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Effects of NFκB decoy oligonucleotides released from biodegradable polymer microparticles on a glioblastoma cell line

Jagjit S. Gill[a], Xun Zhu[b], Michael J. Moore[b], Lichun Lu[b], Michael J. Yaszemski[b,*], Anthony J. Windebank[a]

[a] *Molecular Neuroscience Program, Mayo Clinic, MS 3-75, 200 First Street SW, Rochester, MN 55905, USA*
[b] *Departments of Orthopedic Surgery and Bioengineering, Mayo Clinic, MS 3-75, 200 First Street SW, Rochester, MN 55905, USA*

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Abstract

The objectives of this study were to investigate a nuclear factor-kappa B (NFκB) decoy oligonucleotide (ODN) strategy on the inhibition of glioblastoma (GBM) cell line growth and to evaluate a poly(DL-lactic-*co*-glycolic acid) (PLGA) microparticle delivery system for the NFκB decoy ODNs in vitro. We have demonstrated that NFκB activation is important in regulating GBM cell line growth. Aberrant nuclear expression of NFκB was found in a panel of GBM cell lines, while untransformed glial cells did not display NFκB activity. Nuclear translocation of NFκB was inhibited by using a "decoy" ODN strategy. NFκB decoy ODNs designed to inhibit NFκB resulted in a significant reduction in cell number (up to 45%) compared to control cultures after 2 days. The reduction in cell number correlated with a decrease in cyclin D1 protein expression and a commensurate decrease in Cdk-4 activity. These results provide evidence suggesting that NFκB mediates cell cycle progression and demonstrates a mechanism linking increased NFκB activity with GBM cell growth and cell cycle disregulation. Decoy ODNs were encapsulated at a yield of 66% in PLGA microparticles and released in a controlled manner in phosphate buffered saline for up to 28 days. Approximately 83% of entrapped ODNs were released by day 28. During 3 days of GBM cell line culture, the released decoy ODNs retained their biologic activity and led to significantly reduced cell number as compared to control cultures. These findings offer a potential therapeutic strategy in the control of human GBM cell line growth in vitro and suggest that PLGA microparticles may be appropriate as delivery vehicles for the "decoy" ODN strategy.

Keywords: Phosphorothioated decoy oligonucleotides (ODNS); Nuclear factor-kappa B (NFκB); Glioblastoma (GBM); Cell growth; Controlled release; Biodegradable microparticles; Poly(DL-lactic-co-glycolic acid) (PLGA)

1. Introduction

Minimal progress has been made in our ability to treat glioblastoma (GBM) over the past century. While surgery, chemotherapy and radiation may remain primary treatment modalities for glioblastomas, they have significant deleterious side effects and drawbacks. The tumor is usually invasive through the normal brain. Incomplete removal of the tumor and development of drug-resistance by the remaining tumor cells ultimately lead to tumor progression [1]. Although many innovative techniques have improved the quality of survival for many patients, the median survival following diagnosis and adjuvant treatment still remains only approximately one-year [2]. Previous studies have focused on identifying the distinct biological features of high-grade human GBM [3–11].

Nuclear factor-kappa B (NFκB) activation has recently been shown to be important in regulating cell proliferation in a variety of tumor cells [12–16]. Activation of NFκB involves phosphorylation and dissociation of the inhibitory IκB protein from a cytoplasmic complex with NFκB [17–19]. After serine phosphorylation of IκB, the inhibitory protein is ubiquinated and degraded by the proteasome pathway [20,21]. Liberated NFκB is translocated into the nucleus where it induces transcription of responsive genes by binding to DNA κB motifs. Activation of NFκB is

*Corresponding author. Tel.: +1-507-284-2667; fax: +1-507-284-5075.
E-mail address: yaszemski.michael@mayo.edu (M.J. Yaszemski).

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correlated with changes in cyclin D1 protein expression and enzymatic activity of cyclin-dependent kinase 4 (Cdk-4). Cyclin D1 is a cell cycle regulatory protein that is expressed in proliferating cells during G_1 phase of the cell cycle. It is not expressed in quiescent cells, such as neurons or mature glial cells, which are in the G_0 phase. Cyclin D1 complexes with Cdk-4 to form an active kinase, which phosphorylates the retinoblastoma gene product [22]. Phosphorylation of Rb promotes progression through the cell cycle [12,22].

If errant NFκB activation in GBM is related to a disregulation in the cell cycle yielding uncontrolled cell growth, blocking the translocation pathway of NFκB might inhibit tumor cell proliferation. We have recently shown that specific inhibition of NFκB can be achieved by using a molecular decoy oligonucleotide (ODN) strategy in dorsal root ganglion neurons [23]. The molecular strategy designed to inhibit NFκB activity required the generation of *cis* decoy ODNs against NFκB binding sites. Binding of liberated NFκB to phosphorothioated decoy deca-nucleotide elements (5′-GGGATTTCCC-3′) within the cytoplasm prevents DNA binding and transactivation by the transcription factor. Therefore, such "decoy" ODN strategies may inhibit the NFκB activity in GBM cell lines and reduce tumor cell growth. Generation and efficiency of NFκB decoy elements have been previously determined [23,24].

