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*Apoptosis Review Series*

# Polyamines and apoptosis

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## Abstract

The natural polyamines putrescine, spermidine and spermine are in multiple ways involved in cell growth and the maintenance of cell viability. In the course of the last 15 years more and more evidence hinted also at roles in gene regulation. It is therefore not surprising that the polyamines are involved in events inherent to genetically programmed cell death. Following inhibition of ornithine decarboxylase, a key step in polyamine biosynthesis, numerous links have been identified between the polyamines and apoptotic pathways. Examples of activation and prevention of apoptosis due to polyamine depletion are known for several cell lines. Elevation of polyamine concentrations may lead to apoptosis or to malignant transformation. These observations are discussed in the present review, together with possible mechanisms of action of the polyamines. Contradictory results and incomplete information blur the picture and complicate interpretation. Since, however, much interest is focussed at present on all aspects of programmed cell death, a considerable progress in the elucidation of polyamine functions in apoptotic signalling pathways is expected, even though enormous difficulties oppose pinpointing specific interactions of the polyamines with pro- and anti-apoptotic factors. Such situation is quite common in polyamine research.

**Keywords**: cell death • putrescine • spermidine • spermine • cell signaling

## Introduction

Apoptosis designates genetically-programmed mechanisms of cell death. Damage to DNA or to other vital molecules propagates a cascade of reactions, which activate death programs inside the cell [1]. Physiologically apoptosis is an integral part in embryonic development, and the regulation of organ homeostasis. Apoptotic mechanisms are also exploited for tumour therapy [2].

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The natural polyamines putrescine (Put), spermidine (Spd) and spermine (Spm) are formed and stored by nearly all eukaryotic cells. They are involved in multiple ways in cell proliferation and the maintenance of cell viability [3]. Therefore it is not surprising that aberrations of polyamine metabolism impair normal cell function and may cause cell death.

Major biosynthetic and catabolic reactions of the natural polyamines are shown in Fig. 1. A characteristic of polyamine metabolism is its sophisticated regulation. Growth factors, hormones and trophic factors, as well as the polyamines themselves regulate key biosynthetic (ODC, AdoMetDC) and catabolic enzymes (SAT, PAO, DAO), as well as uptake and release [3]. A high turnover rate of the regulatory enzymes and of other regulatory proteins (*e.g.* antizymes) ensures rapid adaptation to physiological needs in situations of changing environmental and metabolic conditions. Owing to their efficient regulation even the overexpression of ODC and AdoMetDC in mice allows the maintenance of polyamine homeostasis in tissues [4].

In the course of the last decade interrelations between polyamine metabolism and cell death attracted more and more interest. Observations that appear to link the polyamines to apoptosis have been reviewed [5, 6]. The complexity of the processes that decide about life and death of cells, their differences in different cell lines, gaps in our knowledge, together with numerous, apparently contradictory observations prevent at present a uniform interpretation of the role of the polyamines in apoptosis. Therefore an attempt was made to compile the most important facts, and turn attention to the major problems. The rapid accumulation of new facts will hopefully soon clarify major inconsistencies, even though enormous difficulties oppose pinpointing specific interactions of the polyamines due to their ability to form ion bonds with innumerable anionic structures.

## Apoptotic pathways

Apoptotic cell death is the result of the successful competition of multiple pro-apoptotic factors over anti-apoptotic factors. Following Hengartner [1], a (simplified) scheme of the major events in programmed cell death is shown in Fig.2.

Usually two major pathways are distinguished: death receptor-signalled pathways and mitochondrial pathways. Apoptosis can be initiated by stimulating so-called death receptors (*e.g.* CD95 (APO-1/FAS), TRAI, and receptors of the TNF receptor gene family). By binding of appropriate ligands, (*e.g.* CD95 ligands (FASL), TNF-α TRAIL etc ) to the respective plasma membrane-localised receptors, cell death-inducing signalling complexes are formed. *Via* the FAS-associated death domain protein (FADD) pro-caspase-8 is activated (through "induced proximity"; see ref [1]). By recruitment of the degenerate caspase homologue c-FLIP caspase 8 activation can be blocked. Caspase-8 activates caspase-3 and hydrolysis apoptotic substrates.

The mitochondrial pathway is initiated by extracellular apoptogenic compounds, and by intracellular insults (*e.g.* DNA damage, release of stress-induced molecules, such as JNK, MAPK/ERK, NF-B, ceramide). The diverse responses converge on mitochondria through the activation of pro-apoptotic members of the Bcl-2 family, which seem to be mostly attached to intracellular membranes. Several pro-apoptotic molecules can shuttle between cytosol and organelles. They are activated by proteolysis or dephosphorylation. Pro-apoptotic signals direct these proteins to the mitochondrial surface, where pro-apoptotic (Bax, Bad, Bim, Bid) and anti-apoptotic members (Bcl-$X_L$) of the Bcl-2 family compete. If the pro-apoptotic side wins, an array of apoptogenic factors (cytochrome *c*, AIF, Smac/DIABLO, Omi/Htr A2, endonuclease G, caspase-2, caspase-9 etc.) are released from mitochondrial compartments. Among these cytochrome *c* is most important. It associates with Apaf-1 and then with procaspase-9 to form the apoptosome. (For the roles of Apaf1 and apoptosomes in programmed cell death see *e.g.* [7]).

Death receptor and mitochondrial pathways converge at the level of caspase-3 activation. Caspase-3 activation is antagonised by the inhibitory apoptosis proteins (IAPs), which themselves are antagonised by the Smac/DIABLO and Omi/Htr A2 proteins. Abrogation of the activity of IAPs promotes caspase-3 activation. Cross talk and integration between death-receptor and mitochondrial pathways is provided by Bid. Caspase-8 mediated cleavage of Bid increases its pro-apoptot-

