Circular dumbbell AP-1 and E2F decoy oligodeoxynucleotide based antiproliferative gene therapy

Review Article

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Abbreviations: activator protein 1, (AP-1); c-Jun NH$_2$-terminal kinase, (JNK); Extracellular signal-regulated kinase, (ERK); fibroblast growth factor, (FGF); methylphosphonate, (MP); oligodeoxynucleotides, (ODNs); phosphorothioate, (PS); retinoblastoma, (Rb); transforming growth factor, (TGF)-β; plasminogen activator inhibitor-1, (PAI-1)

Summary

Excessive proliferation of cells is a characteristic finding in a wide variety of diseases including post-angioplasty restenosis, diabetic nephropathy, and malignant disease. It is well known that the transcription factors AP-1 and E2F play a critical role in cell proliferation and cell cycle regulation. Therefore, sequence-specific inhibition of AP-1 and E2F by decoy oligodeoxynucleotides (ODNs) is an attractive method to treat the above mentioned diseases. However, one of the main limitations of the conventional decoy ODNs is that they are easily degraded by intracellular nucleases. To rectify this problem, we have developed novel circular dumbbell (CD) decoy ODNs for AP-1 and E2F. The CD decoy ODNs for AP-1 and E2F significantly decreased the expression of genes that are transactivated by these factors and blocked cell proliferation in vascular smooth muscle cells, mesangial cells, and U2OS and C33A cancer cell lines in vitro. Moreover, we demonstrated the effectiveness of CD-AP-1 and CD-E2F decoy ODN based gene therapy in animal models of restenosis and diabetic nephropathy. Therefore, our data suggest that CD-AP-1 and CD-E2F decoy ODN based antiproliferative gene therapy could provide a new therapeutic strategy for the treatment of restenosis following angioplasty, diabetic nephropathy and cancer.

I. Introduction

Precise control of the cell cycle is essential for normal development and prevention of diseases which involve excessive cell proliferation. Excessive cell proliferation is a characteristic finding in cardiovascular diseases such as atherosclerosis and postangioplastic restenosis and in renal diseases such as glomerulonephritis and diabetic nephropathy, as well as in malignant growths (Klahr et al, 1988; Liu et al, 1989; Striker et al, 1991; Pauletto et al, 1994). Faulty transcriptional regulation has been implicated in the misexpression of numerous genes mediating cell proliferative responses and extracellular matrix (ECM) production in the development of cell proliferative diseases (Black and Azizkhan-Clifford, 1999; Akira, 2000; Williams, 2000; Nevins, 2001; Shaulian and Karin, 2001; 2002; Thiel and Cibelli, 2002). Therefore, an understanding of cell activation and cell cycle regulation at the transcriptional level may provide more effective gene therapy targets for treatment of these diseases. Two transcription factors, activator protein 1 (AP-1) and E2F, play critical roles in the development of cellular proliferative diseases. The activity of AP-1, a transcription factor influenced by the cellular redox state, is increased in diabetic and atherogenic conditions (Sen and Packer, 1996; Hamaguchi et al, 1998; 2000; Kikkawa et al, 2003). AP-1 binds to specific consensus sequences present in many genes associated with cell proliferation and ECM production, such as cyclin D1, transforming growth factor (TGF)-β, endothelin-1, and plasminogen activator inhibitor-1 (PAI-1), genes which are also implicated in neointimal formation (Shi et al, 1994; Daley and Gotlieb, 1996; DeYoung et al, 2001) and diabetic nephropathy. E2F regulates cell cycle progression through the expression of proteins required for the G1/S phase transition and DNA synthesis (Watson et al, 1987; Wagner...
E2F forms complexes with a number of cell cycle progression related proteins, including the products of the retinoblastoma (Rb) gene family and several CDKs (Krek et al., 1993; Kato et al., 1993; Kim et al., 1994). As cells progress toward S phase, E2F is bound to Rb family proteins, which are phosphorylated by G1 cyclin-CDK complexes and release E2F, directly leading to cell cycle progression.**Figure 1**

