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Polyamine biosynthesis as a target to inhibit apoptosis of non-tumoral cells

Review Article

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Summary. Growing evidence suggests a role for polyamines in apoptosis, although the relationship appears to be complex. α-Difluoromethylornithine (DFMO), a largely used ornithine decarboxylase inhibitor, is cytostatic, hardly cytotoxic and may even increase the resistance of tumour cells to some apoptotic stimuli. This may represent a problem in cancer therapy, where the killing of tumoral cells would be a desired effect, but could be an advantage in other pathological contexts related to an excess of apoptosis, such as cardiovascular diseases, stem cell transplantation, arthritis and infections. In different cellular models, polyamine depletion following treatment with polyamine biosynthesis inhibitors appears to inhibit mitochondrial and death receptor pathways of apoptosis by affecting key proteins. These studies indicate that inhibition of polyamine biosynthesis may prevent or reduce the apoptotic response triggered by a variety of stimuli in non-tumoral cells, such as cardiac cells, stem cells, chondrocytes, macrophages and intestinal epithelial cells.

Keywords: Polyamines – Apoptosis – Signal transduction – Difluoromethylornithine – Caspases – Non-tumoral cells

Abbreviations: ASK, apoptosis signal-regulating kinase; CHX, cycloheximide; DFMO, α-difluoromethylornithine; ERK, extracellular signal-regulated kinase; JNK, c-Jun-N-terminal kinase; ODC, ornithine decarboxylase; PP, protein phosphatase; SAM, S-adenosylmethionine; STAT, signal transducer and activator of transcription; TNF-α, tumor necrosis factor-α

Introduction

The aliphatic polyamines putrescine, spermidine and spermine are small, flexible molecules, which can specifically bind to nucleic acids and proteins "in vitro" and thus affect their conformation and biological activity (Thomas and Thomas, 2001; Bachrach, 2005). The concentration of these organic polycations in cells can be finely modulated by enzymatic and transport systems. Ornithine decarboxylase (ODC), the key enzyme in polyamine biosynthesis, is rapidly induced by growth stimuli with consequent intracellular accumulation of polyamines (Pegg et al., 1995; Thomas and Thomas, 2001). Although it has been known for some time that ODC and polyamines are essential for cell proliferation, growing evidence suggests a role for polyamines even in other cell responses, including apoptosis (Seiler and Raul, 2005) and the regulation of inflammatory reactions (Moinard et al., 2005). Some studies in intact cells indicate that polyamines may modulate signal transduction pathways and the expression of specific genes at the transcriptional and/or translational level (Bachrach et al., 2001; Yoshida et al., 2004; Moinard et al., 2005).

DFMO, cell survival and apoptosis

Several studies published in recent years have shown that polyamines can doubtless affect the apoptotic process, however the relationship appears to be complex and dependent on the cell type and death stimulus, as well as on the actual levels and activated polyamine pathways present in the particular models (for recent reviews see: Wallace et al., 2003; Pignatti et al., 2004; Seiler and Raul, 2005). Whereas excessive intracellular polyamines in overproducing cells or following exogenous addition can induce apoptosis, polyamine depletion caused by a single

specific polyamine biosynthesis inhibitor like DFMO, a largely used ODC inhibitor, is cytostatic, but hardly cytotoxic, although important exceptions have been reported (Gerner and Meyskens, 2004; Seiler and Raul, 2005). We and others have shown that polyamine depletion by DFMO can reduce or enhance the susceptibility to apoptosis even in the same cell type, depending on the specific death stimulus, thus hinting at the importance of the death pathway engaged (Li et al., 2001; Stefanelli et al., 2001). A recent review on polyamines and apoptosis (Seiler and Raul, 2005) reports a partial list of papers showing protective effects of DFMO in a variety of experimental models of apoptosis, including several types of neoplastic cells.

