Effect of Antisense Mediated BCL-2 Suppression on the Expression of the Androgen Receptor and Coactivating p300 and CREB Binding Proteins

Marvin Rubenstein1,2,3,4,∗, Courtney M. P. Hollowell2, and Patrick Guinan1,2,4,5

1 Division of Cellular Biology, Hektoen Institute for Medical Research, Chicago, IL 60612, USA
2 Division of Urology, Stroger Hospital of Cook County, Chicago, IL 60612, USA
3 Department of Biochemistry, Rush University Medical Center, Chicago, IL 60612, USA
4 Department of Urology, Rush University Medical Center, Chicago, IL 60612, USA
5 Department of Urology, University of Illinois at Chicago, Chicago, IL 60612, USA

∗Corresponding Author: Marvin Rubenstein; email: DrMarv@Prodigy.net

Received 10 August 2013; Accepted 22 October 2013

Academic Editor: Nianjun Liu

Copyright © 2013 Marvin Rubenstein et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract. Antisense oligonucleotides (oligos) have been employed against in vivo and in vitro prostate cancer models. While most oligos target growth factors or their receptors, others are directed against inhibitors of apoptosis or mediators of androgen activity. In previous experiments, mono- and bispecific oligos directed against bcl-2 suppressed both the targeted bcl-2 protein (an inhibitor of apoptosis) and non-targeted caspase-3 (a promoter of apoptosis), potentially negating the effect of therapeutic bcl-2 inhibition. Subsequently we reported that AR and p300 expression were significantly enhanced by these oligos. In a continuation of this study, we now report that the expression of another androgen receptor co-stimulatory protein, CREB binding protein (CREBBP), is not similarly increased. These data suggest that oligo treatment directed against bcl-2 can be evaded through compensatory increases in AR and p300 expression. Increased AR and p300 expression may transition the tumor to a more dedifferentiated and aggressive phenotype. However, not all co-stimulating proteins (CREBBP) are involved, and this may be important when controlling unanticipated (compensatory) effects of gene therapy.

Keywords: Antisense, bcl-2, Androgen receptor, p300, Prostate cancer, Therapy.

1. Introduction

Effective therapeutics directs their activity towards unique characteristics of etiologic agents (like bacterial cell walls, ribosomes or viral encoded enzymes). However, even dedifferentiated cancer cells are not substantially different from non-cancerous (differentiated cells). They use the same biochemical pathways and, unless virally induced, most are (even antigenically) similar to normal cells. The effectiveness of chemotherapy capitalizes on the fact that within a tumor a greater proportion of cells are in the process of replicating. Therefore, some aspect of DNA synthesis or repair is usually targeted. In prostate and breast cancers, growth factor (hormonal or protein) deprivation provides another type of therapy where orchiectomy or biochemical analogs or agonists block/interfere with steroid production or activity. Another approach could target transcriptional activity initiated by the DNA hormone response elements recognized
by the hormonal binding receptors (androgen receptor [AR], or estrogen receptor [ER]) or their co-activators (p300 and cAMP response element binding [CREB] binding protein [CREBBP]) which are more prevalent in advanced, hormone insensitive, disease [1]. However, for most chemotherapy tumor cell specificity is relative, often lacking, and most agents have significant toxicity towards other replicating cells, producing side effects in tissues or organs (gut or bone marrow) with rapid cell replenishment.

Gene therapy is based on a similar premise and while effective protocols require either translational suppression (antisense oligonucleotides [oligos]) or replacement (of inactivated, mutated or deleted suppressor genes like phosphatase and tensin homolog (PTEN) technology [2] both tumor and normal cells still express mostly the same genes. Targets for gene therapy are found in many pathways and it is likely that hundreds (or thousands) of genes are involved in malignant transformation. Although tumors can express an overall altered pattern of gene expression, the levels of many growth regulatory genes are similar to normal cells. Resistance develops because the biochemical pathways involved are complex and highly regulated by stimulatory and inhibitory factors, many altered by therapy. In addition, we report that tumors can alter their dependence upon single gene influences by relying upon others through compensation [3].