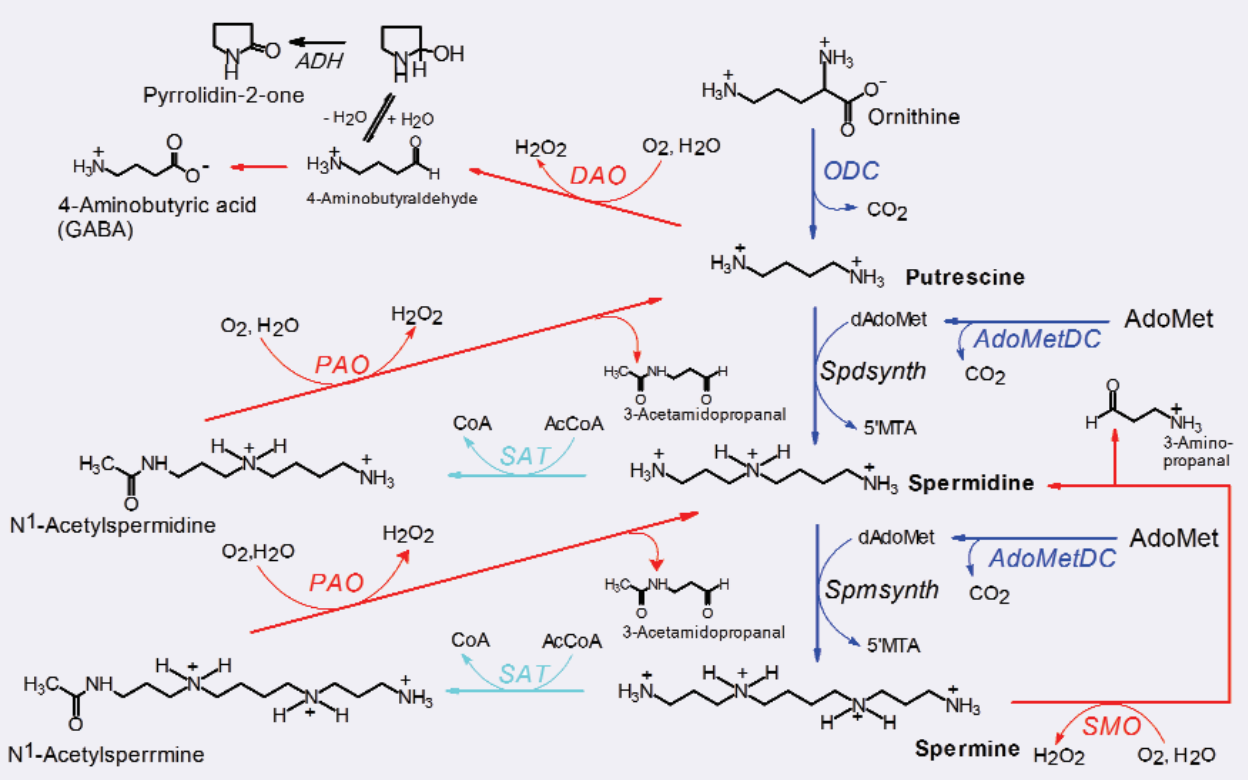


**Fig. 1** Major reactions of the natural polyamines. AdoMet S-adenosylmethionine, AdoMetDC S-adenosylmethionine decarboxylase, d-AdoMet decarboxylation product of S-adenosylmethionine, AcCoA acetylcoenzyme A, DAO diamine oxidase, ODC ornithine decarboxylase, 5´MTA 5´-methylthioadenosine, PAO polyamine oxidase (FAD dependent), SAT AcCoA: spermidine $N^1$-acetyltransferase, SMO spermine oxidase (FAD dependent), SpdSynth spermidine synthase, SpmSynth spermine synthase.

ic activity, and results in its translocation to mitochondria, where it promotes among others the release of cytochrome *c*.

Downstream of caspase-3 the apoptotic programme branches into several sub-programmes. By cleaving different substrates in the cytoplasm and nucleus, caspases produce (directly and indirectly) the typical morphologic features (cell shrinkage, chromatin condensation, oligonucleosomal DNA fragmentation, membrane blebbing) of apoptotic cell death [8]. In addition to caspases other proteolytic enzymes are involved in the regulation of apoptosis [9].

## Methodical considerations

Intracellular polyamine concentrations may be reduced by starvation. More selectively they are depleted by inhibition of the biosynthetic enzymes ODC, AdoMetDC, spermidine synthase and spermine synthase. [10, 11]. Polyamine depletion can also be achieved by structural analogues, which mimic Spd and Spm in regulatory properties, and cause the activation of catabolic pathways and release [12]. MGBG and structural analogues of this AdoMetDC inhibitor deplete Spd and Spm pools, enhance Put concentrations due to induction of ODC, and cause apoptosis of various cancer cell lines [5]. But since they are not selective for AdoMetDC, and in addition mitochondriotoxic [13], (or their mitchondriotoxicity has not been excluded) they are not suited for studying apoptotic mechanisms of polyamines. The same is true for $N^1$,$N^{12}$-diethylspermine and its homologs (see *e.g.* ref. [12, 14]). These drugs will, therefore, not be considered in the present context. For a brief discussion of potential apoptotic mechanisms of structural analogues of the natural polyamines see ref. [15].

Most investigators used DFMO for the depletion of polyamines. This selective inactivator of ODC

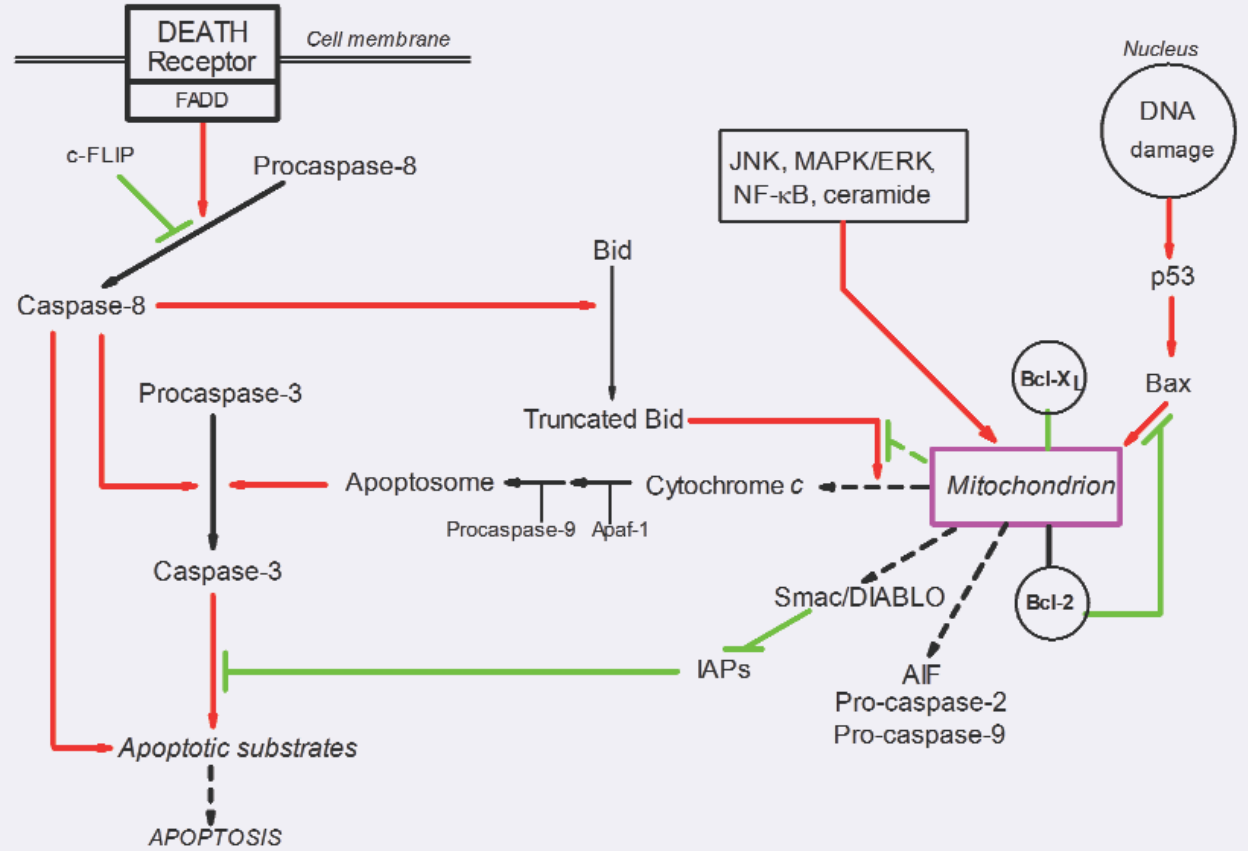


**Fig. 2** Major apoptotic pathways (according to Hengartner 2000 [1]). (For details see text).

[16] is known to diminish Put and Spd concentrations of a wide variety of cells, and cause cell cycle arrest, mostly in G1 [11]. It allowed identifying Spd as the actual growth-promoting compound among the polyamines. Polyamine depletion by DFMO affects cell function at multiple sites. Among many other effects protein synthesis is impaired [17, 18], and topoisomerase II, an enzyme necessary for normal cell proliferation, looses its functionality if polyamines are depleted [19].

