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J. N. Andrews Honors Program
Andrews University

HONS 497
Honors Thesis

The Effect of Degrading the Transcription Factor NF- κ B Subunit Proteins on NF- κ B's Oncological Activity

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Mar. 28, 2016

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Abstract

Nuclear factor-kappa B (NF- κ B) is a transcription factor that becomes functional when any two of its five component proteins (p50, p52, p65, c-Rel, and RelB) join together. NF- κ B plays an important role in bringing out cell proliferation, or cell growth. When NF- κ B malfunctions and becomes hyperactive, excessive NF- κ B activity promotes abnormally high cell growth, which is a symptom of cancer. Because of its tie to cancer, NF- κ B is commonly subjected to modification to curb cancer growth. In this project, each component protein of NF- κ B was degraded via a method called RNAi to see if it would have any negative influence on NF- κ B activity of glioblastoma, or brain cancer, cells. It was found that degradation of p50 and p52 significantly reduced NF- κ B activity while the remaining three failed to produce significant reduction.

Introduction

Nuclear factor-kappa B (NF- κ B) is a transcription factor that functions as a dimer unit, and its component proteins include p50, p52, p65, c-Rel, and RelB. Once activated, NF- κ B plays an important role in initiating the activation of genes responsible for apoptosis (cell death), cell proliferation, and drug resistance. NF- κ B: 1) induces resistance to apoptosis through upregulating expression of Inhibitors of Apoptosis (IAPs) [Takahashi et al. 1998]; 2) promotes cell proliferation by affecting several genes involved in cell cycle such as those of cyclin family [Guttridge et al. 1999]; and 3) bestows chemo/radio-resistance to cells by causing expression of multidrug resistance P-glycoprotein [Dolcet et al. 2005]. Since NF- κ B is involved in cell growth and drug resistance, abnormal NF- κ B puts cells in danger of becoming cancer cells.

NF- κ B has four roles in tumor initiation and development: 1) it promotes cancer cell survival through stimulating cell proliferation and preventing apoptosis, 2) it regulates proteins involved in tumor angiogenesis (synthesis of blood vessels) such as vascular endothelial growth factor (VEGF), 3) it plays an important role in metastasis through modulating epithelial-mesenchymal transition (EMT), and 4) it can change normal cells' metabolism to that of tumor cells through affecting components involved in glucose uptake such as glucose transporter 3 (GLUT3) [Xia et al. 2014].

This project analyzes the relative importance of each of NF- κ B's five component proteins on the transcription factor's activity. Each subunit protein is degraded via a method called RNA interference (RNAi). RNAi is a convenient way of silencing a target protein through binding and destroying the protein's mRNA, thereby preventing it from being translated. To summarize the mechanism shortly, double-stranded RNAs (dsRNAs) get processed into 21- and 22-nucleotide fragments, which is the optimum length to carry out RNAi. These fragments are then inserted

into cells, bind with complimentary mRNAs, and mediate RNA nuclease attachment which leads to the target sequence degradation [Elbashir, et al. 2001].

Extensive research has already been done on the connection between NF- κ B and breast cancer. A study done on 82 breast cancer patients found that 55 patients' cancer cells exhibited higher level of NF- κ B activity than normal breast cells ($p < 0.005$) [Hou, et al, 2003]. Another study performed a mutation analysis on 36 candidate breast cancer genes in 96 human breast cancer tissues. The genes were amplified via polymerase chain reaction (PCR) and sequenced. Compared to normal breast tissue genes, the cancer genes, which included NF- κ B, contained 28 mutations, implying that mutations in NF- κ B family are likely linked to breast cancer development [Jiao, et al, 2012].

Analysis of five breast cancer cell lines (MCF7, T47D, MDA213, SKBR3, and BT474) through electrophoretic mobility shift assays (EMSAs) revealed that although these cell lines showed increased activation of NF- κ B, not all of NF- κ B's subunits were increased in proportional amount. Namely, p50, p52, and c-Rel were shown to be the predominant subunits in these cell lines [Cogswell, et al. 2000]. A similar study that analyzed the expression of breast tumors' several markers, which included NF- κ B, found that expression levels of p50 and p65 were correlated with breast cancer [Shapochka, et al. 2012]. These results suggest that NF- κ B's five component proteins may not have an equal contribution in cancer development.

This project is unique in that it attempts to find NF- κ B's extent of influence in glioblastoma. Along with the previous studies done with breast cancer, this research will add further knowledge on the relationship between NF- κ B and cancer.