Tumors are essentially heterogeneous masses of rapidly growing and selectively adapted cells whose sole purpose is to survive and replicate. While doing so, those bearing mutations able to evade therapeutic interventions are clonally selected. The best example is the emergence of hormone insensitive prostate cancer cells following androgen deprivation therapy, resulting in the increased expression of the apoptotic promoter caspase-3. In another form of compensation, we also found that expression of the AR was increased [8] as was the transcriptional co-activator p300 [9], which is usually associated with and more highly expressed in advanced prostate tumors [1]. Our earlier studies [8, 9] evaluated expression of several co-activators of the AR, the cytokines IL-4 and IL-6. The results identified an increased IL-6 expression but no similar effect in the IL-4 co-activator [9]. In this study we evaluate another co-activator of the AR, CREBBP. CREBBP is universally expressed and acts in association with many transcriptional factors including the AR. Functionally, the protein has histone acetyltransferase activity and provides a scaffold to the transcriptional complex and shares some sequence homology with p300 [10]. Enhanced AR activity could not only select cells which again evade apoptosis and enhance tumor progression (even with bcl-2 suppression), but could also indicate the emergence of a more aggressive (hormone insensitive) phenotype. For gene therapy to ultimately be successful it must be made more specific or mechanisms of compensation identified and suppressed.

2. Materials and Methods

2.1. Oligonucleotides. Oligos (mono- or bispecific) were purchased from Eurofins MWG Operon (Huntsville, AL). Each was phosphorothioated on three terminal bases at 5’ and 3’ positions. Stock solutions were made to a final concentration of 625 µM in sterile Dulbecco PBS.

Base Sequences

Each oligo contained at least one CAT sequence and targeted the area adjacent to the mRNA AUG initiation codon for the respective targeted protein (EGFR or bcl-2).


Cell Culture

LNCaP cells were grown in RPMI 1640 supplemented with 10% bovine serum, 1% L-glutamine and 1% penicillin/streptomycin in a 5% CO2 incubator. Log phase cells were harvested using EDTA/trypsin and equally distributed into 75 cm2 flasks (Corning, NY). At intervals media were either supplemented or replaced with fresh.

Oligo Treatment Prior to RT-PCR

Fours days prior to oligo addition, when cell density approached 75% confluence, 10 ml of fresh media was added. Cells were incubated for an additional 3 days before 5 ml of media was replaced with fresh the day before oligos were added. 100 µl of stock oligos were added to bring the final concentration to 6.25 µM. Incubation proceeded...
for an additional 24 hours in the presence or absence of monospecific MR₄, or the MR₂₄ and MR₄₂ bispecifics.

**RNA Extraction**

Following treatment, media was removed, a single ml of cold (4°C) RNAzol B was added to each 75 cm² culture flask and the monolayer lysed by repeated passage through a pipette. All procedures were performed at 4°C. The lysate was removed, placed in a centrifuge tube to which 0.2 ml of chloroform was added, and shaken. The mixture stayed on ice for 5 min, was spun at 12,000 g for 15 min, and the upper aqueous volume removed and placed in a fresh tube. An equal volume of isopropanol was added, the tube shaken, and allowed to stay at 4°C for 15 min before similar centrifugation to pellet the RNA. The supernatant was removed, the pellet washed in a single ml of 75% ethanol, then spun for 8 min at 7500 g. The ethanol was pipetted off and the formed pellet air dried at −20°C.

**RNA Quantitation**

RNA was resuspended in 250 µl of diethylpyrocarbonate (DEPC) treated H₂O, and quantitated using a Qubit fluorometer and Quant-iT RNA assay kit (Invitrogen; Grand Island, NY). DEPC is an inhibitor of RNase activity.

**Reverse Transcriptase-PCR**

Extracted RNA was diluted in DEPC treated water to 40 µg/µl. 1–4 µl of this RNA was added to 1 µl of both sense and antisense primers (forward and reverse sequences from RealTimePrimers: Elkins Park, PA) for bcl-2, AR, p300 and CREBBP. From a kit purchased from Invitrogen the following reactants were added for Reverse Transcriptase-PCR (RT-PCR): 25 µl of 2X reaction mixture, 2 µl SuperScript III RT / platinum Taq mix, tracking dye, and 3 µl MgSO₄ (of a 5mM stock concentration). DEPC treated water was added to yield a final volume of 50 µl. RT-PCR was performed for 2 X 25 cycles using the F54 program in a Sprint PCR Thermocycler. As a control for RT-PCR product production, 3 µl of a molecular marker (Invitrogen) which contained a sequence of bases in 100 base pair increments (Invitrogen) was included, the difference compensated for by 3 µl of DEPC treated water.

**Primers**

**Actin**

Forward primer sequence: 5’ CAA ACA TGA TCT GGG TCA TCT TCT C 3’

Reverse primer sequence: 5’ GCT CGT CGT CGA CAA CGG CTC

PCR product produced was 353 base pairs in length

**Bcl-2**

Forward primer sequence: 5’ GAG ACA GCC AGG AGA AAT CA 3’

Reverse primer sequence: 5’ CCT GTG GAT GAC TGA GTA CC 3’

PCR product produced was 127 base pairs in length.