The lack of toxicity of DFMO or other polyamine inhibitors may represent a problem in cancer therapy (Gerner and Meyskens, 2004), where the killing of tumoral cells would be a desired effect, but could be an advantage in other pathological contexts related to an excess of apoptosis. Examples of such contexts may be cardiovascular diseases (cardiomyocyte apoptosis is a cellular mechanism of injury in ischemia and reperfusion, hypertrophy and heart failure, reviewed in Kitsis and Mann, 2005), stem cell transplantation to treat heart infarction (a majority of transplanted stem cells may die of apoptosis, see e.g. Mangi et al., 2003) and osteoarthritis (chondrocyte apoptosis contributes to cartilage damage, reviewed in Goggs et al., 2003). Thus we have recently investigated whether DFMO can inhibit apoptosis in rat H9c2 cardiac cells (Tantini et al., 2006), in stem cells isolated from rat bone marrow (Muscari et al., 2005), in human chondrocyte-derived C-28/I2 cell line and in primary cultures of chondrocytes isolated from osteoarthritis patients (Stanic' et al., 2006). In these cell models, DFMO treatment was performed at concentrations of 0.1–1 mM and for times ranging from 24 to 72 h before addition of an apoptotic stimulus. In these conditions DFMO generally reduced polyamine content (particularly putrescine and spermidine, whereas spermine level was slightly or no affected) and cell death induced by a variety of stimuli including simulated ischemia (in H9c2 cells), staurosporine and a combination of tumor necrosis factor-α (TNF) and cycloheximide (CHX) or MG-132, a proteasome inhibitor. The increase in the number of surviving cells was evaluated by the trypan blue exclusion test and other morphological criteria (DAPI staining). DFMO pre-treatment was also able to prevent or reduce critical apoptotic events, i.e.: 1) activation of effector caspase 3-like enzymes, which represents a no return point in apoptosis (Thornberry and Lazebnik, 1998): the activation was estimated from the

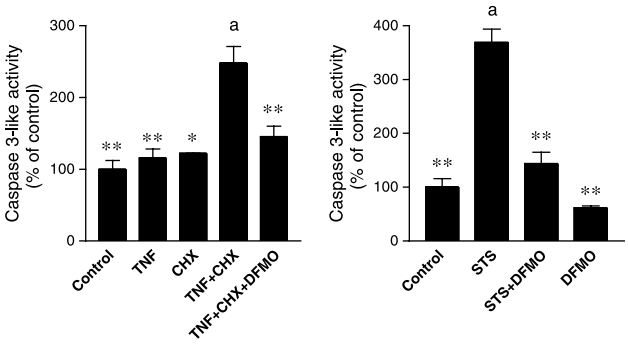

Fig. 1. DFMO pre-treatment inhibits effector caspase activation in primary, monolayer cultures of human chondrocytes isolated from cartilage explants of osteoarthritis patients. Chondrocytes were grown for 3 days after seeding without any addition or in the presence of 1 mM DFMO, and then were treated with 500 U/ml TNF and/or 0.2 mM CHX for 8 h (left), or 1 μM staurosporine (STS) for 5 h (right). Cells were collected and analysed for caspase 3-like activity (DEVDase) as previously detailed (Stanic' et al., 2006). Data are means ± SEM ($N = 4$); * $p < 0.05$ and ** $p < 0.01$ vs. TNF + CHX-treated cells (left) and STS-treated cells (right); [a] $p < 0.01$ vs. control cells

proteolytic cleavage of the inactive procaspase-3 and a substantial increase in catalytic DEVDase activity; 2) internucleosomal DNA fragmentation, evaluated as DNA laddering by agarose gel electrophoresis. The number of apoptotic cells was also reduced as evidenced by TUNEL assay, which detects fragmented DNA. As an example, we show the effect of DFMO pre-treatment on caspase 3-like activity in primary chondrocytes isolated from osteoarthritis patients and stimulated by TNF + CHX or by staurosporine (Fig. 1).

Polyamine levels and apoptosis

Importantly, co-addition of putrescine (at 5–100 μM), which restores the intracellular polyamine levels impaired by DFMO, also prevented the DFMO effects on apoptosis-related events mentioned above. These results show that inhibition of polyamine biosynthesis by DFMO prevents or reduces apoptosis, but can an *increase* in polyamine content favour apoptosis? Actually in the model of H9c2 cardiac cells, the apoptotic stimulus (simulated ischemia) caused a transient increase in ODC activity and putrescine level, followed by a more delayed enhancement in spermidine. Moreover we have reported that primary cardiomyocytes, isolated from neonatal transgenic mice over-expressing ODC in the heart (Shantz et al., 2001), show a higher caspase activity with respect to cells from control mice, particularly after serum deprivation (Tantini et al., 2006). Moreover, this pro-apoptotic effect was abolished when the inactive stereoisomer D-ornithine was supplemented to the cells instead of the natural sub-

strate L-ornithine, resulting in the lack of polyamine synthesis. It should be noted that ODC activity was clearly higher in cardiomyocytes isolated from transgenic animals compared to littermate cardiac cells, but resulted in an about 4-fold increase in putrescine only (i.e. it is not an extreme condition). Thus, these results point at a critical role for ODC activity and polyamine biosynthesis in inducing/potentiating apoptosis in cardiomyocytes.

