Tumor Reversion: Protein Kinase A Isozyme Switching

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ABSTRACT: The regulatory subunit of cAMP-dependent protein kinase (PKA) exists in the isoforms RI and RII, which distinguish PKA isozymes type I (PKA-I) and type II (PKA-II). Evidence obtained from different experimental approaches—such as site-selective cAMP analogs, antisense oligonucleotides, transcription factor decoys, cDNA microarrays, and gene transfer—has shown that PKA-I and -II are expressed in a balance of cell growth and differentiation. Loss of this balance may underlie cancer genesis and progression. DNA microarrays demonstrate that antisense suppression of the RIα, which upregulates RIIβ, downregulates a wide range of genes involved in cell proliferation and transformation while upregulating cell differentiation and reverse transformation genes in PC3M prostate tumors that undergo regression. Conversely, the vector-mediated overexpression of RIIβ, as opposed to those of RIα and Cα, exhibits induction of differentiation genes along with suppression of cell proliferation and transformation genes leading to reversion of tumor phenotype. Thus, switching of PKA isozyme can cause tumor cells to undergo phenotypic reversion of the malignancy.

KEYWORDS: protein kinase A; antisense; gene transfer; gene therapy; site-selective cAMP analog; tumor reversion

INTRODUCTION

Permanent eradication of cancer in the cell can most surely be achieved by tumor reversion. A burgeoning number of reports in the literature describe reverse transformation or redifferentiation of many malignancies by various agents. Promises of tumor reversion were made in the mid-1960s when a cell line of normal mouse fibroblasts, NIH3T3, was established and was found to exhibit a sensitivity to contact inhibition, which is caused by a reversible arrest of growth in G1.1 Such sensitivity to contact inhibition is lost when NIH3T3 cells are transformed by polyoma virus/simian virus 40 (SV40). In 1968, Pollack, Green, and Todaro2 described for the first time discovery of sublines of polyoma virus/SV40-transformed NIH3T3 that had regained an increased sensitivity to contact inhibition, and importantly, decreased tumor-producing ability. These sublines were called “revertants.”
cAMP has long been considered to play a critical role in the regulation of cell growth and differentiation in a variety of cell types.\textsuperscript{3–5} The potential for clinical applications of cAMP was, however, appreciated only when 8-Cl-cAMP, a site-selective cAMP analog, was selected by the U.S. National Cancer Institute as a preclinical phase I antineoplastic drug (January 27, 1988); since then several phase I clinical studies of 8-Cl-cAMP have been completed.

8-Cl-cAMP exhibits potent growth inhibition \textit{in vitro} and \textit{in vivo} in a broad spectrum of human carcinoma, fibrosarcoma, and leukemia cell lines without causing cytotoxicity.\textsuperscript{6–8} The molecular mechanism for such potency in the growth inhibitory effect of 8-Cl-cAMP and other site-selective cAMP analogs takes advantage of the ability of these analogs to selectively modulate two isoforms of cAMP-dependent protein kinase (PKA-I and PKA-II), the positive and negative intracellular regulators,\textsuperscript{9} respectively, of cell growth at physiologic $\mu$M concentrations as opposed to the previously known analogs that require clinically irrelevant mM concentrations.\textsuperscript{6–8} However, the dual functions of cAMP, positive and negative regulation of cell growth, and the assignment of the PKA-I$\alpha$ and PKA-II$\beta$, respectively, for these opposite functions of cAMP on cell growth, have been the subjects of debate among investigators in the field for more than 20 years.\textsuperscript{9–12} Only during the past decade has experimental evidence revealed distinct functions for PKA-I and -II, providing molecular proof that intracellular balanced expression between the two isoforms of PKA may play a critical role in controlling cell growth and differentiation.\textsuperscript{13–15} It is shown that PKA-I is only transiently overexpressed in normal cells in response to physiologic stimuli of cell proliferation. In contrast, it is constitutively overexpressed in cancer cells and is associated with poor prognosis in human cancers of different cell types. Conversely, PKA-II is preferentially expressed in normal differentiated tissues.

Here, we describe how modulation of the regulatory isoforms (RI versus RII) of PKA influence the ability of PKA to regulate cancer cell growth and to induce tumor reversion. Such approaches not only provide the molecular tools for critically assessing cAMP/PKA signaling in cancer genesis and progression, but they also contribute to the discovery of target-based cancer treatment drugs.

