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REGULATORY ROLES OF POLYAMINES AND ORNITHINE DECARBOXYLASE IN THE GROWTH OF CANCER CELLS

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ABSTRACT
Polyamines play an important role in cellular proliferation. These major amine molecules are natural and biologically active inside and outside the cells, which include putrescine, spermidine, spermine and their metabolites such as N-acetyl spermidine and N-acetyl spermine. The rate limiting step of polyamine synthesis is the decarboxylation of ornithine to form putrescine by ornithine decarboxylase (ODC; EC 4.1.1.17). Increases in ODC activity are one of the early changes observed in cells stimulated to grow and these increases precede changes in DNA synthesis by several hours, making ODC an 'immediate early' response gene. ODC activity has been found to be regulated at several levels, to include the formation of antizyme and antizyme inhibitors as ODC regulatory molecules. This review takes a look into the roles of polyamines and ODC as the regulators of cellular growth and profileration. Their roles in the development of cancer are also discussed in detail.

KEYWORD: polyamines, ornithine decarboxylase, antizyme, antizyme inhibitor, cancer.

INTRODUCTION

For some time now, it has been recognized that polyamines play important roles in the growth and proliferation of mammalian cells and cancer cells. These major amine molecules are natural and biologically active inside and outside the cells, which include putrescine, spermidine, spermine and their metabolites such as N-acetyl spermidine and N-acetyl spermine. The three major polyamines in mammalian cells are synthesized in sequence from ornithine to putrescine to spermidine to spermine.[1] Each of them is small, straight chain aliphatic water-soluble carbon-nitrogen molecules with the amino groups evenly distributed throughout.[1,2] The rate limiting step of polyamine synthesis is the decarboxylation of ornithine to form putrescine by ornithine decarboxylase (ODC; EC 4.1.1.17).[3,7] This is followed by the decarboxylation of S-adenosylmethionine to decarboxylated S-adenosylmethionine by the enzyme S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50). Decarboxylated S-adenosylmethionine is then combined with putrescine to produce spermidine, an enzymatic step catalyzed by spermidine synthetase (EC 2.5.1.16).[4-7] Another decarboxylated S-adenosylmethionine is then added to spermidine by spermine synthetase (EC 2.5.1.22) to produce spermine. The spermidine and spermine can be catabolized by one specific enzyme, spermidine/spermine N1-acetyltransferase (SSAT; EC2.3.1.57) to become N-acetyl-spermidine and N-acetyl- spermine, respectively.[4-6] The N1-acetyl polyamines are then the preferred substrates of FAD-dependent polyamine oxidase (EC1.5.3.11), which converts N-acetyl-spermidine to putrescine and N-acetyl-spermine to spermidine, respectively.[1-7] Although the precise physiological functions of these amines have not been defined, polyamines have been proved to be essential for growth, proliferation and differentiation of virtually all eukaryotic and prokaryotic cells. As such, increases in polyamine biosynthesis via the regulation of ODC usually accompany the increases in cell growth in response to hormones and growth factors.[2,3] In addition to biosynthesis, factors which influence the regulation of intracellular polyamine levels including turnover, uptake into the cell and efflux of these positively charged amines from the cell.[3-6] As such, polyamines are believed to function within the cell by virtue of their high positive charges, which allow them to tightly bind to intracellular anionic macromolecules such as DNA, RNA or membrane phospholipids.[1] This review discussed their role in regulating cellular growth and proliferation related to cancer.

Polyamines roles in the regulation of cellular proliferation

Polyamines play an important role in cellular proliferation. In non-growing cells, the concentration of free polyamines has been found to be low, but as cells enter the DNA synthesis followed by proliferation cycle, a significant rise in polyamine levels has been observed.

The amount of polyamines and the activities of polyamine-synthesizing enzymes, ODC and SAMDC, increased rapidly in late G1 and in the transition of G2 to mitosis.[7] In the S- phase and mitosis, polyamine catabolism by SSAT overdrive the synthesis rate.[7] Polyamine levels in fact regulate the cell proliferation progression. Fluctuations of polyamines occurring in the proliferation cycle are related to temporal activation of cyclins and cyclin-dependent kinases, especially the complexes of cyclin E/cdk2 and cyclin A/cdk2.[8] It has been shown previously that activation of mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways are one of the early signals in mitogenesis, and these regulatory proteins upregulate the transcription and activation of ODC.[9] This stimulation is performed through the activation of the transcription factor c-Myc which binds to specific responsive elements in the ODC promoter region. The resulting elevations of ODC activity and polyamine concentration increase the growth signal by upregulating immediate early genes c-Myc, c-Fos and c-Jun expressions. Difluoromethylornithine (DFMO), an inhibitor of ODC, has been found to deplete cellular polyamines causing a cell cycle block in G1/G0 phase by dysregulating the expression of the above-mentioned transcription factors and cell cycle inhibitors p53, junD/AP-1, p27Kip1, p21Waf1/Cip1 and TGF-β.[10-12]

