In LNCaP cells enhanced expression of the androgen receptor compensates for Bcl-2 suppression by antisense oligonucleotides

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Ther Adv Urol

(2011) 3(2) 51–57

DOI: 10.1177/

1756287211400493

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Abstract:

Background and methods: Antisense oligonucleotides (oligos) have been employed against *in vivo* and *in vitro* prostate cancer models targeting growth stimulatory gene products. While most oligos have targeted growth factors or their receptors, others have been directed against inhibitors of apoptosis. In LNCaP cells we evaluated a set of oligos which targeted and comparably suppressed the expression of the apoptosis inhibitor protein Bcl-2. LNCaP cells adapted to this restoration of apoptosis with a compensatory suppression of caspase-3 expression, a nontargeted promoter of this process. In a continuation of this study we now evaluate the expression of the androgen receptor (AR) following oligo mediated regulation of apoptosis with suppression of Bcl-2.

Results: Monospecific and bispecific oligos directed against Bcl-2 suppressed both the targeted Bcl-2 protein (an inhibitor of apoptosis) and the nontargeted caspase-3 (a promoter of apoptosis), potentially negating the effect on apoptosis produced by specific inhibition of Bcl-2. In contrast, the expression of the AR was significantly enhanced by each type of oligo.

Conclusions: This suggests that when Bcl-2 expression is inhibited there are compensatory changes in the expression of additional proteins which regulate tumor growth, apoptosis and cell survival, and in this scenario might increase or re-establish hormonal sensitivity. If tumors variants are selected which evade gene therapy additional mechanisms of compensation must be identified and subsequently suppressed. These experiments identify pathways by which tumors can develop resistance to gene therapy and suggests additional targets for intervention.

Keywords: antisense, interferon, prostate cancer, therapy

Introduction

Effective antibiotics and antiviral agents direct their activity towards unique characteristics of etiologic agents. However, biochemical resistance to therapeutics is a well-established consequence of treating bacterial and viral infections. While antibiotics often target unique bacterial components (cell walls or ribosomes) or differences between human/bacterial metabolism and synthesis, antivirals target viral encoded proteins (enzymes) facilitating binding, entry, uncoating, nucleic acid replication or DNA integration. Resistance develops as a result of enzymatic breakdown of the therapeutic agent (such as

penicillin by β -lactamase), or mutational events which render the target less susceptible.

In contrast, cancer cells are of human origin and not substantially different from normal (even differentiated) cells. They use the same biochemical pathways and unless virally induced most are (even immunologically) similar to normal cells. The effectiveness of chemotherapy capitalizes on the fact that within a tumor mass a greater proportion of cells are in the process of replicating. Therefore, anticancer drugs frequently target some aspect of DNA synthesis. In prostate and breast cancers, growth factor (hormonal or

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protein) deprivation presents an example of another type of therapy. However, for most chemotherapy, tumor cell specificity is relative, often lacking, and most agents administered (such as paclitaxel) have significant toxicity towards other replicating cells producing side effects in tissues or organs, such as the the gut or bone marrow, associated with rapid cell replenishment.

Gene therapy is based on a similar premise, and while effective protocols can employ either translational suppressive capability (antisense oligonucleotides [oligos]) or replacement (of inactivated, mutated or deleted suppressor genes such as PTEN) technology [Huang *et al.* 2001], both tumor and normal cells express the same genes. Although targets for gene therapy are found in many pathways and probably hundreds of genes can ultimately become involved in the malignant process, the level of individual gene expression may be minimally different from normal cells. Resistance develops because the biochemical pathways involved are complex and highly regulated by many stimulatory and inhibitory factors which are easily altered. It has been suggested that tumors can alter their dependence upon single influences by relying upon others through compensation [Rubenstein *et al.* 2011].

