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Signal transduction pathways linking polyamines to apoptosis

Review Article

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Summary. Polyamines are important multifunctional cellular components and are classically considered as mediators of cell growth and division. Recently polyamines have been also implicated in cell death. Now it appears that polyamines are bivalent regulators of cellular functions, promoting proliferation or cell death depending on the cell type and on environmental signals. This review draws a picture about the role of polyamines in signalling pathways related to apoptotic cell death and the proposed molecular targets of these polycations at the level of the apoptotic cascade. Solid evidence indicates that polyamines may affect the mitochondrial and postmitochondrial phases of apoptosis, by modulating cytochrome *c* release from mitochondria and activation of caspases. Recently, polyamines have been also implicated in the regulation of the premitochondrial phase of apoptosis, during which upstream apoptotic signal transduction pathways are activated. The studies reviewed here suggest that polyamines may participate in loops involving interaction with signal transduction pathways and activation/expression of proteins that may control cell death or cell growth.

Keywords: Spermine – Apoptosis – Spermidine – Mitochondria – MAPK – NF-κB

Abbreviations: AIF, apoptosis inducing factor; CHX, cycloheximide; DFMO, α-difluoromethylornithine; ERK, extracellular signal-regulated kinase; IκB, inhibitor protein-κB; JNK, c-Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear transcription factor-κB; NRE sequences, NF-κB response element sequences; ODC, ornithine decarboxylase; SAMD, S-adenosylmethionine decarboxylase; TNF-α, tumor necrosis factor-α

Polyamines in cell proliferation and cell death

Polyamines, cell cycle and cell growth

Polyamines are ubiquitous cellular components with multiple functions and are essential for life.

The three basic polyamines (putrescine, spermidine and spermine) are small, flexible molecules, that specifically bind to nucleic acids and proteins "in vitro" and thus affect their conformation and biological activity (Thomas and Thomas, 2001).

The metabolic pathways which govern the homeostasis of polyamines in mammalian cells are well established and have been summarized in several reviews (Pegg et al., 1995; Persson et al., 1996; Seiler and Douaud, 1998; Morgan, 1999). The intracellular concentration of these organic polycations is finely regulated by biosynthetic and metabolizing enzymes, as well as by transport systems. Although their relevant interactions with molecular components in the intact cell remain largely to be identified, some studies indicate that polyamines may modulate signalling pathways and the expression of specific genes, some of which may, in turn, regulate polyamine biosynthesis.

It has been proposed that polyamines may participate, under some circumstances, in positive loops, with response reinforcement (Flamigni et al., 1999; Bachrach et al., 2001).

Polyamines are classically known to be important mediators of cell growth and division, in view of their capability to directly bind to DNA and to modulate DNA-protein interactions (Cohen, 1998).

Recently these polycations have been involved in the progression of the cell through the cell cycle, which represents the basis for cell proliferation (Wallace et al., 2003; Oredsson, 2003). Polyamine content is altered during the course of cell cycling, via changes in two key biosynthetic enzymes: ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMD). These enzymatic activities increase bicyclically during the cell cycle, in conjunction with the G1/S and S/G2 transition, through

regulation at transcriptional (mRNA) and post-translational (half-life activities) levels. The consequent variation of polyamine concentrations has been reported to up- and down-regulate important checkpoints within the cell cycle and to affect cyclin degradation (Thomas and Thomas, 2001; Oredsson, 2003). As a consequence, depletion of polyamines would result in cell-growth arrest, mainly at the level of G1 phase (Kramer et al., 2001), because of accumulation of p21 and p27 proteins (Ray et al., 1999; Ackermann et al., 2003), known inhibitors of cyclin-dependent kinases, and p53 protein (Ray et al., 1999; Li et al., 2001b), which plays a role in the transcription of genes involved in growth arrest and cell death.

Polyamines and cell death

Beside the role of polyamines in events linked to cell proliferation, more recently these polycations have been also implicated in the cell death process known as apoptosis (Schipper et al., 2000; Nitta et al., 2002). Apoptosis is a physiological cell death regulated by genetic mechanisms and is principally characterized by morphological and biochemical changes in cell nuclei, including chromatin condensation and internucleosomal DNA fragmentation (Vaux and Strasser, 1996).