**PKA ISOZYME DISTRIBUTION IN CANCER**

The changing ratios of PKA-I and -II have been correlated with ontogenic development and differentiation processes.\textsuperscript{9,16} Evidence suggests an interesting correlation regarding the differential expression of PKA-I and -II subunits and their mRNAs in clinical human tumors and transformed cell lines. Increased expression of RI/PKA-I over that of RII/PKA-II has been shown in several human cancer tissues and cell lines, including retinoblastoma, Wilm’s tumor, renal, breast, and colon carcinomas, transformed BT5C glioma cell line, malignant osteoblasts, in serous ovarian tumors vs. mucinous, and endometrioid or clear cell lesions.\textsuperscript{9,17} Increased RI/PKA-I expression was also shown to be associated with chemical and viral carcinogenesis and oncogene-induced cell transformation.\textsuperscript{9} Furthermore, overexpression of RI$\alpha$, but not the catalytic (C) subunit, in immortalized MCF-10A cells, conferred the ability to grow in serum and growth factor-free conditions,\textsuperscript{18} and RI$\alpha$ but not C$\alpha$ overexpression in CHO cells, provided the growth advantages in monolayer and soft agar conditions.\textsuperscript{19}
These reports suggest that expression of RI isoform of PKA is involved in neoplastic transformation and progression, and therefore suppression of RIα/PKA-Iα and/or induction of RIIβ/PKA-IIβ may restore growth control in these malignancies.

THE FIRST CLINICAL DRUG FOR cAMP

8-Cl-cAMP

Site-selective cAMP analogs, but not parental cAMP, demonstrate selective binding toward either one of two cAMP binding sites, Site A (Site 2) and Site B (Site 1) in the R subunit, resulting in preferential binding and activation of either PKA isozyme. The use of site-selective cAMP analogs that demonstrate high affinity and selectivity toward the protein kinase isozyme make it possible to correlate the specific effect of PKA isozymes with cAMP-mediated responses in intact cells. With respect to growth control, site-selective cAMP analogs have been shown to induce growth inhibition and differentiation in a broad spectrum of human cancer cell lines, including carcinomas, sarcomas, and leukemias, without causing cytotoxicity. Of these, 8-Cl-cAMP, the most potent site-selective cAMP analog, has completed several phase I clinical studies.

8-Cl-cAMP: A PKA-I Downregulator

8-Cl-cAMP, which belongs to the isozyme site discriminator class, activates and downregulates PKA-I owing to its equally high-affinity binding to the A and B sites of RI. On the other hand, this analog binds with high affinity only to the B site of RII, exhibiting a weaker activation for PKA-II than for PKA-I. In HL-60 promyelocytic leukemia cells, 8-Cl-cAMP downregulates PKA-I by promoting truncation of the 48 kDa RIα subunit to a 34 kDa form. The 34 kDa RIα exists in the PKA-I holoenzyme, suggesting that this molecule is truncated at the C terminus. This mode of RIα truncation may facilitate rebinding of 8-Cl-cAMP to the reconstituted holoenzyme, enhancing PKA-I downregulation without allowing the free RIα subunit to accumulate. Truncation of the 48 kDa RIα to the 34 kDa form is a mechanism of action unique to 8-Cl-cAMP; the 34 kDa protein is not induced in PKA-I downregulation by other means, such as treatment with RIα antisense or RIIβ overexpression. Most likely, 8-Cl-cAMP treatment activates a protease that breaks down a 48 kDa RIα into a 34 kDa molecule.

8-Cl-cAMP–Induced Tumor Reversion

In preclinical studies, 8-Cl-cAMP suppresses the expression of c-myc and c-ras, and induces apoptotic cell death in human cancer cells. Results of a phase I clinical trial suggest that effective plasma levels (determined in preclinical studies) of 8-Cl-cAMP can be maintained below the maximum tolerated dose. The mechanism of anti-tumor activity of 8-Cl-cAMP was studied using cells that either overexpressing the Bcl-2 gene or cells treated with ZVAD (a broad-range caspase inhibitor) that specifically blocks apoptotic cell death without affecting cell proliferation. Up to 5 days of 8-Cl-cAMP treatment, Bcl-2 was transiently downregulated and Bad expression continuously in-
creased. Overexpression of Bcl-2 blocked 8-Cl-cAMP-induced apoptosis but had no effect on the accompanying 8-Cl-cAMP-induced inhibition of cell proliferation. In addition, suppression of apoptosis by ZVAD did not abrogate 8-Cl-cAMP-induced inhibition of cell proliferation.