Recently, several new technologies have been introduced which can inhibit target gene expression in a sequence-specific manner, including antisense oligonucleotides, ribozymes, and siRNA. A more successful oligonucleotide-based approach has been the use of synthetic double-stranded oligodeoxynucleotides (ODNs) containing a transcription factor consensus sequence binding element. These decoy ODNs can penetrate cells and interact with DNA-binding proteins in a sequence specific manner, thereby interfering with eukaryotic transcription (Bielinska et al., 1990; Sawa et al., 1997; Morishita et al., 1998; Tomita et al., 2002; 2003). The ODN decoy strategy targeting the transcription factors AP-1 and E2F was recently reported to be quite effective in inhibiting cell proliferation in vitro, in vivo, and ex vivo (Morishita et al., 1995; Mann et al. 1999; Buchwald et al., 2002; Kume et al., 2002). However, one of the major limitations of the unmodified decoy ODN is that they are easily degraded by intracellular nucleases. To circumvent this problem, we have developed a novel circular dumbbell decoy ODN (CD decoy ODN) and have demonstrated the advantage of the CD decoy ODN strategy in vivo, in restenosis and diabetic nephropathy models, and in vitro, in two cancer cell lines. In this article, we review briefly the advantages of circular dumbbell decoy ODNs for AP-1 and E2F, which we will refer to as CD-AP-1 and CD-E2F, and introduce their application to gene therapy for cardiovascular disease, renal disease and cancer.

**II. Construction and advantage of CD decoy ODNs**

The therapeutic effectiveness of double-stranded decoy ODNs in the specific modulation of gene expression largely depends on the stability and specificity of the decoy ODN, as well as the efficiency of tissue and cellular delivery. To improve the effectiveness of double-stranded decoy ODNs, many forms of decoy ODN have been developed. These include unmodified decoy ODN duplexes, α- anomeric (chirally modified) decoy ODNs, phosphorothioate- (PS) and methylphosphonate- (MP) modified decoy ODN duplexes, and dumbbell decoy ODNs (Scholer and Gruss, 1984; Cereghini et al., 1988; Berkowitz et al., 1989; Bielinska et al., 1990; Clusel et al., 1993; Tanaka et al., 1994; Lim et al., 1997; Hosoya et al., 1999). The main limitation of the unmodified decoy ODN is that it is relatively easily degraded by intracellular nucleases. To enhance stability against nuclease activity, decoy ODNs have been chemically modified with sulfur ions (PS decoy ODN), methyl groups (MP decoy ODN), or other substituents. Although the stability of decoy ODNs is enhanced by these chemical modifications, other problems attributable to the substituents, such as target specificity, have been encountered (Gao et al., 1992; Brown et al., 1994; Burgess et al., 1995; Khaled et al., 1996).

**A. Construction of CD-AP-1 and E2F decoy ODNs**

To overcome these problems, we designed a circular dumbbell type decoy ODN containing one or two transcription factor binding sites in a single decoy molecule without an open end. The sequences of the CD decoy ODNs, with transcription factor binding consensus sequences underlined, are as follows: CD-AP-1, 5'-AATTCATGACTCAGAAGACGAAAACGTCTTCTGAAGTCATG-3'; CD-E2F, 5'-GGATCCGTTTCGCGCTATTGCAAAAGCAATAGCGCGAAAC-3'; and a short ring form of CD-E2F

