Perspectives in Cancer Research

Role of Cyclic AMP Receptor Proteins in Growth, Differentiation, and Suppression of Malignancy: New Approaches to Therapy

Yoon S. Cho-Chung

Cellular Biochemistry Section, Laboratory of Tumor Immunology and Biology, National Cancer Institute, NIH, Bethesda, Maryland 20892

Abstract

Two isoforms of the regulatory subunits of cyclic AMP (cAMP)-dependent protein kinase that bind cAMP are inversely expressed during ontogeny and cell differentiation. These cAMP-binding receptor proteins in harmony may regulate the growth of normal cells and their differentiation into nondividing states. Cancer cells can also be made to differentiate and stop growing when the functional balance of these cAMP receptor proteins is restored by treatment with site-selective cAMP analogues or by the use of an antisense oligodeoxynucleotide, suggesting new approaches to cancer treatment.

Introduction

Cancer cells can be unlocked from their biological blockage and be forced to differentiate and cease dividing; once this happens, the cells should eventually die. This idea is not new (1–5) and is now shared widely among the investigators in cancer research. There exists great potential for clinical applications which are targeted toward noncycotoxic induction of differentiation. There is a need for nontoxic biological agents that modulate intracellular regulatory molecules and thereby restore normal control mechanisms in malignant cells.

I will discuss the role of cAMP receptor proteins in ontogeny and differentiation of normal cells and our experimental approaches which made the control and reversal of malignancy possible by the use of nontoxic biological agents. One such biological agent, 8-Cl-cAMP, is now in preclinical phase I studies at the National Cancer Institute.

Two Types of cAMP Receptor Proteins

cAMP, discovered by Sutherland in 1957 as a mediator of hormonal signals, is present in all cells and tissues, from bacteria to humans (6). The primary mediator of cAMP action in eukaryotic cells is cAMP-dependent protein kinase (7, 8). Unlike other protein kinases, cAMP-dependent protein kinase is uniquely composed of two genetically distinct catalytic (C) and regulatory (R) subunits. The activating ligand cAMP, which binds to the R subunit, induces conformational changes and dissociates holoenzyme R2C2 into an R2-(cAMP)4 dimer and two free C subunits that are catalytically active (9, 10). There are two different classes of cAMP-dependent protein kinase, designated as type I and type II, which contain distinct R subunits, RI and RII, respectively, but share a common C subunit (10). Four different regulatory subunits [RI, (11), RII, (12), RII, (RIIα), (13), and RII, (RIIβ)] (14) have been identified at the gene/mRNA level. Two distinct C subunits [C, (15) and C (16, 17)] have also been identified; however, preferential coexpression of either one of these C subunits with either the type I or type II protein kinase R subunit has not been found (17).

The two general classes of R subunits, RI and RII, contain two tandem cAMP-binding domains at the carboxyl terminus the amino acid sequences of which are highly conserved, while the primary structure of the remaining one-third of the molecule at the amino terminus is quite variable among the R subunits (18). RI and RII differ significantly in the amino termini at a proteolytically sensitive hinge region that occupies the peptide substrate-binding site of the C subunit in the holoenzyme complex. In this segment, RII contains the autophosphorylation site (Thr-Arg-Arg-Val-Ser-Val-Cys), whereas RI has a high-affinity binding site for ATP at the pseudophosphorylation site (Arg-Gly-Ala-Ile-Ser). Thus, the type II holoenzyme undergoes autophosphorylation (phosphorylation of the R subunit by the C subunit) while the type I isoenzyme does not, and the type I holoenzyme binds ATP with high affinity while the type II enzyme has a low affinity for ATP (18).