The high selectivity of DFMO for ODC is a decisive advantage of this compound. A disadvantage is the fact that in most cells only Put and Spd concentrations can be lowered by exposure to DFMO. In some cell lines the partial depletion of Spm has been observed, but usually its concentration increases slightly, because depletion of Spd causes the induction of ODC and AdoMetDC. In the presence of appropriate concentrations of DFMO, AdoMetDC remains active, while ODC accumulates as inactive form. Since Put disappears more rapidly than Spd, the excessively formed decarboxylation product of S-adenosylmethionine reacts with Spd, to form Spm. Unfortunately polyamine data of DFMO-exposed cells have not been generated by all workers. In many cases it is not possible to decide, whether the described effects are due to the depletion of Put and Spd, or whether the depletion of Spm was important for the observed effect.

Owing to their unavailability from commercial sources, inhibitors of polyamine biosynthesis other than DFMO have scarcely been in use. On one hand this delimitates the clarification of interrelationships between polyamine pool size and apoptosis, however on the other hand, the use of one and the same tool is a constant parameter of numerous investigations and facilitates the comparison of results from different sources.

## Polyamines and the expression of growth-related genes

More and more evidence points at the involvement of the natural polyamines in gene expression [20, 21],

including genes encoding cytoskeletal proteins [22]. Gene expressions have mainly been investigated following polyamine depletion. In the present context only some selected observations concerning the regulation of the cell cycle will be briefly discussed.

A scheme of a signalling pathway, for which a role of the polyamines has been demonstrated, is shown in Fig. 3. It illustrates the sites of polyamine actions. The polyamines affect several phosphorylation reactions, and the expression of nuclear transcription factors [20]. Disregarding tyrosine kinase the preferential targets of Put and Spd are not identical: Put activates the expression of c-jun and c-fos, while Spd is directed toward c-myc, and the ERKs [20]. Present information does not allow conclusions concerning a direct role of Spm in gene regulation.

c-Myc is a nuclear transcription factor, which plays a central role in the regulation of cell cycle progression, and differentiation. Its activation causes an increase of Put concentration due to the activation of ODC expression, and it may cause apoptosis [23]. In IEC-6 cells depletion of polyamines by DFMO impairs activation of c-myc, c-fos and c-jun gene expression. The presence of Spd in the culture medium prevents the DFMO effect, indicating that the polyamines, not ODC protein is important for gene regulation [20, 24, 25].

The apoptotic effect of DMSO in RPMI 8402 human pre T-cells has been explained by the down-regulation of c-myc expression, which is followed by a decreased expression of ODC, and the depletion of polyamines. However, depletion of polyamines by DFMO in these cells neither provoked apoptosis nor had it an effect on c-myc expression [26], indicating that a diminished rate of Put formation is not the exclusive reason for DMSO-induced apoptosis.

Inhibition of 26S proteasome function prevented dexamethasone-induced depletion of polyamines and apoptosis in thymocytes. This effect was attributed to the prevention of ODC degradation [27]. However, since 26S proteasome is not exclusively involved in ODC degradation, but more generally in the degradation of ubiquinated proteins [9], the suggestion of Grassilli *et al.* [27] is only one among several possible interpretations.

The nuclear phosphoprotein p53 (tumour suppressor) plays a critical role in the transcriptional regulation of target genes and apoptosis of intestinal epithelial cells. Depletion of Put and Spd pools

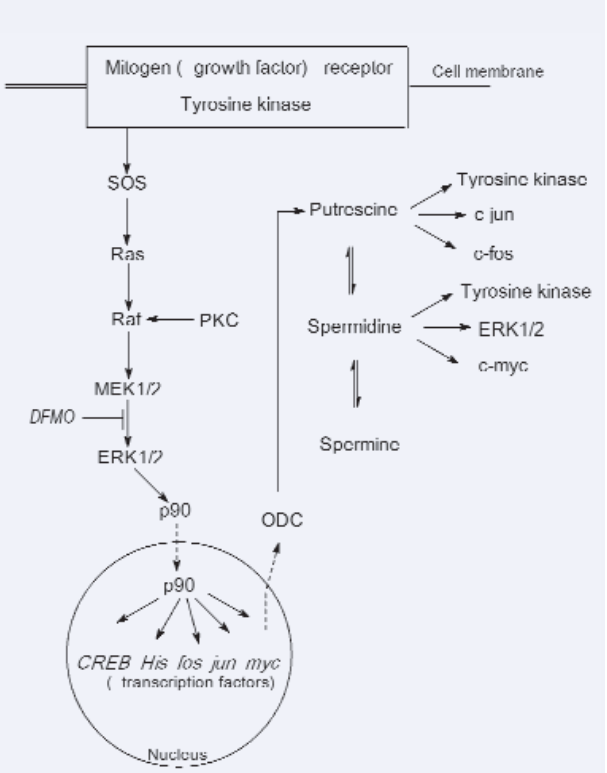


**Fig. 3** A mitogen (growth factor) -activated signal transduction pathway. The mitogen-receptor complex activates several signalling pathways. Some of these are tyrosine-kinase dependent. Phosphorylation of tyrosine activates Ras and this proto-oncogene activates a series of mitogen-activated protein kinases, (MAPKs). Phosphorylation by ERKs initiates the transduction of the signal to the nucleus by p90. Rsk-2, a member of the p90 family phosphorylates histone H3, which causes structural changes of chromatin, and several transcription factors are activated by phosphorylation. These events stimulate growth. Mutated Ras has been identified as oncogene. Src, another mitogen-activated oncogene (not shown) also induces malignant transformation. Put stimulates tyrosine phosphorylation by tyrosine kinase and the expression of c-fos and c-jun. Spd stimulates the phosphorylation of tyrosine and of ERKs and it activates c-myc. Spm seems not participating directly in gene expression regulation. DFMO prevents the expression of c-myc and c-fos. For more details see Bachrach *at al.* [20]. CREB cyclic AMP binding site of the ODC gene, ERKs, MEKs extracellular signal-regulated kinases, PKC protein kinase C, Ras, Raf proto-oncogenes, SOS son-of-sevenless protein.

in IEC-6 cells increased p53 gene expression, as well as the stability of p53 mRNA and p53 protein, but had no effect on p53 gene transcription. Exposure of polyamine-depleted cells to Spd caused a decrease in p53 gene expression, which preceded an increase in DNA synthesis, demonstrating that polyamines are involved in the regulation of p53 gene expression [28].

Polyamine depletion in IEC-6 cells is accompanied by an increase in junD and prevents the p21 promoter expression. These changes correlate with cell cycle arrest in G1 and suggest that an increase in JunD/AP-1 prevents cell proliferation [29, 30]. In addition to JunD, the expression of TGF-β has repeatedly been described in polyamine depleted intestinal cells [31, 32]. Polyamine deficiency was associated with a greater sensitivity to exogenous TGF-β. (Activation of TGF-β triggers signals from the cell surface receptors to the nucleus).

Smad proteins are transcription activators that are critical for transmitting signalling by TGF-β. Polyamine depletion activates the TGF-β-Smad signalling pathway in intestinal epithelial cells, which leads to growth arrest [33].

