in these cells. The histogram (C) shows the relative viability in each group as measured with the spectral MTT assay (mean $\pm$ standard deviation; N=3 per group). No significant loss of neuronal viability was found with IMT.

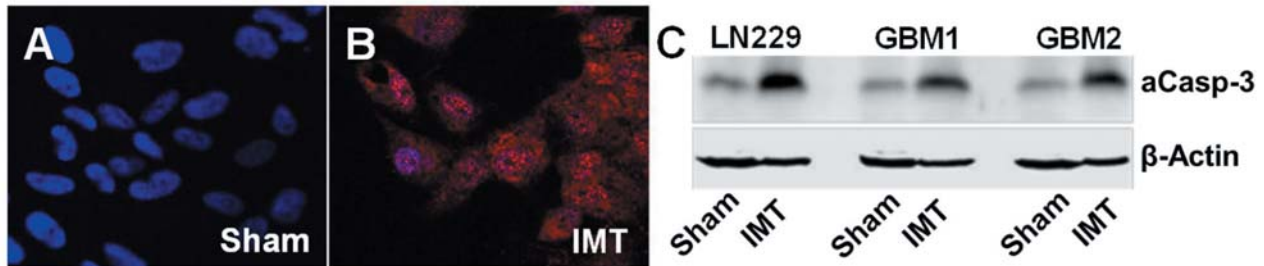


Figure 3. Intratumoral modulation therapy (IMT) induces caspase-3 activation in glioblastoma (GBM) cells. Confocal imaging ( $\times 63$  magnification) of sham-treated (A) and IMT-treated (B) primary patient GBM cells immunolabeled for activated caspase-3 (red) and counterstained with the nuclear dye, 4'-6-diamidino-2-phenylindole (blue). Compared to the low level found with sham conditions, GBM cells exposed to 72 h of IMT exhibited robust caspase-3 activation. C: Representative western blots confirmed the immunocytochemical data and demonstrate robust and consistent activation of caspase-3 in the LN229 GBM cell line and primary GBM cells (GBM1, GBM2) treated with IMT. aCasp-3, Activated caspase-3.

IMT ( $28.5 \pm 14.9\%$ ), and finally to combination treatment with temozolomide and IMT ( $52.4 \pm 21.8\%$ ). The results of the quantitative metabolic MTT assay further confirmed the detrimental impact of each treatment modality and the heightened benefits of combined IMT and temozolomide on reducing primary GBM cell viability (Figure 5B). As stand-alone treatments, IMT ( $52.2 \pm 4.8\%$  viability relative to untreated cells) was significantly more effective than temozolomide ( $69.7 \pm 11.8\%$  viability), as measured by MTT metabolism. The combination of IMT with temozolomide produced further significant GBM cell death compared to either treatment alone ( $29.1 \pm 3.2\%$  viability; Figure 5B). Comparable effects were produced in immortalized LN229 GBM cells (data not shown).

*IMT enhances the efficacy of siRNA-mediated gene knockdown in GBM.* Gene silencing methods in primary, patient-derived GBM cells are hindered by poor uptake of hydrophilic genetic material across lipid membranes. This study tested the notion that IMT may act in concert with *HSP27* siRNA, possibly by enhancing uptake and bio-availability of siRNA in the cells or through a secondary means of impairing cytokinesis and anti-apoptotic mechanisms. There was avid expression of *HSP27* in patient GBM cells that was not notably affected by control or IMT conditions. In contrast, non-viral transfection of *HSP27*-specific siRNA (50 nM) using a cationic polymer resulted in a moderate reduction in *HSP27* levels that was significantly and consistently enhanced with concomitant IMT (Figure 6A). Quantitative densitometry of western blot analyses was performed in triplicate using GBM cells obtained from three patients and confirmed no significant change in the level of *HSP27* expression among sham-treated cells [ $0.40 \pm 0.08$  normalized optical density (OD)], control siRNA-treated ( $0.46 \pm 0.05$  OD), IMT-treated ( $0.45 \pm 0.08$  OD), or the combination of IMT with control siRNA-treated cells