A heat-stable protein inhibitor, protein kinase inhibitor (19), like RI, contains a pseudophosphorylation site, and the protein kinase inhibitor·C subunit complex has a high-affinity binding site for ATP (20). This mechanism for inhibition is used by other protein kinases, e.g., myosin light chain kinase (21) and protein kinase C (22). Therefore, it appears from an evolutionary point of view that type I protein kinase is similar to other protein kinases rather than to type II protein kinase. On the other hand, the RII shares extensive homology (23) with the cAMP-binding domain of bacterial CAP (24). The regulation of gene expression by cAMP in bacteria is achieved by modulating the DNA-binding potential of a single protein, CAP (24). The homologies between CAP and the RII subunit indicate that a large part of this structure has, with some modifications, been conserved during evolution. Thus, RI subunits may have retained the bifunctionality of CAP (i.e., both cAMP and DNA binding) (25). The cAMP-inducible eukaryotic genes have been shown to contain CRE with a consensus palindromic sequence (26) and cAMP-dependent and sequence-selective binding of RII to double helical DNA (CRE) has been shown (27). Accordingly, RII, either in its subunit form or in its holoenzyme complex (type II protein kinase), may regulate gene transcription in eukaryotes as does bacterial CAP (25, 28).

Two isoforms of cAMP-dependent protein kinase in mammalian cells may thus serve distinct physiological functions.

Differential Expression of cAMP Receptor Isoforms in Ontogeny and Differentiation

The relative content of protein kinase type I and type II varies among tissues in adult animals and also in the same tissue among the different species of animals (29–31), although the total R subunit/C subunit molar ratio in all normal tissues examined was found to be 1:1 (31). The ratios of these isozymes in tissues also depend on physiological conditions and hormonal status (for review see Ref. 32). These findings suggest
that protein kinase isozymes may have distinct roles in different physiological processes. In fact, the changes in the amounts or activity of cAMP-dependent protein kinase isozymes have been correlated with highly regulated processes such as ontogeny and cell differentiation. Examples of such studies are described here.

In rat cerebrum the postnatal development of cAMP-dependent protein kinase was studied by measuring the amounts of RI and RII and C subunit activity (33). Neither RI nor RII nor C subunit was found to show any significant change in amount during postnatal development of rat cerebrum, indicating the possibility that the relative distribution of protein kinases may be established sooner, prenatally.

Two studies (34, 35) of the development of cAMP-dependent protein kinase in mouse heart reached similar conclusions. The type I/type II isozyme activity ratio (or RI/RII ratio) decreased from 3.0 in neonates (7- or 14-day-old) to 1.0 in adult hearts. It was noted (35) that in rodents after the third postnatal week no more cardiac cell division occurs such that only cellular hypertrophy is responsible for further cardiac growth (36). This implies that high levels of type I kinase (or RI) are associated with the developmental phase of cell growth.

Both total and cAMP-dependent protein kinase activity of rat testes increased sevenfold between birth and 90 days of age (37). The increase in cAMP-dependent protein kinase was due to type II enzyme because at birth it was mostly absent and type I enzyme was already present at adult levels. The development of type II kinase in testes coincided with acquisition of the capacity for spermatogenesis and steroidogenesis.

One well-studied example of normal cellular differentiation is that of ovarian follicles shown to contain a follicle-stimulating hormone-responsive adenylate cyclase which seems to be involved in mediation of the conversion of immature follicles to ones able to ovulate, luteinize, and produce steroids (38). A study of the differentiation of ovarian follicle granulosa cells from immature hypophysectomized rats treated with injections of estradiol and follicle-stimulating hormone showed a 10- to 20-fold increase in the RII content of granulosa cells, without a corresponding increase in the catalytic activity of cAMP-dependent protein kinase (39). On the other hand, an increase in RI or type I protein kinase with a decrease in RII or type II kinase was shown in tissues for which steroids have a trophic role. Three days postcastration, steroid-dependent tissues, e.g., ventral prostate and levator ani muscle, showed a 50% decrease in type I protein kinase activity and little change in the type II enzyme (40). An inverse relationship between estrogen receptor and RII cAMP receptor has been shown (41) in the growth and regression of hormone-dependent mammary tumors for which estrogen has a trophic role. Thus, the growing tumors contained high ratios of estrogen receptor/RII, whereas in regressing tumors following ovariotomy or dibutyryl cAMP treatment, such estrogen receptor/RII ratios became inverted. These studies suggest an association among type I protein kinase levels, steroidogenesis, and growth, and an association between increased type II protein kinase and tissue differentiation.