The previous experiments indicate that the effects of DFMO on apoptosis were polyamine-specific. However DFMO can also cause accumulation of decarboxylated S-adenosyl methionine (dc-SAM), which may be responsible for some effects of DFMO (Frostesjo et al., 1997). Therefore we have used even the SAM decarboxylase inhibitor CGP 48664, which preventing dc-SAM synthesis also blocks formation of spermidine and spermine. We have found that CGP 48664 was also effective in inhibiting apoptosis in the models tested for DFMO. The relation with polyamine levels was particularly investigated in C28/I2 chondrocytes. In these cells pre-incubation with DFMO for 3 days resulted in a strong reduction of putrescine and spermidine content, whereas spermine was only slightly affected. CGP 48664 alone (1 μM) was able to reduce the level of both spermidine and spermine markedly, and actually increased that of putrescine (Stanic' et al., 2006). Finally the combined treatment of DFMO and CGP 48664 markedly reduced the content of all three polyamines (not shown). The effect of the treatments with these inhibitors on the increase in caspase 3 activity stimulated by TNF + CHX is shown in Fig. 2. CGP 48664 and DFMO + CGP 48664 were effective in inhibiting caspase activation to an extent similar to that of DFMO alone. In the presence of DFMO + CGP 48664, which do not allow conversion of putrescine to spermidine and of spermidine to spermine, co-addition of putrescine was not able to sustain the increase of caspase activity, whereas spermidine was. Exogenous spermine was also effective, but the possibility of intracellular retroversion to spermidine cannot be excluded. Altogether these results suggest that spermidine is the critical polyamine in this context, even if the possibility that spermine may replace spermidine in fulfilling this role may not be ruled out, as suggested in a previous paper for spermine synthase-deficient fibroblasts induced to apoptosis by etoposide (Stefanelli et al., 2001).

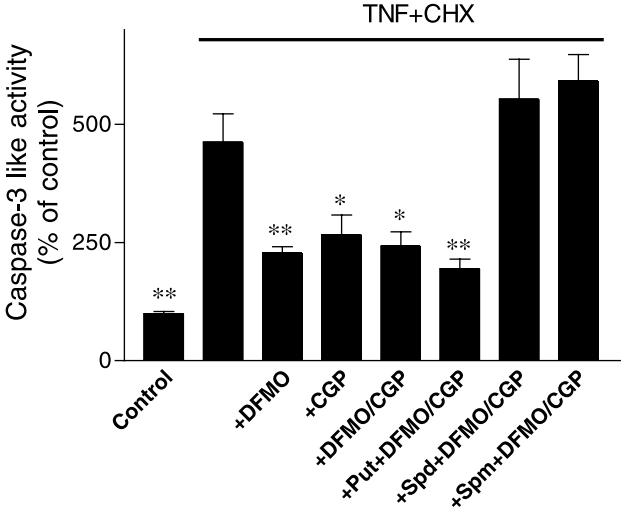

Fig. 2. Reversal by polyamines of the effect of polyamine biosynthesis inhibitors on TNF + CHX induced effector caspase activity. C-28/I2 chondrocytes were grown for 3 days after seeding without any addition (control), or in the presence of 1 mM DFMO and/or 1 μM CGP 48664 (CGP) or in the presence of both inhibitors (DFMO/CGP) plus 5 μM putrescine (Put), spermidine (Spd) or spermine (Spm). Then cells were treated with TNF plus CHX for 8 h. Cells were harvested and assayed for caspase 3-like activity. Data represent means ± SEM ($N = 4$); * $p < 0.05$ and ** $p < 0.01$ vs. TNF + CHX-treated cells. Polyamines alone did not affect caspase activity at these concentrations

