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Spermine Deficiency Resulting from Targeted Disruption of the Spermine Synthase Gene in Embryonic Stem Cells Leads to Enhanced Sensitivity to Antiproliferative Drugs

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ABSTRACT

Polyamines are known to be essential for normal cell growth and differentiation. However, despite numerous studies, specific cellular functions of polyamines in general and individual polyamines in particular have remained only tentative, because of a lack of appropriate cell lines in which genes of polyamine-synthesizing enzymes have been disrupted by gene targeting. With the use of homologous recombination technique, we disrupted the gene encoding spermine synthase in mouse embryonic stem cells. The spermine synthase gene is located on X chromosome in mouse and, because the cells used in this study were of XY karyotype, a single targeting event was sufficient to result in null genotype. The targeted cells did not have any measurable spermine synthase activity and were totally devoid of the polyamine spermine. Spermine deficiency led to a substantial increase in spermidine content, but the total polyamine content was nearly unchanged. Despite the lack of spermine, these cells displayed a growth rate that was nearly similar to that of the parental cells and showed no overt morphological changes. However, the spermine-deficient cells were significantly more sensitive to the growth inhibition exerted by 2-difluoromethylornithine, an inhibitor of ornithine decarboxylase. Similarly, methylglyoxal bis(guanylhydrazone), an inhibitor of *S*-adenosylmethionine decarboxylase, and diethylnorspermine, a polyamine analog, although exerting cytostatic growth inhibition on wild-type cells, were clearly cytotoxic to the spermine-deficient cells. The spermine-deficient cells were also much more sensitive to etoposide-induced DNA damage than their wild-type counterparts.

Polyamines spermidine and spermine and their diamine precursor putrescine are known to be essential for cell growth and differentiation (Jänne et al., 1991; Tabor and Tabor, 1984). The route of polyamine biosynthesis is similar in prokaryotic and eukaryotic cells, except that only the latter cells synthesize and contain spermine. The biosynthesis involves a concerted action of four separate enzymes: ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase, and spermine synthase. The overall rate of polyamine biosynthesis is predominantly regulated by changes in the activities of ornithine and adenosylmethionine decarboxylases, because spermidine and spermine synthases are expressed constitutively and largely regulated by the availability of their common substrate, decarboxylated AdoMet (Pegg, 1986). Although a vast number of studies has linked the polyamines to numerous cellular functions, their specific biological roles, if any, are far from clear. Even less is known about possible physiological functions of individual polyamines. Although experiments with specific inhibitors of polyamine biosynthesis have generated useful information about the roles of polyamines, especially in cell proliferation, they do not answer the crucial question of whether each of the three polyamines is vital for animal cell proliferation to occur.

We have now generated a mouse embryonic stem cell line with targeted disruption of the spermine synthase gene. These cells do not contain any spermine synthase activity nor is the enzyme protein present. Although the cells are totally devoid of spermine, their spermidine content is about doubled. Interestingly, the targeted cells display growth characteristics nearly identical to those of the wild-type cells yet

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[1] The nucleotide sequence of the mouse spermine synthase gene fragment covering the exons 3 to 9 has been deposited in the GenBank database under GenBank accession number AF136179.

ABBREVIATIONS: ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; AdoMet, *S*-adenosylmethionine; DFMO, 2-difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone); DENSPM, diethylnorspermine; kb, kilobase(s); PCR, polymerase chain reaction; pMSPM, plasmid containing a fragment (exons 3–9) of mouse spermine synthase gene; NEO, neomycin phosphotransferase; ES, embryonic stem; SPMKO, spermine synthase-deficient mouse embryonic stem cells.

they were distinctly more sensitive to the growth-inhibitory effects of polyamine antimetabolites, such as 2-difluoromethylornithine (DFMO) and methylglyoxal bis(guanylhydrazone) (MGBG). Similarly, the polyamine analog diethylnorspermine (DENSPM) which was cytostatic to wild-type cells, exerted cytotoxic action on the spermine-deficient cells. The spermine-deficient cells were also more sensitive to etoposide-induced DNA damage.