Other researchers claimed (42) the RI subunit to be a better marker of differentiation because it increased steadily from fetal to adult ages and was higher in well-differentiated hepatoma cells (Morris 9618A) than in those that were poorly differentiated (Yoshida AH130). However, the RII subunit also increased with the degree of terminal differentiation and the RI/RII ratio was lower in the more differentialed cells. Differentiation of neuroblastoma × glioma hybrid cells (43) and neuroblastoma cells (44) after treatment with N2O3-dibutyryl-
cAMP has also been correlated with the increase of RI free subunit; there was no change in the amount of free C subunit or type II protein kinase holoenzyme. It may be that an elevated level of RI is prerequisite to RII development, in which case an increase of both RI and RII would be required during development or differentiation of organs in which RI is at or below its threshold level.

Differential expression of type I and type II protein kinase is also cell cycle specific. In Chinese hamster ovary cells, type I kinase activity is high in mitosis and relatively low during G1 and S phases, while type II activity is low during mitosis and increases at the G1-S phase border, again decreasing by mid- to late S phase (45). Also, it was found that there is a selective activation of type I isozyme during stimulation of mitogenesis in human lymphocytes (46). These data have implicated type I protein kinase as a positive effector of growth. On the other hand, in instances in which cAMP stimulates growth, usually in G1 of nonmalignant cells, the increase of type II protein kinase during late G1 phase was claimed to trigger DNA synthesis (47).

Several examples of changes in cAMP-dependent protein kinase associated with differentiation induced in malignant cells by means other than cAMP stimulus have been described. Friend erythroleukemia cells, virus-transformed murine erythroleukemia cells, can be stimulated by a wide variety of agents to express several characteristics of erythroid cell differentiation. Induction of differentiation by dimethyl sulfoxide was shown to result in a 3-fold increase in RII, a 3-fold decrease in RI, and an RI/RII ratio of 11, compared to 1.2 in control, in treated cells (48). Murine F-9 embryonal carcinoma cells, the pluripotent stem cells of malignant teratocarcinoma, responded to retinoic acid followed by cAMP (but not the reverse order) by differentiating and becoming parietal endoderm cells (49). Because cAMP alone cannot produce this effect, it was reasoned that the retinoic acid induced changes which altered the response to cAMP (50). The treatment of F-9 cells with retinoic acid resulted in an increase in RI, RII, and histone kinase activity in cytosol and a preferential increase in the amount of RII associated with plasma membrane. Similarly, retinoic acid inhibited growth of mouse B16-F melanoma cells and increased their total cAMP-dependent protein kinase activity, whereas it produced neither effect in MR-4 cells, a protein kinase-deficient variant of F1, (51). A selective activation of type II isozyme was also correlated with the growth arrest induced by calcitonin in a human breast cancer cell line (52). These data indicate that cAMP receptors may act as intracellular effector molecules of a common pathway for those different agents that induce differentiation in malignant cells.

These studies illustrate the distinct roles of cAMP receptor isofoms in the regulation of ontogeny and cell differentiation and support a hypothesis that protein kinase type II is primarily involved in differentiation processes, whereas protein kinase type I relates to cell proliferation (53, 54) (Table 1).

Disruption in the Normal Patterns of cAMP Receptor Proteins in Malignancy

Since the changing ratio of type I versus type II protein kinase appears to be involved in the ontogenic and differentiation processes as described above, it is logical to ask if there are in fact any changes or abnormalities in the protein kinase of neoplastic cells.

RI is the major or sole R subunit of protein kinase detected in a variety of types of human cancer cell lines (55, 56). These
differentiated states of the chronic lymphocytic leukemia are with chronic lymphocytic leukemia showed that the poorly blood lymphocytes with those in lymphocytes from patients.

