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Review

Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications

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Abstract. Polyamines are aliphatic cations with multiple functions and are essential for life. Cellular polyamine levels are regulated by multiple pathways such as synthesis from amino acid precursors, cellular uptake mechanisms that salvage polyamines from diet and intestinal microorganisms, as well as stepwise degradation and efflux. Investigations using polyamine biosynthetic inhibitors indicate that alterations in cellular polyamine levels modulate normal and cancer cell growth. Studies using transgenic mice overexpressing polyamine biosynthetic enzymes support a role of polyamines in carcinogenesis. Many, if not all, signal transduction pathways intersect with polyamine biosynthetic pathways and the regulation of intracellular polyamine levels. Direct binding of polyamines to DNA and their ability to modulate DNA-protein interactions appear to be important in the molecular mechanisms of polyamine action in cell proliferation. Consistent with the role of polyamines as facilitators of cell growth, several studies have shown their ability to protect cells from apoptosis. However, polyamines also have a role in facilitating cell death. The basis of these diverse cellular responses is currently not known. Cell death response might be partly mediated by the production of hydrogen peroxide during polyamine catabolism. In addition, the ability of polyamines to alter DNA-protein and protein-protein interactions might be disruptive to cellular functions, when abnormally high levels are accumulated due to defects in polyamine catabolic or efflux pathways. A large body of data indicates that polyamine pathway can be a molecular target for therapeutic intervention in several types cancers. Inhibitors of biosynthesis, polyamine analogues as well as oligonucleotide/polyamine analogue combinations are promising drug candidates for chemoprevention and/or treatment of cancer.

Key words. Polyamines; cell cycle; apoptosis; breast cancer.

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Introduction

Polyamines are present in all living cells, prokaryotes, eukaryotes, plants and animals. In mammalian cells, the

natural polyamines—putrescine, spermidine and spermine—are found in millimolar concentrations [1, 2]. Polyamines are organic cations since the primary and secondary amino groups are protonated at physiological pH (fig. 1). Therefore, putrescine is divalent, spermidine is trivalent and spermine is tetravalent. Putrescine is synthesized by the decarboxylation of ornithine by the enzyme ornithine decarboxylase (ODC) [3–5]. The higher polyamines are synthesized by the sequential addition of aminopropyl groups to putrescine by reactions involving *S*-adenosyl methionine decarboxylase (SAMDC) as well as spermidine and spermine synthases. The unique feature of polyamine structure is the presence of methylene groups that can enter into hydrophobic interactions along with positive charges at defined distances. Polyamines are considered to be essential for life as inhibitors of polyamine biosynthesis block cell growth [6, 7]. Polyamine-depleted cells are stimulated to grow in the presence of exogenous polyamines [6–8]. Polyamines have specific roles in embryonic development [9], cell cycle [10, 11], cancer [12, 13], neurochemistry [14], as well as pulmonary [15] and immune system functions [16, 17]. Cellular polyamines are regulated by a complex circuitry of synthesis, degradation, as well as cellular uptake and efflux [3–5, 18]. Polyamine catabolism is driven by spermidine/spermine acetyl transferase (SSAT) which is induced by natural polyamines and their synthetic analogues as well as

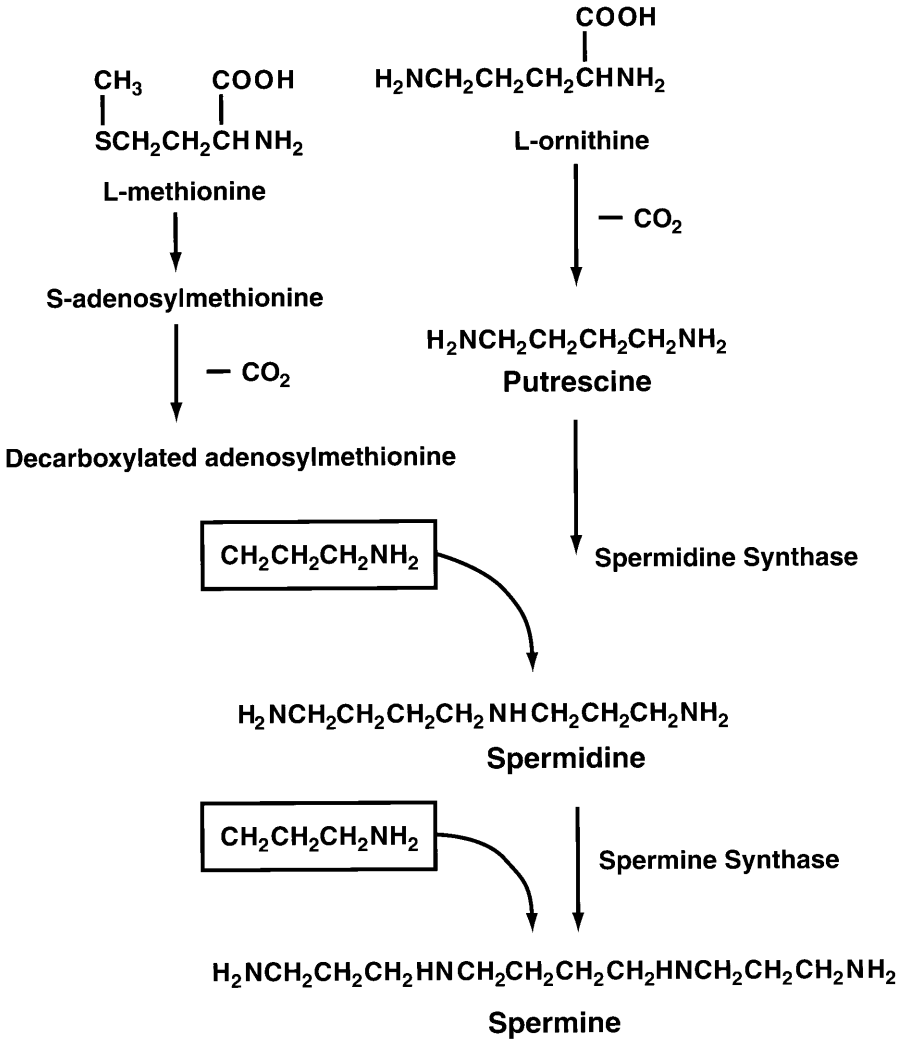

Figure 1. Salient features of polyamine biosynthetic pathway. Putrescine is formed by the decarboxylation of ornithine by ornithine decarboxylase (E.C. 4.1.1.17). Spermidine is formed by the action of spermidine synthase (E.C. 2.5.1.16) that links putrescine to an aminopropyl group derived from decarboxylated *S*-adenosylmethionine, a reaction product of *S*-adenosylmethionine decarboxylase (E.C. 4.1.1.50). Spermine is synthesized from spermidine by a similar process by spermine synthase (E.C. 2.5.1.22).