Tumors are essentially heterogeneous masses of rapidly growing and selectively adapted cells whose sole purpose is to survive, replicate and, while doing so, evade therapeutic interventions. The best example is the emergence of hormone insensitive prostate cancer cells following androgen deprivation therapy, resulting in the increased expression of the autocrine loop consisting of transforming growth factor- α (TGF- α) and its binding site the epidermal growth factor receptor (EGFR) in prostate and breast tumors [Rubenstein *et al.* 1994].

As bacteria and viruses mutate to evade antibiotic and antiviral agents, tumor cells are under similar selective pressure to evade chemotherapy. Although newly developed forms of gene therapy provide specific ways to inhibit uncontrolled growth or promote (re-establish) apoptosis, the unintended consequences of intervention are poorly understood.

Gene therapy is already being clinically applied for the treatment of human tumors including those of the prostate, and antisense oligos have been administered targeting the inhibitors Bcl-2

and clusterin in efforts to restore apoptosis following radiotherapy [Mu *et al.* 2005; Yip *et al.* 2005] or chemotherapy [Yamanaka *et al.* 2004]. If such therapy is to be successful, it is important to examine mechanisms by which tumors can evade this therapy through compensation. We recently reported [Rubenstein *et al.* 2011] that, in the LNCaP tumor model, inhibition of Bcl-2 with antisense oligos suppressed the expression of the apoptotic promoter caspase-3. In this study we evaluated a similar compensation which resulted in an increased reliance upon the expression of the androgen receptor (AR). Enhanced AR expression and increased hormonal sensitivity could select cells which again evade apoptosis and enhance tumor progression. If gene therapy is to be ultimately successful additional mechanisms of compensation must be identified and subsequently suppressed.

Methods

Oligonucleotides

Oligos (monospecific or bispecific) were purchased from Eurofins MWG Operon (Huntsville, AL, USA). Each was phosphorothioated on three terminal bases at 5' and 3' positions. Stock solutions were made to a final concentration of 625 μ M in sterile Dulbecco phosphate buffered saline (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).

MR₄ (monospecific targeting Bcl-2) T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T

MR₂₄ (bispecific targeting EGFR/Bcl-2) G-A-G-G-G-T-C-G-C-A-T-C-G-C-T-G-C-T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T

MR₄₂ (bispecific targeting Bcl-2/EGFR) T-C-T-C-C-C-A-G-C-G-T-G-C-G-C-C-A-T-G-A-G-G-G-T-C-G-C-A-T-C-G-C-T-G-C-T-C

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 was either supplemented or replaced with fresh.

Oligo treatment prior to PCR

Four 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. We added 100 µl of stock oligos 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 milliliter 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 milliliter 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 DEPC treated H₂O, and quantitated using a Qubit fluorometer and Quant-iT RNA assay kit (Invitrogen). DEPC is an inhibitor of RNase activity.

Reverse transcriptase polymerase chain reaction

Extracted RNA was diluted in DEPC treated water to 40 µg/µl. Between 1 and 4 µl of this RNA was added to 1 µl of both sense and anti-sense primers (forward and reverse sequences) for Bcl-2, caspase-3 and AR. From a kit purchased from Invitrogen, the following reactants were added for reverse transcriptase polymerase chain reaction (RT-PCR): 25 µl of 2 × reaction mixture, 2 µl SuperScript III RT / platinum *Taq* mix, tracking dye, and 3 µl MgSO₄ (of a 5 mM stock concentration). DEPC treated water was added to yield a final volume of 50 µl. RT-PCR

was performed for 2 × 25 cycles using the F54 program in a Sprint PCR Thermocycler

Primers

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.

Caspase-3. Forward primer sequence: 5' CCC CTG GAT CTA CCA GCA TA 3'

Reverse primer sequence: 5' TGT CTC TGC TCA GGC TCA AA 3'

PCR product produced was 262 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.

Detection and quantitation of product

Agarose gel electrophoresis

We prepared 1.5% agarose gels in a 50 ml volume of TBE buffer (1 × solution: 0.089 M Tris borate and 0.002 M EDTA, pH 8.3), containing 3 µl of ethidium bromide in a Fisher Biotest electrophoresis system. Samples were run for 2 h at a constant voltage of 70 V 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 bromophenol 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 NIH.