( $0.43 \pm 0.09$  OD). In contrast, GBM cells transfected with *HSP27* siRNA alone ( $0.27 \pm 0.04$  OD) or the combination of IMT and *HSP27* siRNA ( $0.07 \pm 0.02$  OD) exhibited significant reductions in *HSP27* levels of 32.5% and 82.5%, respectively (Figure 6B). With either *HSP27* siRNA or IMT plus *HSP27* siRNA treatment, there was no reduction in the expression of *HSP90*, a related stress-response chaperones, further supporting the specificity of the gene-targeting method and antitumor impact (Figure 6C). GBM cell viability in patient specimens was quantified with MTT and, as in the previous series, demonstrated significantly reduced values following IMT alone ( $60.3 \pm 7.7\%$  viability relative to untreated cells). *HSP27* siRNA alone also produced significant cytotoxic effects ( $70.3 \pm 5.4\%$  viability). The combination of IMT and control siRNA did not further reduce cell viability compared to IMT alone ( $57.1 \pm 8.8\%$  viability); however, IMT with concomitant *HSP27* siRNA produced a robust and significant increase in GBM cell death ( $35.9 \pm 12.8\%$  viability; Figure 7).

## Discussion

New strategies are required to treat GBM and emerging data suggest that electrotherapy may offer a promising adjunct to surgical resection and conventional chemoradiation (2, 3). The present study sought proof-of-concept evidence for the *in vitro* antitumor efficacy of pulsed (90  $\mu$ s pulse width at 130 Hz) low-intensity (4 V) electric stimulation continuously delivered to the epicenter of tumor cell preparations *via* an indwelling electrode. This technique, termed IMT, profoundly reduced the viability of immortalized GBM cells and multiple primary patient specimens but had little impact on primary post-mitotic neurons, consistent with a relative selectivity for proliferating cancer cells. Embryonic rat brains provided adequate numbers of cortical neurons, unavailable from human sources, and comparison of IMT effects in