Increasing evidence indicates that polyamines, cell cycle events and apoptosis are closely connected, but this correlation is quite complex and some results apparently appear contradictory. Now it appears that polyamines are bivalent regulators of cellular functions, promoting cell proliferation or cell death depending on the cell type as well as on environmental signals.

In several cell types, excessive levels of polyamines and/or a deregulation in their accumulation may be the cause of cell death. Exogenous polyamines, in particular spermine, can be toxic at high concentrations and this results to be, at least in part, independent of their oxidative metabolites (Schipper et al., 2000).

Both up-regulation and down-regulation of polyamine levels have been reported during apoptosis (Schipper et al., 2000 and ref. inside). It was observed that the treatment of HL-60 cells with etoposide, a classic inducer of apoptosis, produces an early and transient increase of ODC, which may initiate apoptosis, followed by a decrease, that would sustain this process (Wallace et al., 2003). ODC induction (Schipper et al., 2000) and spermidine accumulation (Monti et al., 1999) have been related to the progression of the cell cycle until a checkpoint from which apoptosis is triggered in the presence of cell death-inducing signals or negative growth factors.

Polyamine depletion may affect cell susceptibility to apoptosis

Inhibition of polyamine biosynthesis can either protect or sensitize cells exposed to death stimuli, depending on the cell type. In particular, depletion of polyamines antagonizes the cytotoxicity of taxol in human breast cancer cells (Das et al., 1997) and of 2–deoxy-D-ribose in HL-60 cells (Monti et al., 1998), prevents apoptosis induced by camptothecin (Yuan et al., 2002) and delays cell death induced by tumor necrosis factor-α (TNF-α) and cycloheximide (CHX) in intestinal cells (Ray et al., 2000). Polyamine depletion also prevents apoptosis induced by green tea extract in bladder carcinoma cells (Facchini et al., 2003). On the other hand, inhibition of polyamine biosynthesis induces apoptosis in neonatal cardiomyocytes (Han et al., 2003) and sensitizes several tumor cell lines to TNF-α-induced apoptosis (Penning et al., 1998). Moreover, polyamine depletion may either enhance or decrease the susceptibility to apoptosis even in the same cell type, depending on the death stimulus. In intestinal cells polyamine depletion does not directly induce cell death, but sensitizes cells to apoptosis induced by staurosporine, and promotes resistance to cell death induced by TNF-α and CHX (Li et al., 2001a). We observed that, in transformed mouse fibroblasts lacking the spermine synthase gene, the complete depletion of polyamines produced by treatment with α-difluoromethylornithine (DFMO), an inhibitor of ODC, increases cell death caused by UV irradiation, but inhibits it when the inducers of apoptosis are cycloheximide or etoposide (Stefanelli et al., 2001).

The main stages of apoptosis: involvement of polyamines

The mechanisms by which polyamines may act as facilitating or counteracting factors to transduce death-signal inside the cells and interact with the apoptotic cascade have been recently investigated. Such mechanisms could operate at different levels in the signal transduction pathways which drive cells toward programmed death. Solid evidence implicates mitochondria in apoptotic signalling. The balance between pro-apoptotic and pro-survival molecules acting at the level of mitochondria determines whether membrane permeabilization occurs and, ultimately, whether cell death ensues.

Loeffer and Kramer (2000) proposed a three-stage model of apoptosis: a pre-mitochondrial phase during which signal transduction cascades or damage pathways

are activated (initiation phase), a mitochondrial phase during which mitochondrial membranes are permeabilized and cytochrome *c* and other proteins are released (decision/effector phase), and a post-mitochondrial phase during which released proteins cause the activation of proteases and nucleases (degradation phase).

In recent years most studies have considered the action of polyamines at the level of mitochondrial and postmitochondrial phases of apoptosis, whereas less is known about the role of these polycations on signal transduction pathways located upstream mitochondrial phase and caspase activation.

