Activation of Multiple Molecular Mechanisms for Increasing Apoptosis in Human Glioblastoma T98G Xenograft

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Abstract

Glioblastoma is the most malignant brain tumor of astroglial origin. It renders poor response or resistance to existing therapeutics. We used all-trans retinoic acid (ATRA) and interferon gamma (IFN-γ) alone and in combination for controlling human glioblastoma T98G xenograft in nude mice. Histopathological examination showed astrocytic differentiation in ATRA group, some apoptosis in IFN-γ group, and occurrence of differentiation and enhancement of apoptosis in ATRA plus IFN-γ group. ATRA plus IFN-γ induced extrinsic pathway of apoptosis by activation of caspase-8 and cleavage of Bid to tBid and also promoted intrinsic pathway of apoptosis due to down regulation of hTERT, c-IAP2, and survivin and upregulation of Smac/Diablo. Mitochondrial release of apoptosis-inducing factor (AIF) induced caspase-independent pathway and also upregulation of calpain and caspase-dependent pathways ultimately activated caspase-3 for apoptosis. Increased activities of calpain and caspase-3 degraded 270 kDa α-spectrin at the specific sites to generate 145 kDa spectrin breakdown product (SBDP) and 120 kDa SBDP, respectively. In situ TUNEL and double immunofluorescent labelings detected apoptosis with increased expression of calpain, caspase-12, caspase-3, and AIF in tumors after treatment with IFN-γ and most effectively with ATRA plus IFN-γ. Results indicated that ATRA plus IFN-γ activated multiple molecular mechanisms for increasing apoptosis in human glioblastoma in vivo.

Keywords: Apoptosis; Calpain; Caspases; Glioblastoma; Xenograft

Abbreviations: AIF: Apoptosis Inducing Factor; ATRA: All-trans Retinoic Acid; BIRC-3: Baculovirus Inhibitor-of-Apoptosis Repeat Containing-3; BIRC-5: Baculovirus Inhibitor-of-Apoptosis Repeat Containing-5; c-IAP2: Cellular Inhibitor-of-Apoptosis Protein 2; DIF: Double Immunofluorescent; ECL: Enhanced Chemiluminescence; FITC: Fluorescein Isothiocyanate; H&E: Hematoxylin and Eosin; HPR: Horseradish Peroxidase; hTERT: Human Telomerase Reverse Transcriptase; IAP: Inhibitor-of-Apoptosis Protein; IFN-γ: Interferon-γ; PBS: Phosphate Buffered Saline; SDS: Sodium Dodecyl Sulfate; SSC: Sodium Citrate; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP Nick-End Labeling; SBDP: Spectrin Breakdown Product; SIF: Single Immunofluorescent; and Smac/Diablo: Second mitochondrial activator of caspases/Direct IAP Binding protein with Low pl

Introduction

Glioblastoma is the most common malignant brain tumor in humans (Rasheed et al., 1999). Current therapy for glioblastoma includes mass-reductive surgery, radiation, and chemotherapy (Benitez et al., 2008) but it eventually proves to be ineffective due to failure in controlling aggressive cell proliferation and metastatic invasion. Thus, development of innovative therapeutic strategies is highly warranted for treating this deadly malignancy. Poor differentiation and uncontrolled cell proliferation in glioblastoma are major problems. These peculiarities should be considered in order to develop a suitable therapeutic regimen for controlling glioblastoma. In the present investigation, we used a combination therapy for induction of differentiation and enhancement of apoptosis in human glioblastoma in vivo.

Retinoids have versatile properties in controlling several essential biological processes (Sun and Lotan, 2002). All-trans retinoic acid (ATRA), a widely known retinoid, induces cell differentiation and causes inhibition of cell proliferation in a variety of cancer cell lines (Breitman et al., 1981; Reynolds et al., 1991). Other retinoids were reported to induce growth inhibitory, differentiating, and apoptotic effects in tumors of mouth, skin, bladder, lung, prostate, and breast in experimental animal models (Niles, 2000; Merino and Hurlé, 2003).

