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No Compensation in VEGF Expression Follows Antisense Suppression of BCL-2 Activity

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Abstract. *Antisense oligonucleotides (oligos) have been employed against prostate cancer models targeting growth-regulatory proteins, and at least one oligo (against bcl-2) has reached clinical trial. We previously found that, in LNCaP cells, mono- and bispecific oligos, which comparably suppressed the expression of bcl-2, compensated with suppression of caspase-3 (apoptosis promoter) activity, and enhanced the expression of the androgen receptor (AR) and its p300 and IL-6 co-activators. In addition, prostate-specific membrane antigen (PSMA) and (possibly its regulator) interferon (IFN) were elevated. A total of 14 proteins distributed between regulators of apoptosis, androgen regulation, differentiation antigens and autocrine-mediated growth have previously been examined. We extend these findings to include vascular endothelial growth factor (VEGF), a promoter of angiogenesis, which is not significantly altered through compensation, and therefore would not need additional regulation for suppressive bcl-2 therapy to be effective (like caspase-3).*

Gene therapy is in theory specific but it encounters difficulties. Although targets are found in many pathways, and tumors alter patterns of expression, the actual activities of most genes are similar to normal cells. Resistance develops because growth pathways are regulated by stimulatory and inhibitory factors, each potentially altered by therapy, and tumors compensate by altering their dependence upon targeted products, relying instead on alternate paths (1). Like bacteria and viruses, which mutate to evade antibiotic and antiviral agents, tumor cells are under selective pressure to evade therapy.

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Gene therapy employing antisense oligos against bcl-2 and clusterin have been clinically applied to treat human tumors, including those of the prostate, in efforts to restore apoptosis following radio- (2, 3) or chemotherapy (4). If such therapy is to be successful, it is important to examine mechanisms by which tumors evade and compensate. We previously found that, in the LNCaP tumor model, inhibition of bcl-2 with antisense oligos suppressed the expression of the apoptosis promoter caspase-3 (1) and simultaneously enhanced both androgen-receptor (AR) (5), p300 (6) and IL-6 expression. This suggests that following bcl-2 suppressive therapy there could be selective pressure within the surviving cells for a more aggressive (androgen sensitive) phenotype. The Gleave group reports similar effects in LNCaP cells, suggesting that androgen-driven compensation following therapeutic castration leads to increased insulin levels which contribute to enhanced *de novo* steroidogenesis and promote transition to castrate-resistant disease (7).

Unexpectedly, altered gene expression also affects non-growth related proteins such as prostate-specific membrane antigen (PSMA) (8), whose expression is enhanced by bispecific oligos targeting bcl-2. However, the capacity to enhance PSMA (not found in a similarly directed monospecific) is not a form of growth regulatory compensation, but instead attributed to a unique double-strand conformation in these bispecifics and their ability to induce IFN (an enhancer of surface antigen expression) (9). For gene therapy to ultimately be successful it must be more specific and mechanisms of compensation must be identified and controlled. The experiments presented here extend our previous findings by evaluating effects on vascular endothelial growth factor (VEGF), the first angiogenesis marker to be evaluated in this manner.

Materials and Methods

Oligo composition, base sequences, cell culture, treatment, RNA extraction, agarose gel electrophoresis and MIPAV quantitation have been previously published (1).

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.

VEGF: Forward primer sequence: 5' AGA CAC ACC CAC CCA CAT AC 3'; Reverse primer sequence: 5' TGC CAG AGT CTC TCA TCT CC 3'; PCR product produced was 231 base pairs in length.

Forward and reverse primer sequences for additional markers (Table I) can be found in their respective references.

Results

Actin control and 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 (10).

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 (10). 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 PCR runs with bcl-2 primers, as well as in repetitive agarose gel quantifications.

Vascular endothelial growth factor (VEGF) 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 VEGF. When background intensity was subtracted, the relative intensity of all bands corresponding to VEGF representing cells treated with MR_4, MR_{24} and MR_{42} compared to controls were 18.8%±31.2 (p=NS), 32.8%±35.0 p=NS) and –25.7%±40.4 (p=NS). These results were pooled from both duplicate PCR runs and gels, and indicate no significant changes. A representative gel is seen in Figure 1.

Discussion

Gene therapy is often promoted as a highly specific and deliverable treatment to control aberrant genes. However, it is apparent it's not as specific as previously thought. Oligos consist of nucleotide bases synthesized complimentary in sequence to mRNA. When hybridized to mRNA, they produce a translational arrest of the targeted gene's mRNA expression. Some (Oncogenex Pharmaceuticals) have reached clinical trials for the treatment of prostate cancer (OGX-011), while others remain in pre-clinical development (OGX-225) in efforts to restore tumor apoptosis by eliminating suppressive bcl-2 (2-4) associated with treatment resistance. Often administered in combination with traditional chemotherapy, these often target bcl-2 or clusterin (OGX-011 in Phase II testing). Others target heat shock protein 27 (OGX-427) or insulin growth factor binding proteins (OGX-225) (11). While it's understandable that genes sharing sequence homology would also be susceptible to treatment directed at common regions what is not expected are effects we report on non-related genes.