Experimental Procedures

Construction of the Targeting Vector. A fragment (14.3 kb) of mouse spermine synthase gene was isolated from mouse strain 129/SvJ genomic library (Stratagene, La Jolla, CA) by PCR screening (Israel, 1993). To distinguish clones carrying fragments of mouse spermine synthase gene from those containing processed pseudogenes (Lorenz et al., 1998), oligonucleotides B699: 5′-TGGCAGAGAGTGACTTGGCA-3′ and B700: 5′-ATCTCCACATGTTCTCCGCA-3′ were designed to amplify a fragment covering exons 6 and 7 and the intron in-between. The exon/intron boundaries of the mouse gene were estimated from the human spermine synthase gene (Grieff et al., 1997). After two rounds of PCR screening, phages from positive wells were plated and individual plaques were picked up for PCR analysis. One positive clone was selected and subcloned into Bluescript KS (Stratagene) and both strands were sequenced (pMSPM). For construction of the targeting vector, pMSPM was digested with *Eco*RI to remove a region from intron 6 to the 3′-end of the clone. The spermine synthase gene was then disrupted by inserting a positive selection marker gene for neomycin phosphotransferase (NEO) from pGT-N28 (New England Biolabs, Beverly, MA) into the *Ehe*I site in exon 5. Herpes simplex virus thymidine kinase gene was used as a negative selection marker and was inserted into the 5′ end of the clone to yield the final targeting vector (pMSPMNEO). A positive control vector was made by inserting NEO gene in the same orientation into EheI site in pMSPM.

Disruption of Spermine Synthase Gene. Mouse embryonic cell line RW-4 (Genome Systems Inc., St.Louis, MO) were grown in an undifferentiated state on mitomycin-inactivated mouse fetal fibroblasts (feeder fibroblasts) in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, Scotland) supplemented with 2 mM L-glutamine, murine leukemia inhibitory factor (1000 U/ml), and 10% fetal bovine serum (Life Technologies). Targeting vector was linearized by *Eco*RI and purified by phenol extraction and ethanol precipitation. Thirty micrograms of linearized plasmid were introduced into RW-4 embryonic stem (ES) cells by electroporation (Gene Pulser; BioRad Life Science, Hercules, CA) as described previously (Jouner, 1993). Clones that survived from neomycin and ganciclovir selection were analyzed by PCR using primers C437: 5′-TGGATGTGGAATGTGTGCGA-3′ and B700. The correct targeting of PCR positive clones were confirmed by Southern blot analysis. For Southern blot analysis, 10 μg of DNA from selected clones was digested with *Vsp*I, and DNA was electrophoresed and transferred onto nylon membranes using capillary transfer. Blots were hybridized with digoxigenin labeled probe external to the targeting vector (Fig. 1, probe a) and chemiluminescent detection was performed according to Engler-Blum et al. (1993). Blots were also hybridized with NEO-specific probe (Fig. 1, probe b) to verify the absence of additional random integration of the targeting vector.

Cell Culture. Parental RW-4 and spermine synthase-deficient cells were adapted to grow without feeder cells in Dulbecco's modified Eagle's medium and 10% fetal bovine serum. The effects of spermine were examined in the presence of 1 mM aminoguanidine. Cells were seeded into 24-h treatments with DFMO, MGBG, DENSPM, or etoposide. Cell number was determined electronically by Coulter Counter (Coulter, Luton, England). DNA fragmentation was determined as described by Nomura et al. (1999).

Determination of Enzyme Activities and Polyamine Concentrations. The enzyme activities and polyamine concentrations were determined from embryonic stem cells adapted to grow without the feeder fibroblasts. For determination of enzyme activities, the cells were lysed in 25 mM Tris·HCl pH 7.4, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Triton X-100, and the homogenates were centrifuged to remove nuclei and cellular debris. Spermidine and spermine synthase activities were measured with methyl-^{14}C- labeled decarboxylated AdoMet as the substrate (Raina et al., 1983). The activities of ornithine and adenosylmethionine decarboxylases were determined as described previously (Jänne and Williams-Ashman, 1971). Polyamine concentrations were measured from cell homogenates with the aid of high-performance liquid chromatography (Hyvönen et al., 1992). The protein concentration was determined as previously described (Bradford, 1976). The MGBG concentrations were determined by high-performance liquid chromatography as described by Yarlett and Bacchi (1988). DENSPM concentrations were measured as described previously (Kramer et al., 1995).

Western Blot Analysis of Spermine Synthase. A synthetic peptide of spermine synthase (EFTYVINDLTAVPISTSP) was coupled to bovine serum albumin using glutaraldehyde as the coupling agent (Harlow and Lane, 1988). Rabbits were immunized four times at 4-week intervals. After the fourth immunization, sera were col-