Interestingly, ATRA and cisplatin combination enhanced cytotoxicity in squamous, head and neck, and ovarian cancer cells (Nehmé et al., 2001). We previously reported that ATRA induced differentiation in glioblastoma cell lines (Das et al., 2007; Haque et al., 2007) and enhanced cytotoxicity of paclitaxel in glioblastoma xenografts (Karmakar et al., 2007; Karmakar et al., 2008). The differentiating and growth inhibitory properties of ATRA indicate that its combination with another cytotoxic agent can be therapeutically useful against glioblastoma.

Interferons (IFNs) are a family of pleiotropic cytokines that typically exhibit antiviral, antiproliferative, immunomodulatory, and antitumor properties. Interferon-gamma (IFN-γ), a member of the IFN family, has been associated with activation, growth, and differentiation of a variety of cells including T cells, B cells, macrophages, NK cells, endothelial cells, and fibroblasts (Haque et al., 2007). Dose-dependently, IFN-γ can also induce major histocompatibility complex (MHC) class II molecules and apoptosis in T98G and U87MG glioblastoma cell lines (Haque et al., 2007). Recently, IFN-γ has been shown to induce STAT1 activation to mediate Bax translocation to endoplasmic reticulum and reduce Ca2+ storage for cell death in airway epithelial cells (Stout et al., 2007). Based on their therapeutic potentials, we adopted an innovative strategy to utilize the differentiating and cytotoxic properties of ATRA and IFN-γ, respectively, for controlling the malignant growth of glioblastoma. We previously reported that ATRA in combination with IFN-γ could work
synergistically to induce differentiation, inhibit cellular proliferation, and enhance apoptosis in human malignant glioblastoma T98G and U87MG cell lines (Haque et al., 2007). Recently, we reported that ATRA plus IFN-γ increased Bax:Bcl-2 ratio, mitochondrial release of cytochrome c, caspase-3 activity and down regulated NF-κB in U87MG cells whereas suppressed PI3K/Akt pathway in T98G cells to facilitate apoptosis (Zhang et al., 2007).

In the present study, we explored the efficacy of the combination of ATRA and IFN-γ in a pre-clinical model of human glioblastoma for validation of this combination therapy one step forward in the developmental process of bench to bedside. We have selected the conventional in vivo ectopic model of glioblastoma that is widely used for its simplicity and reproducibility for relatively fast selection of the best performing compound or new combinations and their optimal sequence of administration. However, evaluation of therapeutics in orthotopic intracranial model of glioblastoma is also needed in the next step of evaluation of therapeutics because of more resemblance of orthotopic model to human brain tumor. Our earlier in vitro data from combination of ATRA and IFN-γ in glioblastoma cell lines showed a new dimension to the existing therapeutic strategy for glioblastoma and also prompted us to explore efficacy of this combination therapy in T98G xenograft. We found that ATRA plus IFN-γ exerted therapeutic efficacy in activation of multiple molecular mechanisms for apoptosis in T98G xenograft.

Materials and Methods

Tumor cell line and culture conditions

Human glioblastoma T98G cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in 75-cm² flasks containing 10 ml of 1x RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin in a fully-humidified incubator containing 5% CO₂ at 37°C. They were serially passaged following trypsinization using a trypsin/EDTA solution.

Human glioblastoma T98G xenograft in nude mice

Six-week-old female athymic nu/nu mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. Human glioblastoma T98G cells (6x10⁶) in 100 µl of a (1:1) mixture of 1x RPMI and Matrigel (BD Biosciences, CA) were implanted by subcutaneous (sc) injection usually on the left flank of each mouse under isoflurane anesthesia condition (Karmakar et al., 2007). Palpable T98G xenografts developed within 6-8 days and tumors were measured using an external caliper and volume was calculated using the formula: 4π/3(length²/2) x (width²/2). Animals with 3 weeks-old glioblastoma xenografts (6 animals per group) were randomly divided into 4 groups: control, ATRA, IFN-γ, and ATRA plus IFN-γ. Animals in control group did not receive any therapy. Each animal in other group received intraperitoneally (ip) a daily dose of ATRA (1.5 µg/kg), or IFN-γ (5000 units/kg), or ATRA (1.5 µg/kg) plus 4 hrs later IFN-γ (5000 units/kg) for 7 days. All animal studies were conducted following guidelines of the NIH Animal Advisory Committee and also approved by our Institutional Animal Care and Use Committee (IACUC). The termination of the treatment at 7 days was used to avoid tumor overlap and rupture related death of untreated animals and also to use the remaining tumor tissues to explore the biochemical mechanisms of the treatments leading to tumor regression in treated animals.