Our laboratory develops and tests bispecific oligos containing dual mRNA binding sites (one against bcl-2). We first reported that mono- and bispecific oligos directed against bcl-2 had comparable activity when evaluated with RT-PCR (10). Subsequent experiments (Table I) tested their activity against other proteins associated with growth and development. As seen in Table I, our program has diverged into several areas of investigation and has so far detected compensation for bcl-2 suppression results in decreased caspase-3 activity and increased expression of the AR and its p300 and IL-6 co-activators. This suggests that following bcl-2 suppression prostate tumors both evade apoptosis-directed therapy and become more aggressive and sensitive to residual androgen. We have also identified increased PSMA expression produced by some bispecific oligos, due to greater IFN expression and suggest that this increase in antigen presentation could enhance the activity of prostate cancer vaccines. 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 replaced (1). We now report that VEGF expression is unchanged following bcl-2 suppression, implying that angiogenesis (at least the one influenced by VEGF) is not adversely stimulated. Such stimulation would complicate treatment and require additional gene regulation if suppressive bcl-2 therapy were optimized.

Gene therapy is a complex process requiring multiple pathways (and the regulatory proteins) to be simultaneously regulated. 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.

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Table I. *Summary of gene expression results directed against bcl-2*

	OLIGOS				
	Monospecific	Bispecific			Ref.
	MR_4	MR_{24}	MR_{42}		
Apoptosis-related					
Targeted bcl-2	↓	↓	↓	Expression	(10)
Inhibitor of apoptosis				suppressed	
Non-targeted bax	---	---	---	Expression	(12)
Promoter of apoptosis				unchanged	
Non-targeted caspase-3	↓	↓	↓	Expression	(1)
Promoter of apoptosis				suppressed	
Non-targeted clusterin	---	---	---	Expression	(13)
Inhibitor of apoptosis				unchanged	
Androgen regulation					
Non-targeted AR	↑	↑	↑	Expression	(5)
Receptor				enhanced	
Non-targeted p300	↑	↑	↑	Expression	(6)
AR co-activator				enhanced	
Non-targeted IL-4	---	---	↓	No increase in expression	
AR co-activator/cytokine					(In Press) SpringerLink date November 8, 2011
Non-targeted IL-6	↑	↑	↑	Expression	
AR co-activator/cytokine				enhanced	(Submitted)
Non-targeted CREB	---	---	---	Expression	
Binding protein				unchanged	(Submitted)
Differentiation antigens					
Non-targeted PSA	---	---	---	Expression	(8)
Differentiation antigen				unchanged	
Non-targeted PSMA	---	↑	↑	Expression	(8)
Differentiation antigen				increased bispecifics	
Non-targeted PAP	---	---	---	Expression	(14)
Differentiation antigen				unchanged	
Non-targeted IFN	---	↑	↑	Expression	(9)
Surface antigen expression/				increased	
Cytokine				bispecifics	
Growth regulation					
Non-targeted IGF1	---	---	---	Expression	(In Press) SpringerLink
Autocrine growth factor				unchanged	date November 8, 2011
Angiogenesis					
Non-targeted VEGF	---	---	---	Expression	
Vascular growth factor				unchanged	

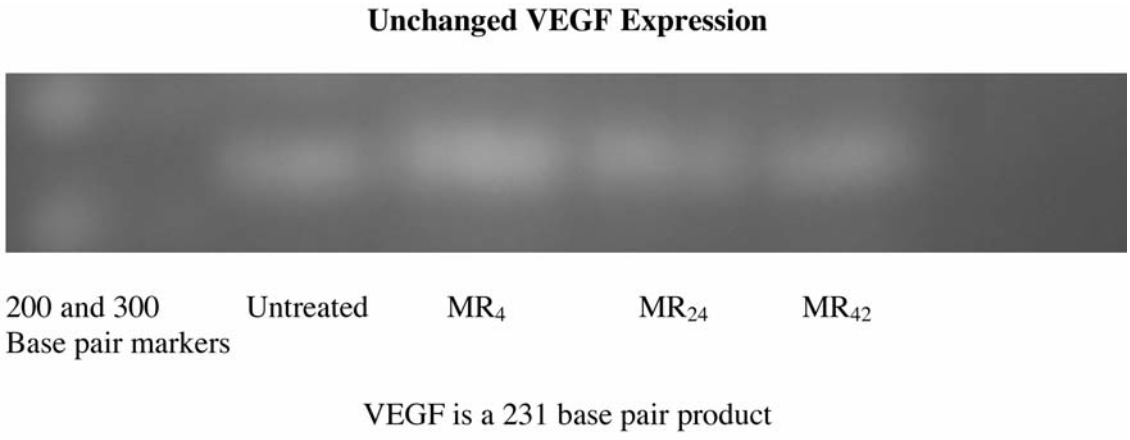

Figure 1. *Unchanged VEGF expression.*

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