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Review paper

Poly(ADP-ribosylation) processing as a target for the anti-tumor effects of the cell differentiating agent, hexamethylenebisacetamide, and the N^6-substituted adenosines

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The cellular process regulating the post-translational poly(ADP-ribosylation) of various nuclear acceptor proteins is discussed in relation to its significance as a target for cancer chemotherapy; and with particular reference to the mechanism underlying the anti-tumor effects of the cell differentiating agent, hexamethylenebisacetamide. Of especial note are the influences which may be exerted on tumor cells expressing certain major types of oncogenically-activated cellular proto-oncogenes (oncogenes). A basis for a pharmacological approach to tumor therapy is further proposed from considerations of the action of a class of anti-tumor agents, the N^6-substituted adenosines, by reason of their possible effects on poly(ADP-ribosylation) processing, as well as on other cellular processes of relevance for tumor therapy.

Key words: Cancer, chemotherapy, hexamethylenebisacetamide, N^6-substituted adenosines, poly(ADP-ribosylation.

Introduction

A recent trend in cancer chemotherapy has been the use of chemicals which cause tumor cells to differentiate to a less malignant phenotype. One such chemical presently undergoing clinical cancer trials in the USA and the UK is the cell differentiating agent, hexamethylenebisacetamide (HMBA); phase 1 clinical trials have already been reported.[1,2] It is therefore important to consider the mechanism(s) by which HMBA may produce its anti-tumor effects.

HMBA has been reported to induce terminal differentiation and growth arrest of cultured mouse erythroleukemic cells; a cascade of molecular changes was also observed, including changes in the expression of the c-*myb*, c-*myc*, c-*fos* proto-oncogenes, and the *p53* tumor suppressor gene.[3] HMBA has further been shown to induce differentiation of cultured colon carcinoma cells, together with the concomitant increase in the expression of transforming growth factor β_1, which appears at least partly to mediate the effect on cell differentiation.[4]

The workers concerned with these and other studies, however, have not yet appreciated a prime aspect of the activity of HMBA, namely, its inhibitory action on the enzyme involved in the regulation of the post-translational poly(ADP-ribosylation) of various nuclear acceptor proteins. This cellular process is now proving to be of special relevance as a target for cancer chemotherapy (see Refs 5 and 6; only central and new references are cited here).

Poly(ADP-ribosylation) reactions

Poly(ADP-ribosylation) is effected by the chromatin-bound enzyme, adenosine diphosphoribosyl transferase (ADPRT) or polymerase, which has an absolute requirement for DNA for its activity and is stimulated by DNA strand breaks. ADPRT specifically catalyzes the cleavage of the oxidized coenzyme, nicotinamide adenine dinucleotide (NAD^+), with the concomitant attachment of its ADP-ribose moiety as a transient post-translational modification of certain nuclear acceptor proteins in the form of linear or branched covalently-linked poly(ADP-ribose) chains.

Poly(ADP-ribosylation) is predominantly confined to the nucleus and exhibits a cell cycle

oscillation with a maximum coinciding with S phase; its removal is effected by the co-operative action of poly(ADP-ribose) glycohydrolase and ADP-ribosyl protein lyase.

Mono(ADP-ribosylation) is a related but distinct process; it occurs in the cytoplasm and is regulated by a different set of enzymes. Apart from the well-defined action of mono(ADP-ribosylation) on various microbial toxins, such as the cholera and diphtheria toxins, its biological significance is otherwise still poorly understood.

Nuclear proteins known to be poly(ADP-ribosylated) by ADPRT are various histones (H1, H2a, H2b, H3 and H5) and non-histones (nuclear lamins and high mobility group proteins) and several DNA enzymes, i.e. DNA ligase I and II, DNA polymerase α and β, deoxynucleotidyl terminal transferase, Ca^{2+}/Mg^{2+}-dependent endonuclease, DNA topoisomerases, and even including the ADPRT enzyme itself. The apparent regulatory roles of the poly(ADP-ribosylation) modification of histones and the DNA replication and repair enzymes have been discussed.[5]