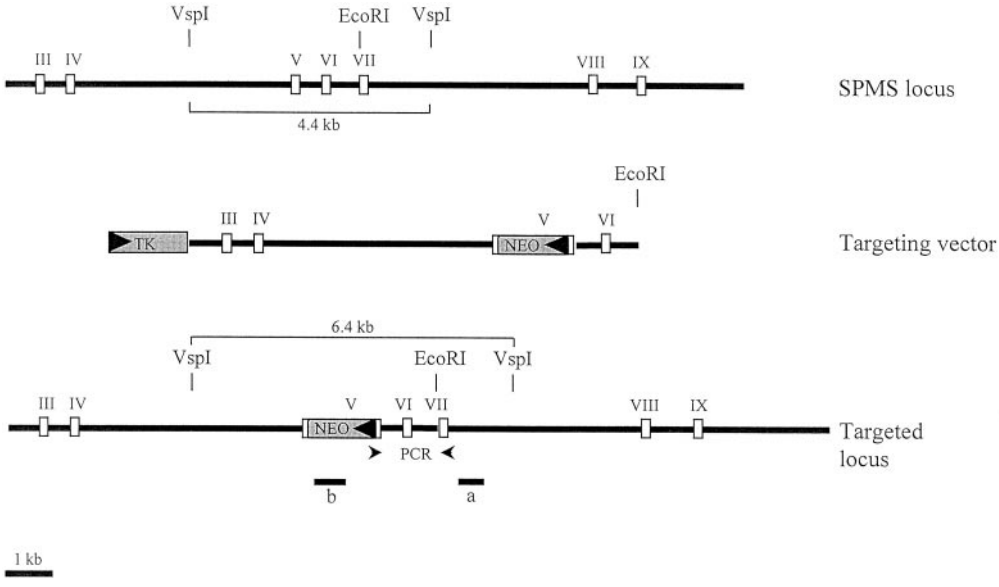

Fig. 1. Targeted disruption of the spermine synthase gene. Structure of wild-type allele, targeting vector, and targeted allele. A portion of the mouse spermine synthase gene was isolated from the mouse 129/SvJ genomic library. The gene was disrupted by inserting the positive selection cassette (NEO) in the opposite orientation into the *Ehe*I site on exon 5. The negative selection marker (TK) was inserted outside of the homology region. The resulting vector featured 1.6 kb of homologous sequence on short arm and 4.6 kb on the long arm. The positions of the probes (a and b) used in Southern blot analysis are shown. The arrows indicate the positions and orientations of oligonucleotides used in PCR analysis of targeted allele. Cleavage sites of restriction enzymes used for linearization of the targeting vector (*Eco*RI) and for Southern blot analysis (*Vsp*I) are shown.

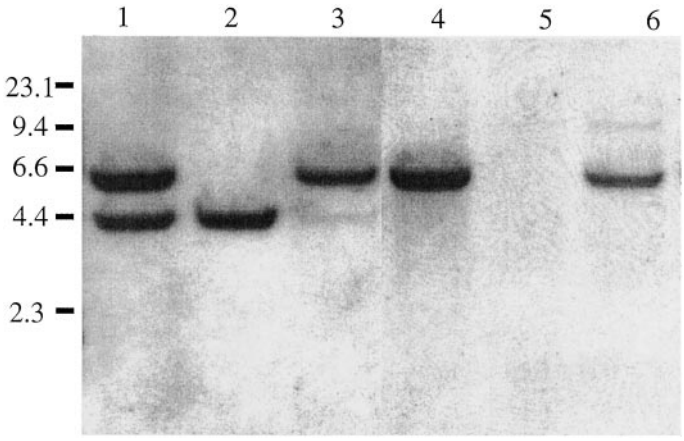

Fig. 2. Southern blot analysis of targeted ES cells. Genomic DNA was isolated from ES cells grown without feeder fibroblasts. DNA was digested with *Vsp*I, electrophoresed in 0.9% agarose, and transferred onto nylon membrane. The blot was hybridized with probes specific to spermine synthase (lanes 1–3) and neomycin phosphotransferase (lanes 4–6) (the positions of the probes are shown in Fig. 1). The lanes were as follows: lanes 1 and 4, positive control plasmid mixed with DNA from control RW-4 cells; lanes 2 and 5, DNA from control RW-4 cells; lanes 3 and 6, DNA from targeted ES cell clone.