**Androgen Receptor**

Forward primer sequence: 5’ CGG AAG CTG AAG AAA CTT GG 3’

Reverse primer sequence: 5’ ATG GCT TCC AGG ACA TTC AG 3’

PCR product produced was 155 base pairs in length.

**p300**

Forward primer sequence: 5’ CGC TTT GTC TAC ACC TGC AA 3’

Reverse primer sequence: 5’ TGC TGG TTG TTG CTC TCA TC 3’

PCR product produced was 167 base pairs in length.

**CREBBP**

Forward primer sequence: 5’ CAC CAG CAG ATG AGG ACT CT 3’

Reverse primer sequence: 5’ TAC ACC GGT GCT AGG AGG AG 3’

PCR product produced was 222 base pairs in length

**Detection and Quantitation of Product**

**Agarose Gel Electrophoresis**

1.5% agarose gels were prepared in a 50 ml volume of TBE buffer (1X solution: 0.089 M Tris borate and 0.002M EDTA, pH 8.3), containing 3 µl of ethidium bromide in a Fisher Biostest electrophoresis system. Samples were run for 2 hours at a constant voltage of 70 using a BioRad 1000/500 power supply source. To locate the amplified PCR product, 3 µl of a molecular marker (Invitrogen) which contained a sequence of bases in 100 base pair increments (Invitrogen) as well as 2 µl of a sucrose based bromphenol blue tracking dye were run in each gel.

**Quantitation**

Gels were visualized under UV light and photographed using a Canon 800 digital camera. Photos were converted to black and white format and bands quantitated using Mipav software provided by the National Institute of Health. Means and standard deviations were compared using Student t-tests to determine significance.

**3. Results and Discussion**

**3.1. Bcl-2 Expression.** As a control (data not shown) for RT-PCR sequence replication human actin expression was tested in RNA extracted from HeLa cells Figure 1.

LNCaP cells incubated for 24 hours in the presence of 6.25 µM of oligos suppressed Bcl-2 expression, and support the finding of comparable biologic activity in both mono- and bispecific oligos measured in the in vitro cell growth inhibition experiments [11]. When photographs of the identified product bands were scanned on agarose gels and quantitated using Mipav software, in a series of runs, the greatest expression of bcl-2 was always found in untreated LNCaP cells. Those treated with oligos, whether mono-
or bispecific, produced bands which indicated obvious (to the naked eye) suppression. For each oligo evaluated, the greatest amount of suppression measured approached 100% for the mono-specific $MR_4$; and for the bispecifics $MR_{24}$ and $MR_{42}$, 86% and 100%, respectively. Suppression was found in both repeat RT-PCR runs with bcl-2 primers, as well as in repetitive agarose gel quantifications. Representative bands are presented Figure 2.

3.2. AR Expression. Comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against bcl-2 (and EGFR in the bispecifics) were then evaluated by RT-PCR using primers directed against AR. When background intensity was subtracted, the relative intensity of all bands corresponding to AR representing cells treated with $MR_4$, $MR_{24}$ and $MR_{42}$ compared to controls were enhanced $31.2\% \pm 26.0 (P = 0.015)$, $58.5\% \pm 51.4 (P = 0.019)$ and $53.1\% \pm 45.9 (P = 0.019)$. These results ($N = 6$) were pooled from both duplicate RT-PCR runs and gels, and indicate similar (significant) enhancement of AR activity is produced by each oligo type. Representative bands are presented Figure 3.

3.3. p300 Expression. Comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against bcl-2 (and EGFR in the bispecifics) were then evaluated by RT-PCR using primers directed against p300. When background intensity was subtracted, the relative intensity of all bands corresponding to p300 representing cells treated with $MR_4$, $MR_{24}$ and $MR_{42}$ compared to controls were increased $82.9\% \pm 51.9 (P = 0.006)$, $93.0\% \pm 87.3 (P = 0.044)$ and $105.4\% \pm 65.9 (P = 0.007)$. These results ($N = 6$) were pooled from both duplicate RT-PCR runs and gels, and indicate similar (significant) enhancement of p300 activity is produced by each oligo type. Representative bands are presented in Figure 4.