Several inhibitors of the ADPRT enzyme are known, such as HMBA, nicotinamide, benzamide, 1,2-benzopyrone (also known as coumarin), luminol and 4-hydroxyquinazoline. Studies with the ADPRT inhibitors, benzamide and nicotinamide, show that they possess little or no inhibitory effect on mono(ADP-ribosylation) reactions catalyzed by mono(ADP-ribosyl) transferases.[7]

By the use of one or more of the ADPRT inhibitors, poly(ADP-ribosylation) processing has been implicated in the regulation of cell proliferation, cell differentiation, gene expression, and DNA replication and repair. Benzamide and 3-aminobenzamide have further been shown to protect cultured mammalian cells from oncogenic transformation induced by various chemical and physical carcinogens.[5]

A requirement for ADPRT activity in DNA excision repair, probably because it is involved in the regulation of DNA ligase II activity,[8] suggests that poly(ADP-ribosylation) processing has a role in DNA ligation, involving such cellular processes as genetic recombination, sister chromatid exchanges (SCE), gene rearrangement, gene transposition and cell differentiation, in all of which some cleavage and rejoining of DNA may take place.[8] Mammalian cells treated with ADPRT inhibitors or cells genetically defective for poly(ADP-ribosylation) processing have a longer cell doubling time and a marked increase in the frequency of SCE; a point to be noted later.

Poly(ADP-ribosylation) processing as a target for cancer chemotherapy

Several studies indicate the significance of poly-(ADP-ribosylation) processing as a target for cancer chemotherapy.

Thus, cultured rat cells transfected with a glucocorticoid-inducible *ras* oncogene are converted to a malignant state only when treated with glucocorticoids. These tumor cells are inhibited for growth by certain ADPRT inhibitors tested, i.e. 1,2-benzopyrone, HMBA and benzamide. The action of thee inhibitors is on the DNA-related site of ADPRT rather than the NAD^+ site; the inhibitor, nicotinamide, which acts only on the NAD^+ site, is ineffective.[9]

ADPRT is inhibited by these drugs to the same extent in both untreated and glucocorticoid-treated cells, but the inhibitory effect on cell proliferation is manifest only when the cells are expressing the *ras* oncogene, which indicates that tumor cells are more acutely sensitive to inhibitory effects produced on poly(ADP-ribosylation) processing.[9]

A similar effect is also claimed on cultured rat cells in which the viral Rous sarcoma oncogene, v-*src*, is also placed under glucocorticoid-inducible transcriptional control.[9]

Also, when rat cells carrying the inducible *ras* oncogene are subcutaneously injected into rats, where the animals' own glucocorticoids induce *ras* oncogene expression, the cells develop into lethal fibrosarcomas. The addition of 1,2-benzopyrone into the drinking water of these rats reduces both tumor size and tumor incidence.[9]

These observations are highly significant, since *ras* oncogenes are found to be present in most types of human tumors, but with varying frequencies in different tumor types, e.g. adenocarcinomas of the pancreas (90%), colon (50%), thyroid (50%), lung (30%) and myeloid leukemias (30%).[10]

An additional property of ADPRT inhibitors is their ability to cause the specific deletion of amplified oncogene and proto-oncogene sequences from cells.

The ADPRT inhibitors, benzamide, 3-aminobenzamide, 1,2-benzopyrone and luminol, induce an efficient and specific loss of various types of amplified oncogene sequences transfected into mouse cells, where they come to exist in multiple copies, i.e. oncogenically-activated forms of rat K-*ras*, human H-*ras*, K-*ras*, *raf* and *retII*; concomitant cellular morphological changes also occur.[11]

HL-60 cells, a human promyelocytic leukemic cell line, have the cellular (c)-*myc* proto-oncogene amplified about 16-fold; amplification of cellular proto-oncogenes, together with an associated increase in their expression, is believed to be responsible for their oncogenic behavior. The ADPRT inhibitors, benzamide, nicotinamide, 1,2-benzopyrone and 4-hydroxyquinazoline, all cause the loss of the amplified c-*myc* sequence from the HL-60 cells, as well as the concomitant induction of granulocytic differentiation of the cells.[12]

A mechanism(s) involving the generation of SCE seems likely to be a cause of the deletion of the amplified c-*myc* sequence following the inhibition of poly(ADP-ribosylation) processing by the inhibitors of ADPRT.[12]

The amplification of cellular proto-oncogenes is found to be a frequent occurrence in various types of human tumors. Examples abound. Thus, in lung tumors, 90% of amplified proto-oncogenes are of the *myc, ras* and *erb* families, which occur in about 20% of tumors examined.