It is probable that such molecular therapeutic strategies will involve local delivery of NFκB decoy ODNs. Even if a potential therapy is effective, it may not work because appropriate delivery has not been achieved. In this regard, incorporation of NFκB decoy ODNs into a biodegradable microparticle delivery scheme would be particularly attractive. Incorporation of biological molecules into synthetic matrices such as microparticles provides a method for sustained and controlled local delivery [25–32]. This approach is an alternative to cell-based delivery systems during the phase of drug development. A major advantage of biodegradable synthetic microparticles is the ability to control the rate of drug release to obtain the desired local therapeutic concentrations by changing the structure and biodegradation rate of the polymers. Using such a delivery system may also offer other advantages such as site-specific delivery and protection of ODNs from degradation prior to release [33].

In the present study, experiments were designed to determine whether NFκB activation would cause disregulation of GBM cell growth, whether decoy ODNs could inhibit NFκB activity, whether NFκB decoy ODNs would inhibit GBM cell line growth, whether ODNs could be released from PLGA microparticles in a controlled fashion, and whether NFκB decoy ODNs released from PLGA microparticles would have inhibitory effects on GBM cell line growth.

2. Materials and methods

2.1. Raw materials

NFκB phosphorothioated decoy ODNs 5′-GGGATTTCCC-3′ and mutated ODNs 5′-GGGCTTTCCC-3′ (underlined base indicates mutation) were purchased from Oligos Etc., Inc. (Wilsonville, OR) on a 50 μM scale, which were purified to $\geqslant 90\%$ with gel electrophoresis and HPLC purification. NFκB decoy and mutant ODNs for electrophoretic mobility gel shift (EMSA) were generated by the Mayo Clinic Molecular Biology Core Facility as follows: 5′-CCTTGAAGGGATTTCCCTCC-3′ and 3′-GGAACTTCCCTAAAGGGAGG-5′ (consensus sequences are underlined). Phosphate buffered saline (PBS, pH 7.4), fetal calf serum, and Dulbecco's modified Eagle medium (DMEM) were obtained from Gibco (Grand Island, NY). Bovine serum albumin was purchased from Bio-Rad Lab (Richmond, CA). Monoclonal anti-Cdk-4 antibody was acquired from Transduction Labs (Lexington, KY) and [γ-^{32}P]adenosine 5′-triphosphate (ATP) was purchased from NEN DuPont (Boston, MA).

PLGA of 50:50 lactic to glycolic acid copolymer ratio (Medisorb®, Alkermes, Cincinnati, OH) was used in this study. The weight average molecular weight (M_w) of PLGA was $72{,}000 \pm 360$ ($n = 4$), as determined by gel permeation chromatography. The polydispersity index (PI), equal to the ratio of the weight average to the number average molecular weight (M_w/M_n), was 1.73 ± 0.05. All other chemical and biochemical reagents were purchased from Sigma (Milwaukee, WI).

2.2. GBM cell culture

A panel of human GBM cell lines (U87, A172, D37) with high proliferative potential were studied. The characteristics of these cell lines have been previously described [34,35]. Primary glial cultures were obtained from adult rat brain tissue (300 g Harlan Sprague Dawley rats; Harlan, Madison, WI). Whole brain tissue was mechanically and enzymatically dissociated and resuspended in DMEM supplemented with 15% fetal calf serum. Dissociated cells were plated onto collagen-coated Petri dishes for 3 h, allowing for neuron attachment to the collagen substrate. Media containing unattached glial cells were collected and replated in fresh collagen coated Petri dishes and cultured in vitro.

2.3. NFκB activation

NFκB activity was determined by EMSA on three human GBM cell lines (U87, A172, D37). Nuclear extracts for EMSA were prepared by the method of Dignam et al. [36]. Briefly, nuclear extracts were

prepared by lysing cells in ice-cold lysis buffer (pH 7.5) containing 10 μM *N*-(2-hydroxyethyl)piperazine-*N*′-(4-butanesulfonic acid) (HEPES), 0.5 M sucrose, 0.5 μM spermidine, 0.15 μM spermin, 5 μM ethylenediaminetetraacetic acid (EDTA), 0.25 M ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 7 μM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 12 000*g* for 30 min at 4°C, each pellet was lysed in one volume of ice-cold lysis buffer containing 0.1% tergitol NP-40. Lysates were centrifuged at 12 000*g* for 30 min at 4°C and the pelleted nuclei were washed twice with ice-cold buffer containing 0.35 M sucrose. After washing, nuclei were extracted with ice-cold lysis buffer containing 0.05 M NaCl and 10% glycerol for 15 min at 4°C. Nuclei were then extracted with lysis buffer containing 0.3 M NaCl and 10% glycerol for 1 h at 4°C and the concentration of DNA was adjusted to 1 mg/ml. After pelleting the extracted nuclei at 12 000*g* for 30 min at 4°C, the supernatant was added to a 45% $(NH_4)_2SO_4$ solution and stirred for 30 min at 4°C. Precipitated protein was collected at 17 000*g* for 30 min, and re-suspended in lysis buffer containing 0.35 M sucrose. Primary glial cultures were used to determine baseline NFκB activity and for comparison with tumor cell lines. Radio-labeled NFκB decoy and mutant ODNs were incubated with nuclear extracts, subjected to electrophoresis and dried.

2.4. Inhibition of NFκB activity

NFκB inhibition was attempted by addition of decoy ODNs to cells in culture. Human GBM U87 cells were maintained in DMEM supplemented with 15% fetal calf serum. Cells were plated in 18-well arrays of 3-row × 6-column 0.5 ml cell wells. Columns accommodated the following formulations: (1) untreated cell cultures, (2) cell cultures exposed to 0.5 μM decoy ODNs, and (3) cell culture exposed to 0.5 μM mutant ODNs. Rows provided for 3 independent data points per iteration. ODNs were added 6 h after re-plating and replenished every 12 h. EMSA was conducted at 24 h of exposure of cell line.