Histological examination of tumor xenografts

After completion of treatment schedule, xenografts were excised. One half of each tumor was immediately frozen in liquid nitrogen and stored at -80°C. The other half was immediately frozen (-70°C) in Optima Cutting Temperature (OCT) media (Fisher Scientific, Suwanee, GA) and 5 µm sections were cut with a cryostat (Leica, Deerfield, IL). These sections were used for hematoxylin and eosin (H&E) staining for routine histological and immunofluorescent studies (Karmakar et al., 2008).

In situ immunofluorescent labeling for detecting level of pro-apoptotic protein expression

For in situ immunofluorescent labelings, tumor sections were fixed to slides using 95% (v/v) ethanol for 10 min and rinsed twice for 10 min each in phosphate buffered saline (PBS), pH 7.4. Fixation and all subsequent steps were conducted at room temperature (RT). Sections were blocked with 2% (v/v) horse and goat sera (Sigma, St. Louis, MO) for 30 min. Samples were probed with primary IgG antibody (1:100) for 1 h and slides were rinsed twice in PBS for 5 min each followed by incubation with (1:75) secondary Texas Red or fluorescein isothiocyanate (FITC)-conjugated IgG antibody (Vector Laboratories, Burlingame, CA) for 30 min. Slides were then rinsed twice in PBS for 5 min and in distilled water for 5 min. The slides were immediately mounted with VectaShield (Vector Laboratories, Burlingame, CA) and viewed rapidly under a fluorescence microscope at 200x magnification (Olympus, Japan), and digital pictures were taken with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD), as we described previously (Karmakar et al., 2008; Karmakar et al., 2009).

Combined TUNEL and double immunofluorescent staining for detection of apoptosis with upregulation of pro-apoptotic protein

Combined TUNEL and double immunofluorescent staining was performed with modification of our previously reported method (Karmakar et al., 2008; Karmakar et al., 2009). Briefly, 5 µm sections of tumor xenograft were cut, mounted on slides, and fixed in 95% (v/v) ethanol for 10 min. The sections were then rinsed in PBS for 10 min and were stored at 4°C in fresh PBS. The tissue sections were fixed in 4% formaldehyde for 15 min at room temperature. Then the sections were rinsed twice in PBS for 5 min each. Next, specimens were placed in 0.2% Triton X-100 for 5 min on ice. The sections were rinsed twice in PBS for 5 min each and then incubated in equilibration buffer (Promega, Madison, WI) for 5 min at RT. The buffer was replaced by TUNEL reaction mixture and slides were incubated for 1 h at 37°C in an OmniSlide Thermal Cycler (Hypaid, UK). The TUNEL reaction was stopped by placing the slides in 2xSSC for 15 min at room temperature. The slides were then rinsed twice in PBS for 5 min each. Following the TUNEL reaction, slides were then blocked with 2% normal goat and horse sera (described previously) for 30 min. Each section was blocked in 100 µl of a primary IgG antibody (1:100) for 1 hr. Sections were then washed in PBS for 5 min and incubated with fluorescein-conjugated secondary antibodies such as 1:50 dilution of Texas Red-conjugated anti-DIG antibody (Roche Molecular Biochemicals, Indianapolis, IN) and 1:75 dilution of FITC-conjugated goat IgG antibody (Vector Laboratories, Burlingame, CA) for 30 min. Finally, the slides were washed twice in PBS and once in water for 5 min each. Slides were immediately mounted using coverslips and VectaShield (Vector Laboratories), and examined under a fluorescence microscope at 200x magnification and images were captured using ImagePro Plus Software (Media Cybernetics, Silver Spring, MD).

Western blotting and ECL detection

The total protein samples were extracted following the...
lysis of control and drug-treated tumor tissues, quantitated spectrophotometrically, denatured in boiling water for 5 min, and loaded onto the SDS-polyacrylamide gradient (4-20% or 5%) gels (Bio-Rad), as we reported recently (Karmakar et al., 2008; Karmakar et al., 2009). All proteins were resolved by electrophoresis in Laemmli buffer and then electroblotted to the polyvinylidene fluoride (PVDF) membrane. For detection of each of specific proteins on the blots, appropriate dilutions of the primary antibodies were used. The blots were incubated with a primary antibody followed by incubation with an alkaline horseradish peroxidase (HRP)-conjugated secondary antibody. Subsequently, specific protein bands were detected by the HRP/H2O2 catalyzed oxidation of luminol in alkaline conditions using enhanced chemiluminescence (ECL) system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for autoradiography. The autoradiograms were imaged on a UMAX PowerLook Scanner using Photoshop software (Adobe Systems, San Jose, CA) for analysis.