Methods

Cell Culture

U87MG glioblastoma cells (ATCC) were grown in Minimal Essential Medium (MEM) (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin antibiotics (Invitrogen). Stock dishes of cells (100 mm) were maintained at 37⁰C and 5% CO₂ and media was changed three times per week. Approximately twice a week cells were sub-cultured using 0.05% trypsin-EDTA for five minutes to remove cells from the dishes and dividing them into new 100 mm dishes. Experimental dishes were sub-cultured at 500,000 cells/ml in 60 mm cell culture dishes (Nunc).

Transfection

Transfection reaction was set up using 100 µl of MEM without FBS, 6 µl of FuGene 6 (Roche) transfection reagent and 5 µl of DNA (p50, p52, p65, c-Rel or RelB). This was incubated at room temperature (RT) for 30 minutes. Media was removed from the 60 mm cell culture dish containing 500,000 cells/ml and replaced with 1.5 ml of MEM without FBS. Absence of FBS ensured that the cells wouldn't divide. At the end of the incubation period, the transfection mixture was added to the cells. After 4 hours, 1.5ml of MEM with FBS was placed onto the cells to allow them to resume division. Cells to be used for reporter gene assay additionally had a pTAL control plasmid or pNF-kB plasmid transfected at the same time as the DNA. Control reactions contained everything except the DNA.

Western Blot

24-hours post-transfection cells were collected for protein analysis. Media was removed from the dishes, which were washed with ice-cold phosphate buffered saline (PBS). 1 ml of cell lysis solution (20mM Tris HCl pH 7.5, 150 mM NaCl, 1mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1mM b-glycerophosphate, 1mM Na₃VO₄, 1 mg/ml leupeptin; Cell Signaling) was added to the cells in the dish, and the dish was incubated for 5 minutes on ice. Cells were scraped off of the plate and transferred to a 1.5 ml microcentrifuge tube. The tube was centrifuged for 5 minutes at maximum rpm. The supernatant was transferred to a new 1.5 ml microcentrifuge tube and stored at -80⁰C until needed.

Electrophoresis of the protein samples was done using an X Cell SureLock electrophoresis chamber (Novex) and a Bio-Rad power pac basic. 20µl of protein were placed into individual 0.65ml tubes and 1/3 volume of Nupage LDS 2x sample buffer (Invitrogen) was added to each tube. The samples were boiled for 5 minutes to induce denaturation and then loaded into a 4-12% polyacrylamide gel (Invitrogen) with 1x Nupage MOPS SDS running buffer (Invitrogen) with 0.0025% Nupage antioxidants (Invitrogen). The gel was electrophoresed for 2 hours at 200 V in an ice bath.

The next step was to transfer the proteins to a PVDF (polyvinylidene fluoride) membrane. The gel was removed from the cassette and placed into an electric transfer apparatus with the PVDF membrane and Tris-glycine transfer buffer (84mM Tris, 1M glycine). The protein transfer was conducted for 3 hours, 20 volts in an ice bath.

At the end of the transfer, a western blot was performed. The PVDF membrane was blocked using 2% skim milk for 1 hour with shaking at RT followed by incubation with the primary antibody (p50, p52, p65, c-Rel or RelB) 1:1000 dilution for 1 hour with shaking at RT.

The membrane was washed with TBST (Tris buffered saline with 1% Tween 20) for 5 minutes, 10 minutes and 15 minutes with shaking. The membrane was then incubated for 1 hour with shaking in the secondary antibody (goat anti-rabbit or goat anti-mouse) 1:1000 dilution at RT. The membrane was again washed as above. Detection of bands was performed in a darkroom using the ECL chemiluminescent kit (Amersham) according to directions. Briefly, 1ml of each solution (A and B) were mixed together and incubated on the membrane for 1 minute in the light. The membrane was exposed to Kodak x-ray film for 30 seconds to 1 minute. The film was then developed and fixed for 3 minutes each with washing between developing and fixing.