2.5. Cell growth study of GBM cell lines treated with decoy ODNs

Human GBM cell lines (U87, A172, D37) were maintained in DMEM supplemented with 15% fetal calf serum. Cells were plated in 18-well arrays of 3-row × 6-column 0.5 ml cell wells. Columns accommodated the following formulations: (1) U87 cell cultures exposed to 0.5 μM decoy ODNs, (2) U87 cell culture exposed to 0.5 μM mutant ODNs, (3) D37 cell cultures exposed to 0.5 μM decoy ODNs, (4) D37 cell culture exposed to 0.5 μM mutant ODNs, (5) A172 cell cultures exposed to 0.5 μM decoy ODNs, and (6) A172 cell culture exposed to 0.5 μM mutant ODNs. Rows provided for 3 independent data points per iteration. Cells were plated at a concentration of 20×10^3 cells per 50 mm Petri dish. ODNs were added 6 h after re-plating and replenished every 12 h. Cells were detached after 48 h from culture plates with a trypsin-EDTA solution and the cell numbers were counted with a hemocytometer and the means of 8 counted 0.1 mm^2 squares were calculated.

2.6. Cyclin D1 immunoblotting

Human GBM A172 cells were maintained in DMEM supplemented with 15% fetal calf serum. Cells were plated in 18-well arrays of 3-row × 6-column 0.5 ml cell wells. Columns accommodated the following formulations: (1) untreated cell cultures, (2) cell cultures exposed to 0.5 μM decoy ODNs, and (3) cell culture exposed to 0.5 μM mutant ODNs. Rows provided for 3 independent data points per iteration. ODNs were added 6 h after re-plating and replenished every 12 h. Whole cell lysates of GBM cell lines were obtained after 24 h in culture. Western blot analysis was completed on whole cell lysates of GBM cell lines using specific monoclonal antibodies to cyclin D1. Immunoblotting and detection of cyclin D1 was completed as described previously [37].

2.7. Immune complex kinase assay

Human GBM 172 cells were treated in cell culture as above. Endogenous activity of the enzymatic counterpart to cyclin D1, cyclin-dependent kinase-4 (Cdk-4), was completed as described [38]. Whole cell lysates were suspended in 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiotretiol (DTT), 0.1% Tween-20, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg leupeptin/ml, 20 U aprotinin/ml, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate and sonicated at 4°C. Lysates were clarified by centrifugation at 10 000*g* for 5 min and total protein concentrations were determined using the Bradford method using bovine serum albumin as a standard. One hundred micrograms of protein was precipitated for 4 h at 4°C with protein A-Sepharose beads precoated with saturating amounts of monoclonal anti-Cdk-4 antibody. Immunoprecipitated proteins were washed 4 times with 1 ml lysis buffer and twice with 50 mM HEPES containing 1 mM DTT. The precipitated beads were suspended in 30 μl kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 1 mM DTT) containing 2 μg of soluble histone protein and 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 20 μM ATP, and 10 μCi of [γ-^{32}P]ATP. After incubation for 30 min at 30°C with occasional mixing, the samples were boiled in polyacrylamide gel sample

buffer containing sodium dodecyl sulfate (SDS) and separated by gel electrophoresis. Phosphorylated proteins were visualized by autoradiography of the dried slab gels.

2.8. Microparticle preparation

PLGA microparticles were fabricated using a double-emulsion-solvent-extraction technique ((water-in-oil)-in-water or W/O/W) as previously described [30]. Briefly, PLGA with a total weight of 500 mg was first dissolved in 2 ml dichloromethane. Then, 400 μl of 100 μM ODN solution was added to a flint glass tube containing the polymer solution. The entire mixture was emulsified on a vortexer for 20 s. This solution was then re-emulsified in 100 ml of 0.3% aqueous poly(vinyl alcohol) (PVA) solution resulting in a double emulsion. The second emulsion was added to 100 ml of 2% isopropyl alcohol (IPA) solution and maintained on a magnetic stirrer for 2 h. The extraction of the dichloromethane to the external alcoholic phase resulted in precipitation of the dissolved polymer and subsequent formation of microparticles. The supernatant was decanted off and settled microparticles were washed 3 times with double distilled water. The fabricated microparticles were finally collected, centrifuged, lyophilized to dryness, and stored at -70°C before use. The procedure was applied to both decoy and mutant ODNs.

2.9. Phosphorothioated ODN release kinetics

The entrapment efficiency of the ODNs in PLGA microparticles was determined by normalizing the amount actually entrapped to the starting amount, using an established solvent extraction technique [30]. Approximately 20 mg of microparticles were dissolved in 1 ml dichloromethane for 6 h at 37°C. The entrapped ODNs were then extracted from the organic phase to the aqueous phase by incubation with 1 ml PBS for an additional 24 h. The concentrations of ODNs were determined by absorption at 260 nm in a Beckman DU-600 UV spectrometer.

