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Killer Polyamines?

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Abstract Mammalian cells can rapidly make large changes in their rate of polyamine biosynthesis in response to mitogenic and trophic signals. However, cultured cells seem to grow adequately as long as they are supplied a steady but unregulated supply of polyamines. This implies that complex and rapid changes in polyamine synthesis serve a function in a special rather than a general biological context. We suggest that the appropriate context in which regulation of polyamines mediates crucial functions is the mammalian embryo and that one function of polyamines is to act as substrate in an oxidative pathway that arbitrates programmed cell death.

Key words: programmed cell death, development, spermidine, spermine, acetylase, oxidase, peroxide

What do polyamines do? Why are the enzymes that carry out their biosynthesis controlled in such complex ways and capable of such rapid changes? We do not have clear answers to these questions but will suggest that conventional views of polyamines should be altered or augmented as follows: Polyamines can be killers, killing cells is among their essential roles, and rapid and complex changes in their biosynthesis are necessary to keep their lethal potential under control.

Let us first summarize a more conventional view of the nature and role of polyamines [reviewed in 1–3]. The polyamines are small positively charged molecules, among the most abundant in cells. They are found ubiquitously in all living organisms. Cells can be depleted of polyamines by either genetic or pharmacologic means, and a sufficient degree of depletion is toxic or even lethal to organisms ranging from bacteria up through higher eukaryotes. In vertebrates, biosynthesis of polyamines begins with ornithine (alternative pathways starting with arginine exist in some organisms, e.g., bacteria and plants). Two key and highly regulated enzymes control biosynthesis of polyamines. The first is ornithine decarboxylase, which converts ornithine to putrescine, a diamine. The second is S-adenosylmethionine decarboxylase, responsible for making available aminopropyl groups, addition of which sequentially converts putrescine to spermidine and then to spermine. Two observations regarding polyamine biosynthesis have remained durable: First, activity of one or both of these enzymes, and hence polyamine synthesis, goes up when cells are stimulated to increase their rate of growth (or, in some cases, when stimulated to differentiate), and, second, both enzymatic activities can undergo enormous changes, going up and/or coming down very fast in response to diverse stimuli. Contributing to the lability of these activities is the extraordinary lability of the two enzymes themselves. Both are among the most rapidly degraded proteins in mammalian cells. Consequently, changes in their synthetic rate are very rapidly reflected in changes in steady-state levels [4]. In addition, their degradation is itself subject to regulatory change [5,6].

In the aggregate, these findings support the following general conclusions. Polyamines are essential for life. Cells seem to make them at a greater rate when they grow faster or significantly alter their function. The enzymes that control polyamine biosynthesis are poised to fulfill this task rapidly and, as rapidly, to shut down when they have fulfilled it. This seems a generally valid view, but a rather unsatisfying one. The details are a bit fuzzy. Polyamines are doing some important things, but we do not know what they are.

One of the underpinnings of this conclusion, i.e., that polyamines are important even if we do

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not know what they are doing, is the observed complexity of regulation of the two key biosynthetic enzymes. Their activities change enormously and quickly. These changes are mediated not only by induction of transcription but also by posttranscriptional mechanisms (processing of a precursor peptide in the case of S-adenosyl methionine decarboxylase [7], poorly understood immediately posttranslational events in the case of ornithine decarboxylase [8]). Both enzymes are responsive to polyamines. S-adenosyl methionine decarboxylase is activated by putrescine, whereas ornithine decarboxylase is negatively posttranslationally regulated by cellular polyamine pools. A prodigious assemblage of diverse machinery is deployed to get polyamines right, but to what end?

If polyamines are merely benign molecules that help cells to realize fully their potential for growth, why put their synthesis under such complex control? Experimentally, this question can be approached by relaxing control and seeing what happens. This has been done using cultured mammalian cells, by taking advantage of a mutant cell devoid of ornithine decarboxylase activity and consequently unable to produce its own polyamines [9]. These cells, the mutant progeny of a Chinese hamster ovary cell line, die when grown in normal tissue culture medium. If putrescine is added to their medium, the cells grow entirely normally. Over a broad range of concentrations of putrescine in the medium, the cells seem to do equally well. At the cellular level, enough of polyamines is enough, and nothing succeeds like excess. However, it could be argued, perhaps cells have clever, discriminating and appropriate ways of letting polyamines in and out, thereby defeating efforts to fix them at inappropriate levels. This possibility invites another experimental approach: restore to the mutant cells the capacity to make an enzymatically active ornithine decarboxylase [10] but one that is as inert as possible from a regulatory point of view. Nature herself has provided such an ornithine decarboxylase, that from *Trypanosoma brucei* [11]. This extracellular bloodstream parasite is transmitted by a fly vector. In Africa *T. brucei* and related parasites are a cause of nagana, a disease of cattle, and human sleeping sickness. When the parasite-derived ornithine decarboxylase gene is expressed in the mutant hamster cells referred to above, under the control of a constitutive promoter, ornithine decarboxylase activity within cells is stable [12] and does not respond to polyamines, nor, so far as we know, to other forms of regulation (in preparation). Again, the mutant cells transfected with such a maladroit gene seem to proliferate entirely normally. At the cellular level, regulation of ornithine decarboxylase activity is an unneeded luxury.