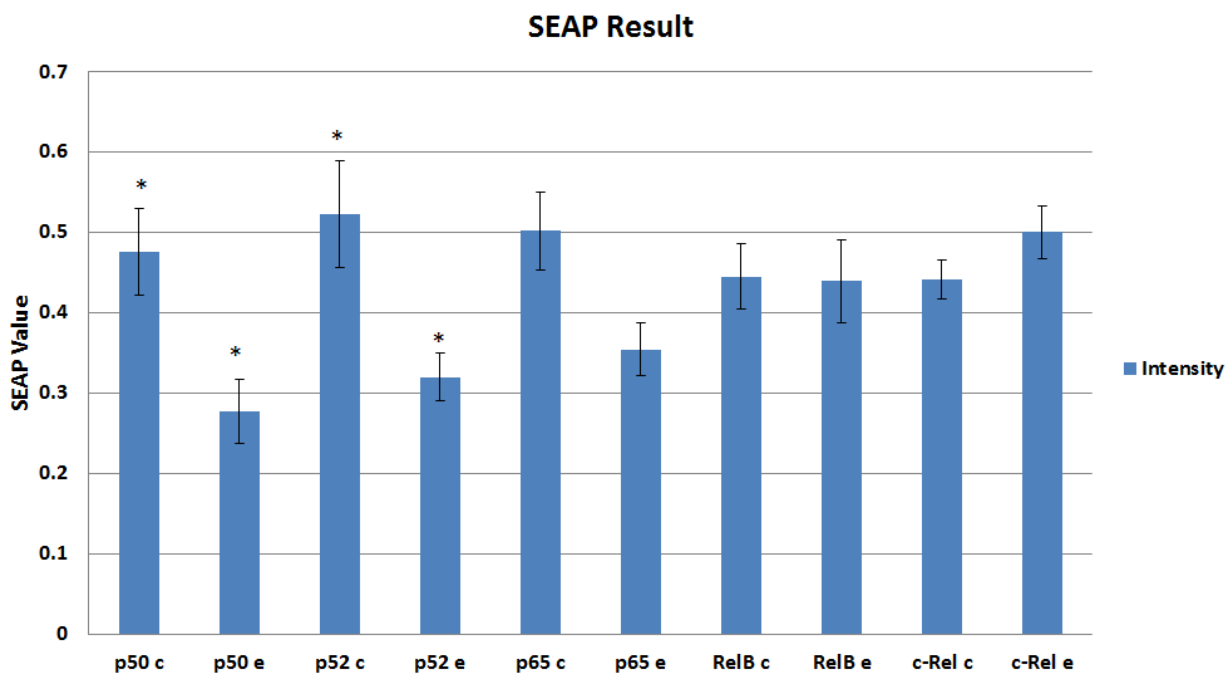
Antibody	Manufacturer	Grown In
c-Rel	Santa Cruz	Rabbit (polyclonal)
p50	Santa Cruz	Rabbit (polyclonal)
p52	Santa Cruz	Rabbit (polyclonal)
p65	Santa Cruz	Rabbit (polyclonal)
RelB	Santa Cruz	Mouse (monoclonal)

Secreted Alkaline Phosphatase Assay (SEAP)

U-87 MG cells were transfected with 2 μ g of shRNA plasmid DNA (p50, p52, p65, c-Rel or RelB) and 2 μ g of reporter plasmid DNA (either pNF-kB SEAP or pTAL; Clontech) in 60 mm dishes. Twenty-four hours after transfection the medium was removed and centrifuged for 5 minutes at room temperature and maximum rpm. The supernatant was placed into a new tube. 200 μ l supernatant and 200 μ l of 1X dilution buffer from the Great Escape kit (Clontech) were put into 0.65 ml tubes and vortexed. These tubes were incubated at 65⁰C for 30 minutes. 100 μ l

of the content was placed into a 1.5 ml microcentrifuge tube and 99 μ l of assay buffer (1M diethanolamine, 0.05M MgCl₂, 0.1M homoarginine) and 1 ml of 4-methylumbelliferyl phosphate (Clontech) were added to each tube. The tubes were incubated at room temperature in the dark for 1 hour. At the end of the incubation, the plate was read with a fluorometer with excitation at 360nm wavelength and emission at 449nm wavelength. The assay was read in triplicate for each tube and averaged. A graph was generated from the data. A Student's t-test, standard deviation and standard error tests were performed. This assay was repeated in triplicate. The remaining medium was stored at -80⁰C.