Results

Bcl-2 Expression

As a control (data not shown) for RT-PCR product production, human actin expression was tested in RNA extracted from HeLa cells [Rubenstein and Guinan, 2010].

LNCaP cells incubated for 24 h in the presence of 6.25 µM of oligos suppressed Bcl-2 expression, and support the finding of comparable biologic activity in both monospecific and bispecific oligos measured in the *in vitro* cell growth inhibition experiments [Rubenstein and Guinan, 2010b]. 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 monospecific 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 monospecific MR₄;

and for the bispecifics MR₂₄ and MR₄₂, 86% and 100%, respectively. Suppression was found in both repeat PCR runs with Bcl-2 primers, as well as in repetitive agarose gel quantifications. Figure 1 presents a Bcl-2 product band in the expected 127 base pair region which in this run was inhibited 23% by treatment with the monospecific MR₄, and 86% and 74%, respectively by bispecifics MR₂₄ and MR₄₂, as measured by Mipav software.

Caspase-3 expression

Comparable amounts of extracted RNA from LNCaP cells treated with either monospecific or bispecific oligos directed against Bcl-2 (and EGFR in the bispecifics) were then evaluated by RT-PCR using primers directed against caspase-3. A representative band for caspase-3 is presented in Figure 2 and appears below the marker representing 300 base pairs in the expected 262 base pair region.

When background intensity was subtracted, the relative intensity of the bands corresponding to

Figure 1. Bcl-2 Expression is suppressed by oligos as indicated in agarose gel RNA from treated LNCaP cells.

Figure 2. Caspase-3 expression is suppressed by oligos as indicated in agarose gel RNA from treated LNCaP cells.

caspase-3 representing cells treated with MR₄, MR₂₄ and MR₄₂ compared with controls were $-35.8\% \pm 12.5\%$ ($p=0.0002$), $-40.3\% \pm 16.6\%$ ($p=0.0006$) and $-43.5\% \pm 26.3\%$ ($p=0.006$). These results were pooled from both duplicate PCR runs and gels, and indicate similar (significant) suppression of caspase-3 activity is produced by each oligo type.

While gene therapy is often aimed at suppressing Bcl-2, caspase-3 expression is essential for the re-establishment of apoptosis. These experiments identify a mechanism for tumors to select variants which (again) evade apoptosis through the diminished expression of an apoptosis promoter (caspase-3). It also identifies caspase-3 as a necessary gene for expression (or replacement therapy) when oligos target Bcl-2 for suppression.

AR expression

Comparable amounts of extracted RNA from LNCaP cells treated with either monospecific or bispecific oligos directed against Bcl-2 (and EGFR in the bispecifics) were then evaluated by RT-PCR using primers directed against AR. To present the range of variability encountered, four replicate bands from a single RT-PCR product run for AR are shown in Figure 3. They appear approximately midway between markers representing 100 and 200 base pairs, in the expected 155 base pair region. The respective enhancement of AR expression was 35%, 71%, 9% and 1.0% produced by MR₄; 94%, 12%, 16% and 20%

produced by MR₂₄; and 113%, 80%, 11% and 4.0% produced by MR₄₂.

Additional bands were also quantitated from a repeat RT-PCR. When background intensity was subtracted, the relative intensity of all bands corresponding to AR representing cells treated with MR₄, MR₂₄ and MR₄₂ compared with controls were $31.2\% \pm 26.0\%$ ($p=0.015$), $58.5\% \pm 51.4\%$ ($p=0.019$) and $53.1\% \pm 45.9\%$ ($p=0.019$). These results were pooled from both duplicate PCR runs and gels, and indicate similar (significant) enhancement of AR activity is produced by each oligo type.

Discussion

In 2010 the American Cancer Society (ACS) estimated that in spite of early detection, screening for prostate-specific antigen (PSA) and effective treatments for localized disease, in the United States there will be 32,050 deaths from prostate cancer with 217,230 newly diagnosed cases [Jemal *et al.* 2010].