CREBBP Expression

Comparable amounts of extracted RNA from LNCaP cells treated with either mono- or bispecific oligos directed against bcl-2 (and EGFR in the bispecifics) were then evaluated by RT-PCR using primers directed against CREBBP. When background intensity was subtracted, the relative intensity of all bands corresponding to CREBBP representing cells treated with $MR_4$, $MR_{24}$ and $MR_{42}$ compared to controls were varied. Although significant decreases were found in both the monospecific $MR_4$ ($-32.1\% \pm 5.7; P < 0.05$) and bispecific $MR_{42}$ ($-26.8\% \pm 32.3; P < 0.05$) there was no significant change in $MR_{24}$. This pattern (where similar changes are not seen in both bispecifics) is unique in the many proteins we have examined, and we conclude that unlike the increased expression of AR and p300, there is no enhancement of this transcription co-activator. These results ($N = 6$) were pooled from both duplicate PCR runs and gels. Representative bands are presented in Figure 5.

The androgen receptor (AR) (also known as NR3C4; nuclear receptor subfamily 3, member 4) plays a principle role in male sexual development, prostate function, cancer
progression and target for treatment strategies. Following cytoplasmic binding of the AR to testosterone or its dihydrotestosterone metabolite, it undergoes a conformational change accompanied by dissociation of heat shock proteins with translocation into the cell nucleus. AR dimerizes, binds to hormone response elements of the DNA, and acts as a transcription factor enhancing synthesis of growth stimulating proteins, including insulin-like growth factor (IGF) [12]. In addition to IGF, other growth factors like transforming growth factor-alpha (TGF-\(\alpha\)) acting through their respective receptors (like the epidermal growth factor receptor [EGFR] which binds TGF-\(\alpha\)) contribute to unregulated prostate cancer growth. These protein factors and their receptors are targets for oligo mediated suppressive therapy [4]. Although disruption of this process by androgen deprivation provides the rationale for most types of prostate cancer treatment, most tumors recur in an androgen insensitive form within a few years. At this stage, genes are driven towards transcription by AR, coactivating transcription factor p300 and its homolog, CREBBP. p300 is essential for cell growth and governs the expression of cyclins regulating the transition between \(G_1\), S, \(G_2\) and M phases of mitosis [13]. Acting with IL-6, p300/CREBBP plays a role in the androgen independent expression of prostate specific antigen (PSA) [14]. In the LNCaP model, administration of R1881 reduces both CREBBP mRNA and the encoded CREBBP protein and suggests, that following androgen ablation, the expression of some coactivators increase and contribute to a state of AR hypersensitivity [15]. Treatment of prostate cancer cells with siRNA directed against p300 reduces cancer cell growth [15], and eliminates the ability of IL-6 to induce PSA [15]. Since both transcriptional coactivator proteins p300 and CREBBP are expressed to a greater extent in advanced prostate cancer [13], well differentiated, androgen sensitive, LNCaP cells would be expected to have relatively low expression of p300, and this has been demonstrated [9]. The enhanced expression seen following oligo treatment [9] makes its induction appear more impressive, and implies transition to a gene expression pattern associated with later stage (androgen insensitive) disease. This suggests that oligo treatment directed against bcl-2, not only can be evaded through compensatory changes in expression which encourage tumor growth, but may also contribute to further dedifferentiation and hormone insensitivity.

Innovative protocols to disrupt androgen driven tumor progression have employed antisense oligos directed against the enzyme for conversion of testosterone to dihydrotestosterone (5-alpha reductase), heat shock proteins, p300 and the AR itself. LNCaP cells express an AR which is mutated in the binding domain however the Eder [16] and Rubenstein groups [17] have separately demonstrated growth inhibition in this \textit{in vitro} model employing oligos.

Gene therapy is a complex process requiring multiple pathways (and the regulatory proteins) to be simultaneously regulated. In addition, the “driver genes” (usually kinases) which greatly influence tumor growth must be distinguished...
and targeted rather than similarly mutated “passenger genes.”

To further complicate antisense mediated gene therapy, some oligos are able to bind to either targeted or non-targeted proteins as aptamers, although in these studies, no such selection or capacity was evaluated. Lastly, in addition to the effects upon non-targeted proteins, no studies have yet been directed to evaluate additional regulation of protein activity at the microRNA level.

Oligos (produced by Oncogenex Pharmaceuticals) have reached clinical trials for the treatment of prostate cancer (OGX-011), while others remain in preclinical development (OGX-225). Often administered in combination with traditional chemotherapy, these oligos target bcl-2, clusterin (OGX-011 in Phase II testing), heat shock protein 27 (OGX-427) or insulin growth factor binding proteins (OGX-225) (16). Many represent efforts to restore tumor apoptosis by eliminating apoptosis inhibitors bcl-2 [5–7], or clusterin (OGX-11) associated with treatment resistance. For (tumor suppressor) genes which are either diminished or lacking in expression gene transfection has been attempted in prostate cells which contain a mutated PTEN [2].