Quantitation and statistical analysis

Images were used to quantify the total number of pixels above the background by using the public domain NIH Image 1.63 software (available at URL: http://rsb.info.nih.gov/nih-image/ last accessed on January 24, 2010). Data were analyzed using StatView software (Abacus Concepts, Berkeley, CA), expressed as arbitrary units ± standard error of mean (SEM) of separate experiments (n ≥ 3), and compared by one-way analysis of variance (ANOVA) followed by the Fisher post hoc test. A significant difference between control and treatment was indicated by *P < 0.05 or **P < 0.001.

Results

Histological evaluation of xenografts after therapeutic treatments

T98G xenografted mice were treated with ATRA and IFN-γ alone or in combination (Figure 1). Treatment with ATRA plus IFN-γ showed more regression of T98G tumor volume than either drug used alone or from control (CTL) xenograft (Figure 1A, 1B). To evaluate potential histological changes, tumor sections from all treatment groups were subjected to histological evaluation by hematoxilin and eosin (H&E) staining and examination at 200x magnification under light microscope (Figure 1C). Examination of H&E stained tumor sections showed the characteristic feature of glioblastoma in control tumors, ATRA monotherapy inhibited tumor cell proliferation and induced astrocytic differentiation, IFN-γ monotherapy induced moderate apoptosis, and ATRA plus IFN-γ treatment caused substantial amounts of apoptosis in differentiated cells.

ATRA plus IFN-γ combination therapy activated receptor mediated pathway of apoptosis

Western blotting demonstrated that ATRA plus IFN-γ stimulated the receptor-mediated pathway of apoptosis for activation of caspase-8 that caused cleavage of Bid to tBid to trigger mitochondrial intrinsic pathway in T98G xenograft (Figure 2). Translocation of tBid to mitochondria could promote mitochondria-mediated activation of the effector caspases, including caspase-3 to cleave a number of cytoplasmic and nuclear substrates leading to apoptosis (Karmakar et al., 2006).

Down regulation of anti-apoptotic signaling network by ATRA plus IFN-γ

We employed Western blotting to explore the molecular mechanisms leading to apoptosis after treatment with ATRA plus IFN-γ combination in T98G xenograft (Figure 3). Expression of 42 kD β-actin was used as internal control. We found that ATRA plus IFN-γ dramatically down regulated 125 kD hTERT, the catalytic subunit responsible for telomerase activity. Similarly, expression of two potent cancer survival proteins such as 68 kD BIRC-3 (c-IAP2), and 17 kD BIRC-5 (survivin) were markedly reduced in the tumors due to treatment with ATRA plus IFN-γ, compared with other treatment groups (Figure 3).

ATRA plus IFN-γ combination therapy caused mitochondrial release of pro-apoptotic molecules for activation of proteases leading to substrate cleavage

We used Western blotting to determine involvement of the mitochondrial pathway in apoptosis following ATRA plus IFN-γ treatment in T98G xenograft (Figure 4). Expression of 42 kD β-actin was used as internal control. We found over expression of 25 kD BIRC-3 (c-IAP2), and 17 kD BIRC-5 (survivin) were markedly reduced in the tumors due to treatment with ATRA plus IFN-γ over expression of 25 kD BIRC-3 (c-IAP2), and 17 kD BIRC-5 (survivin) were markedly reduced in the tumors due to treatment with ATRA plus IFN-γ, compared with other treatment groups (Figure 3).

Figure 1: Treatment of T98G xenograft and histopathological evaluation. Combination therapy with ATRA plus IFN-γ caused a significant reduction in tumor volume, compared with control. (A) T98G xenografts following treatment with ATRA, IFN-γ, and ATRA plus IFN-γ. Inset picture shows the tumor in respective group. (B) The bar diagram represents the tumor volume following treatment. (C) Histopathological features of H&E stained tumor sections showed that control group maintained characteristic growth pattern of glioblastoma, ATRA alone inhibited tumor cell proliferation and caused astrocytic differentiation, IFN-γ alone induced notable amounts of apoptosis, and ATRA plus IFN-γ enhanced amounts of apoptosis.