Polyamines and the mitochondrial and post-mitochondrial phases of apoptosis

The mitochondrial and post-mitochondrial phases of apoptosis classically culminate with the activation of proteolytic cascades involving a family of proteases, called caspases, which play a central role in signalling and executing the cell death program (Thornberry and Lazebnik, 1998). In particular, proteases of the caspase-3 subfamily act as effectors in the execution stage of apoptosis (Thornberry and Lazebnik, 1998), by cleaving specific cellular substrates. During apoptosis the activation of caspase-3 is regulated by multiple distinct pathways, such as death receptors like Fas/CD95 and alteration of mitochondria (Daniel, 2000). Apoptotic signals can induce mitochondria to release cytochrome *c* which represents a crucial step directly related to the onset of the proteolytic caspase cascade and to the induction of apoptosis (Daniel, 2000; Loeffler and Kroemer, 2000). In addition to cytochrome *c*, another mitochondrial apoptogenic factor is the apoptosis inducing factor (AIF), which can activate caspase-3 or translocate from mitochondria to nucleus thus inducing DNA fragmentation, independently of caspase activation (Loeffler and Kroemer, 2000).

The involvement of polyamines in apoptosis-related pathways at the level of mitochondria has been investigated in different cell models. Some groups, including ours, have initially studied the effects of polyamines on events directly related to the activation of the caspases. We observed that, in whole cells and in cell-free models of apoptosis, polyamines, particularly spermine, can trigger the activation of caspases, and we showed that polyamines can directly induce the release of cytochrome *c* from mitochondria and activate the death program (Stefanelli et al., 1998, 1999, 2000).

The release of cytochrome *c* from mitochondria may be, in turn, modulated by Bcl-2 family proteins, such as Bax and truncated Bid, which, upon translocation to mitochondria, cause the opening of mitochondrial permeability transition pores and the subsequent release of cytochrome *c* into cytosol (Gros et al., 1999; Tsujimoto and Shimizu, 2000). In a cell line from intestinal cells, polyamine depletion antagonizes camptothecin-induced apoptosis by preventing the translocation of the proapoptotic Bcl-2 family member Bax to mitochondria and inhibiting the release of cytochrome *c* (Yuan et al., 2002). Moreover, in ODC overproducing mouse myeloma cells, accumulation of putrescine provokes apoptotic death, that is inhibited by DFMO and involves the release of cytochrome *c* from the mitochondria, followed by the activation of caspase cascades (Erez et al., 2002).

On the other hand, in various lymphoid cell lines, the complete depletion of polyamines provoked by the combined use of ODC and SAMD inhibitors, causes the disruption of the mitochondrial membrane potential, resulting in caspase activation and apoptotic cell death (Nitta et al., 2002). A recent work shows that spermine inhibits the release of cytochrome *c* from the mitochondria of dexamethasone-treated thymocytes, but it does not totally prevent the dexamethasone-induced DNA fragmentation (Hegardt et al., 2003). The authors hypothesize that spermine inhibits the release of cytochrome *c*, but does not affect the release of AIF, thus conferring only a partial survival advantage on the cells.

Polyamines and the pre-mitochondrial phase of apoptosis

Contrary to the large number of studies concerning the involvement of polyamines in regulating the mitochondrial and post-mitochondrial phases of apoptosis, only a few reports have been published about the role of these polycations in the pre-mitochondrial phase, characterized by the activation of upstream apoptotic signal transduction cascades.

In fact, normal physiological processes and pathological stimuli induce apoptosis via different signal transduction pathways (Ellis et al., 1991; Corcoran et al., 1994; Steller, 1995; Vaux and Strasser, 1996), some of which seem to be affected by polyamines (Bachrach et al., 2001). Many extracellular stimuli induce apoptosis by affecting the same signalling transduction pathways implicated in control of cell growth and it is generally thought that activation of a proliferative process sensitizes cells toward apoptosis (Evan and Littlewood, 1998). Components of these signalling pathways are the mitogen-activated protein kinase (MAPK) cascades, which are

generally activated in response to mitogenic signals, cytokines and environmental stress, and lead to activation of the MAPK members extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK) and p38 MAPK. MAPK cascades have been shown to participate in a wide array of cellular functions, such as cell growth, cell differentiation and cell death (Schaeffer and Weber, 1999; Robinson and Cobb, 1997; Cross et al., 2000). Interestingly we have reported that ERK pathway is specifically involved in ODC induction and expression caused by a large variety of stimuli in different cell types (Flamigni et al., 1999, 2001; Tantini et al., 2002; Facchini et al., 2003). Conversely, ODC overproducing cells show enhanced MAPK activity (Flamigni et al., 1999); polyamines, in turn, are implicated in the phosphorylation of MAPK and this activation triggers the expression of nuclear oncogenes *c-fos*, *c-jun* and *c-myc* (Bachrach et al., 2001).