**Figure 1. A cascade in activation of E2F**
(Ring-CD-E2F), 5’-ATGCGCGAAACGCGTTTTCGCGTTTCGCGCATAGTTTTCT-3’. CD decoy ODNs were predicted to form stem-loop structures. The stem is formed by the consensus sequence and its complement. In CD-AP-1 and CD-E2F, two oligonucleotide molecules were predicted to anneal at the complementary 6-base overhanging sequence of the 5’ ends. The decoy ODNs were annealed by cooling from 80 °C to 25 °C over a 2 h period. One unit of T4 DNA ligase was added to the mixture and it was incubated for 24 hours at 16 °C to generate a covalently ligated dumbbell shaped decoy ODN molecule. Thus, CD-AP-1 and CD-E2F consist of two loops and one stem, containing two transcription factor consensus sequences in tandem. In the case of Ring-CD-E2F, the 5’ terminus of the stem does not contain the six base overhang, but a single molecule forms the dumbbell structure with 5’ and 3’ ends juxtaposed in one of the loops. Following the addition of T4 DNA ligase (1 unit), the mixture was incubated for 24 h at 16 °C to generate a covalently ligated circular dumbbell decoy molecule (Figure 2).

B. Advantage of CD-AP-1 and CD-E2F decoy ODNs

Recently, we and others have reported that CD decoy ODNs have increased nuclease resistance and enhanced cellular uptake. Additionally, CD decoy ODNs cannot undergo strand separation and have a non-toxic, unmodified backbone which resembles natural DNA (Hosoya et al, 1999; Ahn et al, 2002a, b). These characteristics make CD decoy ODNs excellent candidates for transcription factor binding and competition studies. Accordingly, we designed a CD decoy ODN containing one or two transcription factor binding sites in a single closed end decoy molecule. As expected, CD-AP-1 and CD-E2F were structurally more stable and effective than chemically modified decoy ODNs. Consistent with recent reports, our CD decoy ODNs were more stable than PS decoy ODNs in the presence of serum, exonuclease III and S1 nuclease (Ahn et al, 2002a, b). These studies also showed that the CD decoy ODNs were more stable than PS decoy ODNs in vivo. The half-life of fluorescein-labeled CD-AP-1 in transfected kidney was about 11 days (Ahn et al, 2004). In addition, the sequence-specificity of the CD decoy ODNs, as assessed by an in vitro competition assay using electrophoretic mobility shift assay was nearly 10 times greater than that of the PS decoy ODNs. The inhibitory effect of the CD decoy ODNs on the DNA binding activity of transcription factors was also greater than that of PS decoy ODNs. These results indicate that the CD decoy ODNs have a greater affinity for their cognate transcription factors than the PS decoy ODNs.

A previous study suggested that the transcription factor recognition sequence in a PS decoy ODN inhibited expression of target genes by binding specifically and competitively to transcription factors regulating these genes and thereby provided therapeutic effects (Bielinska et al, 1990). However, studies by other investigators have shown that PS decoy ODNs exhibit sequence-independent

A. CD-AP-1 decoy ODN

\[
\begin{align*}
\text{A} & \quad \text{ATTTCAAGCTCAAGACG} \\
\text{A} & \quad \text{GCTTTTCAGCTCATG} + \text{GTACTGATCTTCTG} \\
\text{A} & \quad \text{GGCGAAGACTCAGTACTTAA} + \text{GAGAGCGCGATAAGC}
\end{align*}
\]

**ligation**

B. CD-E2F decoy ODN

\[
\begin{align*}
\text{A} & \quad \text{GGATCCGTTTCGGGAATG} \\
\text{A} & \quad \text{GCAATAGCGCGAAC} + \text{CAAGCGCGATAACG} \\
\text{A} & \quad \text{CGTATCGCGTTGCCCTAGG} + \text{T}
\end{align*}
\]

**ligation**

C. Ring-E2F decoy ODN

\[
\begin{align*}
\text{T} & \quad \text{T} \\
\text{T} & \quad \text{T}
\end{align*}
\]

(Figure 2). Construction of CD-AP-1 and E2F decoy ODN (A) CD-AP-1 decoy ODN: circular dumbbell type AP-1 decoy oligodeoxynucleotides, (B) CD-E2F decoy ODN: circular dumbbell type E2F decoy oligodeoxynucleotides, (C) Ring-E2F decoy ODN: short ring form of circular dumbbell type E2F decoy oligodeoxynucleotides. The bold letters indicate consensus sequences for each transcription factors.
effects due to non-specific protein binding (Brown et al., 1994; Hosoya et al., 1999). Brown et al. (1994) suggested that this non-specific binding activity resulted from the polyanionic characteristics of the phosphorothioate backbone of these decoy ODNs. These non-specific interactions appear to be dependent on the length of modified nucleotides. In our own studies, we have shown that CD-AP-1 and CD-E2F had greater sequence specificity than the PS decoy ODNs (Ahn et al., 2002a, b).