other toxicants [4, 19, 20]. Acetylation removes the positive charge on the amino group, and consequently acetylated polyamines are less potent than the parent molecules. Acetylated spermine is cleaved into spermidine through the action of a flavin adenine dinucleotide (FAD)-dependent polyamine oxidase [5, 14, 21]. Similarly, spermidine is acetylated and cleaved by this polyamine oxidase back into putrescine, completing the backward conversion of spermine to putrescine. Polyamines are also acted upon by diamine oxidases, which are copper-containing amine oxidases. Products of this catabolic process include γ-aminobutyric acid, 3-acetamidopropanal, hydrogen peroxide (H_2O_2) and ammonia. H_2O_2 and aminoaldehydes produced during the degradation of polyamines also have biological significance as they trigger programmed cell death or apoptosis in certain cell types [22–24].

Unlike many other nutrients, well-orchestrated synthetic and catabolic pathways of polyamine metabolism are complemented by cellular uptake and efflux mechanisms [18]. These processes are then fine tuned with hormonal regulatory pathways unique to individual organ system [2, 5]. Thus, organisms have evolved with numerous regulatory pathways in controlling intracellular polyamine levels, and when these pathways fail, disease processes seem to set in [25]. The purpose of this review is to illuminate the complexity of the role of polyamines in cell growth and cell death and delineate possibilities in cancer therapeutics. Emphasis is on the understanding of polyamine functions at the molecular level, since aspects of polyamine metabolism and general biological functions are often covered in other reviews [4, 5, 13–15]. It is a challenge for the biologist to decipher the circuitry involved in polyamine regulation and function and utilize it for prevention or treatment of specific diseases.

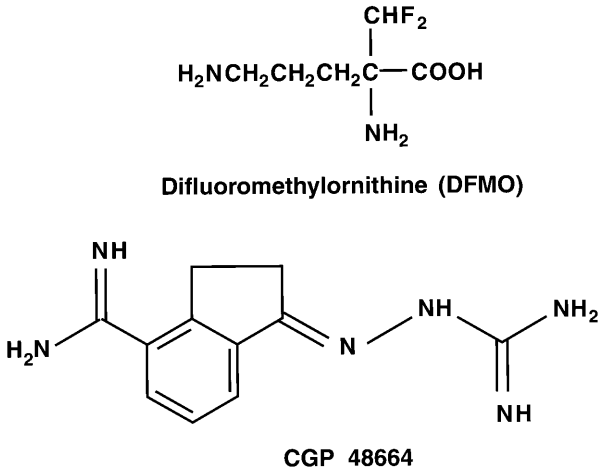

Figure 2. Chemical structures of the ornithine decarboxylase inhibitor, α-difluoromethylornithine (DFMO) and the *S*-adenosylmethionine decarboxylase inhibitor, CGP 48664.

Polyamines in embryonic development

Polyamines are important in embryonic development as evidenced from early data on sea urchin eggs [9]. When ODC was inhibited by a competitive inhibitor, α-methylornithine, first egg cleavage was reduced to 70%, second cleavage to 41.7%, and third cleavage to 5.8%. This inhibition of egg cleavage was reversible in the presence of 5 mM ornithine or different concentrations of polyamines. It is important to note that the reversal was effective only under narrow concentrations of putrescine, spermidine or spermine.

Another example of results of inhibition of polyamine synthesis can be seen from an experiment using chick embryos [26]. In this case, an irreversible inhibitor of ODC, DL-α-difluoromethylornithine (DFMO) was used. The synthesis of DFMO has been a major milestone in polyamine research (fig. 2) [27]. In the chick embryo system, primary cultures of embryonic mesoderm were used [26]. In control cultures, mesodermal cells retained their in ovo outgrowth behavior and differentiation pattern. Addition of DFMO retarded attachment and outgrowth, and delayed the expression of differentiation phenotypes such as beating heart tissue, erythroid cells and adipocyte-like cells. The major ultrastructural effect observed in the arrested embryos was an interference with nucleolar formation. The effect of DFMO was reversible in the presence of exogenous polyamines, indicating that DFMO is not cytotoxic by itself, and its effects were mediated by polyamine depletion. The effects of DFMO on embryonic development were also evident from its ability to abort pregnancies. Pregnancies in rats and rabbits could be terminated by treatment with DFMO [28, 29]. However, the treatment had to be conducted during days 4–7 of pregnancy to cause inhibition of embryogenesis in rats. DFMO did not cause abortion if rats were treated during days 1–3 of pregnancy [28].

Recent studies on *Caenorhabditis elegans* further illustrate the role of polyamines in development [30]. A mutant worm was isolated with defective odc-1 gene and no detectable ODC activity. These worms developed normal in a medium containing polyamines, but showed deficiencies in development if transferred to polyamine-free medium. Effects of polyamine deficiency was dependent on the developmental stage at which larvae were transferred to polyamine-free medium. If polyamines were removed at L1 larval stage, the mutant worms developed into adults, but produced very few or

no eggs. If the mutant larvae were transferred to polyamine-free medium at the L4 stage of development, animals developed and laid eggs. However, embryos from these eggs failed to develop at stage 3 unless polyamines were supplemented. These results demonstrate that polyamines are essential at certain stages of larval growth and that exogenous polyamines can be used to correct developmental defects produced by deficiency in polyamine biosynthesis.