The ODN release kinetics from PLGA microparticles was studied in pH 7.4 PBS. Approximately 20 mg of microparticles were placed into 1.8 ml microvials containing 1 ml PBS. The samples were maintained at 37°C with shaking ($\sim$100 rpm) for various time periods up to 28 days. At the end of each time point: 4, 8, 12, 16 h, 1, 2, 3, 4, 5, 6, 7, 10, 14, 18, 22, 28 days, the microparticle suspension was centrifuged and the supernatant was collected for analysis. The pellet was re-suspended in fresh PBS. The amount of ODNs released from microparticles was determined by quantifying the UV absorption at 260 nm of collected sample solutions.

2.10. Delivery of NFκB ODNS from microparticles into cell culture

Human GBM cells were maintained in DMEM supplemented with 15% fetal calf serum. Cells were plated in 18-well arrays of 3-row × 6-column 0.5 ml cell wells. Columns accommodated the following formulations: (1) a positive control of 0.5 μM aqueous decoy ODNs, (2) a negative control of mutant ODN-loaded microparticles (175 mg microparticles per well) left floating in culture medium, (3) a second negative control of blank microparticles left floating in culture medium, and (4–6) decoy ODN-loaded microparticles in three varying quantities (17.5, 87.5, or 175 mg microparticles per well) left floating in culture medium. Decoy, mutant, and blank microparticles were applied directly to cell culture while still wet from the final filtration. Weights of 17.5, 87.5, and 175 mg were chosen to approximate effective ODN concentrations of 0.5, 2.5, and 5 μM in medium after 72 h. Rows provided for 3 independent data points per iteration. Cells were plated at a concentration of 20×10^3 cells per 50 mm Petri dish. Microparticles were added 6 h after re-plating. One single array was harvested at each of 24, 48, and 72 h time periods. Cells were detached from culture plates with a trypsin-EDTA solution and the cell numbers were counted with a hemocytometer and the means of 8 counted 0.1 mm^2 squares were calculated. Harvest of each array yielded data regarding total cell numbers per well.

2.11. Statistical analysis

All data are reported as means ± standard deviations for $n = 3$ or 4. Single factor analysis of variance (ANOVA) was used to assess the statistical significance of results. Scheffé's method was employed for multiple comparison tests at significance levels of 95% and 99%.

3. Results

3.1. NFκB activity in GBM cell lines

In the first line of experiments, the level of NFκB activity in three GBM cell lines was compared to that in primary glial cultures. EMSA revealed NFκB activity was detected in all GBM cell lines (arrow) but not in primary glial cultures (Fig. 1). It suggests that the disregulation of tumor cell growth could be caused by NFκB activation. The NFκB activity was most significant on the U87 cell line. Therefore, we selected this cell line for the evaluation of the inhibition of NFκB activity.

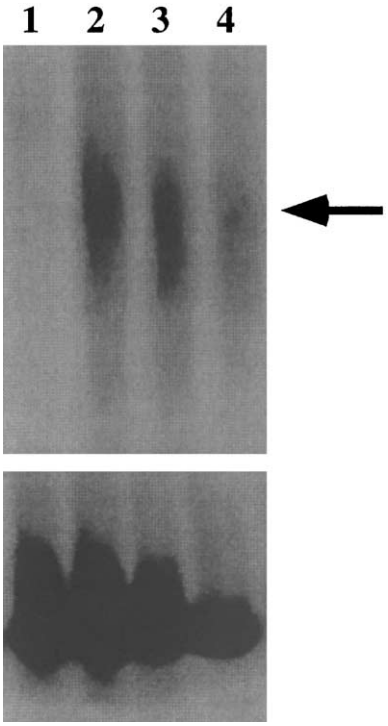

Fig. 1. Aberrant activation of NFκB in GBM cell lines. EMSA of NFκB was performed on nuclear extracts from rat primary glial cultures (lane 1) and from the GBM cell lines U87 (lane 2), D37 (lane 3), A172 (lane 4). Unbound radiolabeled ODNs were revealed in the bottom panel and served as a control for sample loading.

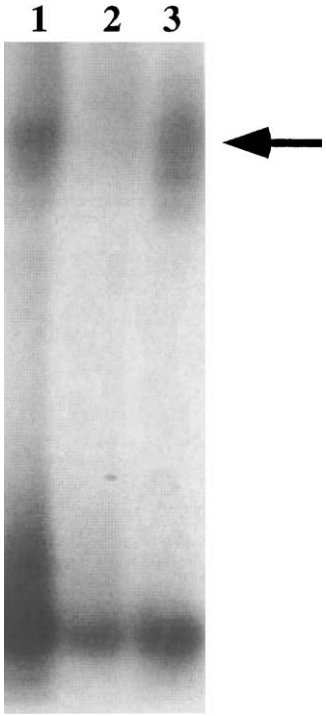

Fig. 2. NFκB inhibition in GBM cell line U87. EMSA of NFκB was performed on nuclear extracts from untreated GBM cell line U87 (lane 1) and GBM cell line U87 which had been exposed to NFκB decoy (lane 2) or mutant (lane 3) ODNs for 24 h. Unbound radiolabeled ODNs were revealed in the bottom panel and served as a control for sample loading.

3.2. Inhibition of NFκB activity by decoy ODNS

Molecular decoy oligonucleotides (0.5 μM, GGGATTTCCC) corresponding to the NFκB DNA binding domain were generated and exposed to the GBM cell line U87 (Fig. 2). These decoy ODNs sequester liberated NFκB within the cytoplasm and prevent nuclear translocation and gene transactivation [24]. EMSA revealed a reduction in nuclear NFκB activity (arrow) in cells exposed to decoy ODNs (lane 2) as compared to control cultures (lane 1) or mutant ODNs (lane 3). Similar inhibition of decoy ODN-mediated inhibition of NFκB activity was observed in the D37 and A172 cell lines. Therefore, NFκB activity of GBM cell lines can be inhibited by being exposed to decoy ODNs.