These results indicate that 8-Cl-cAMP inhibits cancer cell growth through anti-proliferation and pro-apoptotic mechanisms. Most likely, 8-Cl-cAMP, via a selective activation of PKA-I, promotes the phosphorylation of Bcl-2 but not Bad (Bad phosphorylation in mitochondria was found to be via PKA-II activation), leading to Bcl-2 inactivation and apoptosis. Use of cDNA microarrays will further refine the mechanism of action of 8-Cl-cAMP in tumor growth inhibition.

ANTISENSE OLIGONUCLEOTIDES

Antisense Protein Kinase A RIα

The possibility that the RI cAMP receptor is a positive regulator of cancer cell growth has been explored using the antisense strategy. A synthetic RIα antisense oligodeoxynucleotide (ODN) corresponding to the N-terminal seven codons of human RIα inhibits growth in breast (MCF-7), colon (LS-174T), and gastric (TMK-1) carcinoma cells, neuroblastoma (SK-N-SH) cells, and HL-60 leukemia cells with no sign of cytotoxicity. The antisense RIα induced changes in cell morphology to one typical of the flat reverted phenotype in SK-N-SH neuroblastoma and HL-60 leukemia cells. Furthermore, treatment with an RIα antisense phosphorothioate ODN (PS-ODN) brings about a marked reduction in RIα levels with a concomitant increase in RIIβ levels. Strikingly, a single injection of RIα antisense PS-ODN targeted against codons 8–13 of human RIα reduces RIα expression and produces sustained growth inhibition in LS-174T colon carcinoma in nude mice at up to 14 days of examination. Tumor cells behave like untransformed cells by making less PKA-I.

The Second Generation RNA-DNA Mixed Backbone Antisense RIα

To address the issue of nonspecific toxicity and side effects associated with antisense PS-ODNs, the polyanionic nature of the antisense RIα PS-ODN has been minimized, and the immunostimulatory GCGT motif has been blocked in a second-generation RNA-DNA mixed-backbone (MBO) RIα antisense ODN. This ODN improved antisense activity over the PS-ODN, was more resistant to nucleases, formed more stable duplexes with RNA than the parental PS-ODN, and retained the capability to induce RNAse H. Thus, in addition to reducing nonspecific effects, the RNA-DNA RIα antisense ODN facilitated the exploration of sequence-specific antisense effects. This modulation ultimately inhibits growth and induces apoptosis in various cancer cell lines and in tumors in nude mice.

Antisense RIα: Target Specificity and Clinical Utility

The target specificity of RNA-DNA MBO RIα antisense has been thoroughly addressed. Pulse-chase experiments have revealed that RIα has a relatively short half-life: 17 hours in control cells and 13 hours in antisense-treated cells (i.e., LS-174 co-
The short half-life of RII\(\alpha\), along with its message downregulation, is consistent with the rapid RII\(\alpha\) downregulation observed in antisense-treated tumors.\(^3\) In addition, levels of RII\(\beta\) protein increased because of an increase in half-life of 2 h to 11 h (5.5 fold),\(^4\) leading to a decrease in the PKA-I-to-PKA-II ratio in tumor cells. The half-lives of RII\(\alpha\) and C\(\alpha\) were unchanged in antisense-treated cells.\(^4\) The RII\(\alpha\) antisense-induced stabilization of the RII\(\beta\) protein was consistent with results in RII\(\beta\) and RII\(\beta\) knockout mice, in which compensatory stabilization-induced elevation of the RII\(\alpha\) protein appeared in tissues that normally expressed \(\beta\) isoforms of the R subunit.\(^5\) These results showed a clear correlation between RII\(\alpha\) antisense-induced growth inhibition and the target-specific antisense effect—namely, RII\(\alpha\) downregulation.

The RNA-DNA MBO second-generation antisense RII\(\alpha\) has demonstrated increased biologic activity, minimal polyanionic or immunostimulatory side effects, improved \textit{in vivo} stability and oral efficacy.\(^4\) The MBO antisense RII\(\alpha\) (GEM-231, Hybroadon, Inc.) has completed phase I clinical studies\(^4\) and currently is under phase II study.

**MOLECULAR PORTRAIT OF A TUMOR REVERSION**

**Antisense Approach**

Using cDNA microarrays, the sequence-specific antisense effects were examined on global gene expression in PC3M prostate and LS-174T colon carcinoma cells exogenously treated with RII\(\alpha\) antisense ODNs or these cells endogenously overexpressed with the antisense RII\(\alpha\) gene. Expression is altered for approximately 10% of the total cDNA elements (2304) on the array, and these changes in gene expression are comparable in prostate and colon cancer cells, which have vastly different gene expression profiles. Strikingly, the gene-expression profile, altered by the antisense ODNs, exactly mirrored the profiles elicited by endogenous antisense gene expression.\(^4\) Affected genes include genes for transcription factors, protein kinases and phosphatases, cell-cycle regulators, proteins involved in DNA synthesis and regulation, G-proteins, and cytoskeleton regulatory proteins.