Another factor implicated in the control of cell growth, survival and apoptosis is the nuclear transcription factor-κB (NF-κB) (Barkett and Gilmore, 1999; Karin and Ben-Neriah, 2000). In some cell types activated MAPK may provoke phosphorylation and activation of NF-κB subunits, thus regulating the transcription of genes involved in both cell proliferation and apoptosis (Vermeulen et al., 2002). The NF-κB family of transcription factors consists of five different subunits in mammalian cells, i.e. p50, p52, p65/RelA, c-Rel and Rel-B, which are able to form homodimers or heterodimers (Baeuerle and Baltimore, 1996). In basal conditions, NF-κB dimers are sequestered in most cells in the cytoplasm by a member of the inhibitor protein-κB (IκB). In response to various stimuli, IκB is phosphorylated and then degradated by the ubiquitin-proteasome pathway (Brown et al., 1995; Lee et al., 1997), thus allowing NF-κB to translocate to the nucleus, bind to NF-κB response element sequences (NRE sequences) and activate transcription of specific genes involved in cell proliferation or in cell death. A further mechanism of NF-κB activation is independent of IκB and involves the phosphorylation of NF-κB subunits, such as the p65 and p50 subunits, which may affect NF-κB binding activity and transcriptional competence (Schmitz et al., 2001).

The transcription factor NF-κB has been reported to have both apoptotic and antiapoptotic functions, depending on cell type and death stimulus (Qin et al., 1998; Beg and Baltimore, 1996) and the precise factors that determine the ability of the transcription factor to regulate these divergent biological actions are unknown. Many of the genes that are activated in the initiation of apoptosis are target genes of NF-κB, such as *Fas ligand*, *p53*, and *c-myc* (Baeuerle and Baltimore, 1996). In this regard, ODC is known to be a transcriptional target for *c-myc*, a transcriptional activator whose deregulated expression may sensitize cells to apoptosis. In leukaemia cells *c-myc* plays a role in triggering apoptosis induced by medium starvation and polyamines may act as facilitating factors (Tiberio et al., 2001).

At the present, the role of polyamines in signal transduction pathways related to apoptosis at the level of the pre-mitochondrial phase, upstream caspase activation, has been investigated in few experimental models, such as intestinal epithelial cells, mouse fibroblasts and human breast cancer cells, with conflicting results.

In intestinal cells and fibroblasts polyamines may facilitate the process of apoptosis by affecting the transcription factor NF-κB and signalling pathways mediated by MAPK family members. In normal intestinal epithelial cells, polyamines appear to be negative regulators of NF-κB activation (Li et al., 2001a). In these cells depletion of polyamines by treatment with DFMO, increases NF-κB activity, through the degradation of IκB (Pfeffer et al., 2001) and this event alters the susceptibility of the cells to apoptotic stimuli. In particular, NF-κB activation after polyamine depletion sensitizes intestinal cells to apoptosis induced by staurosporine, but promotes resistance to cell death induced by TNF-α and CHX (Li et al., 2001a). In these studies, however, the exact downstream targets of activated NF-κB following polyamine depletion have been not investigated. A recent work of Zou et al. (2004) shows that activated NF-κB by polyamine depletion stimulates the expression of genes of the inhibitor of apoptosis family proteins, potent natural suppressors of apoptosis, which directly inhibit the activation of caspases, protecting the cells from TNF-α/CHX induced apoptosis.

In conflict with these findings other reports indicate that, in breast cancer cells, polyamines, in particular spermine, facilitate the binding of NF-κB with its response element, contributing to up-regulation of genes involved in cancer cell proliferation (Shah et al., 1999, 2001). These discrepancies have been explained by the possible differential responses of normal cells versus cancer cells to polyamine effects. Alternatively, it has been proposed that activation of NF-κB observed in intestinal cells treated with DFMO could be determined by a decrease of putrescine level, since spermidine and spermine levels resulted poorly affected by the ODC inhibitor.