ODC as a regulatory enzyme

ODC is in itself an interesting enzyme with several novel regulatory features. It is a highly inducible, cytosolic, subunit enzyme that responds to a range of trophic stimuli.[13] It has a short half-life (10 min–1 h) compared with many mammalian enzymes whose half-lives are more often expressed in days.[14] ODC requires pyridoxal phosphate as a cofactor and thiol-group reducing agents are necessary for enzyme activity, possibly owing to the high number of cysteine residues in the protein. ODC contains two PEST (proline-, glutamate-, serine- and threonine-rich) regions that are rich in proline, glutamic acid, aspartic acid, serine and threonine.[15] The PEST region located at the C-terminus of ODC is essential for the degradation of the enzyme, and truncations and mutations in this region result in stabilization of the enzyme.[16] ODC activity is dependent upon the formation of a dimer with the active site, occurring at the interface between the two subunits.[17] Residues at the active site which are critical to ODC activity including Lys169 and His197.[18] ODC expression is also regulated by transcription, stability and the efficiency of translation of the mRNA. At a transcriptional level, ODC expression can be regulated by oncogenes. The hODC gene contains three CACGTG regions: one at the 5 promoter region and two others in intron 1[19] that bind the protein product of the c-Myc oncogene.[20] Overexpression of c-Myc and other oncogenes such as v-mos, Ha-Ras, and c-Fos.[21-23] can lead to overexpression and induction of ODC and, ultimately, carcinogenesis. ODC mRNA has long of 5' and 3' untranslated regions (UTRs) and, whereas neither region seems to be involved in polyamine-mediated feedback control of ODC activity.[24] The 3 UTRs may have a role in regulation under special circumstances, such as hypotonic shock.[25]

Increases in ODC activity are one of the early changes observed in cells stimulated to grow and these increases precede the changes in DNA synthesis by several hours, making ODC an 'immediate early' response gene.[26] ODC is subject to both positive and negative feedback regulation by polyamines: high polyamine concentrations decrease and low polyamine concentrations increase, activity. The feedback regulation appears to be a mixture of post-transcriptional regulation and the induction of a unique ODC-specific inhibitor termed 'antizyme' (AZ).[27]

Antizyme regulation of ODC

Antizymes (AZs) are proteins which sequester monomeric ODC molecules. AZs prevent the dimerization and formation of enzymatically active ODC.[28] Binding of AZ leads to conformational changes in ODC and exposure of the C-terminal PEST sequence that provokes translocation to the 26S proteasome for degradation without ubiquitination.[29] AZ itself is not degraded together with ODC, but is recycled back to the cytoplasm. Due to antizyme-induced degradation, the half-life of ODC is 10–20 min, which is one of the shortest known for proteins in mammalian cells.[28,29] An increased polyamine content protects AZ from ubiquitin-mediated degradation and enhances AZ expression by affecting the rate of ribosomal frame-shifting.[30] AZ1 contains also a mitochondrial targeting motif. It is transported to the mitochondrial membrane where it depolarizes the membrane and activates the caspase cascade leading to the induction of apoptosis.[31] Over-expression of AZ1 inhibits cell proliferation and growth via ODC inhibition and reduction of the polyamine content.[28,29] AZ can be considered a tumor suppressor. Depletion of AZ in cultured cells leads to over-duplication of centrosomes, whereas the silencing of antizyme inhibitor (AZI) reduces centrosome abnormalities.[32] These data suggest that AZ and AZI are connected to the early stages of carcinogenesis in which the loss of tumor suppressors triggers defects in centrosome functioning. Recent data suggests that antizyme is not solely a regulator of ODC but also controls the degradation of other proteins that participate in growth regulation, e.g. cyclin D1, Smad1 and Aurora-A.[33]