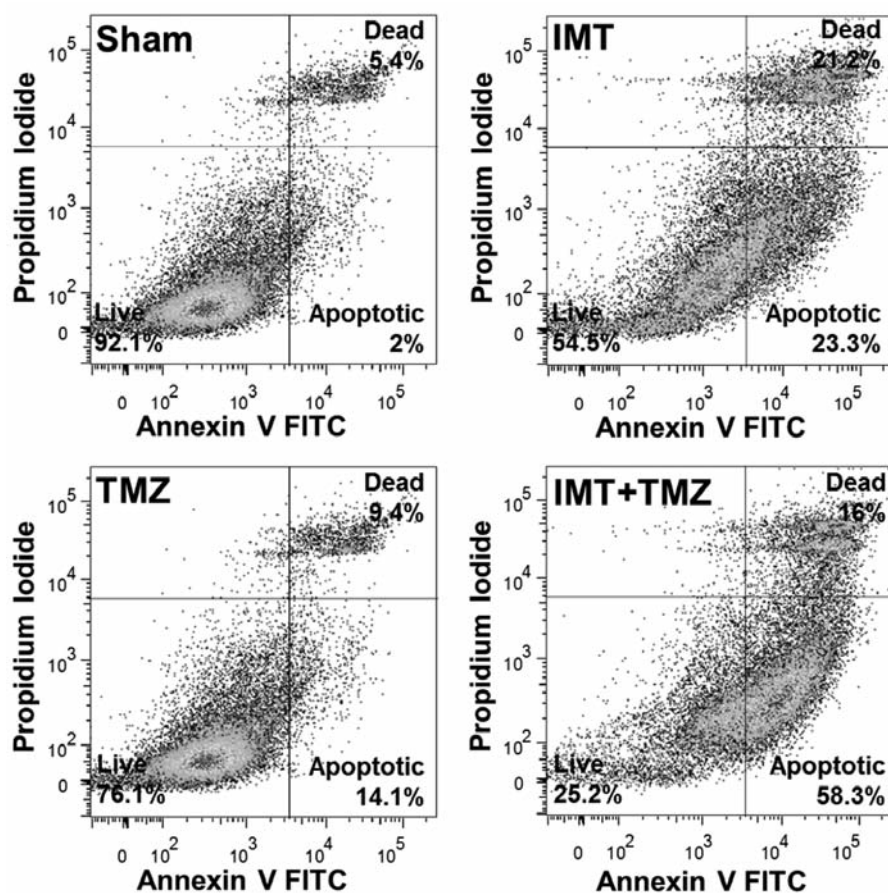


Figure 4. Apoptosis and death of glioblastoma (GBM) cells treated with concomitant intratumoral modulation therapy (IMT) and temozolomide (TMZ). Representative flow cytometric scatterplots showing annexin and propidium iodide (PI) labeling of apoptotic and dead patient GBM cells, respectively, after 72 h of the indicated treatment. There were approximately 30,000 cells analyzed for each treatment condition. Note the markedly elevated fractions of apoptotic (annexin<sup>+</sup>) and dead (annexin<sup>+</sup> and PI<sup>+</sup>) cells with the combination of IMT and temozolomide, relative to those observed with sham or either stand-alone treatments. These studies were performed in triplicate using primary GBM cells from three different patients and the quantified data are summarized in Figure 5.

preparations of primary central nervous system neurons and GBM cells. GBM cell death was largely apoptotic and associated with caspase-3 activation and annexin labeling. The viability studies produced consistent and complementary data that demonstrated the potent efficacy of IMT in GBM cell lines and primary cells. The flow cytometric values may actually underestimate the death fraction, as GBM cells that had degenerated following IMT are not measurable, thus skewing the overall ratio in favor of viability. The combination of IMT with physiological concentrations of temozolomide produced a significant increase in GBM apoptosis relative to that achieved with either treatment alone. In addition, IMT markedly enhanced target knockdown and antitumor efficacy of *HSP27* siRNA treatment when delivered concomitantly in GBM cells obtained from multiple patients.

Electrical stimulation has been used for decades to enhance the uptake of large or charged molecules into tumor cells and various forms have been tested for direct efficacy against GBM. The related fields of electrochemotherapy and electrogene therapy have arisen from evidence that perturbations of the bio-electromagnetic environment can improve cell membrane permeability of drugs and ribonucleic acids (11-14). Long duration/low-intensity pulses drive migration of charged molecules across cell membranes (*i.e.* electrophoresis) whereas short-duration/high-intensity stimuli produce hydrophilic pores through which charged substances may pass (*i.e.* electroporation) (11, 12). Studies have shown that four to eight pulses at a frequency of 1 Hz, intensity of 1,000-1,750 V/cm and pulse width of 0.1 ms produce marked cytotoxicity and enhance sensitivity to chemotherapy in GBM cells *in vitro* (15-17). Rodent *in vivo*