Polyamines in cell cycle

Polyamines are known to have specific peaks of induction during cell cycle progression [10, 31]. The cell cycle is generally divided into four phases: G1, the first gap or growth phase; S, the DNA synthetic phase; G2, the second gap or growth phase; and M, the mitotic phase (fig. 3) [32]. Early studies indicated increased levels of polyamines in G1 and S phases and an inhibition of G1- to S-phase transition due to polyamine depletion [31]. In Chinese hamster ovary (CHO) cells, putrescine, spermidine and spermine increased twofold during cell cycle progression [33]. This study was conducted by separating mitotic cells and reseeding them. During progression from the mitotic phase, putrescine levels were increased during S and G2 phases, spermidine increased during the entire cell cycle, and spermine mainly during G1 and S phases. Continuous increase in polyamine levels observed in this study is in contrast to other instances where cell cycle specific fluctuations were observed [10, 31, 33]. In MCF-7 breast cancer cells synchronized in G1 phase, we found that estradiol, which stimulates the proliferation of these cells, increased intracellular polyamine levels approximately twofold at 8 h after the initiation of cell cycle [34]. By the 12-h time point, there was about 50% reduction from the peak levels of polyamines which remained at the reduced level for the rest of the cell cycle. These results illustrate the importance of polyamines in cell cycle, although there are differences in the details of polyamine regulation, either due to cell type specificity or the use of different synchronization methods.

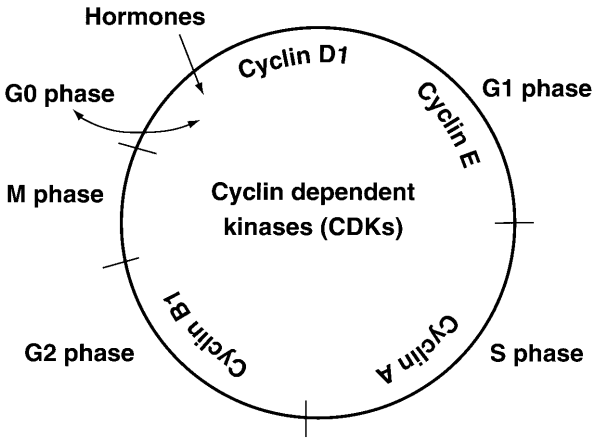

Figure 3. Schematics of different phases of the cell cycle and mediators of the cell cycle. Quiescent cells are usually in the G0 phase. Hormones and/or growth factors signal cells to enter the G1 state and initiate the process of cell division. Hormonal and growth factor signalling results in increased synthesis of cyclins. Polyamines augment the signalling process in many cases. Binding of the cyclins to CDKs activates these kinases to phosphorylate numerous substrates to accelerate the progression of cells through the cell cycle.

Inhibitors of polyamine biosynthesis have been useful in dissecting the role of polyamines in the cell cycle. Methylglyoxal bis(guanylhydrazone) (MGBG) is an inhibitor of SAMDC, introduced in 1972 [35]. Cell culture and other studies were suggestive of target sites and toxicities in addition to the inhibition of SAMDC [36]. A new generation of SAMDC inhibitors was synthesized recently, and one of these compounds, referred to as CGP 48664, appears to be particularly useful as a novel cancer therapeutic agent (fig. 3) [37, 38]. MCF-7 cells treated with 1 mM DFMO or 1 μM CGP 48664 showed comparable levels of growth inhibition [39]. If DFMO was added to the cells with spermidine, growth inhibition could be prevented [7]. Similarly, when spermine was included during the incubation of CGP 48664, growth inhibition was not seen [38]. These results indicate the specificity of DFMO and CGP 48664 in inhibiting the appropriate biosynthetic enzymes. DFMO caused a 50–70% decrease in putrescine and an up to 90% decrease in spermidine levels in MCF-7 cells [34, 39]. However, spermine levels were not significantly altered.

Treatment of MCF-7 cells with CGP 48664 resulted in an $\sim 50\%$ decrease in spermidine and spermine, but a sixfold increase in putrescine level, compared with the control [38, 39]. Inhibition of SAMDC by CGP 48664 in other cell types has also resulted in compensatory increases of putrescine levels [37]. These perturbations in polyamine levels resulted in a retardation of the rate of cell cycle progression in both G1 and S phases of MCF-7 cells [40]. In CHO cells, inhibition of cell cycle by CGP 48664 increased S phase duration [41]. Recent studies also showed that treatment of cells with the polyamine analogue N^1,N^{11}-diethylnorspermine also led to a retardation of S-phase progression [42]. Taken together, these results indicate that polyamines are required at different phases of cell cycle progression. The regulation of polyamine levels during cell cycle and the association of cell cycle arrest to the disruption of polyamine biosynthesis raise the possibility that polyamine pathways might intersect with proteins that regulate cell cycle.

Polyamines and mediators of cell cycle: cyclins, cyclin-dependent kinases and inhibitors

Cyclins were originally named by their unusual pattern of synthesis and degradation during early embryonic cycles of sea urchins and clams [43, 44]. Subsequently, cyclins were identified as the catalytic partners of a class of protein kinases termed cyclin-dependent kinases (CDKs) [45, 46]. Recent studies have revealed CDKs as integral parts of the decision-making points or 'checkpoints' in cell cycle progression through the G1/S phase boundary for duplication of the genome and the G2/M boundary for execution of cell division. Activation of CDKs serves to integrate extracellular signals and intracellular events necessary for proceeding with cell cycle. Generally, signals from growth factors and hormones that cause an acceleration of the cell cycle act by increasing the level of cyclins. Six different cyclin types have been identified in mammalian cells, and these molecules act at specific phases of the cell cycle. In the G1 phase, cyclins D1, D2 and D3, and cyclin E are responsible for activating the appropriate CDKs [47]. For example, cyclin D1 forms complexes with CDK4 and CDK2. Cyclin E forms a complex with CDK2. Cyclin A, and cyclin B1 and B2 form complexes with CDK1 mainly in the S and G2/M phases. Activated CDKs phosphorylate a network of substrates and facilitate cell cycle progression. Immediately after activation of the designated CDK, cyclins are downregulated by proteolytic degradation. In addition, a group of molecules called CDK inhibitors (CDKIs) are involved in blocking the activity of cyclin-CDK complexes and thus breaking the cell cycle progression [46, 47]. Signal transduction processes that alter the synthesis, degradation or activation of these cell cycle regulatory proteins facilitate or inhibit the cell cycle. The critical role of polyamines in cell growth and the cell cycle calls for identification of interaction sites between cyclins, CDKs and CDKIs with polyamines.