Figure 2: Western blotting to examine activation of receptor-mediated caspase cascade for apoptosis. Treatments (7 days): CTL, ATRA (1.5 μg/kg/day), IFN-γ (5000 units/kg/day), and ATRA (1.5 μg/kg/day) plus 4 h later IFN-γ (5000 units/kg/day). Representative Western blots show levels of 42 kD β-actin, 20 kD caspase-8 active fragments, and 15 kD tBid. Activation of caspase-8 caused cleavage of Bid to tBid for translocation to mitochondria to promote mitochondria-mediated activation of the caspases leading to apoptosis in T98G xenograft.
pro-apoptotic protein Smac/Diablo following combination treatment, indicating the involvement of mitochondrial pathway in apoptosis in T98G xenograft. Smac/Diablo upregulation could down regulate anti-apoptotic BIRC proteins, as shown previously (Figure 3), to promote apoptosis in T98G xenograft following activation of proteases (Du et al., 2000; Verhagen et al., 2000). Compared with control or ATRA or IFN-γ monotherapy, we found ATRA plus IFN-γ combination therapy caused a robust release of pro-apoptotic molecule 67 kD AIF into the cytosolic fraction (Figure 4), which could be self-sufficient for DNA fragmentation and apoptosis (Daugas et al., 2000; Cregan et al., 2004), confirming the mitochondrial involvement in the apoptotic process.

We found overexpression of the Ca2+-dependent cysteine protease calpain in combination treatment group (Figure 4). Calpain activation is well known for promoting activation of caspases for apoptosis. Western blotting was performed to examine activation of caspase-9 (Figure 4). We found marked increase in caspase-9 activation in generation of 35 kD caspase-9 active fragment from its 45 kD pro-enzyme. Caspase-9 could then activate caspase-3 (Figure 4) for final execution of cells. Thus, mitochondria activated both caspase-dependent and caspase-independent pathways of apoptosis in T98G xenograft following treatment with combination of ATRA and IFN-γ.

Calpain and caspase-3 activities cause 270 kD α-spectrin cleavage to calpain-specific 145 kD spectrin breakdown product (SBDP) (Nath et al., 1996) and caspase-3-specific 120 kD SBPD (Wang et al., 1998). We examined the calpain and caspase-3 activities concurrently in all treatment groups by examining production of 145 kD SBPD and 120 kD SBPD, respectively, on the Western blots (Figure 4). ATRA plus IFN-γ combination caused more increases in both 145 kD SBPD and 120 kD SBPD than other treatment groups, indicating involvement of both calpain and caspase-3 activities in increasing apoptosis in T98G xenograft (Figure 4).

Immunofluorescent labeling showing ATRA plus IFN-γ combination therapy upregulated calpain and caspase-12 for apoptosis in T98G xenograft

T98G tumor sections from all groups were subjected to single immunofluorescent (SIF) or double immunofluorescent (DIF) stainings and examined under fluorescent microscope. SIF detected specific pro-apoptotic protein expression and whereas DIF showed co-localization of both a specific pro-apoptotic protein expression along DNA fragmentation (Figure 5). SIF staining showed an increase in calpain expression after ATRA plus IFN-γ combination therapy, compared with control xenograft (Figure 5A). Subsequently, the use of combination of DIF staining with TUNEL detected calpain overexpression as well as increased DNA fragmentation indicating more increase in apoptosis in T98G xenograft after ATRA plus IFN-γ treatment than all other treatment groups (Figure 5A). These observations suggested that calpain activation was an important event to induce apoptosis in T98G xenograft following combination therapy.

Our SIF stainings showed more caspase-12 overexpression in combination treatment than control group (Figure 5B). We identified co-localization of increased caspase-12 expression and DNA fragmentation in T98G tumor sections following ATRA plus IFN-γ combination treatment, compared with control group (Figure 5B). Caspase-12 activation, which might be due to calpain activation, suggested involvement of endoplasmic reticulum stress following ATRA plus IFN-γ combination therapy in T98G xenograft.