AZIs have arisen from ODC by gene duplication and thus share a high degree of sequence similarity with ODC.[28,29] Due to the homology, AZIs bind AZs, with even higher affinity than ODC and thus liberate ODC from the heterodimer complex with AZ resulting in the formation of active homodimers and increased ODC activity.[32] The binding of AZI to AZ also blocks the inhibition of polyamine transporters mediated by AZ, and the uptake of polyamines is enhanced.[33] The binding of AZ to AZI or ODC is reversible, and the

equilibrium is constantly monitored and adjusted by the concentration of polyamines. In biochemical assays, AZI binds all known AZs, AZ1-3.[35] AZI remains a monomer under physiological conditions and it is unable to bind the cofactor, pyridoxal-L-phosphate, which is needed for the enzymatic activity of ODC.[36]

The transcription of AZI is increased by growth stimuli prior to induction of ODC. During the cell cycle, AZI is activated similarly to ODC in late G1 and again in G2/M, and during mitosis (M) it is located in the centrosome analogously to AZ.[37] The growth-promoting activity of AZI may not be solely dependent on the neutralization of AZ, since AZI has been demonstrated to stabilize cyclin D1 independently of the AZ-binding. The expression of AZI1 is elevated in human gastric cancer compared to normal gastric tissue as well as in ras-transformed fibroblasts.[38] AZI also promotes the survival of various types of cancer cells via activation of ODC under hypoxic conditions.[39] In carcinogenesis, activation of ODC is considered to be an early step in malignant transformation. It has, however, been postulated that the activation of ODC might actually proceed via induction by AZI.[38] ODC activation itself leads to a rapid increase in the amount of AZ, leading to the reciprocal diminution of ODC activity and polyamine uptake. In contrast, the activation of AZI blocks AZ and promotes cell growth via sustained polyamine accumulation.

Compartmentalization of ODC

Intracellular compartmentalization of polyamines is functionally of great importance, since polyamines participate simultaneously in various cellular functions in the nucleus, mitochondria, plasma membrane, secretory vesicles and cytoplasm.[1] Investigations on the localization of ODC have proven difficult due to its extremely short half-life, and thus minute amounts of detectable protein. On the other hand, problems have been encountered in determining the compartmentalization of polyamines with the larger pool of bound polyamines compared to freely recruitable ones.[2,3] Polyamines take part in the modulation of various cellular signalling cascades for which they are either synthesized *de novo* or transported into extracellular spaces.[2-6] Polyamines bound to nucleic acids and proteins are considered rather inactive in the event of the rapid recruitment for signalling. Mitogenic signalling translocates ODC to the nucleus[1], possibly in connection with antizyme that is considered to regulate the nucleocytoplasmic shuttling of ODC.[7] Indeed, AZ contains two nuclear export signals.[1,7] Immunochemical stainings from different cell lines indicate that AZ is mainly localized to the nucleus in actively proliferating cells. Epitope-tagged AZI1 has also been detected in the nucleus of proliferating, cultured cells, suggesting that reciprocal activities of AZ and AZI1 mediate the fluctuations in ODC activity during the cell cycle.[40] However, the role of ODC in the synthesis of nuclear polyamines needs to be investigated further, since the presence of other enzymes needed for polyamine synthesis have not been described for the nucleus. If the function of nuclear proteasomes is restricted, ODC accumulates in the nucleus, indicating that ODC degradation targeted by AZ also occurs in the nucleus.[7] AZ might have a more potent role in the degradational targeting in the nucleus, since AZ also mediates the degradation of the oncogene Aurora-A, which is related to the progression of mitosis.[33] During transition from prophase to telophase in mitosis, the proportions of AZ and AZI1 are located in the centrosomes where they facilitate the completion of mitosis.[37] Overactivity of AZ leads to a decrease in the number of centrosomes, whereas the increased activity of AZI1 is followed by an accumulation of excessive centrioles.[32] After mitosis, the entire orchestra of polyamine regulators, including ODC, AZ1 and AZI1, is detected in the perinuclear space.[32]

The induction of apoptosis is accompanied by an increase in ODC activity and the resulting accumulation of putrescine is assumed to contribute to the activation of apoptotic signalling cascades. However, polyamines have also been proposed to play an antiapoptotic role.[1,7] Conversely, the inhibition of ODC by DFMO and apoptosis initiated by both extrinsic (receptor-induced) and intrinsic (mitochondria- derived) pathways.[7] Although ODC itself has not been detected in mitochondria, AZ1 contains an N-terminal motif for mitochondrial targeting. An overexpression of AZ1 in hematopoietic cells leads to its accumulation in mitochondria, which is subsequently followed by caspase cascade- and cytochrome c-mediated apoptosis.[31] Apoptosis, in which partly overlapping in signalling cascades with growth induction are activated, exemplifies the necessity of compartmentalization and localized regulation of polyamines and the regulators of their synthesis.