A comparison of RI and RII patterns in normal peripheral amounts of RII with decreased levels of RI (64).

distant and adjacent mucosa contained detectable cAMP-binding R subunits, with no detectable levels of RII, whereas both high- and low-affinity cAMP-binding sites, whereas RII in tumors exhibited only the low affinity site, and these changes in the cAMP-binding property correlated in degree with tumor size and extent of anaplasia. In contrast, a decreased expression of type I protein kinase and RI subunit was found in tumor cell lines of lung epithelial origin as compared to the immortalized nontumorigenic cell lines (73).

Changes in cAMP-dependent protein kinase isozyme patterns have been commonly observed in in vitro cell transformation. SV40 viral transformation of BALB 3T3 fibroblasts was accompanied by an increase in type I protein kinase activity and RI subunit; normal BALB 3T3 cells contain only type II kinase isozyme (74). Transformation of rat 3Y1 cells by the highly oncogenic human adenovirus type 12 correlated with 3- to 6-fold increase of type I kinase activity and RI subunit (75). A marked increase in RI with a decrease in RI subunit was detected in Harvey murine sarcoma virus-transformed NIH/3T3 clone 13-3B-4 cells (76), and in NRK cells transformed with TGFα or v-Ki-ras oncogene (77). TGFα-induced transformation of mouse mammary epithelial cells brought about a marked increase in the RI mRNA level along with a decrease in RIIα and RIIβ mRNA levels (78).

As these studies illustrate, there is abnormal expression of type I and type II protein kinases and their regulatory subunits associated with malignant transformation (Tables 1 and 2).

Restoration of Normal cAMP Receptor Patterns in the Suppression of Malignancy by Site-selective cAMP Analogues

cAMP binds to its receptor proteins, the regulatory subunits of protein kinase, at two different binding sites, termed Site A.

<table>
<thead>
<tr>
<th>Tissue, cell line</th>
<th>Development, differentiation, dedifferentiation</th>
<th>cAMP receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat cerebrum</td>
<td>Synapses</td>
<td>←*</td>
</tr>
<tr>
<td>0–4 wk</td>
<td></td>
<td>←</td>
</tr>
<tr>
<td>Postnatal</td>
<td></td>
<td>←</td>
</tr>
<tr>
<td>Mouse heart</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td>7 days → adult</td>
<td>End cellular proliferation</td>
<td>↑</td>
</tr>
<tr>
<td>14 days → adult</td>
<td>Onset cellular hypertrophy</td>
<td>↓</td>
</tr>
<tr>
<td>Rat testes</td>
<td></td>
<td>←*</td>
</tr>
<tr>
<td>0–90 days</td>
<td>Spermatogenesis</td>
<td>↑</td>
</tr>
<tr>
<td>Postnatal</td>
<td>Steroidogenesis</td>
<td>↓</td>
</tr>
<tr>
<td>Rat ovarian granulosa cells</td>
<td>Differentiation</td>
<td>←*</td>
</tr>
<tr>
<td>Neuroblastoma cells</td>
<td>Differentiation</td>
<td>↑</td>
</tr>
<tr>
<td>Friend erythroblasts</td>
<td>Differentiation to hemoglobin-containing cells</td>
<td>↑</td>
</tr>
<tr>
<td>Rat mammary gland</td>
<td>N-Methyl-N'-'nitro-N-nitrosoguanidine carcinogenesis</td>
<td>↑</td>
</tr>
<tr>
<td>Rat stomach</td>
<td></td>
<td>↑</td>
</tr>
<tr>
<td>BALB 3T3 fibroblasts</td>
<td>SV40 viral transformation</td>
<td>↓</td>
</tr>
<tr>
<td>NIH/3T3 fibroblasts</td>
<td>Harvey murine sarcoma virus transformation</td>
<td>↑</td>
</tr>
<tr>
<td>Normal rat kidney fibroblasts</td>
<td>v-Ki-ras or TGFα transformation</td>
<td>↓</td>
</tr>
</tbody>
</table>