We first examined the effect of polyamine depletion on cyclin B1 messenger RNA (mRNA) and protein levels in MCF-7 breast cancer cells [48]. Ectopic expression of mitotic cyclins has been reported previously in breast cancer cells [49]. We found high levels of cyclin B1 in G1-synchronized cells, in addition to its presence in the mitotic phase. Other investigators have found that degradation of the mitotic cyclins is required for induction of G1 cyclins [50]. Our results showed that DFMO prevented estradiol-stimulated degradation of cyclin B1 in early G1 phase. Stabilization of cyclin B1 mRNA was found in MCF-7 cells treated with 0.5 and 1 mM DFMO. Addition of putrescine or spermidine could reverse the effect of DFMO on cyclin B1. These results show that polyamines are required for the degradation of cyclin B1 and suggest pathways for cell cycle retardation by DFMO.

In the G1 phase, cyclin D1 is a major mediator of cell cycle progression of MCF-7 cells [47, 51–53]. The link between polyamine levels and cyclin D1 was examined by using a bis(benzyl)spermine analogue, $C_6H_5CH_2NH(CH_2)_3NH(CH_2)_7NH(CH_2)_3NHCH_2C_6H_5$ [54]. Treatment of cells with this polyamine analogue caused a 43, 38 and 45% decrease in putrescine, spermidine and spermine levels, respectively [54]. This decrease in polyamine levels was associated with a decrease in cyclin D1 levels and cell cycle arrest in the G1 phase. Furthermore, our studies showed diverse effects of DFMO and CGP 48664 on the level of cyclin D1 in MCF-7 cells progressing in the cell cycle [40]. In cells treated with DFMO, there was a 50% reduction of cyclin D1. In contrast, a twofold increase in cyclin D1 occurred in cells treated with CGP 48664. This is consistent with the elevation of putrescine levels in cells treated with CGP 48664, suggesting the regulation of cyclin D1 by putrescine. Evaluation of the effects of DFMO and CGP 48664 on cyclin E showed that DFMO had no effect on cyclin E, whereas CGP 48664 caused a 50–60% decrease in cyclin E protein levels [40]. This result supports a selective role for putrescine and spermidine in the regulation of cyclin D1 and cyclin E, respectively. An increase in putrescine alone was unable to satisfy the polyamine requirement of cell cycle progression, even though there was a significant increase in cyclin D1. Activation of specific CDKs by cyclin D1 and cyclin E appears to be necessary for the progression of MCF-7 breast cancer cells through the G1 phase.

While the acceleration of cell cycle is mediated by cyclins, cell cycle inhibition is often mediated by specific proteins that inhibit the activity of CDKs. Recently, Kramer et al. [55] found that polyamine analogues induce growth arrest of melanoma cells with an increase in the expression of p21, an inhibitor of CDK. Polyamine analogues offer an alternative to inhibitors of polyamine biosynthesis in altering intracellular polyamine levels. Several polyamine analogues have antitumor effects in experimental models of melanoma, lung cancer and breast cancer [56–59]. One of the pathways for induction of their action in melanoma cells involves the upregulation of the tumor suppressor gene product p53, and induction of the CDK inhibitor p21 [55]. Interestingly, polyamine analogues are also active in p53-negative cell lines, indicating the interaction of polyamines with several targets in the cell death pathway. Our studies on the role of polyamines in epidermal growth factor (EGF)-induced apoptosis of MDA-MB-468 breast cancer cells also illustrate an inverse relationship between polyamine levels and induction of p21 [60]. Thus, the mechanism of the antiproliferative action of polyamine analogues and biosynthetic inhibitors includes disruption of the func-

tions of cyclin D1, cyclin E, cyclin B1 and/or the induction of the CDK inhibitor p21. Molecular mechanisms by which these gene expression patterns are regulated by polyamines need to be elucidated by further research.

Polyamines and apoptosis

Polyamines have paradoxical roles in inducing apoptosis and in its prevention. Early studies by Brune et al. [61] showed that apoptosis induced by the Ca^{2+} mobilization agent ionomycin could be prevented by spermine. This effect may be, due at least in part, to polyamine action to counter calcium mobilization in T cells [62]. Other target site interactions are also likely to be involved since dexamethasone-induced apoptosis in thymocytes could be inhibited by the addition of polyamines [63]. In the dexamethasone-induced apoptosis of thymocytes, a decrease in cellular polyamine levels occurred in spite of an induction of ODC [64]. Thus, decrease in polyamine levels due to catabolism may also be involved in regulating polyamines and driving cells to the apoptotic pathway. The protective role of polyamines in apoptosis is consistent with their growth-stimulatory effects and promotion of the cell cycle, so that increased levels of polyamines appear to drive cells into the proliferative pathway.

In contrast to the protective role of polyamines in apoptosis, it was found that the ODC gene is actively involved in apoptosis induced by overexpression of c-Myc [65]. Like ODC, c-Myc is also a double-edged sword, playing important roles in cell proliferation and apoptosis [66]. Enforced expression of ODC, like c-Myc, was sufficient to induce accelerated cell death following IL-3 withdrawal from murine myeloid cells [65, 66]. ODC-induced cell death was dose dependent and amenable to inhibition with DFMO [66]. In this context, ODC was a mediator of c-Myc-induced apoptosis since c-Myc is a potent transactivator of ODC. Studies using reporter constructs showed that ODC was regulated by c-Myc protein at the level of transcription initiation [66].