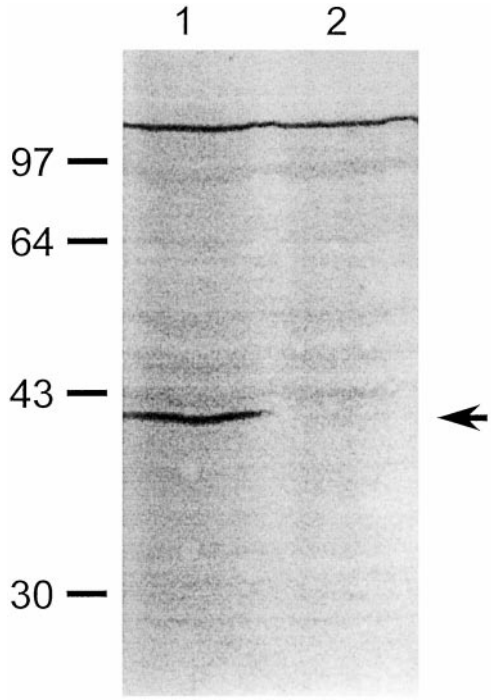

Fig. 3. Western blot analysis of spermine synthase-deficient cells. Total cell lysates were prepared from feeder fibroblast free cell cultures. Fifty micrograms of protein was electrophoresed on a 12% SDS-polyacrylamide gel, transferred onto polyvinylidene difluoride membrane (Millipore). Spermine synthase was detected with antibodies raised against synthetic peptide. The blot was developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining. Lane 1, control RW-4 cells; lane 2, spermine synthase-deficient clone. The arrow indicates the position of spermine synthase subunit. The molecular size markers shown are in kilodaltons.

lected by heart puncture, and spermine synthase-specific antibodies were purified by peptide-affinity-chromatography as described by Harlow and Lane (1988). For Western blot analysis, protein samples were electrophoresed in 12% SDS-polyacrylamide gel as described previously (Laemmli, 1970) and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were blocked with 5% (w/v) nonfat dried milk in Tris-buffered saline and incubated with peptide-specific antibody. Alkaline phosphatase-labeled anti-rabbit IgG (Zymed, San Francisco, CA) was used as a detection antibody. The blots were developed using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining.

Results

Disruption of Spermine Synthase Gene. PCR-based screening method was used to clone a portion of the mouse spermine synthase gene. The ultimate aim was to isolate a gene fragment coding for the protein region proposed to be involved in the binding of decarboxylated AdoMet (Korhonen et al., 1995). Therefore, oligonucleotides were designed to amplify a region of mouse spermine synthase gene between exons 6 and 7. Sequencing of the clone isolated from 129/SvJ genomic library revealed a 14,318-base-pair fragment containing the exons 3 to 9 of spermine synthase gene encoding amino acids 58 to 315.[1] Comparison of the exon sequences with mouse cDNA (GenBank accession numbers Y09419 and AF031486) revealed no base differences, suggesting that this gene fragment was a part of the functional spermine synthase gene. The spermine synthase gene was disrupted by inserting a neomycin phosphotransferase gene into exon 5 at opposite orientation to the gene (Fig. 1). Altogether, 44 neomycin and ganciclovir resistant cell clones were analyzed and one of them was correctly targeted and contained no additional integrations of the targeting vector as indicated by Southern blot analysis (Fig. 2).

Effects of Spermine Deficiency on Polyamine Synthesis. There is one functional spermine synthase gene in mouse genome located on chromosome X (Lorenz et al., 1998). Hence, disruption of the spermine synthase locus in RW-4 embryonic stem cells, which are of XY karyotype, led to hemizygous spermine synthase deficiency. As expected, the targeted cells did not show any detectable spermine synthase activity (Table 1) and Western blot analysis with spermine synthase peptide-specific antibodies confirmed the lack of spermine synthase protein (Fig. 3). The concentration of

TABLE 1
Polyamine synthesising enzyme activities and polyamine concentrations of parental (RW-4) and spermine synthase-deficient (SPMKO) cells. Cells were cultured in the absence of feeder fibroblasts and culture medium was changed 4 h before collecting the samples. The values represent means ± S.E.M. of triplicate cultures.

	Enzyme Activities				Polyamine Pool[a]		
	ODC	SAMDC	SPDS	SPMS	Putrescine	Spermidine	Spermine
	nmol/h mg of soluble protein				*nmol/mg of protein*		
RW-4	11.4 ± 1.1	0.37 ± 0.2	13.14 ± 1.5	3.88 ± 0.4	2.2 ± 0.4	21.2 ± 1.5	12.8 ± 1.6
SPMKO	39.4 ± 3.4***	1.40 ± 0.1***	13.33 ± 1.2	n.d.	n.d.	47.1 ± 7.5***	n.d.

[a] Protein concentration was determined from sulphosalicylic-acid-precipitate after dissolving it into 0.1 M NaOH.
n.d., not detectable. *** $p < 0.001$.