* ↑, increase; ↓, decrease; ←, no change.
The effect of the analogue on growth of various cell types, including breast, colon, and lung carcinoma, as well as on growth in athymic mice of human cancer xenografts, cell lines, and nude mouse tumors, it is essential that these kinases can be selectively modulated to identify any specific function of these protein kinase isozymes, with cAMP-mediated regulation (Site B selective) are combined with C-6 analogues (Site A selective). Thus, C-8 amino analogues are optimally synergistic for Site A, whereas analogues modified at C-2 or C-8 (C-2 and C-8 analogues, respectively) are selective for Site B. Importantly, Site B-sensitive analogues further exhibit specificity for either type I or type II protein kinase (81, 82); CAMP analogues with nitrogen attached at C-8 of the adenine ring exhibit higher affinity toward type I kinase, and C-8 analogues with sulfur or halogen attached possess higher affinity for type II enzyme. Moreover, such selectivity toward type I and type II kinase is synergistically enhanced when these C-8 analogues (Site B selective) are combined with C-6 analogues (Site A selective). Thus, C-8 amino analogues are optimally synergistic with C-6 analogues for type I kinase, whereas a sulfur- or halogen-attached C-8 analogue is most effective toward type II kinase when combined with the C-6 analogue (81, 82).

CAMP-dependent protein kinase is usually present in tissues as a mixture of type I and type II isozymes (29–31). In order to identify any specific function of these protein kinase isozymes, it is essential that these kinases can be selectively modulated in intact cells. With the use of site-selective cAMP analogues it became possible to correlate the specific effect of cellular protein kinase isozymes with cAMP-mediated responses in intact cells (83).

It was found that site-selective cAMP analogues demonstrate a major regulatory effect on growth in a broad spectrum of human cancer cell lines, including breast, colon, lung, and gastric carcinomas, fibrosarcomas, gliomas, and leukemias, as well as on growth in athymic mice of human cancer xenografts of various cell types, including breast, colon, and lung carcinomas (55, 56, 84, 85). The effect of the analogue on growth inhibition appeared to be selective toward transformed cancer cells as opposed to nontransformed cells. The analogues produced little or no growth inhibition of NIH/3T3 cells, normal rat kidney fibroblasts, normal mammary epithelial cells, and normal peripheral blood lymphocytes (84).

The growth-inhibitory effect of the site-selective cAMP analogues was not due to the cytotoxic effect of its adenosine metabolites as experimentally documented using an adenosine analogue (86). Thus, the growth inhibitory effect of site-selective cAMP analogues is different from that in previous reports that have shown a strong cytotoxicity using some of the amino-substituted C-8 analogues (87) and cyclic nucleotides of purine analogues (88); the cytotoxicity was mainly due to the adenosine metabolites of their nucleotides.

The growth inhibition induced by the analogues brought about biochemical (oncogene and transforming growth factor α suppression) and morphological changes, differentiation, and reverse transformation (56, 84). Despite the appearance of markers of mature phenotype and definitive growth arrest as shown in the analogue-treated leukemic cells, the cell cycle phase between the treated and untreated cells was unmodified; namely, the treated cells were not G2-M arrested (89). Thus, it appears that site-selective cAMP analogues produce growth inhibition while allowing the cells to progress through their normal cell cycle, albeit at a slower rate, and this may lead to eventual restoration of a balance between cell proliferation and differentiation in cancer cells.

The site-selective cAMP analogues were able to inhibit the growth of cultured cancer cell lines that are resistant to the cAMP analogues previously studied, such as dibutyryl-cAMP. This important property of site-selective analogues was reproduced by the effect of 8-Cl-cAMP on in vivo growing tumors. The growth of transplantable hormone-independent DMBA-1 and metastatic NMu-2 rat mammary carcinomas that are completely resistant to dibutyryl-cAMP was markedly inhibited by 8-Cl-cAMP treatment, and in the treated NMu-2 tumor-bearing animals no metastatic lesions were detected at the time of sacrifice, while the animals with untreated control tumor displayed metastatic lesions (56).