Amplification of *myc* is detected in breast carcinomas, where it appears to be associated with a particularly aggressive form of the disease. Amplification of *erbB-2* (also known as HER-2/neu) is also present in 22% of node-positive invasive breast tumors, and is an indicator of early relapse and death. Amplification of either *myc* or *erbB-2* is found in about 40% of breast tumors sampled. The *erbB-2* proto-oncogene is also amplified in about 8% of stomach cancers, showing a range from 2- to 8-fold, and an associated overexpression of *erbB-2* mRNA from 8- to 32-fold. The amplified proto-oncogene K-*sam* is also detectable in poorly differentiated stomach cancers.[13] The *int-2* proto-oncogene has been reported to be frequently amplified in head and neck squamous cell carcinomas.[14] Overexpression of *erbB-2* is reported in advanced ovarian cancer[15] and that of the *fos* proto-oncogene in the majority (61%) of osteosarcomas.[16] Other examples have been reported, together with a discussion on the amplification of proto-oncogenes as a predictor of the clinical outcome for the cancer patient.[17]

From the foregoing considerations, it is evident that inhibitory effects produced on poly(ADP-ribosylation) processing in tumor cells present an important new strategy for cancer chemotherapy. Moreover, it is significant that greatly elevated ADPRT activities are found in tumors of basal cell epithelioma, malignant myeloma, cervical carcinoma, and both colorectal carcinoma and its premalignant polyp.

ADPRT inhibitors, such as the cell differentiating agent HMBA, therefore appear to be capable of inducing a wide range of cellular effects of particular relevance for tumor therapy. Especially notable are the influences exerted on tumor cells expressing certain major types of oncogenes and which are represented in a broad spectrum of human tumor cell types.

By contrast, effects produced on normal cells may be more limited, since the inhibition of poly(ADP-ribosylation) processing by nicotinamide on cultured rat pituitary cells[18] and that of benzamide on liver cells following treatment of the intact rat[19] seem to be relatively inconsequential, apart from the induction of certain enzymes.

A metabolic approach to cancer chemotherapy

As an approach to cancer therapy, the simple application of ADPRT inhibitors, such as HMBA, may not realize the overall therapeutic potential it is possible to achieve, particularly as toxic plasma levels of the inhibitors need to be maintained over some period of time to attain appropriate intracellular concentrations.[1,2]

Accordingly, a more metabolically-directed approach has been proposed for producing effects on poly(ADP-ribosylation) processing, besides affecting various other cellular processes of relevance for tumor therapy.[5,6]

Two adenine ribonucleotide regulatory cellular metabolites, diadenosine tetraphosphate (Ap4A) and the 2′,5′-oligoadenylates [2′5′(A)$_n$], have been specifically identified as appropriate intracellular targets.[5]

Ap4A consists of two adenosine molecules joined together through their 5′ ribose positions by a tetraphosphate linkage. The 2′5′(A)$_n$ are series of adenosine molecules linked together through their 2′ and 5′ ribose positions by a phosphate bond, with a triphosphate group at the 5′ end; the major species is the trimer, ppp2′5′(A)$_3$, although longer polymers may be formed. The linkage in the 2′5′(A)$_n$ contrasts with the 3′–5′ phosphate linkage found between nucleosides in the nucleic acids.

The synthesis and biological significance of Ap4A and the 2′5′(A)$_n$ have been extensively discussed, and notable among their manifold roles in cellular metabolism is their evident involvement in the regulation of poly(ADP-ribosylation) processing.[5]

Various studies have identified the structural

requirements for the activity of the 2′5′(A)$_n$, including the presence of the N^6-amino groups at the 5′ end of 2′5′(A)$_3$ and that of the third adenine residue.[5] Studies on the structural requirements for the activity of Ap4A have been limited and less informative, although the presence of one or both of its intact N^6-amino groups appears to be necessary.