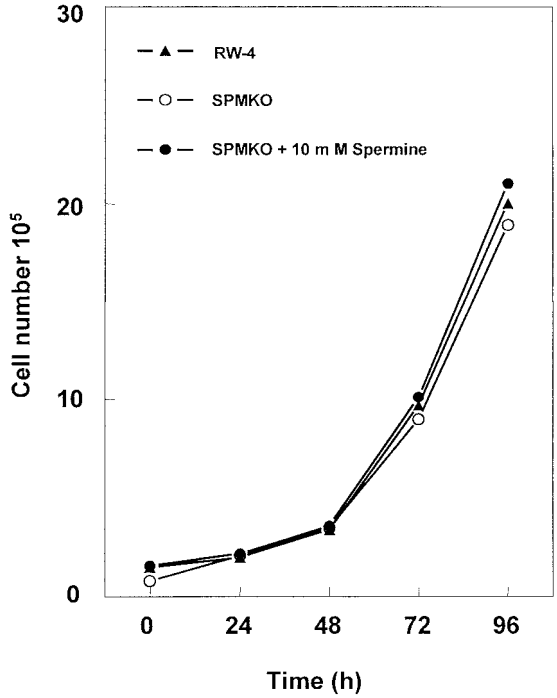

Fig. 4. Growth of spermine synthase-deficient RW-4 cells. Cells were grown without feeder fibroblasts in ES-cell medium supplemented with 1 mM aminoguanidine and counted with Coulter Counter (Coulter, Luton, England). The values represent means of duplicate cultures.

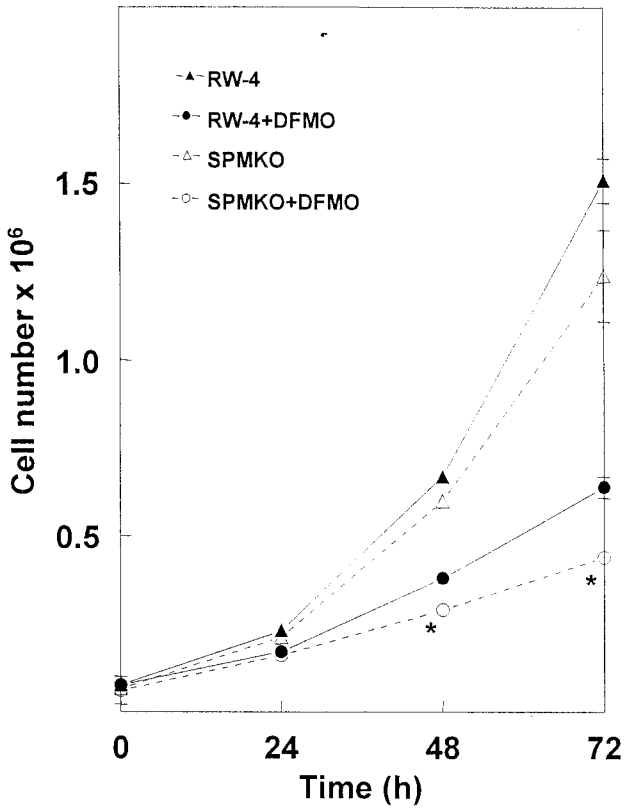

Fig. 5. Effect of 1 mM DFMO on the growth of RW-4 parental and spermine synthase-deficient SPMKO cells. The cells were exposed to the drug for time periods indicated. The values are means ± S.E.M. obtained from triplicate cultures. $*p < 0.05$, statistical significance between drug-exposed parental and targeted cells calculated by comparing the percentage values of untreated cells at each time point.

spermine was below detection level in the targeted cells grown without the feeder fibroblasts (Table 1). Spermine depletion apparently led to a compensatory increase in spermidine content (Table 1), retaining the total polyamine pool virtually unaltered. As shown in Table 1, spermine deficiency likewise led to 3- and 5-fold increases in activities of ODC and AdoMetDC, respectively. This suggests that spermine may have a major role in the regulation of ODC and AdoMetDC expression, whereas spermidine, despite the substantial expansion of its pool in the targeted cells, may be less important in this sense. Also, the addition of spermine in culture medium repressed the activities ODC and AdoMetDC of mutated cells to the level of control cells grown without spermine (results not shown). In all likelihood, the mutated cells maintained their total (spermidine plus spermine) pool by enhancing the synthesis of spermidine. The activities of spermidine and spermine synthases are assumed to be mainly regulated by the availability of their common substrate, decarboxylated *S*-adenosylmethionine (Pegg, 1986), probably leading to a competition between spermidine and spermine synthases for the use of available decarboxylated AdoMet. Hence, in cells without functional spermine synthase, the increase in spermidine and decrease in putrescine concentration may simply be attributable to improved availability of decarboxylated AdoMet for spermidine synthase.

TABLE 2

Polyamine concentrations of control RW-4 and mutated SPMKO cells grown in the absence or presence of 1 mM DFMO. The values are means ± S.E.M. of triplicate wells.