In contrast to the previously studied cAMP analogues, the site-selective analogues demonstrated their growth-inhibitory effect at micromolar concentrations. The analogues appeared to work directly through the cAMP receptor protein by substituting for endogenous cellular cAMP. Among the site-selective analogues tested, 8-Cl-cAMP, which has a high Site B selectivity for type II protein kinase (90), exhibited the most potency in all human cancer cell lines tested (55, 56, 84). The analogue effects, in fact, correlated with a selective modulation of two types of cAMP receptor protein, i.e., a marked reduction in the RI receptor with an increase in the RII receptor. Thus, site-selective cAMP analogues restore normal cAMP receptor patterns in cancer cells.

This selective modulation of the RI and RII cAMP receptor protein is not mimicked by the cAMP analogues previously studied, by cAMP itself, or by agents that increase cellular cAMP level. cAMP at high levels, having no site selectivity (80), activates both type I and type II isozymes maximally and equally without discrimination (91, 92). Selective modulation of type I versus type II protein kinase isozymes clearly correlates with the potency of growth inhibition demonstrated among the site-selective cAMP analogues. The analogues with a lower concentration, inducing 50% inhibition of cell proliferation, bring about a greater RII/RI cAMP receptor ratio in cancer cells. Thus, 8-Br-cAMP, despite its high Site B selectivity toward the type II isozyme, when compared with 8-Cl-cAMP, exhibits greater IC50 (50% inhibitory concentration) and brings about less increase in the RII/RI ratio in cancer cells. The synergistic effect of the C-6- and C-8-substituted analogue combinations on growth inhibition (55, 86) and differentiation (93) of cancer cells further supports the idea that the efficacy of an analogue is dependent on its ability to selectively bind to type II protein kinase.

The affinity and activation potency of some cAMP analogues for purified preparations of protein kinase are shown in Table 3. Among C-8-substituted (Site B-selective) analogues (79, 80), the halogen derivatives, 8-Cl- and 8-Br-cAMP, which demonstrate a higher affinity binding for Site B of RI than RI receptor, are the potent growth inhibitors (55, 90), while the amino derivatives, 8-amino- and 8-aminoxylylamino-cAMP,
Table 3  Affinity and activation potency of cAMP analogues for protein kinase

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Relative affinity ($K'_A$)</th>
<th>Relative activation constant ($K'_I$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site A</td>
<td>Site B</td>
<td>Site A</td>
</tr>
<tr>
<td>8-Cl-cAMP</td>
<td>2.7</td>
<td>2.0</td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>8-NH₂-cAMP</td>
<td>0.15</td>
<td>3.9</td>
</tr>
<tr>
<td>8-Aminohexylamo-</td>
<td>0.11</td>
<td>1.6</td>
</tr>
<tr>
<td>cAMP</td>
<td>2.3</td>
<td>2.0</td>
</tr>
<tr>
<td>N⁶-Benzoyl-cAMP</td>
<td>3.5</td>
<td>0.19</td>
</tr>
<tr>
<td>N⁶-Monobutyryl-</td>
<td>3.6</td>
<td>0.093</td>
</tr>
</tbody>
</table>

which show higher affinity Site B binding for RI than RII, are the weak growth inhibitors (55). Among C-6-substituted (Site A-selective) analogues (79, 80), N⁶-benzoyl-cAMP, which shows higher affinity binding for Site A of RII than RI, is more potent than N⁶-monobutyryl-cAMP, which exhibits higher affinity Site A binding for RI than RII receptor (55).

In the activation of protein kinase, 8-Cl-cAMP exhibits 2.3-fold greater potency than cAMP toward type I protein kinase while showing 30% less potency than cAMP for type II protein kinase (90). In contrast, 8-Br-cAMP, which not only exhibits a high-affinity Site B binding but also shows potent activation for type II protein kinase (1.7-fold of cAMP), is less potent in growth inhibition than 8-Cl-cAMP (55). Thus, the efficacy of cAMP analogues in growth inhibition is dependent on two important properties: high-affinity binding (either Site A or Site B) toward RII but not RI; and low activation capacity for type II protein kinase. This latter property implicates type II protein kinase holoenzyme in the growth inhibition by cAMP.