An appropriate class of anti-tumor agents which could be utilized in the synthesis of Ap4A and the 2′5′(A)$_n$ is the N^6-substituted adenosines, where one hydrogen atom of the extranuclear 6-amino group of adenosine is substituted by some other group.

Many N^6-substituted adenosines have been shown to have anti-tumor effects (growth inhibitory or cytotoxic) on mammalian tumor cells.[5] In fact, it seems that any N^6-substitution of adenosine confers it with anti-tumor properties, e.g. N^6-methyl, -hydroxyl, -isopentenyl, -isoamyl, -isopropyl, -allyl, -benzyl, -benzoyl, -hydroxymethyl, -propargyl, -methylallyl and -furfuryl derivatives.

The presence of the ribose sugar is crucial to the activity of the N^6-substituted adenosines, since those corresponding N^6-substituted adenines tested show no anti-tumor activities, probably because the N^6-substituted adenines are not metabolized to the nucleoside in mammalian cells. This restriction excludes the subsequent triphosphorylation needed at the 5′ position of the ribose sugar for the utilization of N^6-substituted adenosines in the synthesis of Ap4A and the 2′5′(A)$_n$, and other ribonucleotide metabolites, such as cyclic AMP, acetyl CoA and NAD, as well as the nucleic acids. The cellular uptake and metabolism of the N^6-substituted adenosines have previously been discussed.[5,6]

Mechanisms have been proposed which might form a basis for the anti-tumor effects of N^6-substituted adenosines following their utilization for the synthesis of Ap4A and the 2′5′(A)$_n$ to form variously N^6-substituted derivatives of both these ribonucleotide metabolites *in situ* to affect their subsequent intracellular behavior.[5] Among these effects, an inhibitory effect on poly(ADP-ribosylation) processing is considered to be of prime importance.

Cellular effects produced, some of which may arise by mechanisms other than on poly(ADP-ribosylation) processing, include effects on the growth and differentiation of tumor cells; influences on the genetic stability of oncogenic amplified proto-oncogene sequences; effects on protein synthesis, viral yield, and DNA replication and repair; and stimulatory effects on certain effector cells of the immune system, i.e. the capacity of monocytes to mediate antibody-dependent cytotoxicity and the tumoricidal activity of natural killer (NK) cells.[5]

N^6-substituted adenosines as normal cellular components

Some N^6-substituted adenosines are normal components of tRNAs, mRNAs, small nuclear (sn)RNAs and even DNA. They are produced by post-translational modifications of the nucleic acid by specific enzymes, e.g. N^6-methyladenosine receives its methyl group from the coenzyme, S-adenosylmethionine, by a specific methyl transferase. N^6-isopentenyl adenosine is also found in some tRNAs and is located adjacent to the 3′ site of anti-codons in those tRNAs that recognize codons starting with uracil; failure to N^6-isopentenylate these adenosines decreases tRNA translational efficiency both *in vitro* and *in vivo*. The source of the isopentenyl group is believed to be from dimethylallyl pyrophosphate, an isomer of isopentenyl pyrophosphate, both of which are formed as intermediate and successive steps in the biosynthesis of cholesterol. Its condensation with adenosine, catalyzed by either dimethylallyl or isopentenyl transferase, produces the N^6-isopentenylated product.

A group of about 14 natural plant hormones, the cytokinins, are N^6-substituted adenines, where the substitution is a 5-carbon moiety; virtually all of them can be regarded as derivatives of N^6-isopentenyladenine. Substitutions at the N-9 position of the adenine ring by a ribose (N^6-isopentenyladenosine) or a methyl group generally result in a 10-fold reduction in their activities and substitutions at other positions almost always reduce their activity.

About 200 not naturally-occurring cytokinin-like compounds have been synthesized; the most active of these are N^6-substituted adenines, such as N^6-benzyladenine and N^6-furfuryladenine (kinetin).

The cytokinins were originally identified as compounds which, in the presence of optimal amounts of another class of plant hormones, the auxins, induce cell division in plant tissue. Subsequently, the cytokinins have been shown to be involved, together with the auxins, in regulating virtually every aspect of plant growth and development. The mechanism(s) of action of the cytokinins (and the auxins) is so far unknown.