Are there circumstances under which an excess of polyamines is not good for cells? Indeed there are. The mutant hamster cells can make use of exogenous putrescine by taking it up from the culture medium and converting it to spermidine, yet, if the thorough but unwitting investigator provides the cells with spermidine itself, rather than putrescine, they quickly die, far faster than if they had merely been denied polyamines. The explanation is that the cells are commonly cultured in fetal calf serum, which, unlike some other sera, such as horse serum, contains high levels of an oxidative enzyme, copper amine oxidase. Spermidine, but not putrescine, is a substrate [13], and one product of the reaction is hydrogen peroxide, which kills the cells.

The lethality of spermidine for cells cultured in fetal calf serum [14] has been "discovered" many times and, sadly, will be again, but this is of uncertain physiologic relevance, because the amine oxidase is in serum and spermidine is (mostly) in cells. However, there is another related enzyme, termed polyamine oxidase, that *is* found in cells. This enzyme can oxidatively cleave spermidine and spermine, but the N^1-acetyl derivatives are much better substrates than the unmodified polyamines. Acetylation is carried out by a cytosolic enzyme, acetyl-CoA: spermidine/spermine N^1-acetyltransferase [reviewed in 15]. The acetyl transferase activity is normally low or absent in cells, but it is highly inducible, notably by chemicals that are toxic for cells [16] or by thermal stress [17].

A product of oxidative cleavage of N^1-acetylspermidine is putrescine and of N^1-acetylspermine, spermidine. Seiler [15] has demonstrated the metabolic importance of these activities. Operating on polyamine pools, the combination of acetylation and oxidation has, as one effect, the ready interconversion of polyamines: The biosynthetic pathway carries putrescine to spermidine and then to spermine, while the acetylation-oxidation reactions perform these conversions, but in the reverse direction. It has been generally assumed that the physiologic role of the latter pathway has been to provide for such

interconversion, thereby making available to cells still another dexterous means for apportioning polyamines. However, another and commonly ignored product of the oxidative reaction is potentially deadly: hydrogen peroxide. A recent series of papers makes it likely that polyamines indeed incite production of hydrogen peroxide in mammalian embryos and participate thereby in a process of programmed cell death.

Polyamines have been implicated as a cytotoxic agent in two embryonic settings where programmed cell death occurs: The mammalian blastocyst and the limb bud of the 14-day-old mammalian embryo. Parchment and Pierce [18] observed malignant cells placed in either setting are killed or regulated to a more normal phenotype, depending on their differentiative potential. When malignant melanoma cells are implanted under the skin of the embryonic day 14 limb or cultured in media conditioned by minced limb buds, tumor cell growth is suppressed. This suppression was found to be caused by the cytotoxic products of polyamines catabolized by amine oxidase. Inhibitors of serum amine oxidase (aminoguanidine) and polyamine toxicity (β-mercaptoethanol) abolished this cytotoxicity. Although serum amine oxidase in the culture medium, contributed by the bovine serum supplement, was probably responsible for catabolism of polyamines, polyamine oxidase activity was readily detectable in the embryonic limb bud homogenates and is likely to be responsible for the generation of cytotoxic products in the limb. Massive programmed cell death occurs in the 14 day limb bud during morphogenesis of the joints and digits. The conclusion by these authors is that catabolism of polyamines by polyamine oxidase is the agent of this morphogenetic sculpting.