3.3. Activation of NFκB: role in cell growth and cell cycle

3.3.1. Inhibition of NFκB results in reduced cell number

We examined the effect of molecular inhibition of NFκB activity on GBM cell growth. Three panels of GBM cell lines were exposed to NFκB decoy and mutant ODNs for 48 h (Fig. 3). After 2 days of exposure to decoy ODNs, only 77 (±4), 59 (±5), and 54 (±6) % of cells were found for the GBM cell lines U87, D37 and A172, respectively, compared to untreated cell culture. The inhibitory effects of decoy ODNs were most

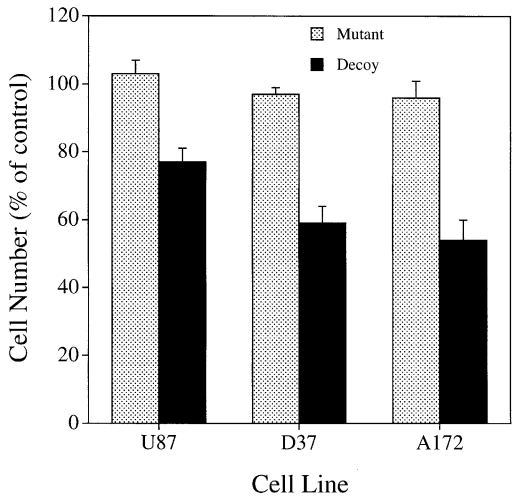

Fig. 3. Inhibition of NFκB resulted in reduced cell number in GBM cell lines. Cell numbers were quantified in a panel of GBM cell lines (U87, D37, A172) and expressed as a percentage of untreated (control) cultures. Cultures were exposed to mutant and decoy ODNs for 48 h at which time cell numbers were determined. Error bars represent means ± SD for $n = 3$.

significant on the A172 cell line. Therefore, we selected this cell line for the following studies: disregulation in cyclin D1 and p16 protein expression and the evaluation of released ODNs from microparticles.

3.3.2. NFκB mediated disregulation in cyclin D1 and Cdk-4 activation

GBM cell lines exposed to NFκB decoy ODNs demonstrated a reduced level of cyclin D1 protein expression compared to untreated cultures and cells treated with mutant ODNs (Fig. 4A). A decrease in cyclin D1 protein expression in cell lines exposed to NFκB decoy ODNs correlated with a decrease in Cdk-4 activity compared to untreated cultures (Fig. 4B). Cdk-4 activity in cultures exposed to mutant NFκB ODNs was comparable to control cultures. A reduced level of cyclin D1 protein expression indicated that more cells are in the G0 phase, which suspends the cell cycle after mitosis and prior to DNA synthesis and, therefore, prevents cell proliferation.

3.4. ODN release kinetics from PLGA microparticles

The initial loading density of NFκB phosphorothioated decoy ODNs in PLGA microparticles was 300 ng/mg. After fabrication, 66.4 (±1.6) % of ODNs were entrapped. The actual loading density was 200 ng of ODN per mg of microparticle. The ODN release profile (Fig. 5) was comprised of three phases: (a) an initial burst during which 23.9 (±1.3) % of entrapped ODNs were released after the first 2 days, (b) a linear steady release phase that lasted up to day 18, during which 22.1 (±1.1) % of entrapped ODNs were released, and (c) a final release phase for the rest of the time frame. Nearly 83% of entrapped ODNs (168 ng/mg) were released at the end of day 28. These results show that the encapsulated ODNs were released in a slow and controlled manner from the PLGA microparticles.

3.5. Microparticle based strategies for ODN delivery

Cell growth studies were performed in the GBM cell line A172 exposed to three sets of ODN-loaded PLGA microparticles to obtain local concentrations of 0.5, 2.5, and 5 μM of NFκB decoy ODNs after 3 days (Fig. 6). Unloaded microparticles and microparticles loaded with mutant ODNs (5 μM) served as negative controls. Unencapsulated decoy ODNs in aqueous solution (0.5 μM) served as a positive control. Quantitation of cell numbers revealed a significant reduction at 48 h (79±6% and 62±6%) and 72 h (74±2% and 55±6%) in cultures exposed to microparticles loaded with 2.5 and 5 μM NFκB decoy ODNs, respectively ($p<0.01$). This dose dependent inhibition of cell growth was comparable to that observed in cultures exposed to 0.5 μM NFκB decoy ODNs (61±3%) delivered aqueously after 3 days. Unloaded microparticles and microparticles loaded with NFκB mutant ODNs did not have a significant effect ($p>0.05$) on cell growth as compared to untreated cultures.