Time	Culture	Polyamine Concentration (pmol/10^6 cells)		
		Putrescine	Spermidine	Spermine
0 h	RW-4	263.8 ± 43.90	1828.7 ± 188.7	770.7 ± 93.7
	SPMKO	116.7 ± 20.7	2533.9 ± 9.0	n.d.
24 h	RW-4	324.8 ± 74.4	2195.2 ± 160.0	847.7 ± 94.3
	RW-4 + DFMO	n.d.	292.6 ± 19.8	947.4 ± 103.5
	SPMKO	133.3 ± 11.0	3189.3 ± 13.9	n.d.
	SPMKO + DFMO	n.d.	1216.1 ± 117.6	n.d.
48 h	RW-4	557.8 ± 72.5	1983.1 ± 145.7	759.6 ± 130.0
	RW-4 + DFMO	n.d.	n.d.	786.1 ± 110.7
	SPMKO	181.3 ± 39.8	2834.7 ± 372.3	n.d.
	SPMKO + DFMO	n.d.	1065.7 ± 43.0	n.d.
72 h	RW-4	261.2 ± 98.0	1281.2 ± 216.4	572.6 ± 185.6
	RW-4 + DFMO	n.d.	n.d.	670.6 ± 100.6
	SPMKO	n.d.	2615.3 ± 154.5	n.d.
	SPMKO + DFMO	n.d.	1140.8 ± 56.1	n.d.

n.d., not detectable.

TABLE 3

Polyamine concentrations of control RW-4 and mutated SPMKO cells grown in the absence or presence of 10 μM MGBG. The values are means ± S.E.M. of triplicate wells.

Time	Cell line/treatment	Polyamine concentration		
		Putrescine	Spermidine	Spermine
		pmol / 10⁶ cells		
0 h	RW-4	263.8 ± 43.90	1828.7 ± 188.7	770.7 ± 93.7
	SPMKO	116.7 ± 20.7	2533.9 ± 9.0	n.d.
24 h	RW-4	324.8 ± 74.4	2195.2 ± 160.0	847.7 ± 94.3
	RW-4 + MGBG	959.7 ± 65.8	1266.0 ± 25.7	323.5 ± 78.1
	SPMKO	133.3 ± 11.0	3189.3 ± 13.9	n.d.
	SPMKO + MGBG	293.8 ± 51.2	1391.3 ± 129.1	n.d.
48 h	RW-4	557.8 ± 72.5	1983.1 ± 145.7	759.6 ± 130.0
	RW-4 + MGBG	1186.1 ± 143.8	1104.4 ± 75.4	340.8 ± 29.7
	SPMKO	181.3 ± 39.8	2834.7 ± 372.3	n.d.
	SPMKO + MGBG	49.0 ± 5.5	1177.0 ± 237.2	n.d.
72 h	RW-4	261.2 ± 98.0	1281.2 ± 216.4	572.6 ± 185.6
	RW-4 + MGBG	678.0 ± 211.0	1179.6 ± 95.5	342.0 ± 29.5
	SPMKO	n.d.	2615.3 ± 154.5	n.d.
	SPMKO + MGBG	138.3 ± 13.7	2300.2 ± 360.4	n.d.

n.d., not detectable.

Effect on Cell Growth and Morphology. The spermine synthase-deficient and parental RW-4 cells were adapted to grow in ES cell medium without feeder fibroblasts. The growth rate of spermine-deficient cells was nearly similar to that of parental RW-4 cells (Fig. 4). Addition of spermine (in the presence of 1 mM aminoguanidine) had no effect on the growth rate of spermine synthase-deficient cells (Fig. 4). Therefore, although spermine may have some specific cellular functions, spermine synthesis is not essential for animal cell growth to occur in vitro. Moreover, spermine deficiency may be circumvented by an enhanced cellular accumulation of spermidine. Although the amount of spermine in mutated cells was under the detection limit, this does not necessarily exclude the possibility that cells may have acquired minor but adequate supplies of spermine from the culture medium.

Both the wild-type and spermine synthase-deficient cells were subjected to transmission electron microscopy, which did not reveal any overt morphological changes in the targeted cells or their cell organelles (results not shown).

Effects of Inhibition of ODC. The parental and spermine-synthase deficient cells were subsequently exposed to 1 mM DFMO, a specific inhibitor of ODC (Metcalf et al., 1978). As indicated in Fig. 5, the mutated cells were significantly more sensitive to the drug as regards cell growth. Interestingly, this growth inhibition was apparently not related to a more rapid decrease in cellular spermidine content in the targeted cells, because the drug-depleted spermidine, but not spermine, pools more efficiently in the wild-type cells (Table 2). Therefore, under conditions of profound putrescine and spermidine deprivation, spermine alone is sufficient to support growth.