ODC in cancer cells

ODC has been found to function at the junctions of signal transduction pathways downstream of the oncogenes src, myc and ras, including raf/ERK/MEK and PI3K pathways.[1,7,41] In cells transformed by oncogenes or carcinogens, the activity of ODC remains constantly elevated.[7,8,22] The inhibition of ODC activity by DFMO, however, retained the normal morphology of src-transformed cells, thus emphasizing the role of ODC and polyamines in the transformation process.[22] Experiments on transgenic mice have provided substantial evidence to support the participation of ODC and polyamines in tumorigenesis. The most studied model is skin tumorigenesis in these mice, in which ODC is expressed under the K6 keratin promoter. The overexpression of ODC in these mice promotes tumor formation after their treatment with carcinogens, UV-radiation or ras-activation.[7,8] Oral administration of DFMO delayed the formation of skin tumors and regressed the existing ones in transgenic mice with MEK1 overexpression under the keratin 14 promoter. This indicates that ODC activity is needed for the

initiation and maintenance of tumors.[7] However, ODC-transgenic mice in which ODC is under its own promoter and overexpressed in all tissues are not prone to spontaneous tumorigenesis in life-long surveillance with the exception of skin papillomas after two-stage chemical induction.[8] Elevated levels of polyamines have been measured in human colon, breast and prostate cancers.[7,42] Acetylated catabolic derivatives of polyamines in urine have been studied and used as a diagnostic marker for cancer. In addition, single-nucleotide polymorphism in ODC promoter is reported to predict a risk for colon polyps and cancer.[43]

The inhibitors of polyamine biosynthesis and polyamine analogs are the focus of study as potential chemotherapeutic agents. The ODC inhibitor, DFMO, lowers the concentration of putrescine and spermidine, and inhibits proliferation of malignant cells in cultures. DFMO can be administered orally or peritoneally, and it is non-toxic.[7] The efficacy of DFMO in clinical trials has, however, been disappointing. Polyamine metabolism is regulated by various factors, and thus the inhibition of ODC has proved inadequate for achieving a significant decrease in the polyamine content of tumors *in vivo*. DFMO is now being studied in combination with non-steroidal anti- inflammatory drugs as a potential chemopreventive agent of epithelial cancers.[1,7,44] Polyamine analogs, various forms of which have been synthesized, have a multi-level impact on polyamine metabolism, and thus seem to be more potent chemotherapeutic agents. In order to function efficiently as a chemotherapeutic agent, a polyamine analog needs to influence polyamine synthesis, catabolism and uptake. It can also be presumed that part of the efficacy is explained by the binding of polyamine analogs to the same structures as the naturally occurring polyamines.[7,45] Polyamines and their analogs are also used as vehicles to provide non-viral routes inside a cell for other chemotherapeutic and anti-parasitic compounds.[45,46]

ODC and polyamines in cancer patients

Because polyamine synthesis is up-regulated in actively proliferating cells, including neoplastic cells[1-3], therefore polyamine concentration as well as gene expression and activity of enzymes involved in polyamine biosynthesis, especially ODC, are higher in cancer tissues than in normal surrounding tissues.[1,7] In the 1960s, Russell and Snyder first demonstrated high levels of ODC enzyme activity in human cancer.[47] ODC activity and polyamine levels were subsequently found to be increased in familial adenomatous polyposis, a genetic predisposition to colon cancer due to an adenomatous polyposis coli mutation.[48] In addition, a correlation between a single nucleotide polymorphism (SNP) in the ODC intron 1 and human colon cancer risk was assessed. These studies revealed that individuals taking aspirin who were homozygous G or heterozygous G/A at the (G315A) SNP site were more likely to develop colon polyps than homozygous A individuals taking aspirin. The A-allele was further shown to reduce the risk of colon cancer because the c-Myc antagonist, MAD1, selectively repressed ODC expression in an A-allele-specific manner.[49] Pioneering work in the field of polyamines and skin carcinogenesis established that ODC is both necessary and sufficient for the onset of tumors in mice.[8,50] ODC has been shown to be elevated in human non-melanoma skin cancer.[51] Moreover, ODC induction and increased levels of polyamines have been associated with breast cancer[52] and prostate cancer.[53] Other metabolic enzymes such as spermidine synthase and spermine synthase have yet to be convincingly coordinated with tumorigenesis in humans.