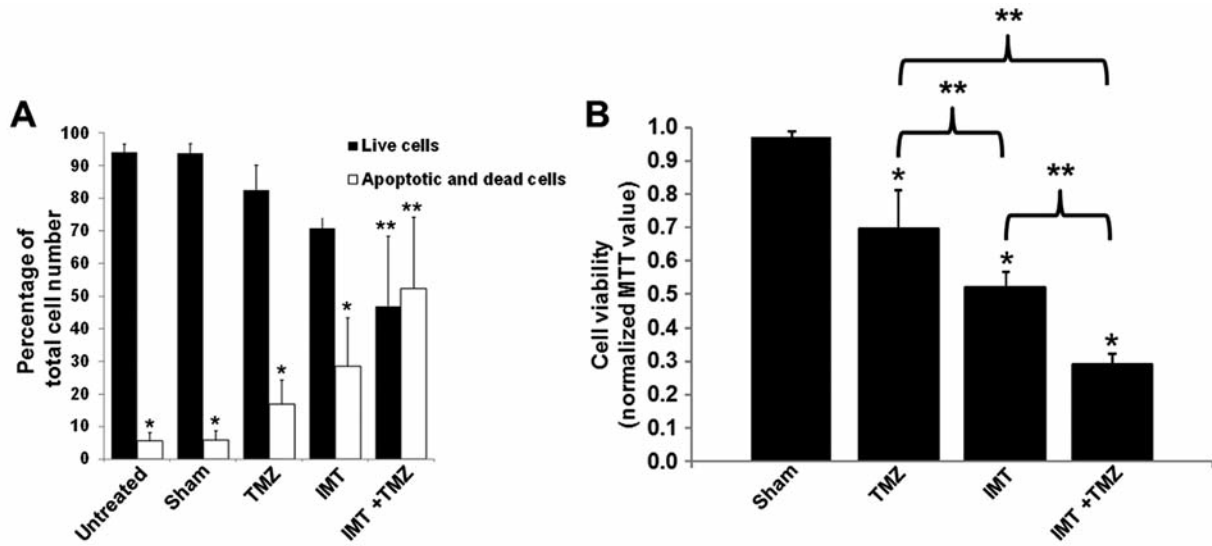


Figure 5. Quantitative effect of combined intratumoral modulation therapy (IMT) and temozolomide (TMZ) on patient glioblastoma (GBM) cells. A: Flow cytometric data showing the percentage of live and apoptotic/dead GBM cells following the indicated treatments. There was a significant difference between the percentage of live and apoptotic/dead cells within all groups (\* $p < 0.05$ , ANOVA), with the exception of the combined IMT plus temozolomide group, where treatment produced greater than 50% mean fractional GBM cell death. \*\* $p < 0.05$  (ANOVA), Significant difference between the live or apoptotic/dead fractions and the respective value obtained from untreated cells. Each treatment condition was analyzed in triplicate using approximately 30,000 GBM cells per run. B: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) spectral analysis measured GBM cell viability following control, single-agent temozolomide or IMT, and concomitant IMT and temozolomide treatments. Relative values were normalized to those of untreated cells. There was a superior antitumor effect using the combination IMT with temozolomide, with overall loss of GBM cell viability greater than 70%. Significant difference: \*from sham treatment values, \*\*between the indicated treatment pairs ( $p < 0.05$ , ANOVA). For both flow cytometric and MTT studies, the duration of treatment was 72 h and each measure shown represents the mean  $\pm$  standard deviation for primary GBM cells from three patients.

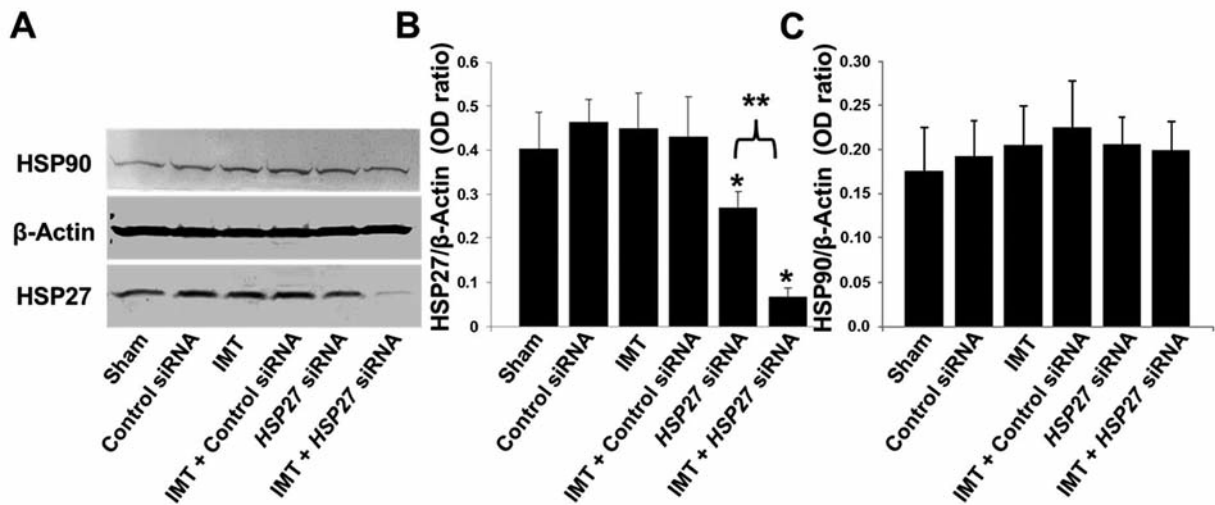


Figure 6. Intratumoral modulation therapy (IMT) enhances heat-shock protein 27 (HSP27) knockdown in glioblastoma (GBM). A: Representative western blot analysis using primary GBM cells derived from an operative tumor specimen. HSP27 siRNA transfection produced a modest target knockdown that was markedly potentiated with concurrent IMT. Sham conditions, IMT and control siRNA were ineffective at reducing the HSP27 level. Mean densitometric values of HSP27 (B) and HSP90 (C) levels in GBM cells from three patients confirmed the robust and specific knockdown of HSP27 that was significantly enhanced with the co-administration of IMT. HSP90 levels were not notably affected by any of the treatment conditions. Values represent the mean  $\pm$  standard deviation. Significant difference: \*from the protein expression measured under sham conditions, \*\*between the indicated treatment pair ( $p < 0.05$ , ANOVA). OD, Optical density.