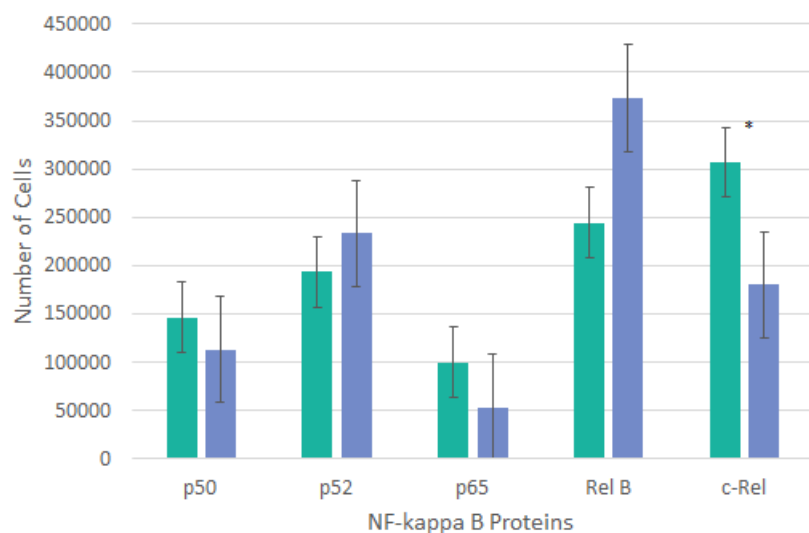
Results



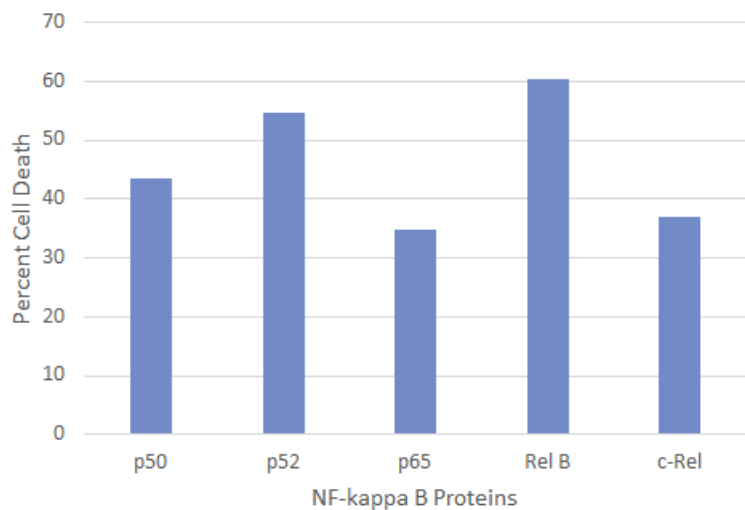
Graph 1) The graph shows the result of t-test done on SEAP result. Only p50 and p52 produced significant NF- κ B reduction. Obvious reduction is not seen for RelB and c-Rel

Graph 1 shows SEAP result. SEAP assay indicated that only degradation of p50 and p52 managed to significantly reduce NF- κ B activity ($p \leq 0.05$). Although degradation of p65 produced

drop in NF- κ B activity, the decrease was not statistically significant. Degradation of RelB and c-Rel failed to produce any noticeable influence on the transcription factor.



Graph 2) The graph shows the cell count of the transfected cell cultures. Only c-Rel produced significant reduction in cell count ($p \leq 0.05$). Green bar indicates # of alive cells, and blue bar indicates # of dead cells



Graph 3) The graph shows the percentage cell death

The cell count was somewhat surprising in that it did not reflect the result of SEAP assay. As Graph 2 summarizes, only degradation of c-Rel brought about significant reduction in cell number. But it is worth noticing in Graph 3 that the percentage cell death of p50 and p52 is 45% and 55%, respectively.

Western blot failed to confirm whether or not proper protein degradation had taken place. Only cells subjected to p65 degradation gave a readable Western image, while those subjected to

the remaining four did not produce a good image. On top of this, the p65 Western image did not show that the expected protein degradation had occurred. Theoretically lighter bands should appear for the experimental than for the control. But not much of a color difference between p65's experimental and control could be detected. Data not shown.

Discussion

The main weakness of the project is the discrepancy between the SEAP data and cell count. Although the SEAP data and cell count did not match up, this difference is not a discouraging one. First of all, the SEAP result somewhat corroborated previous studies on breast cancer that found that p50, p52, and c-Rel seemed to play comparatively more important role for NF-kB activity than p65 and RelB. SEAP assay and cell count indicated that p50, p52, and c-Rel were correlated with U87MG glioblastoma cells' NF-kB activity. While this finding does not perfectly reflect that of breast cancer studies, the common ground between the two suggests that there is some reason to believe in the validity of this research.

In addition, the SEAP result does not actually prove that the project entails problems. Two things have to be considered: to begin with, transfection efficiency is quite low; second, during the transfection step, cells that are being transfected express different amounts of NF-kB. Say that there are two dishes, for instance, in which half the cells is overexpressing NF-kB and another half is only slightly overexpressing NF-kB. It may very well result that in Dish 1, majority of transfected cells came from those overexpressing NF-kB, while transfected cells of Dish 2 equally came from those overexpressing and only slightly overexpressing NF-kB. Performing SEAP assay on these two plates would yield very different results, with Dish 1 appearing to have a better outcome. The same situation applies for Western blot as well. As a

result, the inconsistent SEAP result and failure to procure supporting Western images could be attributed to the nature of the assays. The only way to eliminate this variability is having a large enough sample size. This means that another experiment with a bigger sample size is necessary in order to validate the current project's findings and to get more accurate results.

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