Polyamine biosynthesis, signaling and apoptotic pathways

Two main pathways of apoptosis have been described: the mitochondrial pathway and the death receptor pathway (Ashkenazi and Dixit, 1998; Green and Reed, 1998; Jin and El-Deiry, 2005). Briefly, the mitochondrial pathway involves Bcl-2 family members, cytochrome *c* release and activation of caspase-9, an initiator caspase that leads to generalized activation of caspase-3 and other effector caspases. In the death receptor pathway, on the other hand, death receptors, like TNFR1, activate different initiator caspases, particularly caspase-8, which in turn can directly activate effector caspases. In some cell models however, induction of apoptosis following death receptor ligation requires also a cross-talk with the mitochondrial pathway (e.g. through caspase-8 mediated truncation of Bid or regulation of protein kinases), which amplify the signal (Varfolomeev and Ashkenazi, 2004). Therefore we addressed the question whether polyamine depletion may interfere with the mitochondrial and/or the death receptor pathway of apoptosis.

In the H9c2 model of apoptosis caused by simulated ischemia, polyamine depletion following DFMO appears to inhibit two critical steps of the mitochondrial pathway of apoptosis: cytochrome *c* release from mitochondria and the subsequent caspase 9 activation, possibly by affecting the expression of Bcl-xL, an anti-apoptotic member of the Bcl-2 family (Tantini et al., 2006). We have also consid-

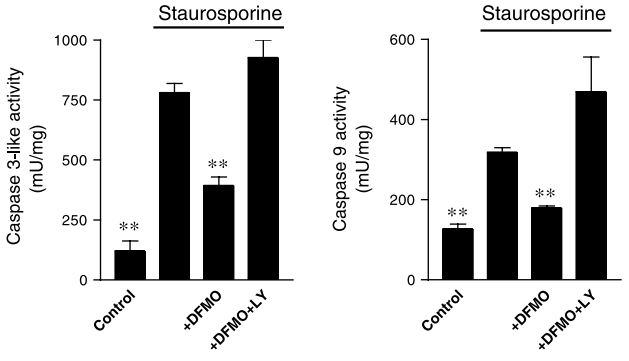

Fig. 3. Effect of the inhibition of Akt pathway on caspase response to polyamine depletion. C-28/I2 chondrocytes were grown for 3 days after seeding without any addition (Control) or in the presence of DFMO and then further incubated with staurosporine for 5 h. The specific PI3K inhibitor LY 294002 (LY; 20 μM) was added 24 h before staurosporine (this treatment prevented Akt phosphorylation). After collection, the cells were assayed for caspase 3-like activity (left) or caspase 9 activity (right). Caspase 9 activity was assayed as described for caspase 3 activity (Stanic' et al., 2006) except that Ac-LEHD-AMC was used as fluorogenic peptide substrate. Data are means ± SEM ($N = 4$); ** $p < 0.01$ vs. staurosporine-treated cells

ered C-28/I2 chondrocytes stimulated by TNF or staurosporine, to investigate the involvement of polyamines in the death receptor and the mitochondrial pathways, respectively, in a same cell type. The results showed that in TNF-stimulated chondrocytes, polyamine depletion, obtained either with DFMO or CGP 48664, can inhibit the activation of effector caspase 3-like enzymes by reducing the protein level (and thus the activity) of the key initiator pro-caspase 8 (Stanic' et al., 2006). On the other hand, DFMO pre-treatment prevented the increase in caspase 9 and caspase 3 activity, following the stimulation of C-28/I2 chondrocytes with staurosporine (Fig. 3).