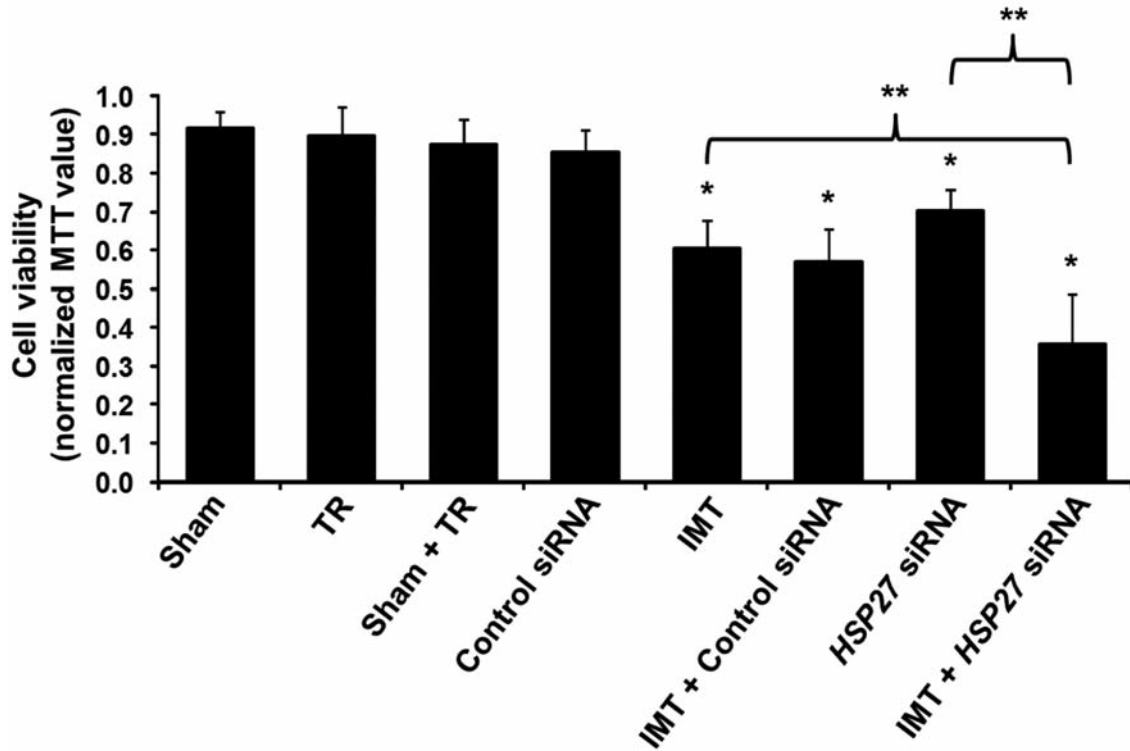


Figure 7. Intratumoral modulation therapy (IMT)-enhanced tumoricidal effect of heat-shock protein 27 (HSP27) gene silencing in glioblastoma (GBM). Adjuvant IMT enhanced the tumoricidal effect of targeted HSP27 knockdown in patient GBM cells. Individual measurements show the normalized MTT viability after 48 h of the indicated treatment. IMT alone produced marked loss of GBM viability that was robustly potentiated with HSP27-specific siRNA, but not control siRNA. Significant difference: \*from the sham-treated group, \*\*between the indicated treatment pair ( $p < 0.05$ , ANOVA). Samples were assessed in triplicate using primary GBM cells from three different patients and shown as mean  $\pm$  standard deviation. TR, Transfection reagent.

studies demonstrated that pulse trains of 400 V delivered within the glioma mass, combined with bleomycin, significantly prolonged animal survival (18). Unfortunately, stimulation with such high intensities has significant limitations on its use in human patients, particularly when the tumor is diffuse or in eloquent brain areas (19). To our knowledge, neither electrophoresis nor electroporation have been described with IMT-type stimulation in GBM.