Clustering analysis demonstrated that the antisense RII\(\alpha\) downregulates one cluster of coordinately expressed genes, or signature, involved in cell proliferation while upregulating the other involved in cell differentiation, i.e., reverse transformation.\(^4\) Similar proliferation and differentiation signatures are found in antisense-treated PC3M tumors, but an expression profile distinct from that seen in antisense-treated cells is also apparent.\(^4\) Genes in the transformation signature, such as oncogenes and genes for tyrosine and serine/threonine kinases that are usually overexpressed in tumors, were specifically downregulated following antisense treatment.\(^4\) These expression signatures modulated by the antisense RII\(\alpha\)—namely, downregulation of proliferation signature and upregulation of the differentiation signature—reflect the profile of the nonmalignant or reverted phenotype.\(^4\)

Importantly, these signatures are quiescent and unaltered in the host livers of antisense-treated animals. This observation clearly indicates that separate and distinct cAMP signaling pathways regulate growth for normal cells versus cancer cells. Thus, RII\(\alpha\) antisense induces the molecular redifferentiation signatures in cancer cells in a sequence-specific manner, leading to induction of a new reverted phenotype.\(^4\)
This microarray study was the first demonstration in the field of antisense research that an antisense, in a sequence-specific manner, can modulate a wide set of genes beyond its targeted gene.\textsuperscript{46} Antisense-directed depletion of R1\(\alpha\) thus modulates the signal transduction signatures of multiple pathways beyond that of cAMP signaling—leading to induction of tumor reversion.

\textbf{Gene-Overexpression Approach}

Experimental evidence shows that the RII\(\beta\) cAMP receptor is essential for cAMP-induced growth inhibition and differentiation in various cancer cell lines. An RII\(\beta\) antisense ODN blocks cAMP-induced, but not phorbolester-induced, growth inhibition and differentiation; cells become refractory to the cAMP stimulus and continue to grow without differentiation in the presence or absence of cAMP analog.\textsuperscript{48}

The relationship between RII\(\beta\) expression and malignancy has been tested using vector-mediated RII\(\beta\) overexpression. The RII\(\beta\) overexpressing cells, including SK-N-SH neuroblastoma, MCF-7 breast and LS-174T colon carcinoma, and Ki-ras–transformed NIH/3T3 clone DT and PC12 mutant-A-126-1B2 cells, exhibit growth inhibition with no sign of cytotoxicity.\textsuperscript{49–52} The growth inhibition correlated with the expression of RII\(\beta\) and accompanied changes in cell morphology. SK-N-SH neuroblastoma, DT and A-126 cells, after infection with MT-RII\(\beta\) retroviral vector, demonstrated striking changes in morphology: cells became flat, exhibiting enlarged cytoplasm and an increased cytoplasm-to-nucleus ratio.\textsuperscript{50,52} Importantly, this changed morphology was similar to that induced by exposure of these cells to R1\(\alpha\) antisense, which induces RII\(\beta\) upon blocking R1\(\alpha\) expression.\textsuperscript{52}

PC3M prostate carcinoma cells were used as a model to overexpress wild-type and mutant R and C subunit genes of PKA, and the effects of differential expression of these genes on PKA isozyme formation, cell morphology, cell proliferation, genome-wide expression profile, and tumor phenotype were examined.\textsuperscript{53} The mutant genes used in this study included: the R1\(\alpha\) mutant, R1\(\alpha\)-P, which gains an autophosphorylation site (Ala\(\rightarrow\)Ser) by point mutation of G\(\rightarrow\)T at R and C interaction site\textsuperscript{54,55} to functionally mimic RII; the RII\(\beta\) mutant, RII\(\beta\)-P, which loses an autophosphorylation site (Ser\(\rightarrow\)Ala) by point mutation of T\(\rightarrow\)G;\textsuperscript{49,51} and the N-terminal myristate-lacking mutant, C\(\alpha\)-m, which has been shown to be as fully active in the cell as wild-type C\(\alpha\), but lacks the ability to secrete into extracellular space.\textsuperscript{56}