4. Discussion

This study was conducted to answer the following five questions: (1) Could NFκB activation be the reason for disregulation of GBM cell growth? (2) Could the decoy ODNs inhibit the NFκB activity? (3) Do the decoy ODNs have inhibitory effects on GBM cell line growth? (4) Could the ODNs be released from PLGA micro-

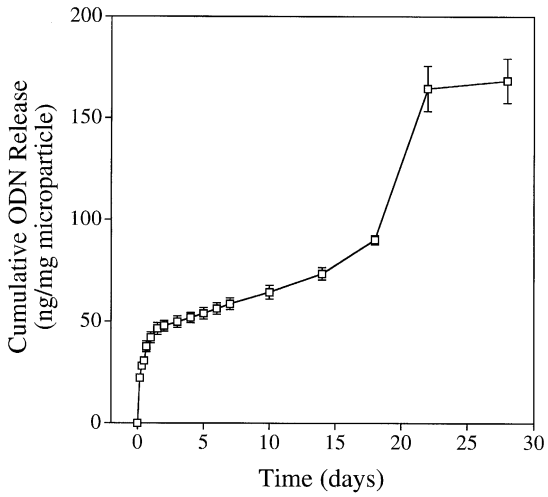

Fig. 5. Cumulative release of decoy ODNs from PLGA microparticles in pH 7.4 PBS. The initial loading density was 300 ng ODN per mg microparticle. Error bars represent means±SD for $n=4$.

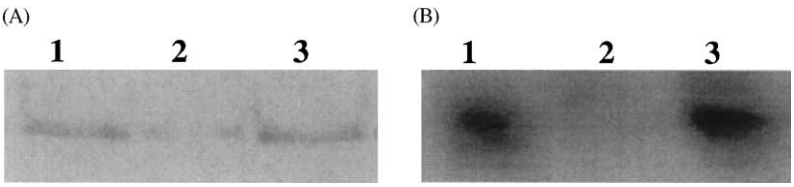

Fig. 4. NFκB mediated disregulation in cyclin D1 and Cdk-4 activation. (A) Western blot analysis using a monoclonal antibody directed at cyclin D1 protein was completed on whole cell lysates of the A172 cell line. (B) Kinase assays for Cdk-4 activity were completed on GBM A172 cells. GBM A172 cell line had been untreated (lane 1), exposed to NFκB decoy ODNs (lane 2), or mutant ODNs (lane 3) for 24 h.

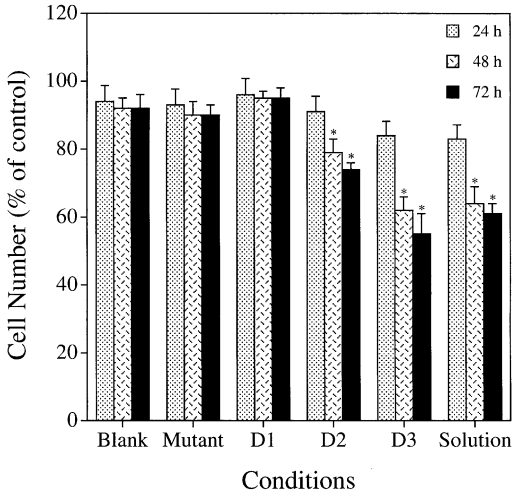

Fig. 6. PLGA microparticle-based delivery of NFκB ODNs. Microparticles were loaded with decoy ODNs to obtain concentrations of 0.5 μM (D1), 2.5 μM (D2), and 5 μM (D3) after 3 days. The GBM cell line A172 was exposed to unloaded microparticles (Blank), microparticles loaded with mutant NFκB ODNs (Mutant) of 5 μM, microparticles loaded with decoy ODNs (D1, D2, D3), as well as 0.5 μM NFκB decoy ODNs in aqueous solution (Solution). Cell numbers were quantified after 24, 48 and 72 h of exposure and normalized to untreated (control) cultures. Error bars represent means ± SD for $n = 3$. * Values significantly ($p < 0.01$) lower than control groups.

particles in a controlled manner? (5) Do the ODNs released from PLGA microparticles also have inhibitory effects on GBM cell line growth?

We have demonstrated that NFκB activation is important in regulating GBM cell line growth. Aberrant nuclear expression of NFκB was found in a panel of GBM cell lines, while untransformed glial cells did not display NFκB activity. NFκB decoy ODNs designed to inhibit NFκB resulted in a significant reduction in cell number compared to control cultures. The reduction in cell number correlated with a decrease in cyclin D1 protein expression and a commensurate decrease in Cdk-4 activity. These results provide evidence suggesting that NFκB mediates cell cycle progression. We demonstrated a mechanism linking increased NFκB activity with GBM cell growth and cell cycle disregulation.

The proposed role of NFκB in GBM oncogenesis and proliferation is supported by other studies implicating its role in tumor biology [12–16]. One member of the NFκB family, Rel, has been characterized as a viral oncogene [14]. Ras and Raf-1 are known to be important regulators of cell proliferation and survival in a number of models. Activation of both results in the activation of NFκB [12]. It has also been determined that matrix metalloproteinase, MMP-9, has κB binding sites in its promoter region [39]. Expression of this gene has been implicated in the appearance of the transformed phenotype in OST tumor cells. In our study, we have demonstrated NFκB mediated regulation of the G1 phase-cell cycle element cyclin D1 and its catalytic counterpart Cdk-4. This is also consistent with other reports describing over-expression of cyclin D1 in a variety of human cancers, including glioblastomas [40,41]. Cyclin D1 plays a critical role in carcinogenesis by enhancing genomic instability and thereby the process of tumor progression. Clearly, strategies to inhibit the effects of cyclin D1 function may be useful in both cancer chemoprevention and therapy.

Our results support a role for NFκB activity in human glioblastomas. There are several potential upstream regulators of NFκB. NFκB activity can be induced by tumor necrosis factor, interleukins, and growth factors [42]. The growth of human glioblastomas is also highly influenced by the above growth factors and cytokines. Thus, it is possible that NFκB activity is a significant post-receptor event induced by binding of growth factors or cytokines that regulate cellular proliferation and differentiation.