An important pathway in polyamine-induced apoptosis is the oxidation of spermidine and spermine either by serum amine oxidase (copper containing) or by the intracellular FAD-dependent polyamine oxidase [24]. H_2O_2 and aminoaldehydes produced in these reactions are strong inducers of apoptosis [22, 23]. The role of H_2O_2 in polyamine analog-induced apoptosis was indicated when coaddition of catalase with polyamines or analogues inhibited apoptosis [22, 23]. Use of a specific inhibitor of FAD-dependent polyamine oxidase, MDL 72,527, has also helped to resolve the role of amine oxidases in provoking apoptosis [67–69]. Results of these studies and recent results from a spermidine/spermine acetyl transferase (SSAT) overproducing transgenic mice [25] indicate that polyamines and/or their analogues do not need to be oxidized for the induction of apoptosis, at least in some cell types. Excessive accumulation or depletion of polyamines may disrupt many cellular functions, including DNA-protein interactions [70], protein-protein interactions [39] and mitochondrial integrity [71], leading to apoptosis. In addition, cell types that undergo apoptosis in the presence of ODC overexpression might be highly sensitive to apoptosis. In other cases, a response to ODC overexpression is the transformation of the cell to a malignant phenotype [72, 73].

Recent studies also reveal another pathway by which the accumulation of putrescine induces apoptosis [74, 75]. Formation of hypusine, in which the butylamine moiety of spermidine is transferred to the lysine residue in eukaryotic initiation factor (eIF-5A), appears to be an important part of the normal function of spermidine. In the presence of excess putrescine, there was an inhibition of hypusine formation and a decrease in modified eIF-5A. The lack of hypusine along with other functional abnormalities caused by accumulation of putrescine led to apoptosis in a hepatoma cell line selected for DFMO resistance. The generality of this phenomenon in other cell types remains to be determined.

In spite of these examples of ODC- and polyamine-induced apoptosis, the major role of polyamines in most cell types is to stimulate cell proliferation. Supporting this general proliferative role of polyamines, ODC overexpressing transgenic mice were susceptible to carcinogen-induced skin tumor formation without the administration of a tumor promoter [76, 77]. Considering the numerous genes involved in facilitating cell cycle progression and cancer phenotypes, the ability of polyamines to regulate gene expression is likely to be a large component their function. Changes in gene expression might be due to direct effects on DNA-protein interactions [70, 78, 79] or indirect effects due to modulation of signal transduction pathways [80, 81]. There have been extensive physical chemical and biochemical studies on the consequences of polyamine-DNA interactions, including modulation of protein-DNA interactions.

Molecular mechanisms: polyamine-DNA interactions

One of the early discoveries about polyamine-DNA interactions was the observation that polyamines could stabilize double-stranded DNA [82]. Putrescine, spermidine and spermine can increase the melting temperature (T_m) of DNA in a concentration-dependent manner by as much as 40 °C in low salt (10 mM Na^+/K^+)

buffers compared with that in the absence of polyamines [82, 83]. Since DNA is a polyelectrolyte with negative charges on the phosphate groups, a major factor in polyamine-DNA interactions is electrostatic in origin [84]. In addition to this charge neutralization, polyamines interact with nucleic acid bases, dock into the major or minor grooves, and enter into multisite interactions depending on the ionic environment [85]. At physiologically compatible ionic conditions (150 mM NaCl), increases in T_m are of the order of 10–20 °C at 1–3 mM concentrations of spermine [82–85]. Since the physiological concentrations of polyamines are in the millimolar range, polyamines are believed to play a significant role in stabilizing the DNA structure. Indeed, a recent immunofluorescence study using an antibody specific to spermine (SPM-2) showed intense staining of the nuclei of mitotic cells, indicating the accumulation of spermine on the chromosomes of mitotic cells [86].

Another consequence of polyamine binding to DNA is the condensation of DNA [84, 87–89]. Polyamine-induced condensation of DNA is part of the mechanism by which 1-m-long phage DNA is condensed into the phage head in the absence of histones. Monomolecular condensation of large DNA molecules and multimolecular condensation of plasmid-size or smaller DNA (>400 bp) has been observed in dilute solutions using laser light scattering and electron microscopic investigations [84, 87–89]. Polyamine-induced condensates show toroidal shaped or rodlike molecules when examined under the electron microscope [87, 88]. Liquid crystalline packing of mononucleosomal size DNA has been reported recently, with an interesting phenomenon associated with the precipitation and resolubilization of DNA in the presence of high concentrations of polyamines [90, 91]. In eukaryotes, the structural organization of chromatin includes histones, nonhistone proteins as well as polyamines. Immunocytochemical study of spermidine and spermine during the cell cycle showed that these polyamines were associated with highly compacted mitotic chromosomes [86, 92]. These studies indicate that polyamine-induced DNA condensation is important to the cellular functions in vivo.

Another interesting phenomenon associated with polyamine-DNA interactions is the ability of certain DNA sequences to undergo conformational transitions in the presence of polyamines [93, 94]. The right-handed B-DNA can be converted to a left-handed Z-DNA in the presence of a micromolar concentration of polyamines [94, 95]. Z-DNA is induced mainly in blocks of alternating purine-pyrimidine sequences and is believed to play a role in transcriptional control [95]. The structures of B-DNA and Z-DNA have distinct features in that the phosphate groups are more exposed in Z-DNA; there are 12 bp per turn compared with 10 bp per turn in B-DNA; and the major groove of the B-DNA is replaced by a shallow groove [95–97]. These changes would affect the reactivity of DNA to proteins, carcinogens and other ligands. X-ray crystallographic evidence shows tight binding of spermine to DNA in the Z-DNA conformation [98]. Spermine winds around the DNA with ionic contacts on both the phosphate charges as well as hydrophobic contacts with the bases [98]. These different modes of interactions provoke Z-DNA formation at a lower concentration than inorganic cations such as Mg^{2+}. Unlike polyamines, Mg^{2+} is a point charge and contacts through hydrating water molecules are continuously exchanged with the solvent [99].