Although antisense oligos are specifically directed through complementary base pairing to inhibit mRNA translation of genes, there can be secondary or downstream effects on non-targeted genes following oligo mediated bcl-2 suppression [3, 8]. The effectiveness of bcl-2 and overall apoptosis activity is highly regulated and dependent upon the expression of many stimulatory, inhibitory, stabilizing factors, as well as the ratio between these proteins. As demonstrated, the specific suppression of one apoptosis inhibitory protein (bcl-2) is compensated by the suppression of a non-targeted promoter, caspase-3 [3]. Clinically these types of experiments are important because they suggest that for oligo mediated bcl-2 suppression to be effective caspase-3 activity should be either maintained or enhanced [3]. Other compensatory alterations are being evaluated, and could involve the enhanced expression of non-targeted apoptosis suppressors, producing a similar effect (evasion of re-established apoptosis following bcl-2 suppression). Growth stimulatory proteins like TGF-α, EGFR or IGF could also be enhanced. In these experiments bispecific oligos also targeted EGFR. However, in previous experiments we found that growth inhibition produced by monospecific oligos directed against EGFR was not accompanied by decreased mRNA expression [19]. Currently Genta is conducting a phase 3 test using oligos (Genasense; oblimersen) directed against bcl-2 for treating melanoma, chronic lymphocytic
leukemia and various solid tumors [20], but compensatory effects produced by this agent have not (yet) been reported. Tumors are resilient in their efforts to overcome (even newly developed) therapeutics and become resistant. If gene therapy is to be effective, we must understand how primary effects evoke compensatory changes. It would also be significant to see whether these changes are replicated in an in vivo model. If these lead to enhanced expression of undesired proteins, the oligo approach can again be applied. If antisense technology is to be fully exploited multivalent forms (as proposed) can be developed to suppress multiple proteins [21].

This year (2013) the American Cancer Society (ACS) estimates that in spite of early detection, screening for prostate specific antigen (PSA) and more effective treatments for localized disease, in the United States there will be 29,720 deaths from prostate cancer in addition to 238,590 newly diagnosed cases [22]. New types of treatment, including gene therapy and translational inhibition must be developed and employed (probably in combination with traditional androgen ablation).

Acknowledgments

The Cellular Biology laboratory at the Hektoen Institute is supported, in part, by the Blum Kovler Foundation, the Cancer Federation, Safeway/Dominicks Campaign for Breast Cancer Awareness, Lawn Manor Beth Jacob Hebrew Congregation, the Max Goldenberg Foundation, the Sternfeld Family Foundation, and the Herbert C. Wenske Foundation.

References

[18] (Oncogenes website) www.Oncogenes.ca.
Dear Colleagues,

Although publications covering various aspects of nuclear receptors (NRs) appear every year in high impact journals, these publications are virtually buried among an overwhelming volume of articles that are only peripherally related to NRs. The latter fact prompted a group of prominent scientists active in the field of nuclear receptor research to conclude that gathering publications on this superfamily of receptors under one umbrella would provide an invaluable resource for a broad assemblage of scientists in the field; thus the idea for a new journal, Nuclear Receptor Research, was born.

I am pleased to share with you that Nuclear Receptor Research is now a reality as an open access peer-reviewed journal devoted to publishing high-quality, original research and review articles covering all aspects of basic and clinical investigations involving members of the nuclear receptor superfamily. Nuclear Receptor Research has an editorial board comprised of a group of renowned scientists from around the world. Board members are committed to make Nuclear Receptor Research a vibrant forum showcasing global efforts in this ever-expanding area of research.

We believe that the impact and visibility of papers related to nuclear receptors will be significantly enhanced by appearing in a journal devoted exclusively to nuclear receptors. In addition, it is hoped that Nuclear Receptor Research will serve as a catalyst to encourage collaborative studies as well as to foster interdisciplinary initiatives within this expansive and dynamic field. For these reasons, I invite you to consider Nuclear Receptor Research (http://www.agialpress.com/journals/nrr/) as a vehicle to share your novel research findings as well as your vision for the future of nuclear receptor research with your colleagues around the world.

Mostafa Badr
Editor-in-Chief
Nuclear Receptor Research