These few examples illustrate the requirement of polyamines for the expression of certain genes. The mechanisms by which they exert there role remains speculative [20, 21]. A very similar situation holds for the role of polyamines in apoptotic signalling.

## Apoptosis activation due to selective depletion of intracellular polyamine pools

Irradiation (UV, γ) and a great number of apoptogenic compounds, including glucocorticoids, polyphenols and TNF-α deplete polyamine pools. They are likely to act, among others, along similar pathways as the above-mentioned DMSO, namely by down-regulation of c-myc (see section 4). From these observations it was concluded [34] that polyamine depletion may be a general event in apoptosis activation. However, depletion of polyamines may have opposite effects on gene expression in different cell types. For example, the exposure of IEC-6 and HeLa-TV cells to DFMO was associated with the decrease of transglutaminase mRNA expression [35], whereas in CaCo-2-cells transglutaminase mRNA and enzyme activity were elevated by the same treatment [36]. These observations are important for the present considerations, because transglutaminases have been shown to have in some cell lines a protective function in ceramide and TNF-α- induced apoptosis [37]. The apparently contradictory results may be explained by differences in the regulation of the transglutaminase gene; or by different preferential apoptogenic pathways in different cell lines.

Only certain cell lines undergo cell death by exposure to DFMO. For instance DFMO does not induce apoptosis in intestinal epithelial cells (IEC-6), but it induces the tumour suppressor gene (p53) expression. In the case of human lung cancer cells the sensitivity to DFMO appears to be related to the steady state level of ODC mRNA, and the state of anchorage dependence of the cells [38, 39]. A predisposition of the cells is obviously a condition for DFMO-induced apoptosis.

Triggering of apoptosis in pre-cancerous cervical epithelial cells [40], uterine leiomyoma cells [41], HC11 mouse mammary epithelial cells [42], and human lung carcinoma (A549) cells [43], are examples of apoptosis induction due to exposure to DFMO. In addition there are examples of synergistic effects between known apoptogenic compounds and polyamine depletion: DFMO enhances the apoptosis rate of $Zn^{2+}$-deficient oesophageal cells [44] and stauroporin-induced apoptosis of IEC-6 cells [45]. Whenever tested, exogenous polyamines attenuated the effect of DFMO, emphasising a specific role of the polyamines.

From the induction of apoptosis by inhibitors of AdoMetDC (MGBG, CGP 48664) and the polyamine mimetic $N^1$,$N^{11}$-diethylnorspermine it was concluded that the sensitisation of T-cell hybridoma cells to TNF-α-induced apoptosis depends on the depletion of Spm [46]. However, transgenic cells which lack spermine synthase and Spm (Gy cells) show normal caspase activation by etoposide (a DNA topoisomerase II inhibitor), stauroporin, and cycloheximide, but were sensitised to UV light. If UV-irradiated Gy cells were exposed to DFMO, caspase activation increased [47]. Obviously the presence or absence of Spm had no effect on the induction of apoptosis.

Bcl-2 proteins are a family of anti-apoptotic (Bcl-2, Bcl-$X_L$, Mcl-1) and pro-apoptotic molecules (Bax, Bak, etc.) that link the death recep-

tor pathway to the mitochondrial pathway [48]. In HC11 cells polyamine depletion was associated with the down-regulation of Bcl-2 protein, and an increase of reactive oxygen species [42]. Expression of Bcl-2 antisense oligonucleotide RNA did not induce apoptosis of the human leukaemia cell line HL-60. However, exposure of these cells to low concentrations of DFMO caused cell death [49]. The induction of mitochondrial apoptosis pathways by selective polyamine depletion was also demonstrated, using a murine (WEHI231) and a human (Ramos) B cell line, as well as a T-cell line (Jurkat) [50]. Over-expression of anti-apoptotic proteins of the Bcl-2 family prevented, according to expectations, caspase-3 activation in the presence of DFMO, and the disruption of the mitochondrial membrane potential, whereas inhibition of the caspases prevented only the nuclear changes. In this connection it should be mentioned that the apoptotic effect of flavonoid procyanidins correlated in SW620 colon cancer-derived metastatic cells with the down-regulation of PKC, a decrease of ODC activity and the activation of caspase-3 [51]. Epigallocatechin-3-gallate, another flavonoid, caused a rapid decline of Put in HL-60 cells, which correlated with the formation of apoptotic DNA fragments: Cell types that show a higher resistance to apoptosis had higher Put concentrations than the HL-60 cells [52].

Cross-linking of the B cell antigen receptor causes apoptosis, which is accompanied by changes in gene expression: Genes involved in polyamine biosynthesis were down-regulated, whereas those involved in catabolism were up-regulated, and polyamine pools decreased. Inhibition of receptor cross-linking or addition of Spm attenuated the apoptotic events [53]. This example is a demonstration of the deregulation of polyamine metabolism by an apoptotic stimulus. The fact that the presence of Spm attenuated the apoptotic effect of receptor cross linking underlines the repeatedly mentioned protection against apoptotic stimuli by polyamines.

NF-κB is a transcription factor involved in the integration of survival signalling pathways, including up-regulation of Bcl-$X_L$, XIAP and cIAP-2. It also regulates immune and inflammatory responses [54]. In IEC-6 and breast cancer cells (MCF-7) NFκB was activated if Put and Spd were depleted by exposure to DFMO. This effect is accompanied by the translocation of p65 (the DNA binding subunit of NF-κB) from the cytoplasm into the nucleus. Exposure to DFMO inhibited also selectively a gene reporter construct that is dependent of the κB site in the HLA-B7 gene [55, 56]. Polyamine depletion and NF-κB activation correlate with caspase-3 activation in etoposide-treated fibroblasts [57], and activation of NF-κB by polyamine depletion was accompanied by the sensitisation to stauroporin - induced apoptosis, and the desensitisation to a combination of TNF-α/cycloheximide. Inhibition of NF-κB binding activity by sulfasalazine reversed the pro-apoptotic and anti-apoptotic effects of DFMO [45]. Recently Zou *et al.* [58] demonstrated that the depletion of polyamines in IEC-6 cells by DFMO not only activated NF-κB, but increased at the same time the expression of endogenous caspase inhibitors c-IAP and XIAP. Thus it follows that the resistance of polyamine deficient cells to TNF-α/cycloheximide-induced apoptosis is at least in part due to inhibition of caspase-3.

In MCF-7 breast cancer cells Spm facilitated the binding of NF-κB to the estrogen receptor α (a transcription factor implicated in breast cancer cell proliferation) suggesting a function of Spm in the regulation of certain proliferation-related genes [59]. The opposite, namely the inhibition of NF- B activation by polyamine depletion was also reported, namely for transformed mouse fibroblasts, which were exposed to etoposide [60]. Exposure of cells to etoposide causes DNA strands breaks [19].

CD95 (FAS) is a type I membrane receptor which mediates apoptosis [61]. DFMO induces FAS expression in HEP-2 cells [62]. Similarly Fas mRNA was up-regulated after exposure to DFMO in the human lung carcinoma cell line A549. Concomitantly cells accumulated in G1, expressed the human lung carcinoma-associated antigen, and ras P21 protein was down-regulated [43].

Polyamine depletion by DFMO in IEC-6 cells decreases the expression of ERK-2 kinase (see see Fig. 3). Cell cycle arrest was accompanied by an increase of p53 protein and other cell cycle inhibitors (p21 (Waf1/Cip1) and p27 ( Kip1). Concomitantly DFMO-induced stress activated MAPKs (protein kinase/c-Jun, JNK ($NH_2$-terminal kinase)). Activation of JNK-1 was the earliest event [63]. From these observations one has to conclude that MAPKs and JNKs are involved in the regulation of cell cycle inhibitors.