Recent studies also show that spermine induces conformational transitions in a short (41-mer) oligonucleotide containing binding sites for specific regulatory proteins [100, 101]. We studied an oligonucleotide harboring the estrogen response element (ERE), the DNA sequence present in the upstream regions of estrogen-responsive genes. Circular dichroism (CD) spectroscopic studies showed spermine-induced conformational changes of this oligonucleotide, indicative of the A-DNA form [100]. Spectral features of A-DNA have been reported in calf thymus DNA in the presence of polyamines [102]. However, spermidine and putrescine were unable to induce spectral characteristics of A-DNA in ERE. A control oligonucleotide lacking the ERE sequence also did not undergo this change, indicating that certain gene-regulatory segments of DNA are particularly sensitive to polyamine-induced changes in conformation.

DNA bending is another mechanism by which polyamines may intervene in the recognition of gene regulatory proteins by their response elements [103, 104]. Polyamines promote DNA bending by neutralizing the negative charges on DNA phosphate, reducing the energy requirement for bending, and thus facilitating enhanced protein-DNA interactions. Molecular modelling studies have illustrated the versatility of natural and synthetic polyamines in bending DNA [104]. Recently, Rouzina and Bloomfield [105] proposed a mechanism by which small multivalent cations induce DNA bending by binding at the entrance to the B-DNA major groove between the two phosphate strands, thus repelling sodium counterions from the neighboring phosphates, which then get strongly attracted to the groove-bound cation leading to groove closure and DNA bending around the cationic ligand. Indeed, DNA bending itself is a major pathway for transcriptional regulation of gene expression [106]. In bacteria, promoter geometry becomes a key regulatory element, allowing cells to sense metabolic changes [107]. In higher organisms, cooperative bending produced by multiple transcription factors produce the required response in transcription. The versatility of polyamines in bending

DNA may work in concert with transcription-factor-induced bending in facilitating transcription [108].

Effects of polyamines on protein-DNA interactions

A potential target of polyamines in gene regulation is at the site of transcription factor binding to DNA. Transcription factor binding to DNA and DNA bending are related processes, since many DNA binding proteins exert their action by their ability to bend DNA [109]. This is particularly important for proteins involved in transcriptional initiation, including RNA polymerase II [110], TATA binding protein (TBP) [111], and transcription factors such as ER [112] and NF-κB [113]. Recent studies on transcription-induced stress in circular DNA show that the stiffness and bending induced by the interaction of polyamines within the major/minor grooves of DNA could not be mimicked by other polycations: polylysine, polyarginine or histones [114].

A model system we used for studies on the role of polyamines in DNA-protein interactions is the estrogen receptor (ER) and its response element, ERE. The effect of spermine on ER-ERE interactions was demonstrated by electrophoretic mobility shift assay as well as a competitive displacement assay [78, 115]. MCF-7 cellular ER, rabbit uterine ERα as well as purified recombinant ERα were used in these assays [115, 116]. A two- to threefold increase in the level of ER-ERE complex formation was observed in the presence of 100–500 μM spermine. At 1000 μM spermine, however, there was a decrease in ER-ERE complex formation, indicating that polyamines may act as regulatory factors in modulating DNA-protein interactions.

We also examined the effect of polyamines on the binding of the transcription factor NF-κB (nuclear factor κB) to its DNA response element (NRE). Our results showed a sixfold increase in the binding of NF-κB to NRE in the presence of 1000–2000 μM spermine [70]. Spermidine and putrescine were less effective, increasing the binding by two- to threefold at 1000–2000 μM concentration. We used cellular extracts prepared from MCF-7 cells for these studies. Antibody super-shift assays were used to identify the proteins involved. Among the NF-κB family of transcription factors, antibodies specific to p65, p50, c-rel and p52 were used. Antibodies specific to p50 showed a supershift, indicating the presence of p50 as a the major component of the protein-DNA complex formed in the presence of spermine. Presence of p65 and c-rel was also indicated because of a partial inhibition of NF-κB binding to NRE in the presence of these antibodies [70]. Experiments using a reporter gene containing NRE showed that polyamine-induced increase in NF-κB-NRE interactions resulted in increased transactivation in the presence of spermine [70].

Pangiotides et al. [117] examined the effects of polyamines on the interaction of several gene-regulatory proteins, including basic-helix-loop-helix/leucine zipper (the adenovirus major late transcription factor, transcription factor E3), basic/leucine zipper (Ig/EBP, NF-IL6), zinc finger (YY1) and helix-turn-helix (Oct-1, λ repressor) proteins. The majority of these proteins showed increased binding in the presence of polyamines, although the concentration-dependence of the response differed. However, the binding of Oct-1 to its response element was inhibited by the presence of polyamines [117]. Thus, the extent of polyamine-induced stimulation of DNA-binding activity was dependent on the particular protein involved. On the other hand, the reduction of cellular polyamine levels led to a decrease or increase in the DNA-binding activity of activator protein (AP-1) transcription factors, depending on the subunit proteins involved [118, 119]. These results are consistent with our observation of the ability of polyamines to modulate ER-ERE and NF-κB-NRE interactions and suggest that the regulation of intracellular polyamines may have a large role in the dynamics of gene regulation.