Our recent works implicate NF-κB and MAPKs in the signal transduction pathways by which polyamines

facilitate the process of apoptosis in etoposide-treated transformed mouse fibroblasts (Tantini et al., 2004). We observed that, in these cells, etoposide elicits MAPK activation and a progressive and sustained NF-κB activation, accompanied by enhancement of p65 NF-κB subunit phosphorylation and p65 DNA-binding activity, which precede the activation of caspases and the induction of apoptosis. In various cells NF-κB and IκB phosphorylation may involve signalling pathways mediated by members of the MAPK family, such as ERK, JNK and p38 MAPKs (Vermeulen et al., 2002). Accordingly, treatment of fibroblasts with inhibitors of ERK or JNK reduces p65 phosphorylation and partially prevents the increase in NF-κB DNA-binding activity caused by etoposide (Tantini et al., 2004). We also showed that increased ERK and JNK phosphorylation as well as NF-κB activation are correlated to caspase activation and induction of apoptosis in etoposide-treated fibroblasts and that all these events are blocked by polyamine depletion (Stefanelli et al., 2002).

These observations do not seem fully extendible to the model of intestinal epithelial cells. In these cells the role of polyamines is that to favour TNF-α/CHX-induced apoptosis by enhancing JNK phosphorylation, which seems to increase mitochondrial permeability, cytochrome *c* release and caspase-3 activity (Bhattacharya et al., 2004). Polyamine depletion prevents cell death by favouring NF-κB activation (Zou et al., 2003) and inducing a sustained ERK phosphorylation, which, in turn, decreases JNK activation and protects cells from apoptosis (Bhattacharya et al., 2004). Recently, an involvement of Akt kinase, which transduces signals downstream growth factor-stimulated tyrosine kinase receptors, has been proposed as another mechanism by which polyamine depletion mediates suppression of apoptosis in normal intestinal epithelial cells (Zhang et al., 2004). In addition, our recent findings suggest that early activation of ERK is needed for ODC induction in bladder cancer ECV 304 cells and that both events are required for late caspase activation induced by green tea extract (Facchini et al., 2003).

In conclusion the overall picture regarding the implication of polyamines in signal transduction related to apoptosis is quite complicate, also because of the intricate cross-talk among signalling pathways and the ODC/polyamine system. A schematic illustration depicting possible relationships linking ODC/polyamines with signal transduction pathways in the regulation of cell growth or cell death is shown in Fig. 1. Although the identification of the direct and specific intracellular molecular targets of polyamines remains largely elusive, these studies indicate that ODC and polyamines could be located between signalling pathways and the expression/activation of proteins that control cell growth and death.

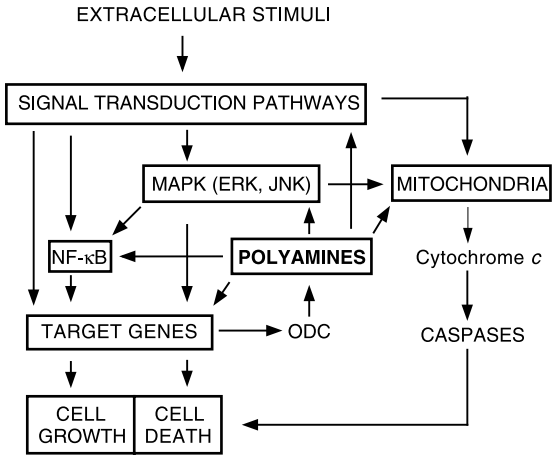

Fig. 1. Proposed involvement of polyamines in the network of signal transduction pathways controlling the balance between cell growth and cell death. Polyamines may affect cell death by modulating the release of cytochrome *c* from mitochondria, which triggers the activation of caspases and the induction of apoptosis. Polyamines may also affect signal transduction pathways mediated by the nuclear transcription factor-κB (*NF-κB*), mitogen activated protein kinase (*MAPK*) family members and, perhaps, other kinases which modulate the expression of genes implicated in the control of cell growth and cell death. Genes controlled by signalling pathways may regulate the expression of ornithine decarboxylase (*ODC*), the first enzyme of polyamine biosynthesis, thus forming reciprocal loops

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