The principles of apoptosis activation by depletion of polyamines in cultured cells appear to be also relevant *in vivo*: Expression of antizyme (an inactivator and feedback regulator of ODC synthesis) in mice, or treatment of mice with DFMO reduced cell proliferation rate and increased the rate of apoptosis [64].

DFMO-induced activation of apoptosis is usually prevented in the presence of exogenous polyamines as has repeatedly bee indicated. But apoptosis induced by non-specific depletion of polyamine pools can also be prevented by exogenous polyamines. In most of these cases Spm was more potent than Spd and Put. For instance in cell death induced in cerebellar granule cells by serum starvation the activation of caspase 3 and Yama/apopain proteases was prevented by polyamines [65]. Other examples are selenomethionine induced apoptosis in A549 lung and HT29 colon cancer cells [66], dexamethasone and $Ca^{2+}$-ionophore apoptosis of liver cells and thymocytes [67, 68], and tyrosine kinase inhibitor (herbimycine)-induced DNA fragmentation of CTLL2 cells [69]. The prevention of endonuclease activation by Spm (upstream caspase-9 activation), respectively the prevention of tyrosine phosphorylation by Spm (in analogy to inhibition of tyrosine phosphorylation by Spd and PPut (Fig. 3)) were suggested by the authors of these works as potential explanations for their observations. In contrast with these examples of mitochondrial cell death, Spm had no effect on a death receptor-mediated apoptosis pathway (FAS cross ligation of the FAS (CD95) receptor) in a human leukaemia T-cell line (Jurkat) [70].

## Delay and prevention of apoptosis induction by selective polyamine depletion

In contradiction to the observations discussed in the previous paragraph, evidence exists also in favour of the prevention (or delay) of apoptosis induction due to selective depletion of polyamines. In Table 1 examples are compiled. Similar to DFMO the down-regulation of c-myc (and of ODC) was made responsible for apoptosis in serum starved leukaemia cells [71], and again, addition of exogenous polyamines prevented the DFMO effect.

Several studies have focused on IEC-6 cells. TNF-α/cycloheximide apoptosis is delayed by DFMO treatment (but DFMO failed to inhibit camptothecin-induced apoptosis) [72, 73]. (Camptothecin is a DNA topoisomerase I inhibitor). As far as mechanisms are concerned, several apoptotic processes have been implicated in TNF-α/cycloheximide apoptosis, but interest has particularly focused on the involvement of MAPK pathways. These are known to involve signalling with ERK1/2, JNK and p38 [74]. Direct evidence for the activation of ERK1/2 in DFMO-exposed IEC-6 cells (in contradiction to the effect of DFMO, as depicted in Fig. 3) was presented by Bhattacharya *et al* [75].

In fibroblasts caspase activation correlated with ERK1/2 phosphorylation, which is upstream caspase activation. Exposure to DFMO blocked caspase and ERK activation and it abolished phosphorylation of c-Jun NH2-terminal kinases in etoposide - treated cells. Replenishment of exogenous Put restored their ability to undergo caspase activation and ERK1/2 phosphorylation in response to etoposide [57]. It should be noted that etoposide causes a loss of polyamines in promyelogenous human leukaemia (HL-60) cells, without producing a great increase in catabolic acetylation. Its cytotoxic effect is thought to be due to the formation of toxic metabolites of polyamine oxidation [76].

An increase of NF-κ activity may have both, a pro-apoptotic and anti-apoptotic effect on intestinal IEC- cells. Pro- and anti-apoptotic effect is determined by the nature of the death program [45]. Polyamine depletion increased the basal level of NF-κ proteins, induced NF-κ nuclear translocation, and activated its sequence specific DNA binding. Inhibition of NF-κ binding by sulfasalazine prevented the increase in susceptibility to stauroporin-induced apoptosis, and blocked resistance to cell death by TNF-α/cycloheximide. Polyamine depletion in several human melanoma cell lines by a structural analogue of Spm gave evidence for the activation of MAPK phosphorylation [77].

In contrast with the above-mentioned observation, two reports [73, 78] suggest the prevention of camptothecin apoptosis of IEC-4 cells by DFMO. According to the observations of Yuan *et al.* [78] exposure to DFMO decreases caspase-3 and caspase-

**Table 1** Examples of prevention of apoptosis induction due to selective polyamine depletion

| Inducer of apoptosis | Cell line | Reference |
|---|---|---|
| Serum starvation | Cerebellar granule cells | [65] |
| Serum starvation | CEM T leukemia | [71] |
| Hydrogen peroxide | human promyelocytic leukemia HL-60 | [139] |
| 2-Deoxyribose | human promyelocytic leukemia HL-60 | [140] |
| Dexamethasone | lymphoblastic leukemia CEM-C7 | [141] |
| Taxol | human breast cancer MCF-7 | [142] |
| Green tea extract | Bladder carcinoma cells | [143] |
| Hepatocyte growth factor | hepatic carcinoma Hep G2 | [144] |
| TNF-α/cycloheximide | human cervical carcinoma MEZ-180 | [145] |
| Selenomethionine | lung cancer A549<br>colon cancer HT29 | [66] |
| Nonsteroidal anti-inflammatory drugs | colon carcinoma CaCo-2 | [146] |
| Etoposide | mouse fibroblasts | [147] |
| TNF-α/cycloheximide | Intestinal epithelial IEC-6 | [75] |
| Camptothecin | intestinal epithelial IEC-6 | [78] |
| Heat shock | thymocytes | [34] |
| γ-Irradiaion | thymocytes | [34] |
| Dexamethasone | thymocytes | [148] |
| Herbimycin A | thymocytes CTLL2 | [69] |

Polyamines were depleted by exposure to DFMO. Exogenous polyamines prevented the anti-apoptotic effect of DFMO

9 activities, and decreases the translocation of Bax to mitochondria, thus diminishing cytochrome *c* release. Finally polyamine depletion increased the expression of the anti-apoptotic proteins Bcl-$X_L$ and Bcl-2, and decreased caspase 8 activity and cleavage of Bid.

Akt is a serine-threonine kinase that has been established as an intracellular signalling factor. It regulates cell survival. The depletion of Akt following exposure of IEC-6 gut mucosal cells to DFMO increased the level of phosphorylated Akt, and increased Akt kinase activity. In addition, the phosphorylation of glycogen synthase kinase-3, a downstream target of Akt, was also increased in DFMO-treated cells. Polyamine depletion had, however, no effect on total Akt, phosphorylated ERKs, p38 and Bcl-2 proteins. Activated Akt was associated with both, a decreased level of caspase-3 and an increased resistance to TNF- α/cycloheximide apoptosis. Ectopic expression of Akt prevented the enhancement of caspase-3 activation, and prevented the DFMO effect [79].

In HL-60 cells 2-deoxy-D-ribose-triggered cell death is switched to necrosis by polyamine depletion. These cells are blocked in G1, whereas cells with normal polyamine content progress through G1 and S phase to G2/M. It was suggested that cells blocking in G1 undergo necrosis [80]. A generalisation of this suggestion awaits confirmation.