If one considers the function of DNA as the fundamental determinant in the choice between cell growth and cell death pathways, the ability of polyamines to alter DNA function will be critical to this ultimate choice. Polyamine-induced DNA conformational changes, sequence-specific interactions and DNA bending are salient features that modulate the interaction of transcription factors with DNA and thus control the expression of a network of genes. If the expressed network of genes forms part of the cascade of growth-stimulatory response, an enhanced rate of cell cycle progression and cell growth can be expected. Alternatively, in the presence of polyamine analogues or metabolic inhibitors, decreased expression of growth-stimulatory genes or increased expression of genes in the cell death pathway can force cells to the choice of cell death. In addition, specific associations between transcription factors, coactivators and the transcription machinery appear to be sensitive to polyamine action [39]. Similarly, interactions between mediators of cell death, loss of the integrity of mitochondria [71] as well as activation of caspases [120] might be part of cell death induced by excessive accumulation or depletion of polyamines under specific cellular contexts. Whereas extreme accumulation or depletion of polyamines in an organism can lead to toxicity, understanding the defects in polyamine homeostasis in cancer cells will be useful in specifically reverting the defects by therapeutic interventions. Furthermore, cell growth and cell death pathways modulated by polyamines can be expected to work in concert with other elements of signal transduction, such as kinase cascades, redox signalling, glutathione levels, ex-

pression of antioxidant genes, the level of Ca^{2+} and so on. Our knowledge of the interplay of different signal transduction pathways is limited, and extensive research is needed to establish the hierarchy of signalling processes in cell death and cell growth and the specific role of polyamines in these processes.

Polyamine transport and ODC antizyme

In recent years, it has become clear that a highly versatile transport system is involved in regulation of cellular polyamines, in addition to the elaborate biosynthesis and degradation pathways [18, 121–123]. Hormones and growth factors regulate the efficiency of the transport system so as to complement the changes in the levels of biosynthetic enzymes [121]. Interestingly, transport is also coupled with a protein known as antizyme which binds to ODC and accelerates its degradation, in response to abnormally high accumulation of polyamines in the cell [123–125]. The induction of antizyme negatively regulates polyamine uptake [124]. The first antizyme gene cloned in 1992 by Hayashi et al. [123] is now termed antizyme 1. A peculiar feature of antizyme 1 is that coding sequences are in two different reading frames, and ribosomes need to shift reading frames to synthesize the functional protein [126, 127]. The efficiency of ribosomal frameshifting is sensitive to polyamine levels, and therefore it acts as a sensor for autoregulation of the antizyme. A second antizyme gene was cloned which also requires ribosomal frameshifting for function [128]. Antizyme 2 mRNA is reported to be less abundant than that of antizyme 1, but both are present in most mammalian tissues tested. Recently, a third antizyme was discovered, and this gene product also appears to have additional functions on polyamine homeostasis during spermatogenesis [129]. Further studies on the roles of antizyme molecules in the tumorigenesis process may provide insights into the basis of paradoxical results of ODC gene overexpression in different cell types.

Effect of polyamines on ligand-receptor interactions

Polyamines can also modulate ligand-receptor interactions and function. For example, the biological action of estradiol is mediated by the interaction of the ligand, with its specific receptor, ER. The stability or dissociation rate of the ligand-receptor complex is important in the potency of estrogenic action [130, 131]. The dissociation rate was determined by adding excess unlabelled steroid to disturb the equilibrium between free receptor, free hormone and the bound ligand-receptor complex. In the presence of polyamines, the rate of dissociation of estradiol from the ligand-receptor complex decreased [132]. The biological consequence of this result is that estradiol-bound ER will be available for a longer time for transcriptional stimulation of responsive genes.

The effect of polyamines on other ligand-receptor systems has also been demonstrated. Polyamines alter the binding of ligands to excitatory amino acid receptors, namely *N*-methyl-D-aspartate (NMDA) receptors [133–135]. The binding of MK801, an antagonist of the NMDA receptor, was increased in the presence of polyamines. Spermine and spermidine produced biphasic dose-response curves for binding in rat brain membrane NMDA receptor, with low concentrations (< 100 microM) enhancing [^{125}I]-MK-801 binding and higher concentrations (> 100 microM) inhibiting the binding. Furthermore, the polyamine binding sites of NMDA receptor were heterogeneous in human cerebral cortex, and show a high degree of regional and individual variability, reflecting differences in ion channel activity [135]. Consequences of polyamine function at these neural cell receptors may include an analgesic effect due to polyamine deprivation [136]. A comprehensive review of the role of polyamines in neurochemistry was recently published [14].

Structural specificity and therapeutic implications

The structural specificity of polyamines arises from the participation of the methylene carbon chain in its interaction with biomacromolecules. Therefore, electrostatic interactions between the positive charges of the polyamine and the negative charges of the biomacromolecule are inadequate to describe the system. The structural requirement of spermidine in supporting cell proliferation was first reported by Porter and Bergeron [8], although the target sites were not identified. If DFMO-treated cells are supplemented with spermidine, they resume growth, but spermidine homologues with a greater than two-carbon extension compared with spermidine had decreased ability to rescue cells form the effect of DFMO [8]. The cellular uptake of polyamines is mediated by transport proteins or putative receptors, and these sites appear to have specific affinities for natural polyamines [137–139]. Consequently, polyamine analogues with minimal changes in the carbon chain compared with the natural polyamines are taken up more efficiently than those with more dramatic changes [8, 59].

In addition to the specificity of polyamine transport, a number of target site interactions have structural requirements. For example, the B-DNA to Z-DNA transition [94], interaction of transcription factors with their response elements [115] and DNA bending [59, 104] are highly dependent on the distance between primary and secondary amines in the polyamine analogues. How-

ever, interactions involving duplex DNA stabilization by polyamines are not altered dramatically by changes in the methylene bridging region [82, 83, 140]. The reason for this lack of structural specificity is that electrostatic interactions between polyamines and DNA phosphates are the dominant force in stabilizing duplex DNA. These differences in structural specificity of polyamine functions might be useful for developing polyamine analogues for cancer therapy. It is to be expected that the cationic requirements of the cell might be met by the presence of certain polyamine analogues in the cell, whereas specific gene-regulatory effects may not be replaced by these analogues. This possibility, in conjunction with the occurrence of a higher level of polyamines in cancer cells compared with normal cells, makes them important targets for cancer therapy [56–59, 141].