The synthesis of free N^6-isopentenyladenine has not been convincingly demonstrated in animal cells; it is unlikely to be represented as a distinct metabolic pool and any small amounts detected may simply have arisen from intracellularly degraded tRNAs. However, a role for N^6-isopentenyladenine (but not for N^6-isopentenyladenosine) has been reported in the regulation of S phase DNA replication in mammalian cells.[20]

Two plant cytokinins, N^6-isopentenyladenosine and N^6-benzyladenosine, have been reported to be capable of inducing clinical remission in some cancer patients.[21]

Effects of N^6-substituted adenosines on normal cells have been studied on human lymphocytes in culture. N^6-substituted adenosine cytokinins generally show an inhibitory effect on RNA synthesis and on phytohemagglutinin (PHA)-induced stimulation at doses of 10^{-4} and 10^{-5} M, but a stimulatory effect at a lower dose (10^{-6} M); the adenine derivatives are also active, but higher doses are required to produce a comparable effect. Since PHA stimulation induces the adenylate cyclase enzyme, cAMP was tested and found to reverse the inhibitory effect of N^6-isopentenyladenosine on PHA stimulation; an effect on cAMP metabolism was therefore proposed.

Pharmacological effects of N^6-substituted adenosines

A further interesting aspect of the biological effects of the N^6-substituted adenosines is the pharmacological properties which some of them are known to possess.

Many of the physiological functions performed by adenosine result from its binding to cell surface receptors,[22] i.e. the A1 and A2 adenosine receptors, defined as either inhibitory (A1) or stimulatory (A2) for adenylate cyclase activity, and which are present on virtually all tissues and cell types.

Through these receptors, adenosine mediates a variety of effects, i.e. coronary vasodilation; vasoconstriction in the kidney; inhibition of the processes of lipolysis, platelet aggregation, lymphocyte function, insulin secretion and neurotransmitter release from nerve endings; also the stimulation of steroidogenesis, as well as the increased release of glycogen from the pancreas and of histamine from mast cells.

Some N^6-substituted adenosines are found to be agonists for binding to the A1 adenosine receptor. This specificity varies from a 2-fold greater A1 selectivity for N^6-benzyladenosine to almost 800-fold for N^6-cyclopentyladenosine.

Various 2-substituted adenosines, such as 2-(phenylamino)adenosine, prove to be the most selective A2 adenosine receptor agonists. However, some N^6-substituted adenosines are now being found to be potent A2 adenosine receptor agonists, notably N^6-(9-fluoroenylmethyl)adenosine. N^6-benzyladenosine is also found to show equal affinity in A1 and A2 adenosine receptor binding.

NK cell activity also appears to be modulated through the adenosine receptors, where an inhibitory effect on the lytic activity of the NK cell is associated with a build-up of cAMP within the cell; A2 adenosine receptor agonists have an inhibitory effect, whereas potent A1 agonists are more stimulatory.[23]

NK lymphocytes exhibit a spontaneous and inherent cytotoxic effect by a process of lymphocyte-mediated cytolysis (LMC) against tumors, and cell lines derived from them, as well as against virally-infected cells, without any apparent prior need for immunization. NK cells are represented as a functionally distinct population of large granular lymphocytes of lymphoid and myeloid cells present in the peripheral blood and lymphoid organs, where they appear to play a role in surveillance against the development of tumor cells.[24]

N^6-substituted adenosine receptor agonists have now been recognized as a potentially novel class of anti-hypertensive agent.[25]

Therapeutic considerations

In view of the wide range of effects indicated for the action of the N^6-substituted adenosines, it is evident that they should be administered at doses which are not directly cytotoxic at either a cellular or systemic level, but treated as pharmacological agents. Otherwise, desirable therapeutic responses may be precluded. Similar considerations also apply to a further aspect of the anti-tumor activities of the N^6-substituted adenosines, but not discussed here, i.e. their possible effects through polyisoprene metabolism (cholesterol biosynthesis).[6]

The N^6-substituted adenosines therefore appear to represent a class of anti-tumor agents which might allow a radically new approach to cancer chemotherapy. Further research may well initiate a pharmacological basis for the treatment and control of cancer manifestly less aggressive for the patient than the current practice of using barely tolerated doses of drugs to produce cytotoxic effects.

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