The RII\(\alpha\)-, RII\(\beta\)-, and RII\(\beta\)-P–overexpressing cells increased PKA-II and almost completely abolished PKA-I, whereas R1\(\alpha\)-, C\(\alpha\)-, and C\(\alpha\)-m–overexpressing cells increased PKA-I but could not suppress PKA-II. Most strikingly, cells overexpressing R1\(\alpha\)-P, the functional mimic of RII, increased PKA-I and markedly downregulated PKA-II. It has been shown that PKA-II is the favored form of PKA holoenzyme rather than PKA-I.\textsuperscript{57} Thus, a single point mutation of R1\(\alpha\) at the R and C interaction site, i.e., the pseudophosphorylation site, brought about mutant PKA-I (R1\(\alpha\)-P–containing PKA-I) functionally mimicking PKA-II, and consequently, its overexpression suppresses the endogenous PKA-II.\textsuperscript{53}

Importantly, RII\(\beta\) and R1\(\alpha\)-P transfectants exhibited changes in cell morphology—although the changed morphologies between these cells were distinctive—growth inhibition \textit{in vitro}, and \textit{in vivo} tumor growth inhibition.\textsuperscript{53} Indeed, cDNA microassays revealed the molecular portrait of a reverted phenotype in RII\(\beta\)- and R1\(\alpha\)-P–
overexpressing cells.\textsuperscript{53} The differentiation signature, a cluster of genes associated with cell differentiation, was upregulated, while transformation and proliferation signatures were downregulated. In contrast, C\(\alpha\)- and RI\(\alpha\)-overexpressing cells upregulated transformation and proliferation signatures and were incapable of upregulating the differentiation signature.\textsuperscript{53} This positive regulatory action of C\(\alpha\) toward cell proliferation and transformation is in accordance with the findings that the CRE-transcription factor-decoy, which blocks the CRE- and AP-1–directed transcription, inhibits tumor cell growth \textit{in vitro} and \textit{in vivo}.\textsuperscript{58} and dominant-negative CREB, KCREB, inhibits tumor growth and metastasis of human melanoma cells.\textsuperscript{59} The opposed effects between RII\(\beta\) and C\(\alpha\) cells on a genome-wide expression profile was further supported by confocal microscopy.\textsuperscript{53} It was shown that in RII\(\beta\)-overexpressing cells, C\(\alpha\) and RII\(\beta\), appear entirely colocalized in the cytoplasm and nucleus. Thus, RII\(\beta\) overexpression sequesters C\(\alpha\) in the holoenzyme with RII\(\beta\), blocking C\(\alpha\) activation and resulting in altered cAMP signaling in these cells. These results suggest that PKA isoyme switching via eliciting differential cAMP signaling gives rise to tumor reversion.

CONCLUSIONS AND PERSPECTIVES

In these different experimental approaches, namely the use of 8-Cl-cAMP, a site-selective analog of cAMP, antisense oligonucleotides, and gene overexpression, the reversion of tumor phenotype was successfully achieved in a wide variety of tumor cell lines including breast, prostate, colon, lung, gastric, and ovarian carcinomas, neuroblastoma, gliomas and leukemias, sarcomas, k- \textit{ras}–transformed NIH 3T3 clone DT cells, and mutant PC12, A-126 cells. The underlying mechanism was the switching of the PKA-I holoenzyme to a PKA-II isozyme.

Because of the persistent increase found in PKA-I over PKA-II in the clinical primary tumors and tumor cell lines of various cell types as described in this review, and the secreted PKA-free catalytic subunit (ECPKA) found in a cancer patient’s serum, which has been correlated with the increase in the intracellular PKA-I in cancer cells,\textsuperscript{56} PKA-I has been recognized as the molecular target for the restoration of normal physiology in cancer cells.

The data reported to date suggest that PKA-I acts as a positive growth regulator, whereas PKA-II acts to inhibit cell proliferation and to induce cell differentiation. Some exceptions have been described, however, especially with Carney complex, the spotty skin pigmentation that can accompany multiple endocrine neoplasia and which is attributed to the mutational loss of the RI\(\alpha\) regulatory subunit of PKA-I\textsuperscript{60}—though no direct causal relationship of the loss of RI\(\alpha\) with the production of these endocrine neoplasia has been established.

Thus, modulation of PKA isoymes can lead to regulation of tumor growth, restoring the balance between cell proliferation and apoptosis/differentiation. Because the PKA-I- to -PKA-II ratio is reversed in many cancer cells as compared to their normal counterparts, PKA isoyme switching in cancer cells could provide tumor-targeted therapy for cancer treatment, eventually restoring a normal phenotype, namely, tumor reversion.

PKA isoyme switching could be achieved in many ways, including via 8-Cl-cAMP and other site-selective cAMP analogs, viral and non-viral vector-mediated
gene transfer/gene therapy, antisense DNAs, interfering RNAs, and targeted gene repair/replacement chimeraplasty methodology.

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