In order to use decoy ODNs as therapeutic agents, the decoy ODNs have to cross the cell membrane and enter the cytoplasmic and nuclear compartments of the cells. The cellular uptake of conventional decoy ODNs is relatively inefficient. However, newly developed CD decoy ODNs should be taken up by cells more effectively due to their circular structure. This hypothesis is supported by results showing that circularization of RNA/DNA chimeric ODNs containing two hairpin loop structures increased their cellular uptake as compared to nicked CD ODNs or linear antisense phosphodiester ODNs (Abe et al., 1998; Yamakawa et al., 1998). Moreover, in a recent review, Tomita et al. (2003b) reported that CD decoy ODNs were more easily transfigured into vascular smooth muscle cells (VSMC) in vitro than conventional decoy ODNs having a smaller molecular weight.

An additional potential therapeutic merit of CD decoy ODNs is that since the nucleotides are unmodified, they have no mutational potential when hydrolyzed and recycled through the salvage pathway.

III. Application of CD-AP-1 and CD-E2F based gene therapy strategies in cardiovascular disease

Excessive proliferation of VSMC is a critical step in the pathogenesis of atherosclerosis and restenosis after percutaneous transluminal coronary angioplasty (Liu et al., 1989; Pauletto et al., 1994). As discussed below, the role of the transcription factors AP-1 and E2F in VSMC proliferation is well established, making these two transcription factors good candidate targets for preventing atherosclerotic lesions and neointimal growth.

Extracellular signal-regulated kinase (ERK), a member of the MAP kinase family, and the c-Jun NH$_2$-terminal kinase (JNK) are rapidly and transiently activated after balloon angioplasty (Hu et al., 1997; Pyles et al., 2001). ERK2 and JNK1 activities in the injured vessel wall rapidly increase and reach high levels by 5 minutes after balloon injury (Hu et al., 1997; Izumi et al., 2001). Furthermore, a sustained increase in ERK2 kinase activity is observed in the arterial wall over a 7-day period and in neointima for 14 days after balloon injury. JNK and ERK translocate to the nucleus and activate c-Jun and c-Fos, which dimerize to form the AP-1 complex (Figure 3). As mentioned before, Several genes involved in ECM production and the cell proliferative response during neointimal formation contain AP-1 consensus binding sequences, including c-myc, FGF, TGF-$\beta$, endothelin-1 and PAI-1 (Shi et al., 1994; Daley and Gotlieb, 1996; DeYoung et al., 2001). Accumulating evidence indicates that activation of the MAP kinase and AP-1 cascades is a key event in the proliferation and growth of VSMC in response to injury (Hu et al., 1997; Pyles et al., 1997; Izumi et al., 2001). It has been reported previously that treatment with AP-1 decoy ODNs abolishes the expression of several genes, including PAI-1, TGF-β and endothelin-1 (Morishita et al., 1998; Lauth et al., 2000; Ahn et al., 2001). Several in vivo studies, by ourselves and other groups, have demonstrated that the AP-1 decoy ODNs dramatically prevented development of VSMC proliferation and neointimal formation in balloon-injured arteries (Ahn et al., 2002a; Buchwald et al., 2002; Kume et al., 2002). Our study used CD-AP-1, which was more stable and had a greater inhibitory effect and sequence specificity than a PS-AP-1 decoy ODN (Ahn et al., 2002a).