The AR (also known as NR3C4; nuclear receptor subfamily 3, member 4) plays a principal role in male sexual development, prostate function, cancer progression and treatment strategies. Following its cytoplasmic binding of either testosterone or its dihydrotestosterone (DHT) metabolite there is a conformational change, accompanied by dissociation of heat shock proteins, and translocation into the cell nucleus. It dimerizes, binds to hormone response elements

Figure 3. Androgen receptor expression is enhanced by oligos as indicated in agarose gel RNA from treated LNCaP cells.

of the DNA and acts as a transcription factor enhancing the synthesis of growth stimulating proteins, including insulin-like growth factor (ILGF) [Pandini *et al.* 2005]. ILGF, TGF- α , and other growth factors which act through their respective receptors contribute to the unregulated growth of prostate cancer and each provides a target for suppressive gene therapy. Disruption of this process by androgen deprivation provides the rationale for most types of prostate cancer treatment, although most tumors recur in an androgen insensitive form within a few years.

Innovative protocols to disrupt the above process have employed gene therapy directed against the enzyme for testosterone to dihydrotestosterone conversion (5- α reductase), heat shock proteins, or the AR itself. One method demonstrated to inhibit AR translation is with antisense oligos. Although LNCaP prostate tumor cells express an AR mutated in the binding domain, the Eder [Eder *et al.* 2000] and Rubenstein groups [Rubenstein *et al.* 2010] have separately demonstrated growth inhibition in this *in vitro* model employing oligos.

Gene therapy for cancer is a complex process requiring multiple pathways (and their 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'. For gene products which are overexpressed, methods to suppress their activities have been developed, including the use of antisense oligos. For the treatment of prostate cancer, some (produced by Oncogenex Pharmaceuticals) have reached clinical trials (OGX-011), while others remain in preclinical development (OGX-225). Often administered in combination with traditional chemotherapy, these oligos target proteins, such as Bcl-2, clusterin (OGX-011 in Phase II testing), heat shock protein 27 (OGX-427) or insulin growth factor binding proteins (OGX-225) (see <http://www.oncogenex.ca>). Some represent efforts to restore tumor apoptosis by eliminating those proteins, such as Bcl-2, associated with treatment resistance [Mu *et al.* 2005; Yip *et al.* 2005; Yamanaka *et al.* 2004]. For those genes diminished or lacking in expression, methods for replacement must be further developed, such as PTEN gene transfection [Huang *et al.* 2001].

Although antisense oligos are specifically directed to inhibit mRNA translation of genes mediated through complementary base pairing, there can be nonspecific (nontargeted) effects. Pathways involving growth or apoptosis are frequently targeted by oligos. However, these are highly regulated paths with expression of many stimulatory, inhibitory and stabilizing factors. Specific inhibition of one protein, such as an inhibitor of apoptosis, could have unexpected compensatory effects in others and, within the apoptosis pathway, compensation could produce nonsequence (oligo-related) effects altering expression of even nontargeted proteins. Specifically (for apoptosis) the original therapeutic effect could be compromised by diminished expression of a promoter gene, or the enhanced expression of a suppressor. 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 [Kling, 2010]. Our data suggest that growth suppressing oligos directed against Bcl-2 (an inhibitor of apoptosis) also suppress the expression of nontargeted caspase-3 (an apoptosis promoter). When Bcl-2 suppression with oligos is used in the above therapy, this secondary effect could adversely affect the original, and suggests that caspase-3 activity should be either maintained or enhanced for optimal results. In addition we show that AR expression is enhanced, suggesting one mechanism to compensate for re-established apoptosis could include more reliance upon AR and greater hormone sensitivity.

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 [Rubenstein *et al.* 2002]. Oligo suppression of EGFR activity, by several suggested mechanisms, could further contribute to compensation and in these cells an increased reliance on the AR.

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 which suppress these additional proteins [Rubenstein *et al.* 2006].

Funding

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.

Conflict of interest statement

None declared.

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