ATRA plus IFN-γ combination therapy activated caspase-3 and also promoted caspase-independent AIF pathway for apoptosis in T98G xenograft

To explore downstream executioners, we performed immunofluorescent labelings to examine the expression of caspase-3 in T98G tumor sections from all groups in vivo. Immunofluorescent labeling for examination of mitochondrial release of pro-apoptotic molecules and proteolytic activity in specific substrate cleavage. ATRA plus IFN-γ combination treatment upregulated calpain and caspase-12 in the cleavage of 270 kD α-spectrin to calpain-specific 145 kD spectrin breakdown product (SBDP) and caspase-3-specific 120 kD SBPD, respectively, in T98G xenograft.
Regression of tumor volume following ATRA plus IFN-γ xenograft. Histopathological observations suggested that the activated multiple molecular mechanisms for apoptosis in T98G (Vaux, 1993; Shah et al., 2003) and also by us (Karmakar et al., 2007; was due to apoptosis (Figure 1), as previously reported by others combination therapy activated upstream receptor-mediated extrinsic mediated apoptosis in T98G xenograft, we examined and found that involvement of caspase-3 mediated apoptosis following ATRA plus IFN-γ therapy in T98G xenograft.

In pre-clinical human glioblastoma T98G xenograft, we examined and found that combination therapy activated upstream receptor-mediated extrinsic pathway with activation of caspase-8 and Bid cleavage (Figure 2) and this observation appeared to be in line with our previous in vitro studies where ATRA plus IFN-γ induced apoptosis in rat glioblastoma C6 cells (Das et al., 2007) and human glioblastoma T98G and U87MG cells (Das et al., 2009).

ATRA plus IFN-γ caused down regulation of a battery of cell survival signaling molecules (Figure 3), which otherwise could act as inhibitors of apoptosis. We observed down regulation of hTERT (Figure 3), the catalytic subunit of telomerase responsible for telomerase activity and regulation of apoptosis (Warner, 1997). It could be a relevant target for therapy in many cancers including glioblastoma (Penna-run et al., 2005; Shervington et al., 2006; George et al., 2009; George et al., 2010). We also observed down regulation of BIRC proteins such as BIRC-3 (c-IAP2) and BIRC-5 (survivin), especially after treatment of T98G xenograft with ATRA plus IFN-γ (Figure 3). It is now known that BIRC-3 (c-IAP2) has an intrinsic ubiquitin ligase feature to overcome the suppression of caspases and therefore down regulation of some of the BIRC proteins is a very promising inhibition of apoptosis, and thus it is considered as a potential tumor marker (Duffy et al., 2007). The fact that ATRA plus IFN-γ induced down regulation of some of the BIRC proteins is a very promising feature to overcome the suppression of caspases and therefore facilitate apoptosis in T98G xenograft. Also, the inhibition of BIRC proteins following ATRA plus IFN-γ combination might be achieved due to the increased mitochondrial release of Smac/Diablo into the cytosol (Figure 4), as reported previously (Du et al., 2000; Verhagen

Discussion

In this investigation, we explored the therapeutic efficacy of ATRA plus IFN-γ in pre-clinical human glioblastoma T98G xenograft. We found that ATRA plus IFN-γ reduced the tumor volume and activated multiple molecular mechanisms for apoptosis in T98G xenograft. Histopathological observations suggested that the regression of tumor volume following ATRA plus IFN-γ therapy was due to apoptosis (Figure 1), as previously reported by others (Vaux, 1993; Shah et al., 2003) and also by us (Karmakar et al., 2007; Kar marcak et al., 2008). To explore the mechanisms of ATRA plus IFN-γ mediated apoptosis in T98G xenograft, we examined and found that combination therapy activated upstream receptor-mediated extrinsic

![Figure 5: Involvement of calpain and caspase-12 in apoptosis. (A) In situ immunofluorescent labeling detected an increase in calpain expression and also cell death with calpain overexpression, suggesting calpain involvement in apoptosis. (B) In situ immunofluorescent labeling showed caspase-12 overexpression and also cell death with caspase-12 overexpression following ATRA and IFN-γ combination therapy.](image)

and AIF in all treatment groups (Figure 6). We found increase in caspase-3 expression by SIF staining in ATRA plus IFN-γ combination group (Figure 6A). DIF staining showed increased DNA fragmentation associated with caspase-3 overexpression in combination treatment group, compared with other groups (Figure 6A), indicating involvement of caspase-3 mediated apoptosis following ATRA plus IFN-γ therapy in T98G xenograft.