## Examples of apoptosis activation by increased polyamine concentrations

As was discussed in the previous paragraphs selective depletion of polyamines by DFMO may prevent or activate death programmes in various cell types. The fact that excessive accumulation of polyamines within cells is also a cause of apoptotic cell death is a further complexity of polyamine actions. For example, the induction of ODC in human non-small cell lung carcinoma cells by a synthetic retinoid (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid) causes apoptosis. Inhibitors of the nuclear transcription factor c-myc, which regulates ODC expression (see section 4), and DFMO prevented retinoid-induced apoptosis, suggesting a role of enhanced Put formation in this process [81].

ODC overproduction together with manipulations that cause over-accumulation of Put or Spd from the environment (*e.g.* due to inhibition of feedback repression of uptake, or in hyposmotic conditions) causes cell death [82–84]. Likewise ODC overproducing mouse myeloma cells undergo cell death that is prevented by DFMO and by aminoguanidine [85]. Since aminoguanidine is a potent inhibitor of DAO, it was suggested that oxidative stress was in this case the cause of cell death. Indeed, formation of $H_2O_2$ and aldehydes, the cytotoxic products of oxidative deaminations of the natural polyamines (see Fig. 1) is a likely cause of cell death in ODC overproducers. Intracellular oxidation of Put is normally not cytotoxic, because catalase copes with the amount of $H_2O_2$ formed by this reaction, and since 4-aminobutyraldehyde cyclises spontaneously to $\Delta^1$-pyrroline (see Fig. 1). If however Put is released in large quantities, and oxidised in the cellular environment, cells may be killed owing to the absence of catalase, even though Put is considerably less toxic than Spd and Spm under such conditions.

Oxidative stress is also presumed to play a role in primary cultures of ODC overproducing keratinocytes from ODC/ras double transgenic mice. The decreased survival time of the transgenic keratinocytes was due to the enhancement of apoptosis rate, not due to terminal differentiation. The green tea polyphenol epigallocatechin-3-gallate, a well known anti-oxidant, decreased paradoxically the growth rate and the survival of these cells, but not the growth of normal or ras-transfected keratinocytes. Under *in vivo* conditions caspase-3 activity was diminished only in epidermal cells with very high levels of ODC activity, suggesting that elevated polyamine levels sensitise tumour cells to apoptosis by the green tea polyphenol. This effect may be explained by the observation that several anti-oxidants are known to have both protective and apoptogenic effects, depending on their concentration [86].

In a DFMO resistant variant of rat hepatoma cells which over-accumulate Put, oxidative stress was excluded, and the prevention of hypusine formation, (the co-factor of the eukaryotic translation initiation factor (eIF-5A)) by high Put concentrations was identified as the cause of programmed cell death [87]. Still another reason for apoptosis appears to play a role in an ODC over-producing mouse myeloma (653-1) cell line. These cells undergo programmed cell death when exposed to ornithine: The rapid decrease of protein synthesis activity in this cell line was considered to induce apoptosis [88].

As the above described observations demonstrate, excessive ODC activity is detrimental for cells. Further support for this idea comes from the forced expression of antizyme in ODC overproducing cell lines. High antizyme levels prevent cell death [89, 90], and exhibit even anti-tumour effects [91].

Induction of apoptosis is not the exclusive consequence of overexpression of ODC in cells. E. Hölttä and his colleagues [92, 93] were the first to demonstrate that ODC over-expressing cells undergo spontaneous malignant transformation. The transformed cells form rapidly growing tumours in nude mice, and the above-mentioned ODC/Ras double transgenic mouse develops spontaneous skin tumours [86]. Earlier observations demonstrate that (at least in mouse skin) elevated Put levels are required for the formation and maintenance of the neoplastic phenotype [94]. The factors which drive ODC overproducing cells into one or the other direction - transformation or apoptosis - have still to be identified

## Possible mechanisms of polyamines in apoptosis

As is obvious from the numerous observations discussed in this and the previous reviews [5, 6] there is no doubt about the existence of multiple links between the natural polyamines and several sig-

nalling pathways of programmed cell death, even though the same change, (*e.g.* depletion of Put and Spd pools by DFMO) may have opposite effects in different cell lines. It is also evident from the prevention or reversion of the effects of DFMO by exogenous polyamines that the amines themselves, not the proteins involved in their metabolism and regulation are responsible for the activation or suppression of pro- and anti-apoptotic factors, respectively of apoptotic cascades. A participation of the natural polyamines in nearly all apoptotic pathways that were illustrated in Fig. 2 was postulated by one or the other worker, as became obvious from reviewing the published work. But since the requirement of polyamines for cell death activation, or their protection to apoptotic stimuli has only been demonstrated by depletion of Put and Spd pools, respectively by replenishment of polyamine deficits by addition of the amines, and since the specific role of the individual amines, Put, Spd, and Spm has usually not been clarified, a scheme including polyamines in apoptotic pathways is at present highly speculative, and contributes little to our understanding. However, it may be useful to briefly consider those mechanisms, by which the polyamines may possibly fulfil their roles in programmed cell death.

The regulation of cellular polyamine concentrations is sophisticated and thorough [3, 4]. In cells major polyamine concentrations changes are physiologically unlikely, except for Put, the product of the highly inducible and rapidly turning over ODC It should however be remembered that Put concentrations are usually lower than those of Spd and Spm by more than an order of magnitude. Contrasting with the physiologic situation, pathological conditions or drugs can provoke considerable concentration changes. These may lead to apoptotic cell death, as has been demonstrated in numerous examples in this review. In other words, aberrant polyamine concentrations are most probably not a primary cause of apoptosis, but the polyamines may promote apoptotic mechanisms, if they attain for some reason concentrations above or below physiological limits. As has already been said, a participation of polyamines in all major pathways of programmed cell death is more than likely, but due to lacking information it is impossible to pinpoint their sites of interactions with apoptotic factors.

The natural polyamines have four different ways of exerting physiological functions in cells (Fig. 4):

1. Binding to anionic sites by forming ion bonds.
2. Formation of covalent bonds by enzyme-catalysed reactions
3. Scavenging radicals (and complexing cations)
4. Formation of cytotoxic aldehydes and reactive oxygen species as products of oxidative deaminations,

These principles should apply in physiological functions of the polyamines, including programmed cell death.

## Binding to anionic structures

Owing to their protonated amino groups, which are arranged along a flexible carbon chain, the natural polyamines form ion bonds with a great variety of negatively charged molecules. Binding energy increases with the number of positive charges from Put to Spd to Spm (see *e.g.* ref [3, 95]). Interactions with polyanions (nucleic acids) have most extensively been studied in the past. Certain base sequences favour binding of Spd and Spm. However, owing to the less monotonous structure of proteins, interactions with anionic groups of proteins are more selective than binding to nucleic acids. Polyamine - protein interactions are most probably very numerous and of profound importance, as is documented for instance by the binding to a great variety of receptors (see *e.g.* ref. [95]).

Electrostatic interactions of polyamines stabilise secondary and tertiary structures of macromolecules or they induce conformational changes, and thus preserve, respectively alter physical and biological properties of polyanions and proteins. Formation of ion bonds is not only the most important general mechanism of polyamine function in cell biology, but most probably also the major domain of polyamine function in programmed cell death, even though specific interactions with known regulators of apoptosis have not been demonstrated.

An apoptosis related example of polyamine - protein interactions is the inhibition of apoptotic endonucleases by Spm (see *e.g.* ref. [96, 97]). Spm is known to prevent endonuclease activation [67]. The stabilisation of an inactive conformation of this enzyme by Spm is a likely mechanism.