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**Figure 3.** Activation of AP-1 by MAP kinase ERK: extracellular-regulated kinase, JNK: c-Jun N-terminal kinase, TCF: Ternary complex factor, SRF: serum response factor, SRE: serum responsive element, TRE: TPA-response element, FRK: c-Fos-regulating kinase.
Several studies have demonstrated that inhibition of cell-cycle regulatory genes successfully blocks VSMC proliferation and neointimal formation in injured vessels (Simons et al, 1992; Morishita et al, 1993; 1994). However, inhibition of a single cell-cycle regulatory gene is insufficient to prevent cell cycle progression. Thus, a cell cycle regulator, such as E2F, that controls expression of many genes, is an attractive therapeutic target. The application of E2F decoy ODNs for therapeutic prevention of neointimal formation after angioplasty was first described in 1995 (Morishita et al, 1995). Although this study provided the possibility of a decoy-based therapeutic strategy to prevent neointimal hyperplasia, the major limitation, rapid degradation of the decoy ODN by intracellular nucleases, still remained to be solved. We designed a novel E2F decoy ODN by the covalent closure of two identical ODN molecules to avoid exonuclease digestion. Our CD-E2F displays markedly enhanced stability and sequence specificity compared to a PS-E2F decoy ODN. Moreover, inhibition of the DNA binding activity of E2F using CD-E2F significantly decreased cell cycle regulatory gene expression and cell proliferation, both in vitro and in vivo (Ahn et al, 2002b).

IV. Application of CD-AP-1 and CD-E2F based gene therapy strategies in renal disease

The proliferation of mesangial cells as well as the accumulation of ECM proteins is a characteristic feature of glomerulonephritis, which eventually leads to glomerulosclerosis and renal failure (Klahr et al, 1988; Striker et al, 1991). Although a variety of therapeutic strategies have been tested to prevent the progression of renal impairment, treatments that can suppress mesangial cell proliferation have not been reported (Glassock, 1992; Clarkson et al, 1993).

Mesangial cells in the adult kidney are normally quiescent, proliferate at low levels, and exist in the G0 phase of the cell cycle. Upon stimulation by growth factors or high glucose, mesangial cells exit G0 and progress through G1 and the G1/S transition of the cell cycle (Schultz et al, 1988; Ruef et al, 1990). The critical role of E2F in cell growth control has been established by several previous studies showing that E2F regulates the expression of genes required for DNA synthesis, as well as regulatory genes such as dihydrofolate reductase, c-myc, DNA polymerase, cdc2, and PCNA (Watson et al, 1987; Wagner and Green, 1991; Dalton, 1992). Several studies have shown that over-expression of E2F can induce cell cycle progression and that dominant negative E2F mutants inhibit cell growth (Johnson et al, 1993; Dobrowolski et al, 1994; Asano et al, 1996). Therefore, the crucial role of E2F in mesangial cell proliferation suggests that the transcriptional regulation of E2F by decoy ODNs could be a target for molecular therapeutics. Maeshima et al, (1998) reported successful inhibition of mesangial cell proliferation by E2F decoy ODNs, both in vitro and in vivo. They used an unmodified E2F decoy ODN, because modified decoy ODNs have been shown to inhibit protein synthesis of non target genes in a nonspecific, sequence-independent manner (Wolff et al, 1992). We transfected Ring-CD-E2F into rat mesangial cells and showed that cell cycle regulatory gene expression and cell proliferation were strongly inhibited (Park et al, 2003). These observations suggest that Ring-CD-E2F is an effective agent in suppression of rat mesangial cell growth and may be of therapeutic value.