Programmed cell death also occurs in the inner cell mass of the early mouse blastocyst, at a time that corresponds with a restriction of differentiative potential of these cells. At this time the cells of the inner cell mass, which will form the embryo, lose the ability to give rise to trophectodermal cells, those cells destined to contribute only to extraembryonic structures. It has been hypothesized that this restriction is the result of programmed cell death of the inner cell mass cells that are pretrophectodermal. An electron micrographic study has estimated that approximately 10% of the blastocyst cells undergo programmed cell death and are endocytosed by their inner cell mass and trophectodermal neighbors [19]. When embryonal carcinoma cells are injected into the blastocoele of this stage, their survival and contribution these cells make to the embryo have been found to depend on their differentiative potential [20]. It was hypothesized that there is a factor in the blastocoele fluid that is cytotoxic to cells with trophectodermal potential and that this factor functions in the normal setting as the agent of the observed programmed cell death. The cells that where used as monitors of the killing activity were two embryonal carcinoma cell lines with distinct differentiative potentials. EC 247 cells have the potential to give rise to trophectodermal cells; P19 cells do not. Pierce and colleagues therefore used the EC 247 cells as a surrogate for the early inner cell mass, which have the potential to give rise to both extraembryonic trophectoderm cells and embryonic cells, and the P19 cells as a surrogate for later inner cell mass, which have the potential to give rise to embryonic germ layers and primary endoderm only. Some of the EC 247 cells when injected into the blastocoele cavity of an early embryo can be regulated but will contribute only to the extraembryonic tissues of the embryo. Many of the EC 247 cells, however, are killed (44%). The P19 cells, in contrast, are regulated and contribute to embryonic tissues of the midgestation chimaeric mice in 60% of the injections. The P19 cells are not killed by the blastocoele fluid. Since the volume of fluid within the blastocoele is very small (about 1 nl) they have used the cystic embryoid bodies of an embryonal carcinoma cell line, C44, as a source of blastocoele fluid for analysis of the cytotoxic factor [21]. The blastocoele-like fluid of the C44 embryoid bodies mimics the differential killing of these two test cells. A low-molecular-weight cytotoxin that exhibited the same target cell selectivity was isolated from this blastocoele-like fluid. Once again, inhibitors of serum amine oxidase (aminoguanidine) and polyamine toxicity (β-mercaptoethanol) abolished this cytotoxicity [22]. The polyamine composition of the blastocoele-like fluid was analyzed by high-performance liquid chromatography (HPLC), and culture medium supplemented with polyamines in the same proportions was found to reconstitute the cytotoxicity. The addition of catalase, which cleaves hydrogen peroxide to O_2 and water, blocks all cytotoxic activity both in

the blastocoele-like fluid and in normal blastocoele fluid (G.B. Pierce, personal communication). It was concluded that the hydrogen peroxide generated by the oxidation of polyamines was the killing agent. However, clearly not all cells of the embryo are killed. The authors hypothesize that the cells that evade being killed do so by means of a glutathione-dependent mechanism that renders them resistant to the hydrogen peroxide. Alternative mechanisms to produce this differential survival could be envisioned. It is also necessary to recall that hydrogen peroxide is not the sole potentially toxic product of polyamine oxidation [15 and references within].

A special role in embryonic development for polyamines remains largely speculation, but it is made more plausible by an enigma: Cellular polyamine pools are elaborately controlled, but this seems not to matter very much in the contexts that are most accessible and most studied. Assuming, as seems likely, that this apparatus serves a necessary biological function, the embryo could be the place were such regulation *does* matter. The developing mammalian embryo constitutes a small assembly of cells undergoing complex and rapid changes. The embryo may be the right context in which to seek an explanation of the meaning of regulation of polyamines. Indeed, the more conventional view of polyamines as concomitants of cell growth, as well as their proposed novel role as killers, may be exemplified in the embryo; mammalian cell division occurs at an otherwise unprecedented rate in the embryo at certain developmental stages [23].

Killing cells with polyamines requires fulfilling many conditions. Polyamines must be present in sufficient amount; they must be N^1-acetylated and then oxidized to generate hydrogen peroxide; target cells must be present that are sufficiently sensitive to that toxic product. Alternatively, unmodified polyamines must be presented to amine oxidases. It is unclear which of these steps might be limiting for cell killing and which might therefore undergo regulatory changes important for inducing properly targeted programmed cell death. A number of possibilities may be considered. It is evident from Parchment and Pierce's work that, among embryonal carcinoma cell lines, there is differential toxicity of hydrogen peroxide. Cells in general have the means to protect themselves from oxidative damage by superoxide and hydrogen peroxide [24]. Some, for example, macrophages, contain large amounts of oxidative enzymes used for such tasks as killing invading microorganisms and must be able to protect themselves against self-induced damage. Differential susceptibility to products of oxidation, therefore, constitutes a plausible regulatory locus. Alternatively, polyamine oxidase may not be distributed uniformly among tissues and is a candidate for regulation. Spermidine/spermine acetyltransferase is normally present in very low levels. It is highly inducible by stimuli that include cellular toxins. It is conceivable that induction of this enzyme could be utilized as a means for administering the coup de grace to damaged cells. Finally, the polyamine biosynthetic enzymes may themselves constitute an activity, regulation of which can elicit or restrain lethal acts. The availability of pharmacologic and genetic means to alter the activities of the relevant enzymatic activities in developing embryos will make it possible to test the validity of the ideas presented here.

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