NFκB normally binds to a double-stranded duplex, which is utilized as a decoy in most of the published examples [43,44]. In this study, we demonstrated that single-stranded decoy ODNs also led to the inhibition of NFκB activation and GBM cell line growth. One possible explanation would be that the decoy ODNs formed a weak self-duplex with a central TT mismatch. The mutant control with a central ATTT mismatch would not be stable enough to act as a decoy. The detailed biochemical mechanism of our decoy strategy will be investigated in future studies.

Our findings were then tested at a therapeutic level by designing PLGA microparticles incorporated with NFκB decoy ODNs. The ODNs were encapsulated in PLGA microparticles with high entrapment efficiency and released in a sustained and controlled manner. The tri-phasic release profile was similar to that previously established for protein release from biodegradable microparticles [30,45]. The initial burst effect was due to the desorption of ODNs at the microparticle surface. Polymer hydration and ODN diffusion led to a linear release phase. Subsequent solubilization and release of low molecular weight PLGA degradation products (polymer erosion) resulted in the final release phase.

In recent work, we showed that the release rate can be varied by changing the microparticle composition, ODN loading density, and local pH to achieve desired local concentrations at delivery sites [32]. ODNs released from such PLGA microparticles were protected from degradation and remained biologically active. The microparticle strategy of ODN delivery produced a significant reduction in cell number compared to unencapsulated ODNs. PLGA microparticles are appropriate as delivery vehicles for controlled release of NFκB decoy ODNs.

Our results suggest that our molecular approach to inhibit NFκB activity in GBM cell lines was effective in vitro and PLGA microparticles are appropriate as delivery vehicles for NFκB decoy phosphorothioated ODNs. The findings presented here warrant the investigation of intra-thecal delivery of microparticles in an animal model of GBM.

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References

[1] Kornblith PK, Welch WC, Bradley MK. The future of therapy for glioblastoma. Surg Neurol 1993;39:538–43.
[2] Loeffler JS, Alexander ED, Shea WM, Wen PY, Fine HA, Kooy HM, Black PM. Radiosurgery as part of the initial management of patients with malignant gliomas. J Clin Oncol 1992;10:1379–85.
[3] Jaros E, Perry RH, Adam L, Kelly PJ, Crawford PJ, Kalbag RM, Mendelow AD, Sengupta RP, Pearson AD. Prognostic implications of p53 protein, epidermal growth factor receptor, and Ki-67 labelling in brain tumours. Br J Cancer 1992;66:373–85.
[4] Reifenberger G, Liu L, Ichimura K, Schmidt EE, Collins VP. Amplification and overexpression of the MDM2 gene in a subset of human malignant gliomas without p53 mutations. Cancer Res 1993;53:2736–9.
[5] Rubio MP, von Deimling A, Yandell DW, Wiestler OD, Gusella JF, Louis DN. Accumulation of wild type p53 protein in human astrocytomas. Cancer Res 1993;53:3465–7.
[6] Montine TJ, Bruner JM, Vandersteenhoven JJ, Dodge RK, Burger PC. Prognostic significance of p53 immunoreactivity in adult patients with supratentorial fibrillary astrocytic neoplasms. Diagn Mol Pathol 1994;3:240–5.
[7] Nishikawa R, Ji XD, Harmon RC, Lazar CS, Gill GN, Cavenee WK, Huang HJ. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. Proc Natl Acad Sci USA 1994;91:7727–31.
[8] Rasheed BK, McLendon RE, Herndon JE, Friedman HS, Friedman AH, Bigner DD, Bigner SH. Alterations of the TP53 gene in human gliomas. Cancer Res 1994;54:1324–30.
[9] Wong AJ, Zoltick PW, Moscatello DK. The molecular biology and molecular genetics of astrocytic neoplasms. Sem Oncol 1994;21:139–48.
[10] Rainov NG, Dobberstein KU, Bahn H, Holzhausen HJ, Lautenschlager C, Heidecke V, Burkert W. Prognostic factors in malignant glioma: influence of the overexpression of oncogene and tumor-suppressor gene products on survival. J Neurooncol 1997;35:13–28.
[11] Wechsler DS, Shelly CA, Petroff CA, Dang CV. MXI1, a putative tumor suppressor gene, suppresses growth of human glioblastoma cells. Cancer Res 1997;57:4905–12.
[12] Finco TS, Baldwin Jr. AS. Kappa B site-dependent induction of gene expression by diverse inducers of nuclear factor kappa B requires Raf-1. J Biol Chem 1993;268:17676–9.
[13] Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994;12:141–79.
[14] Dejardin E, Bonizzi G, Bellahcene A, Castronovo V, Merville MP, Bours V. Highly expressed p100/p52 (NFKB2) sequesters other NF-kappa B-related proteins in the cytoplasm of human breast cancer cells. Oncogene 1995;11:1835–41.
[15] Herrmann JL, Beham AW, Sarkiss M, Chiao PJ, Rands MT, Bruckheimer EM, Brisbay S, McDonnell TJ. Bcl-2 suppresses apoptosis resulting from disruption of the NF-kappa B survival pathway. Exp Cell Res 1997;237:101–9.
[16] Sovak MA, Bellas RE, Kim DW, Zanieski GJ, Rogers AE, Traish AM, Sonenshein GE. Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. J Clin Invest 1997;100:2952–60.
[17] Haskill S, Beg AA, Tompkins SM, Morris JS, Yurochko AD, Sampson-Johannes A, Mondal K, Ralph P, Baldwin Jr. AS. Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. Cell 1991;65:1281–9.
[18] Beg AA, Baldwin Jr. AS. The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. Genes Dev 1993;7:2064–70.
[19] Gilmore TD, Morin PJ. The I kappa B proteins: members of a multifunctional family. Trends Genet 1993;9:427–33.
[20] Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. Genes Dev 1995;9:2723–35.
[21] Baeuerle PA, Baltimore D. NF-kappa B: ten years after. Cell 1996;87:13–20.
[22] Mercurio F, DiDonato JA, Rosette C, Karin M. P105 and p98 precursor proteins play an active role in NF-kappa B-mediated signal transduction. Genes Dev 1993;7:705–18.
[23] Gill JS, Windebank AJ. Ceramide initiates NFkappaB-mediated caspase activation in neuronal apoptosis. Neurobiol Dis 2000;7:448–61.
[24] Neurath MF, Pettersson S, Meyer zum Buschenfelde KH, Strober W. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice. Nat Med 1996;2:998–1004.
[25] Cohen S, Yoshioka T, Lucarelli M, Hwang LH, Langer R. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm Res 1991;8:713–20.
[26] Hollinger JO, Leong K. Poly(alpha-hydroxy acids): carriers for bone morphogenetic proteins. Biomaterials 1996;17:187–94.
[27] Cleek RL, Rege AA, Denner LA, Eskin SG, Mikos AG. Inhibition of smooth muscle cell growth in vitro by an antisense oligodeoxynucleotide released from poly(DL-lactic-co-glycolic acid) microparticles. J Biomed Mater Res 1997;35:525–30.
[28] Crotts G, Park TG. Protein delivery from poly(lactic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues. J Microencapsul 1998;15:699–713.
[29] Cao X, Shiochet MS. Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders. Biomaterials 1999;20:329–39.
[30] Lu L, Stamatas GN, Mikos AG. Controlled release of transforming growth factor beta1 from biodegradable polymer microparticles. J Biomed Mater Res 2000;50:440–51.
[31] Peter SJ, Lu L, Kim DJ, Stamatas GN, Miller MJ, Yaszemski MJ, Mikos AG. Effects of transforming growth factor beta1 released from biodegradable polymer microparticles on marrow stromal osteoblasts cultured on poly(propylene fumarate) substrates. J Biomed Mater Res 2000;50:452–62.
[32] Zhu X, Lu L, Currier BL, Windebank AJ, Yaszemski MJ. Controlled release of NFkB decoy oligonucleotides from biodegradable polymer microparticles. Biomaterials, in press.
[33] Miller KJ, Das SK. Antisense oligonucleotides: strategies for delivery. Pharm Sci Tech Today 1998;1:377–86.