The present study also assessed whether IMT can enhance siRNA-mediated target knockdown and therapeutic effects in patient-derived GBM cells. The heat-shock protein, HSP27, was chosen as a prototypic target for IMT-related studies due to its roles in cancer cell proliferation, migration, anti-apoptotic mechanisms and drug resistance (8, 20, 21). There are no known selective natural or synthetic protein inhibitors for HSP27 and targeted interruption requires gene-silencing strategies. We, and others, have previously shown that siRNA-mediated HSP27 inhibition reduces viability and robustly sensitizes treatment-resistant GBM cell lines to chemoradiation (10, 22, 23). Sub-optimal transfection efficiency makes the same dramatic effects more difficult to

achieve in primary GBM cells (24). The significant increase in siRNA-mediated HSP27 knockdown and primary GBM cell death produced with concomitant IMT is consistent with reported effects of other electrotherapeutic modalities, possibly through enhanced uptake of genetic material to produce a specific and robust response. Improved drug bioavailability may also contribute to the increase in cytotoxic benefits observed with the IMT-temozolomide combination, raising the possibility that IMT may offer broader application to facilitate reduced and more efficacious dosing of a host of chemotherapeutic agents in GBM. Further investigations are needed to delineate the precise mechanism(s) by which IMT produces these effects.

The use of alternating electric fields (AEFs) is another electrotherapeutic strategy that can decrease cell proliferation and viability in various cancer types, including GBM. The biological action is frequency-dependent and anticancer effects may be achieved at rates of between 10-1,000 kHz, above which there is the risk of tissue heating and thermal injury (2, 25). AEFs in this range of frequencies interfere with charged intracellular molecules and thereby



disrupt spindle microtubule organization, leading to ineffective cytokinesis and membrane rupture. Post-mitotic (*i.e.* non-neoplastic) neural cells in the brain are less impacted and AEFs appear to confer a degree of tumor selectivity. A portable device to deliver low-intensity AEFs of 200 kHz across the patient's cranium has now been approved by the U.S. Food and Drug Administration for treating recurrent GBM. A phase III clinical trial was conducted to compare AEFs with physician-choice chemotherapy in recurrent GBM (26, 27). The AEF treatment involved placement of four transducer arrays on the patient's shaved scalp that were connected to a portable generator. The field intensity was  $>0.7$  V/cm at the center of the brain. Treatment cycles were 4 weeks in duration and uninterrupted therapy was recommended, with a 1-hour break twice per day. There were fewer reported severe adverse effects and a higher quality of life with the AEFs compared to chemotherapy. While there was no difference in the overall or progression-free survival between groups, *post hoc* analysis indicated that AEFs may produce better outcomes when groups are controlled for completion of the therapeutic regimen (28). Patient compliance may present a challenge in successful AEF application as adherence to therapy was the main predictor of improved overall survival, with patients who used the device for more than 18 h a day living significantly longer than those who used it less. There is no half-life to AEFs and continuous application is required to maintain the therapeutic effect. The reasons for compliance difficulties have not been defined but may be related to operational aspects (*e.g.* requiring a shaved scalp, dermatological complications, perpetual application) and stigma of using an external treatment system. Treatment efficacy may also be limited by an inability to conform field dimensions to maximize stimulation intensity and avoid off-target injury (28-30).

The underlying rationale for IMT is the premise that an in-dwelling device to deliver electric charge within tumor-affected brain regions may exploit the known electrosensitivity of GBM cells while providing anatomically targeted, sustained and titratable therapy with low maintenance, concealed hardware for improved self-perception and quality of life. The results of this *in vitro* study provide important pilot data that must be interpreted in light of the physiological and technical limitations of the current experimental paradigm and require validation using *in vivo* GBM models. The optimal settings for IMT are expected to vary amongst different models and individuals, and have yet to be defined. It is also not known whether this treatment, as currently described, shares common mechanisms of action with other electrotherapeutic modalities. It is hoped that further advances in this field will ultimately guide development of IMT as an innovative and effective complement to current GBM therapies.

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