Effects of Inhibition of AdoMetDC. Control and spermine synthase-deficient cells were grown in the presence of 10 μM methylglyoxal bis(guanylhydrazone). MGBG is a competitive inhibitor of AdoMetDC (Williams-Ashman and Schenone, 1972) and, as expected, in control cells, the treatment with MGBG reduced the pools of higher polyamines, spermidine, and spermine and increased the amount of putrescine

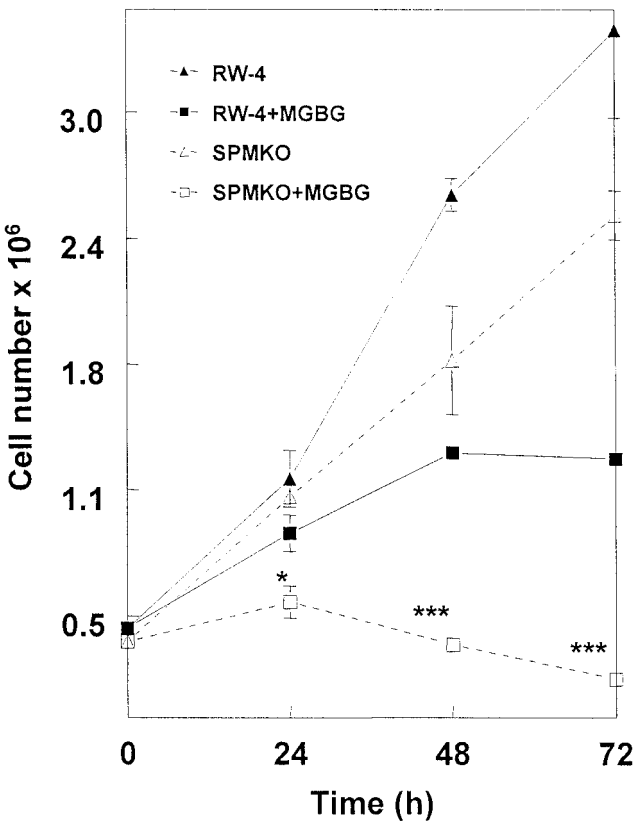

Fig. 6. Effect of 10 μM MGBG on the growth of RW-4 parental and spermine synthase-deficient SPMKO cells. The cells were exposed to the drug for time periods indicated. The values are means ± S.E.M. obtained from triplicate cultures. *$p < 0.05$, ***$p < 0.001$, statistical significance between drug-exposed parental and targeted cells calculated by comparing the percentage values of untreated cells at each time point.

TABLE 4

MGBG concentration of control RW-4 and mutated SPMKO cells grown in the presence of 10 μM MGBG for time periods indicated. The values are means ± S.E.M. of triplicate cultures.

Cell line	MGBG concentration		
	2 h	6 h	12 h
	pmol / 10⁶ cells		
RW-4	n.d.	104.0 ± 28.5	309.7 ± 35.9
SPMKO	147.0 ± 8.9	729.7 ± 83.7***	1087 ± 14.5***

n.d., not detectable.

*** $p < 0.001$ statistical significance between drug-exposed parental and targeted cells.

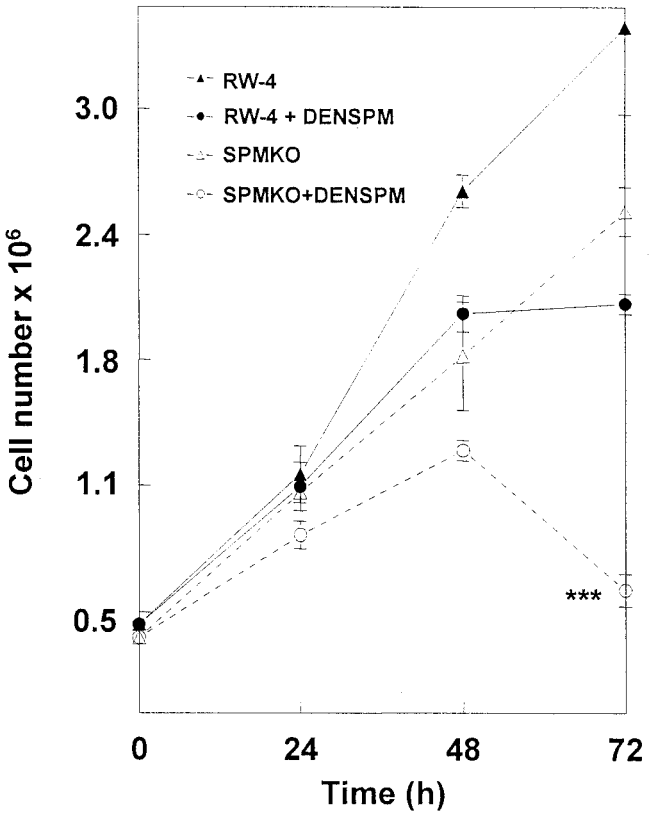

Fig. 7. Effect of 50 μM DENSPM on the growth of RW-4 parental and spermine synthase deficient SPMKO cells. The cells were exposed to the drug for time periods indicated. The values are means ± S.E.M. obtained from triplicate cultures. ***$p < 0.001$: statistical significance between drug-exposed parental and targeted cells calculated by comparing the percentage values of untreated cells at each time point.