Studies on several experimental models of cancer have yielded promising results on the antitumor effects of polyamine analogues and inhibitors of polyamine biosynthesis [6, 56–59, 141, 142]. However, the window of opportunity for low toxicity and high efficacy is narrow because of the multiple roles of polyamines in the structural organization and function of normal cells [143]. Creative clinical trial designs with an appreciation of the target site interactions and combinations with conventional therapy will be necessary to bring polyamine analogues or metabolic inhibitors to the benefit of the patient [144–146].

Another possible application of polyamine analogues in cancer therapy may be that these analogues and related compounds can be used as condensing agents to package genes of interest for efficient transfection into living cells [147–149]. Currently, viral and lipid-based transfection of genes and oligonucleotides is emerging as an important mode of gene therapy for a variety of diseases. Oligonucleotide therapeutics come under two categories, antisense oligonucleotides targeted to segments of mRNAs or triplex-forming oligonucleotides targeted to inhibit the expression of specific genes [150, 151]. We found that a hexamine analogue of spermine was highly efficient in facilitating the uptake of oligonucleotides into the cells [152]. The facilitation of cellular uptake of oligonucleotides was also sensitive to the polyamine structure. Moreover, hexamine analogues were efficient in stabilizing the triplex DNA as measured by electrophoretic mobility shift assay and T_m studies [153]. Despite the lack of structural specificity of polyamines in duplex DNA stabilization, polyamine interactions with triplex DNA are highly structure specific [140, 153]. Thus, certain polyamine analogues may be useful in oligonucleotide delivery as well as triplex DNA stabilization.

Role of ODC overexpression in carcinogenesis

A possible role for ODC in carcinogenesis has been suggested by a series of studies by O'Brien et al. using a skin-tumor model initiated by a carcinogen (initiator) and promoted by phorbol ester [154, 155]. Studies on the structure-activity relationships of tumor promoters in conjunction with stimulation of ODC activity indicated an intimate relationship between tumor promotion and ODC [155]. More recently, the overexpression of ODC in NIH 3T3 cells caused transformation of these cells to a malignant phenotype, in essence qualifying ODC as an oncogene [72, 73]. However, transgenic mice overexpressing the ODC gene did not show increased levels of spontaneous tumorigenesis and were able to maintain normal levels of polyamines in their tissues [156, 157].

Studies of the metabolic flux of polyamines in transgenic mice fibroblasts indicated that polyamine flux is faster in these fibroblasts compared with fibroblasts from normal mice [157]. On the other hand, overexpression of ODC cooperated with Ha-ras oncogene in epithelial tumorigenesis [158, 159]. Thus ODC is one of the most important genes whose overexpression or aberration contributes to the process of carcinogenesis. Taken together with the experimental model where ODC overexpression leads to apoptosis, the function of ODC and polyamines seems to be paradoxical. However, it is possible that cells undergoing transformation with ODC overexpression might have multiple abnormalities in processing excessive levels of polyamines. For example, in the transgenic mouse model of familial adenomatous polyposis, elevation of the expression of the ODC gene was accompanied by a decrease in the expression of antizyme 1 gene, contributing to the increase in polyamine levels [160]. Furthermore, although tumor cells and tissues are reported to have increased polyamine levels compared with normal cells, this increase is often in the range of two- to threefold [161–163]. Putrescine levels in cells undergoing apoptosis are approximately 10-fold higher than those present in cancer cells [75, 164]. Thus, the magnitude of polyamine increase, coupled with alterations in oncogenes and components of the apoptotic pathway [165], might determine different cellular responses to ODC overexpression.

Potential for chemoprevention

The role of ODC in tumor promotion and the relatively low toxicity of DFMO support the exploration of DFMO as a chemopreventive agent [166]. The inability of DFMO to reduce spermine levels may be the key to its low toxicity so that cellular polyamine pools are not completely depleted. Although millimolar concentra-

tions of DFMO are needed to inhibit ODC, clinical trials have now demonstrated that ODC inhibition can be achieved at nontoxic doses [167, 168]. Early results on cervical cancer and colon cancer support the use of this agent in high-risk patients [168, 169]. Cancer syndromes with one or more identified defects in polyamine metabolism or uptake should be particularly amenable to chemoprevention [160]. However, it is to be noted that cells are able to take up polyamines from the diet and from the polyamines produced by the intestinal mucosa and bacteria [170]. Thus, complete control of polyamines may require a polyamine-free diet and inhibition of uptake from gastrointestinal sources. This type of experimental design on an MCF-7 xenograft model indicates that DFMO could deplete all three natural polyamines and reduce tumor growth [170].

Conclusions

Often, evidence on the cellular function of polyamines is complicated by the fact that they have multiple functions. In addition, cellular polyamines are regulated at several levels: synthesis, degradation, uptake and efflux. ODC protein has a rapid turnover rate, and polyamines have a positive regulatory effect on that process. Polyamine transport as well as ODC degradation are also controlled by a family of proteins called antizymes. Cells take up exogenous polyamines when they are ready for growth. Similarly, ODC, SAMDC and SSAT are induced by external stimuli at the transcriptional level, regulated by polyamines at the translational level, and positively regulated at the level of degradation. Numerous mitogenic and hormonal signals are known to induce ODC, illustrating the complexity of polyamine control and function. This exquisite regulation permeates multiple signal transduction pathways as well as choices of cell cycle progression, differentiation or cell death. Studies using the overexpression of ODC has resulted in disparate results on the role of ODC in carcinogenesis and apoptosis. These differences may be either due to cell-type-specific differences in the polyamine uptake/efflux pathway or to differences in the sensitivity to apoptosis. Knowledge of the threshold of switch in their functions from cell growth to apoptosis and the cellular context of polyamines facilitating cell death versus transformation would be particularly useful for drug development. The crosstalk between polyamine signal and hormonal/growth factor signal transduction pathways needs to be elucidated in future studies. Despite these complexities, polyamine biosynthetic inhibitors and polyamine analogues offer many opportunities to control cancer, a disease involving abnormalities of cell growth and cell death.

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