Some studies, mainly performed with intestinal epithelial cells (IEC-6 line), have reported that DFMO affect key signaling proteins involved in apoptosis control, such as Src, extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), Akt, apoptosis signal-regulating kinase (ASK1) and protein phosphatases (PP5 or PP2A) (Stefanelli et al., 2002; Bhattacharya et al., 2003, 2004, 2005, 2006; Zhang et al., 2004; Kutuzov et al., 2005; Ray et al., 2005). Therefore we wondered whether polyamine depletion may affect key signaling protein kinases even in chondrocytes thus mediating caspase inhibition. Among several protein kinases and other key signaling proteins tested, we have found that DFMO induces the phosphorylation/activation of Akt in C-28/I2 chondrocytes. LY 294002, a specific Akt pathway inhibitor, was able to prevent Akt phosphorylation in DFMO-treated cells, but could not restore the TNF + CHX-induced caspase activation blunted by either DFMO or CGP 48664. The reason may be that LY 294002 did not prevent either the decrease in procaspase 8 content caused by DFMO. On the other hand, Akt inhibition prevents the effect of DFMO on caspase 3 and caspase 9 activation induced by staurosporine (Fig. 3). Thus, in C-28/I2 chondrocytes the Akt pathway is not involved in the DFMO inhibiting action on TNF-induced caspases and apoptosis (death receptor pathway), but is involved in the case of caspase activation provoked by staurosporine (mitochondrial pathway). DFMO has been shown to increase Akt phosphorylation at Ser473 in intestinal epithelial cells (Zhang et al., 2004; Bhattacharya et al., 2005) and in neuroblastoma cells (Bachmann et al., 2006). TNF-triggered apoptosis of intestinal epithelial cells appears to require the mitochondrial pathway, and DFMO may interfere with the activation of this intrinsic pathway and caspase 9 by activating Akt kinase and preventing JNK activation (Bhattacharya et al., 2003, 2005; Zhang et al., 2004). Interestingly it has been reported that DFMO inhibits both caspase 8 and caspase 9 activation in intestinal cells, whereas inhibition of JNK pathway affects only the caspase 9 pathway (Bhattacharya et al., 2003). On the other hand C-28/I2 chondrocytes may belong to a type of cells that does not require cross-talk with the mitochondrial pathway for death receptor-induced apoptosis. Thus signalling pathways that interfere with the mitochondrial pathway of apoptosis may be dispensable for and/or not influent on TNF-induced apoptosis in chondrocytes. In fact, the increase of caspase 3-like activity following TNF + CHX in C28/I2 chondrocytes was not significantly inhibited by the specific JNK inhibitor SP600125 (not shown), which on the contrary was able to inhibit caspase 3 and 9 activation in response to TNF + CHX in IEC-6 cells (Bhattacharya et al., 2003). In conclusion, these results with chondrocytes show that DFMO can inhibit both the death receptor pathway by reducing the level of procaspase-8 content, and the mitochondrial pathway by activating key signaling proteins such as Akt.

Finally it has been reported quite recently (Bhattacharya et al., 2006) that DFMO prevented apoptosis of IEC-6 cells by a rapid (within 1 h) activation/phosphorylation of Src, ERK1/2 and signal transducer and activator of transcription (STAT3). However under conditions similar to those described for IEC-6 cells (serum deprivation for 24 h before addition of DFMO for 1 h), we fail to show an increase in the C28/I2 cellular content of phosphorylated Src (pTyr 416), ERK1/2 (pThr202/pTyr204), STAT-3 (pTyr705) by immunoblotting using phospho-specific antibodies.

Concluding remarks

Although the direct targets of polyamines remain elusive and the actual mechanisms may differ among the cell types, the results summarized above indicate that inhibition of polyamine biosynthesis may prevent or reduce the apoptotic response triggered by a variety of stimuli in non-tumoral cells, such as cardiac cells, stem cells and chondrocytes. The role of the enzymes of polyamine catabolism, which can also be involved in cell death (Wallace et al., 2003; Wang and Casero, 2006), remains to be evaluated in these cellular models of apoptosis. Another context where inhibition of apoptosis would be desirable could be gastric infection by *Helicobacter pylori* (Wang and Casero, 2006) or pneumonia by *Pneumocystis* (Lasbury et al., 2006). These micro-organisms can induce apoptosis of macrophages, thus reducing the efficacy of host immune response. Interestingly, polyamine biosynthesis, catabolism or uptake may play a role in the mechanisms of these infections by facilitating macrophage apoptosis (Chaturvedi et al., 2004; Cheng et al., 2005; Liao et al., 2006).

Of course a possible use of these findings in pathological contexts to reduce deleterious apoptosis would require in vivo investigation to validate what found in cell culture. Although the inhibiting effects of DFMO on apoptosis of various cell types (tumoral and non-tumoral) may rise some concern regarding the possibility that it may favour carcinogenesis or tumor progression, this seems to be excluded by the growing evidence of its cancer chemopreventive effects in a variety of animal models (Gerner et al., 2004; Basuroy and Gerner, 2006) and by its well known and universal ability to inhibit cell proliferation. Actually the capability of polyamine biosynthesis inhibitors to interfere with expression or activity of key proteins regulating cell cycle has been suggested to be related to their ability to inhibit apoptosis (Monti et al., 1999; Tantini et al., 2006).

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