Many investigators have shown that both blood and urine polyamine concentrations are often increased in cancer patients.[7,45] A close correlation between blood polyamine levels and the amount of urinary polyamines has also been found in cancer patients.[45] Moreover, these levels were decreased after tumor eradication and increased after relapse[1,7,45], indicating that polyamines synthesized by cancer tissues were transferred to the blood circulation and kidney, where they are excreted into the urine.[54] Polyamines are also produced in other parts of the body and can be transported to various organs and tissues such as the intestinal lumen where polyamines are absorbed quickly to increase portal vein polyamine concentrations.[55] The majority of spermine and spermidine in the intestinal lumen is absorbed in their original forms because there is no apparent enzymatic activity present to catalyze their degradation.[56] Polyamines absorbed by the intestinal lumen are distributed to almost all organs and tissues in the body[57], as demonstrated by the increased blood polyamine levels in animals and humans produced in response to continuous enhanced polyamine intake for six and two months, respectively.[58] However, short-term increased polyamine intake failed to produce such increases[58], possibly because of the homeostasis that inhibits acute changes in intracellular polyamine concentration. On the other hand, reductions in blood polyamine concentration were not achieved only by restricting oral polyamine intake. As such, at least two sources of intestinal polyamines are postulated: foods and intestinal microbiota.[45] Decreased blood polyamine levels can be successfully achieved by eliminating intestinal microbiota in addition to restricting food polyamines.[59] Taken together, these results indicate that polyamines are not only produced by cancer tissues but are also supplied from the intestinal lumen and together appear to influence polyamine levels in the body of cancer patients.[45]

In blood circulation, the majority of polyamines are contained in blood cells, especially in red and white blood cells, and therefore increases in blood polyamine concentration indicate concurrent increases in polyamine levels in blood cells.[60] Similarly, intracellular polyamine concentrations in cells of otherwise normal tissues and organs in cancer patients can be increased.[61]

One examination showed that spermidine and spermine levels are increased in the normal colon mucosa of cancer patients compared to the normal colon mucosa from patients without cancer.[62] Given that polyamine concentrations are increased in the blood cells of cancer patients and numerous blood cells with increased polyamine concentrations exist in normal tissues, the polyamine concentration in normal tissues of cancer patients with increased blood polyamine levels might also be increased.[61] In addition, orally administered radiolabeled polyamines have been shown to be immediately distributed to almost all organs and tissues.[57] Polyamine concentrations in the blood vary considerably among healthy individuals such that concentrations are not necessarily higher in cancer patients than in otherwise normal subjects[61,63] and this wide variation precludes the use of polyamine levels as a tumor marker as well as making detection of differences in polyamine concentrations in normal tissues of cancer patients and normal subjects. The pharmacokinetics of polyamines may allow distant tissues and organs to influence polyamine levels of all cells in an organism.

Conclusion ad Future perspective

Over the past 40 years, much progress has been made in understanding the role of the polyamine pathway and ODC activity in normal cell functioning and carcinogenesis. The knowledge gained is now being used to develop new strategies for the treatment and prevention of cancer. Targeting the polyamine biosynthetic pathway for antitumor therapy started soon after the discovery that normal upregulation of polyamine levels were a hallmark for numerous tumor types. Thus, the original therapies that targeted the polyamine pathway were for the metabolic enzymes ODC and S-adenosylmethionine decarboxylase (AdoMetDC). Difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, was first shown in the 1970s to have antitumor properties. Unfortunately, DFMO, alone or in combination with other agents, was largely ineffective as a chemotherapeutic agent. The lack of effectiveness of DFMO as a chemotherapeutic agent is likely due to its poor transport into the cell, the fact that it is typically cytostatic and not cytotoxic, and compensatory mechanisms such as increased polyamine transport or upregulation of AdoMetDC that occur as a result of the depleted polyamine pools. Thus, tumor cells can overcome the effects of ODC inhibition.

One important area in the polyamine field that continues to be poorly understood is the polyamine transport system, influx and efflux. Very little is currently known regarding the molecular components of the mammalian polyamine transport system. To fully exploit the polyamine pathway and to optimize the use of polyamine analogues in a therapeutic setting will require greater knowledge in this area. The polyamine transport system has been used for cellular entry of molecules that are conjugated to a polyamine backbone, however, a greater understanding of the transporter will be necessary to fully exploit this strategy.

Finally, as the field moves forward and we gain a better understanding of the roles that polyamines play in growth and differentiation in the normal setting, as well as their dysregulation in neoplastic disease, it is likely that more rational targets and better agents to target them will be discovered. There is no doubt that the newly generated animal models, along with a continuing stream of polyamine-based compounds will aid in this endeavor.

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