[34] Pomykala HM, Bohlander SK, Broeker PL, Olopade OI, Diaz MO. Breakpoint junctions of chromosome 9p deletions in two human glioma cell lines. Mol Cell Biol 1994;14:7604–10.
[35] De Hauwer C, Camby I, Darro F, Migeotte I, Decaestecker C, Verbeek C, Danguy A, Pasteels JL, Brotchi J, Salmon I, Van Ham P, Kiss R. Gastrin inhibits motility, decreases cell death levels and increases proliferation in human glioblastoma cell lines. J Neurobiol 1998;37:373–82.
[36] Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl Acids Res 1983;11:1475–89.
[37] Gill JS, Windebank AJ. Cisplatin-induced apoptosis in rat dorsal root ganglion neurons is associated with attempted entry into the cell cycle. J Clin Invest 1998;101:2842–50.
[38] Matsushime H, Quelle DE, Shurtleff SA, Shibuya M, Sherr CJ, Kato JY. D-type cyclin-dependent kinase activity in mammalian cells. Mol Cell Biol 1994;14:2066–76.
[39] Yokoo T, Kitamura M. Dual regulation of IL-1 beta-mediated matrix metalloproteinase-9 expression in mesangial cells by NF-kappa B and AP-1. Am J Physiol 1996;270:F123.
[40] Weinstein IB, Begemann M, Zhou P, Han EK, Sgambato A, Doki Y, Arber N, Ciaparrone M, Yamamoto H. Disorders in cell circuitry associated with multistage carcinogenesis: exploitable targets for cancer prevention and therapy. Clin Cancer Res 1997;3:2696–702.
[41] Li DM, Sun H. PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. Proc Natl Acad Sci USA 1998;95:15406–11.
[42] Trepicchio WL, Dorner AJ. Interleukin-11. A gp130 cytokine. Ann NY Acad Sci 1998;856:12–21.
[43] Blondeau N, Widmann C, Lazdunski M, Heurteaux C. Activation of the nuclear factor-kappaB is a key event in brain tolerance. J Neurosci 2001;21:4668–77.
[44] Kawamura I, Morishita R, Tsujimoto S, Manda T, Tomoi M, Tomita N, Goto T, Ogihara T, Kaneda Y. Intravenous injection of oligodeoxynucleotides to the NF-kappaB binding site inhibits hepatic metastasis of M5076 reticulosarcoma in mice. Gene Ther 2001;8:905–12.
[45] Batycky RP, Hanes J, Langer R, Edwards DA. A theoretical model of erosion and macromolecular drug release from biodegrading microspheres. J Pharm Sci 1997;86:1464–77.