To examine involvement of caspase-independent pathway, we assessed AIF expression by SIF and DIF stainings. SIF exhibited a dramatic increase in AIF expression in ATRA plus IFN-γ group than control group (Figure 6B). Increased DNA fragmentation and AIF overexpression were co-localized in the ATRA plus IFN-γ combination group but not in other treatment groups (Figure 6B). All these observations showed involvement of caspase-independent pathway as well as of caspase-dependent pathway for induction of apoptosis in T98G xenograft following treatment with ATRA plus IFN-γ.

![Figure 6: Calpain and Caspase-12 expression in ATRA and IFN-γ combination therapy in T98G xenograft.](image)
Activation of caspase-8 for cleavage of Bid to tBid (Figure 3), which is well known to be translocated to mitochondria (Li et al., 1998) to release Smac/Diablo (Madesh et al., 2002), can also contribute to inhibition of BIRC proteins.

Release of several pro-apoptotic molecules from mitochondria is a key determining factor for inducing apoptosis, as reported by several groups (Saelens et al., 2004; Kim et al., 2006) and also by us in glioblastoma (Karmakar et al., 2006; Das et al., 2009). It is well known that mitochondria can also induce caspase-independent apoptosis by releasing AIF (Cregan et al., 2002; Cregan et al., 2004). We found that combination therapy dramatically increased level of AIF in the cytosol (Figure 4), suggesting that the traslocation of AIF to the nucleus could cause caspase-independent nuclear DNA fragmentation in human glioblastoma T98G xenograft. All of these in vivo observations such as down regulation of telomerase and BIRC proteins and upregulation of Smac/Diablo in glioblastoma T98G xenograft are well correlated and further supported by our previously reported studies where ATRA plus IFN-γ combination therapy produced similar effects in rat glioblastoma C6 cells (Das et al., 2007) and human glioblastoma T98G and U87MG cells (Das et al., 2009).

We found that ATRA plus IFN-γ combination therapy caused dramatic overexpression of the Ca²⁺-dependent cysteine protease calpain (Figure 4), which could be strongly associated with apoptosis through mitochondrial release of Smac/Diablo (Altznauer et al., 2004), AIF (Cao et al., 2007), and activation of caspases (Karmakar et al., 2006; Das et al., 2009). Since mitochondrial release of Smac/Diablo (Du et al., 2000; Verhagen et al., 2000; Rajalingam et al., 2007) and activation of calpain can cross-talk with caspases (Nakagawa and Yuan, 2000) for apoptosis, we examined and found activation of caspase-9 (Figure 4), which in turn activated the final executioner caspase-3 (Figure 4) leading to cytoskeletal brakedown and apoptosis. The generation of calpain-specific 145 kD SBDP (Nath et al., 1996), and the caspase-3-specific 120 kD SBDP (Wang et al., 1998) in our study (Figure 4) suggested the involvement of both calpain and caspase-3 activities for apoptosis. This observation is consistent with previous studies that demonstrate participation or co-operation between calpain and caspase-3 in apoptosis following ATRA plus IFN-γ combination therapy in glioblastoma cell lines (Das et al., 2007; Das et al., 2009).

Immunofluorescent results indicated involvement of both calpain and caspase-12 overexpression in cell death after combination therapy (Figure 5). Calpain induced activation of caspase-12, as reported previously (Sergeev, 2004; Muruganandan and Cribb, 2006), indicating involvement of endoplasmic reticulum stress in the apoptotic process. Our studies demonstrated that the DNA fragmentation was associated with overexpression of caspase-3 and AIF (Figure 6), strongly implicating the involvement of both mitochondrial caspase-dependent and caspase-independent pathways for induction of apoptosis in T98G xenograft.

Conclusions

Present investigation showed the activation of multiple molecular mechanisms for increasing apoptosis in human glioblastoma T98G xenograft after treatment with ATRA plus IFN-γ. Thus, our findings from this pre-clinical model strongly suggest that ATRA plus IFN-γ may have a significant potential for the treatment of glioblastoma in humans in the near future.
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Conflict of Interest Statement

The authors declare that they have no conflict of interests.

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