The changing levels of RII and RII cAMP receptor proteins were reflected in changes in the mRNA levels and the rates of transcription of these receptor genes. In LS-174T human colon cancer cells, the 8-Cl-cAMP-induced growth inhibition was preceded by an increase in the transcription of RII gene with a decreased transcription of RI gene and with corresponding changing levels of RII, and RI, proteins (90). The inhibition of growth of LX-1 human lung carcinoma in athymic mice by 8-Cl-cAMP (continuous infusion, s.c.) correlated with an increase in RII, mRNA that is not present in RI. It was found that the nuclear translocation of RII, induced by 8-Cl-cAMP is 5-10-fold greater than that induced by 8-Br- or N⁶,N⁸-di(2-butyryl)cAMP (98), indicating that the magnitude of RII, translocation parallels the growth-inhibitory potency of cAMP analogues.

Involvement of cAMP in the control of transcription in mammalian cells is demonstrated by the discovery of a specific DNA sequence, CRE (26), which is common to promoter regions of genes for proteins the synthesis of which is regulated by changes in cellular levels of cAMP. The possibility was examined that in cancer cells, site-selective cAMP analogues increase transcription factors that bind specifically to CRE and whether this effect can be correlated with the ability of the analogues to promote nuclear translocation of RII, and growth inhibition (98, 100). These results suggest that the fundamental basis for the anti-neoplastic activity of site-selective cAMP analogues may reside in the restoration of normal gene transcription in cancer cells in which the RII, cAMP receptor protein plays an important role.

Use of Antisense Oligodeoxynucleotide in the Suppression of Malignancy

Antisense RNA sequences have been described as naturally occurring biological inhibitors of gene expression in both prokaryotes (101) and eukaryotes (102), and these sequences presumably function by hybridizing to complementary mRNA sequences, resulting in hybridization arrest of translation (103). Antisense oligodeoxynucleotides are short synthetic nucleotide sequences formulated to be complementary to a specific gene or RNA message. Through the binding of these oligomers to a target DNA or mRNA sequence, transcription or translation of the gene can be selectively blocked and the disease process generated by that gene can be halted. The cytoplasmic location of mRNA provides a target considered to be readily accessible to antisense oligodeoxynucleotides entering the cell; hence much of the work in the field has focused on RNA as a target. Currently, the use of antisense oligodeoxynucleotides provides a useful tool for exploring regulation of gene expression in vitro and in tissue culture (104).

As described in the preceding sections, the cAMP-dependent protein kinase type I and type II ratio varies among tissues and an increased expression of protein kinase type I or RI correlates with active cell growth, cell transformation, or early stages of differentiation, suggesting that RI may be involved in ontogenic growth. Constitutive expression of RI may disrupt normal ontogenic processes, resulting in a pathogenic outgrowth, such as malignancy.

Antisense strategy was used to determine whether the presence of RI, is essential for neoplastic cell growth. Exposure of human HL-60 promyelocytic leukemia cells to 21-mer RI,
antisense oligodeoxynucleotide brought about growth inhibition and monocyte differentiation, bypassing the effects of an exogenous cAMP analogue, with no sign of cytotoxicity (105). In cells unexposed to RI, antisense oligomer, 8-Cl-cAMP plus N\(^{6}\)-benzyl-cAMP induce a monocyte morphological change characterized by a decrease in nuclear/cytoplasm ratio, abundant ruffled and vacuolated cytoplasm, and loss of nucleoli (89). Strikingly, the same morphological change is induced when cells are exposed to RI, antisense oligodeoxynucleotide, and the morphological change induced by the oligomer is indistinguishable from that induced by 12-O-tetradecanoylphorbol-13-acetate. Moreover, exposure of these cells to RI, antisense oligodeoxynucleotide induces a marked increase (10- to 50-fold) in the expression of monocyte surface antigens (Leu-15 and Leu-M3) along with a decrease in markers related to the immature myelogenous cells (My9) (105). Treatment of these cells exposed to RI, antisense oligomer with 8-Cl-cAMP plus N\(^{6}\)-benzyl-cAMP does not further change the antigen expression.