Presumably the most important example of conformational stabilisation and packaging is in the present context the repeatedly observed destabilisation of the chromatin structure due to depletion of polyamines. Exogenous polyamines stabilise chromatin structure in cell nuclei, while polyamine depletion provokes an increased sensitivity of chromatin to degradation by different nucleases (see *e.g.* ref. [98–100]), impairs DNA repair mechanisms [101], and increases the sensitivity of DNA and chromatin to irradiation, heat and cytotoxic compounds [101–106]. There is evidence for the suggestion that binding of Spd and Spm reduces radiation accessible sites due to compaction of the DNA structure [107, 108], and prevents double strand brakes in the intact nucleus [109]. Furtheron Spm reduces the formation of radiation-induced DNA-protein cross-links of chromatin [110]. Owing to less tight binding and its low concentration, the role of Put appears to be restricted to radical scavenging, while Spd and Spm seem to be part in two protective mechanisms in radiation-induced chromatin damage.

Destabilisation of chromatin is the most likely explanation at least of some of the observations concerning the activation of apoptosis following inhibition of ODC, AdoMetDC or the perturbation of polyamine regulation by structural analogues of Spm. As was pointed out by Hobbs *et al.* [111] nucleosome flexibility and sliding is required to permit polymerases to advance along the DNA strand. Impairment of movement or of the dissociation of chromatin-associating proteins from chromosomal DNA due to lacking polyamines could impede transcription, recombination and repair, and lead to chromosomal instability and an increased rate of mutation. Specific interactions of polyamines with DNA that imply chromatin configuration are also suggested by the observation that DNA methyltransferases are inhibited by polyamines [112]. However, the fact that Chinese hamster ovary (CHO) cells exposed to DFMO had no significant effect on the condensation state of chromatin during the cell cycle [113] is a *caveat* against generalisations concerning polyamine pool size changes and functional changes of chromatin.

## Formation of covalent bonds

The natural polyamines are substrates of transglutaminases. The attachment of Put, Spd or Spm profoundly alters physicochemical and biological properties of proteins. The same is true for crosslink formation (see *e.g.* ref. [114]). In view of their role in apoptosis, structural modifications of proteins by transglutaminase-catalysed linking of polyamines to proteins is a possible reaction in cell death programs [115] that should be particularly important in situations of excessive polyamine accumulation, although research is at present mainly focused on transglutaminase catalysed cross link formation by reaction of ε-amino groups of lysine residues [116].

Spd is a precursor of hypusine, which as cofactor of the eukaryotic initiation factor 5A (eIF-5A) is necessary for cell proliferation [117]. The formation of active eIF-5A can be prevented by depletion of polyamines, using a selective inactivator of AdoMetDC [118] and possibly also by exposure to DFMO [119]. Since the apoptotic effect of high concentrations of Put has been linked with the prevention of hypusine formation [87], the impairment of eIF-5A formation may be a more frequent cause of apoptosis than is presently known.

Lipophilic derivatives of the natural polyamines are cytotoxic and may cause apoptotic cell death (see *e.g.* ref. [15, 95]). Although at present speculative, as far as the vertebrate organism is concerned, there is a probability that apoptogenic polyamine derivatives are formed, for instance by conjugation with fatty acids, amino acids etc., in analogy to wasp and spider toxins, and the lipophilic toxins with a polyamine backbone of other lower species. A systematic search for this structural type should be of considerable interest and may help explaining certain pharmacological properties of the polyamines.

## Scavenging of radicals

During the initial phase of apoptosis a variety of cellular signalling pathways are activated as has briefly been discussed in section 2. Activation of death receptor signalling pathways promote the excessive formation of reactive oxygen species (ROS) (superoxide anion, hydroxyl radical, $H_2O_2$) in mitochondria (see *e.g.* ref. [120–122]). ROS are mainly formed by stimulation of NADPH oxidases and the mitochondrial electron transport chain. While ROS have numerous physiological functions in signalling pathways [123], their excessive formation leads to chromatin degradation,

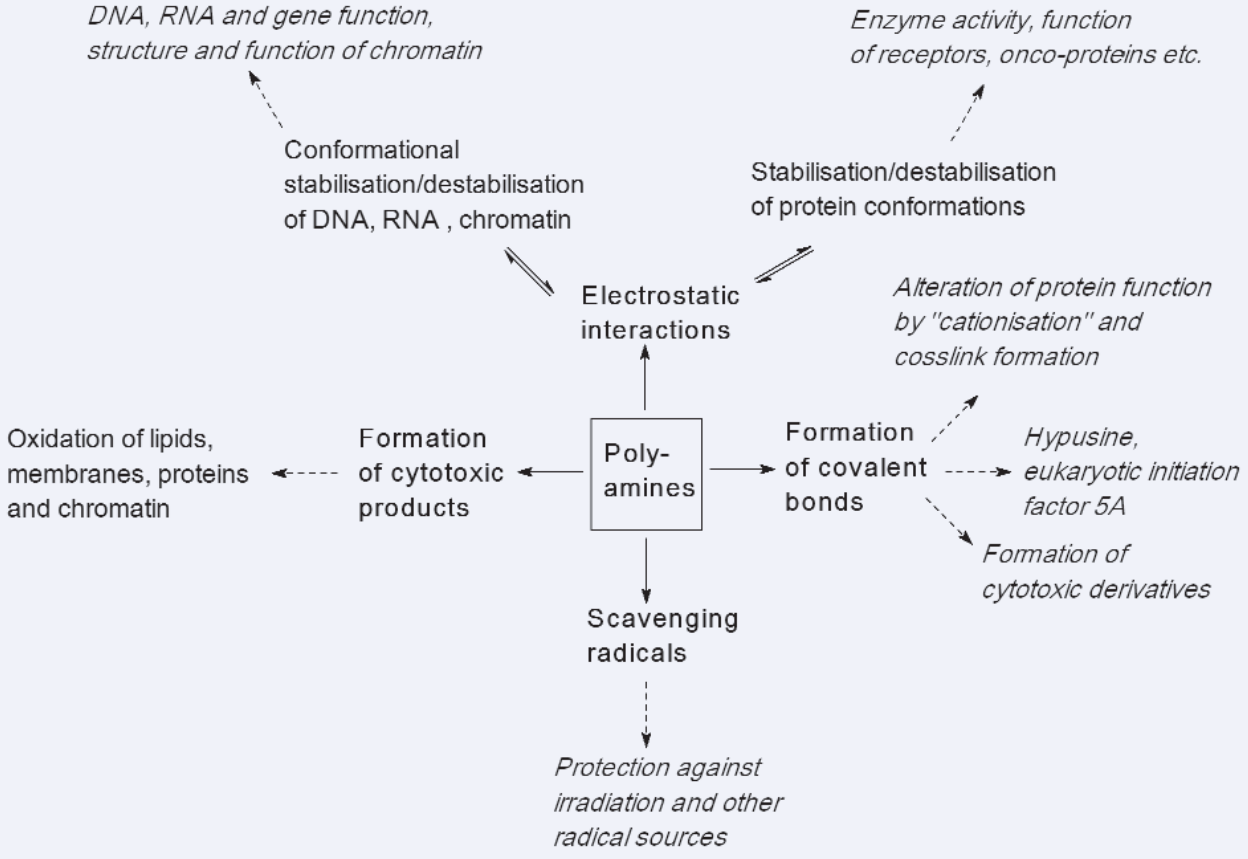


**Fig. 4** Possible consequences of major changes (increase or decrease) in cellular polyamine concentrations. (For details see text).

oxidative damage of various proteins and to membrane lipid peroxidation (see *e.g.* ref. [123, 124]). Intracellular thiols (glutathione, thioredoxin) play a paramount role in neutralising ROS and stabilising cells against apoptotic cell death and senescence (see *e.g.* ref. [125]).