Diabetic nephropathy is a disease characterized by expansion of glomerular mesangium, which is caused by mesangial cell proliferation and excessive accumulation of ECM proteins (Klahr et al, 1988; Steffes et al, 1989). Intracellular oxidative stress, induced by hyperglycemia, is a causative factor in the development of diabetic nephropathy (Hamaguchi et al, 1998, 2000). The activity of AP-1, induced by the intracellular redox state, is increased in diabetic patients. AP-1 stimulated expression of immediate early genes, and results in reentry into the cell cycle and increased proliferation of mesangial cells (Wolf et al, 1992). Activation of AP-1 induced by high glucose is also involved in increased mesangial TGF-β1 gene expression (Weigert et al, 2000). To verify the critical role of AP-1 in the pathogenesis of mesangial cell proliferation and increased extracellular matrix synthesis, we used CD-AP-1 transfection. CD-AP-1 effectively attenuates rat mesangial cell proliferation and expression of TGF-β induced by high glucose (Ahn et al, 2004). Moreover, a single administration of CD-AP-1 in streptozotocin-induced diabetic rats significantly decreased renal expression of ECM genes, including TGF-β1, PAI-1, type I collagen, fibronectin and smooth muscle α-actin, and, consequently, attenuated the increase in kidney weight (Ahn et al, 2004). Our data clearly demonstrated that AP-1 is a key transcription factor mediating mesangial cell proliferation and expression of ECM genes induced by diabetes. Therefore, this new molecular strategy using CD-AP-1 could represent a powerful investigative and potentially therapeutic strategy in the prevention and treatment of diabetic nephropathy.

V. Application of a CD-E2F decoy ODN based gene therapy strategy in cancer cell lines

Over the past decade, several molecular anti-cancer therapies, including antisense oligonucleotides, siRNA, and decoy ODNs, have been developed. Transcription factors are central to cancer related changes in the cell cycle; thus, the decoy strategy is of great interest in order to develop new drug targets to block the progression of malignancies. For example, an NF-κB decoy ODN has been tested in stomach and pancreatic cancer cell lines and melanoma cell lines (Franco et al, 2001; Spalding et al, 2002; Uetsuka et al, 2003; Zhang et al, 2003). Moreover, combinations of decoy ODNs and chemotherapeutic agents have been effective against drug resistant cancer cell lines, in which NF-κB activity was induced by chemotherapeutic agents (Uetsuka et al, 2003; Zhang et al, 2003). In addition to the NF-κB decoy ODN, decoy ODNs against the cyclic AMP responsive element, CRE, have been used in breast and ovarian cancer with promising...
results (Lee et al, 2000; Alper et al, 2001). However, there have been few studies using E2F decoy ODNs for human cancer therapy.

As discussed above, the transcription factor E2F is critical in regulating the initiation of DNA replication. It is also clear that the pathway which is probably the most frequently disrupted pathway in human cancers is composed of E2F proteins, their target genes, and the proteins that regulate E2F activity (e.g. Rb) (Nevins, 2001). To test the clinical potential of E2F decoy ODNs in cancer therapy, we introduced CD-E2F into the human osteosarcoma cell line, U2OS and the human cervical carcinoma cell line, C33A. CD-E2F effectively suppressed transactivation of gene expression by E2F and subsequently decreased cell cycle regulatory gene expression and cell proliferation in both cancer cell lines (Ahn et al, 2003). Moreover, in this study we found that the CD-E2F inhibited serum-induced E2F promoter activity in an Rb-negative human cervical carcinoma, C33A, as well as in an Rb-positive human osteosarcoma, U2OS (Ahn et al, 2003). These data suggest that CD-E2F is effective even in cancer cells deficient in Rb. The studies discussed herein, from our own lab as well as several others, make clear the possibility that a new molecular strategy, using the CD-E2F, could represent a potentially powerful therapy in the treatment of cancer.

V. Conclusion

Many previous studies have collectively demonstrated that CD decoy ODNs display markedly enhanced stability and effective sequence-specific decoy effects, compared to chemically modified decoy ODNs. Moreover, inhibition AP-1 and E2F mediated gene expression using CD decoy ODNs for these proteins significantly decreased the expression of genes that are transactivated by the factors and concomitantly decreased cell proliferation in VSMC, glomerular mesangial cells, and U2OS and C33A cancer cell lines, both in vitro and in vivo. Therefore, CD-AP-1 and CD-E2F based antiproliferative gene therapies could represent a new therapeutic strategy in the treatment of proliferative diseases including restenosis following angioplasty, diabetic nephropathy, and carcinogenesis.

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