The effect of RI, antisense oligodeoxynucleotide correlates with a decrease in RI, receptor and an increase in RI, receptor level. In control cells, treatment with 8-Cl-cAMP plus N\(^{6}\)-benzyl-cAMP brought about a 70% reduction of RI, with a 3-fold increase in RI, resulting in a 10-fold increase in the RI,/RI, ratio. Exposure of these cells to RI, antisense oligodeoxynucleotide brought about marked changes in RI, and RI, levels; an 80% reduction in RI, with a 5-fold increase in RI, resulted in a 25-fold increase in the RI,./RI, compared with that in control cells. Thus, suppression of RI, by its antisense oligodeoxynucleotide results in a compensatory increase in RI, level. Such coordinated expression of RI and RI has also been shown in other cells (106).

This increase in RI, may be responsible for the self-differentiation in these cells exposed to RI, antisense oligodeoxynucleotide. HL-60 cells that were exposed to RI, antisense oligodeoxynucleotide have been shown (107) to become refractory to treatment with cAMP analogues and to continue to grow, indicating the essential role of RI, in cAMP-induced differentiation. The increase in RI, mRNA or RI, protein level has been correlated with cAMP analogue-induced differentiation in K-562 chronic myelocytic leukemic cells (93) and in erythroid differentiation of Friend erythrocytic leukemic cells (108).

The essential role of RI, in differentiation of HI-60 cells was further demonstrated when these cells were exposed to both RI, and RI, antisense oligodeoxynucleotides simultaneously (105). Cells exposed to both RI, and RI, antisense oligomers were neither growth inhibited or self-differentiated nor differentiated upon cAMP analogue treatment. These results may reflect an escape from the cAMP-dependent growth regulatory pathway. Cells are no longer cAMP dependent but survive and proliferate probably through an alternate pathway. Thus, suppression of both RI, and RI, gene expression led to an abnormal cellular growth regulation similar to that in mutant cell lines (109), which contain either deficient or defective regulatory subunits of cAMP-dependent protein kinase and are no longer sensitive to cAMP stimulus.

These results suggest that two isoforms of cAMP-binding receptor proteins, RI, and RI, control the regulatory subunits of protein kinase, in harmony, regulate cell growth and differentiation in HL-60 cells. The RI, antisense oligodeoxynucleotide that appears to restore the functional balance of these cAMP receptor proteins led to terminal differentiation in HL-60 leukemia with no sign of cytotoxicity.

These effects of RI, antisense oligodeoxynucleotide are not limited to leukemic cells. The RI, antisense oligomer is similarly effective toward the growth control of epithelial human cancer cell lines including colon, breast, and gastric carcinoma and neuroblastoma (110). These studies with the use of antisense strategy provided direct evidence for the distinct roles of cAMP receptor isoforms in cell proliferation and differentiation.

Conclusions

The following conclusions can be drawn from studies on the role of cAMP receptor proteins in the molecular control of growth and differentiation in normal development, malignant transformation, and suppression of malignancy: (a) malignancy can be suppressed by inducing differentiation with site-selective cAMP analogues which demonstrate a high affinity for the RII cAMP receptor or with an antisense oligodeoxynucleotide targeted against the RI cAMP receptor mRNA; (b) this suppression of malignancy is due to restoration of the normal functional balance of cAMP receptor isoforms, RI and RII; (c) this functional balance of these cAMP receptor proteins underlies the controlled expression of growth factors and cellular oncogenes; and (d) since cAMP receptor proteins are expressed in every type of cell and tissue, this suppression of malignancy is effective toward a broad spectrum of neoplasia. This suppression of malignancy by restoration of normal function of intracellular regulatory molecules that induce differentiation can be of value in cancer therapy (84).

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