For many years the polyamines are presumed to have anti-oxidant and radical scavenging properties. The model systems in use to demonstrate protection by the polyamines against lipid peroxidation and DNA and cell damage were compiled by Lovaas [126]. Not much has been done in this area during the recent years. It is, however interesting to note that up-regulation of Spd in skin fibroblasts from hemizygous Gy mice, which lack spermine synthase and Spm, causes an increased resistance to oxidative stress, but a decreased resistance to UV irradiation [127].

Based on the fragmentation of [$^{13}$C]-labelled Spm by hydroxyl radicals Ha *et al.* [128] suggested a direct free radical scavenging mechanism by Spm, whereas Das and Misra [129] report results, which are compatible with hydroxyl radical scavenging by Put, Spd and Spm, and singlet oxygen quenching by Spd and Spm.

The available information does not allow one to prove a protective function of the natural polyamines to ROS-induced damage under physiological or pathological conditions. However, the observed activation of apoptosis due to selective and non-selective polyamine depletion, and the prevention of these drug effects by exogenous polyamines (see section 5) is likely to be at least in part the result of the radical scavenging properties of Put, and particularly of Spd and Spm, which are present in cell nuclei at higher concentrations than in the extra-nuclear space [130]. Thus there is a fair probability for a physiological function of the polyamines in protecting chromatin from damage.

## Formation of cytotoxic products

It has been mentioned in section 7 that aminoguanidine prevents apoptosis of ODC over-producing

mouse myeloma cells [85]. The effect of the DAO inhibitor was explained by preventing $H_2O_2$ formation in the environment of the cells.

An extensive literature describes apoptogenic effects of the products of polyamine oxidation. $H_2O_2$ forming reactions of the polyamines are shown in Fig. 1. Non-apoptotic cell death may also be induced by these products [131]. "Functions" of extracellular oxidative deaminations of the polyamines have recently been reviewed [132]. Therefore polyamine oxidation will not be considered here in detail. It should only be pointed out that (a) intracellular formation of toxic products is dependent on the presence of appropriate polyamine metabolising enzymes, *i.e.* DAO, PAO or SMO. Their activities may vary considerable in different cell lines. Tumour cells contain normally lower activities of PAO than their normal counterparts [133], so that oxidative damage in tumour cells is less likely than in their normal counterparts. (b) The toxicity of the products of oxidative deamination of polyamines increase in the order Put<Spd<Spm, indicating that not only $H_2O_2$, but the aldehydes formed from these amines are also important cytotoxic agents. Acrolein appears to be particularly cytotoxic [134]. It is formed by spontaneous β-elimination from 3-aminopropanal, respectively from the aldehydes formed from Spd and Spm by oxidative deamination (Fig. 1). Owing to the absence of catalase and of aldehyde dehydrogenases, the formation of $H_2O_2$ and toxic aldehydes in the extracellular space is considerably more cytotoxic than their intracellular formation, which allows rapid inactivation. In experiments aiming at investigating effects of exogenous polyamines on cell function, serum amine oxidase (fetal calf serum or bovine serum) - containing culture media have frequently been the cause of cell death, and of misinterpretations of "polyamine cytotoxicity".

There is an example for the rapid formation of cytotoxic products inside the cells, with apoptosis as a consequence: Potent inducers of SAT, such as $N^1$-ethyl-$N^{11}$-[(cycloheptyl)methyl]-4,8-diazaundecane and $N^1$,$N^{11}$-diethylnorspermine cause apoptosis of cell lines, which express PAO [77, 135]. Apoptosis by these drugs can be explained by the massive production of N-acetyl derivatives of Spd and Spm, which follows induction of SAT. The acetyl derivatives react with PAO to form $H_2O_2$ and 3-acetamidopropanal (Fig. 1). Their formation and apoptosis is prevented by inhibition of PAO by a selective inactivator of this enzyme. It is known that SAT activity increases in response to an increased production of ROS [136, 137]. The apoptotic effect of SAT inducers may, therefore, be amplified by $H_2O_2$ through a positive feedback mechanism.

## Conclusion

Owing to an enormous interest and the development of appropriate methods, a very large number of factors involved in programmed cell death have been identified within a short period, and a relatively clear, though not yet complete picture has emerged of the rather complex and multifaceted events, which enable the organism to remove cells in a regulated manner. Our preliminary, mostly descriptive knowledge of the role of the natural polyamines in these processes contrasts with the state of the art of apoptosis research, although the participation of Put, Spd ad Spm in programmed cell death is not doubtful. In view of the multiple interactions of the polyamines with factors involved in signal transduction, and the not yet exactly defined functions of the natural polyamines in cell growth and cell cycle regulation, a bleary picture of their role in apoptosis does not surprise. The present situation reminds one of an unfinished puzzle, of which we neither know the number of missing pieces nor the number of those pieces that have already been correctly placed.

One of the not easily solvable experimental problems derives from the fact that the work of individual investigators necessarily focuses on small segments of the complex events involved in apoptotic mechanisms, and work is usually limited to one or a few cell lines. In most cases not even the precise function of the individual amine - Put, Spd or Spm - has been identified because of the difficulty of the task, so that generalisations and extrapolations from one cell line to another is hazardous.

In section 7 several likely mechanisms of apoptosis activation have been discussed for cells, which over-accumulate polyamines, suggesting a variety of potential mechanisms. None of the presently available hypothesis is, however, able to explain the prevention of apoptosis following partial depletion of cellular polyamine pools, disregarding the trivial suggestion that by a lack of polyamine ligands, pro-

apoptotic factors are less efficiently activated than anti-apoptotic factors. Keeping in mind that chromatin damage is a frequent primary cause of apoptosis induction, a polyamine deficit is at least a plausible explanation for the activation of apoptosis in polyamine-deprived cells.

The emerging role of polyamine functions in apoptosis, particularly their role in chromatin stabilisation, which may imply epigenetic changes and tumour formation, requires an improvement of our knowledge. The ability of the polyamines to interact with a very large number of anionic macromolecules is a nearly insurmountable problem in attempts to clarify the precise contribution of polyamines in individual death programs. Pinpointing specific interactions with selected macromolecules and the elucidation of physiological consequences of these interactions will be impossible, as long as exclusively concentrations of the natural polyamines are manipulated, or the natural polyamines are used as tools for the demonstration of interactions. A potential way out of the dilemma, though not a simple way, is perhaps the design and synthesis of structural analogues which mimic polyamine functions, but have more selective binding affinities than the natural polyamines. Melchiorre and his colleagues [138] have demonstrated that it is in principle possible to modify the polyamine backbone, and to attach substituents in such a way to the polyamine structure that compounds are obtained, which bind selectively to certain membrane receptor proteins and enzymes. This approach should also be applicable to regulators of growth, to regulators of the cell cycle and to apoptotic factors. Gene regulation by polyamines should profit from this approach. From the comparison of the effects of the natural polyamines, with those of the artificial analogues new drugs can be expected, or at least new tools, which allow the characterisation of specific functions of the polyamines.

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