(Table 3). In the control cells, the major decrease in higher polyamine concentrations were reached after 24 h of treatment; thereafter, there were only minor changes in the concentrations of spermidine and spermine (Table 3). After 48 h exposure, the control cells ceased to proliferate (Fig. 6). In striking contrast to the wild-type cells, MGBG exerted a cytotoxic effect on the spermine synthase-deficient cells (Fig. 6). In targeted cells, the MGBG treatment decreased the amount of spermidine with concomitant increase in putrescine content after 24-h treatment. Interestingly, MGBG treatment did not decrease the amount of spermidine later; instead, polyamine levels increased nearly to that of untreated cells (Table 3). Because there was clear difference in response to MGBG treatment between cell lines, we measured the intracellular concentration of MGBG at various time points. As shown in Table 4, the MGBG concentration was much higher at each time point in spermine synthase-deficient cells compared with control cells.

Effects of Diethylnorspermine Treatment. Parental and targeted cells were subsequently exposed to 50 μM diethylnorspermine, which is known to deplete polyamine pools by indirectly reducing the levels of ODC and AdoMetDC activities and by up-regulating spermidine/spermine N^1-acetyltransferase activity (Chang et al., 1992). Spermidine/spermine N^1-acetyltransferase is the key enzyme in the interconversion pathway, where spermine and spermidine can be back-converted to spermidine and putrescine, respectively. Both parental and spermine synthase-deficient cells showed reduced growth rates compared with nontreated control cultures (Fig. 7). However, the targeted cells were more sensitive to the treatment and started to die after 48-h exposure. In the targeted cells, DENSPM treatment lead to dramatic decrease in spermidine content after 24 h (Table 5). In control cells, DENSPM reduced the amounts of both spermidine and spermine and after 24-h treatment, the total polyamine pool (spermidine+spermine) was even lower than spermidine content in targeted cells (Table 5). This may indicate that the cytotoxic effect of DENSPM in spermine-deficient cells is not directly related to the depletion of total polyamine pools. We also determined the intracellular concentration of DENSPM at 6, 24, and 48 h after the addition of the drug. As shown in Table 6, the concentration of the drug was substantially higher in spermine synthase deficient cells at 6 h. However, after 24 h, the concentration of the drug was comparable in both cell lines.

Sensitivity to Etoposide. Etoposide inhibits topoisomerase II at the strand rejoining step resulting single and double strand breaks in DNA. As shown in Figs. 8 and 9 the spermine synthase deficient cells were more sensitive to etoposide treatment. The typical cleavage of DNA into a "ladder" of

TABLE 5
Polyamine concentrations of control RW-4 and mutated SPMKO cells grown in the absence or presence of 50 μM DENSPM. The values are means ± S.E.M. of triplicate wells

Time	Culture	Polyamine concentration		
		Putrescine	Spermidine	Spermine
			pmol / 10^6 cells	
0 h	RW-4	263.8 ± 43.90	1828.7 ± 188.7	770.7 ± 93.7
	SPMKO	116.7 ± 20.7	2533.9 ± 9.0	n.d.
24 h	RW-4	324.8 ± 74.4	2195.2 ± 160.0	847.7 ± 94.3
	RW-4 + DENSPM	83.4 ± 7.6	294.8 ± 48.2	274.3 ± 13.7
	SPMKO	133.3 ± 11.0	3189.3 ± 13.9	n.d.
	SPMKO + DENSPM	47.8 ± 8.9	610.1 ± 27.4	n.d.
48 h	RW-4	557.8 ± 72.5	1983.1 ± 145.7	759.6 ± 130.0
	RW-4 + DENSPM	n.d.	108.3 ± 12.8	102.8 ± 23.6
	SPMKO	181.3 ± 39.8	2834.7 ± 372.3	n.d.
	SPMKO + DENSPM	n.d.	293.9 ± 18.4	n.d.
72 h	RW-4	261.2 ± 98.0	1281.2 ± 216.4	572.6 ± 185.6
	RW-4 + DENSPM	n.d.	n.d.	51.2 ± 6.6
	SPMKO	n.d.	2615.3 ± 154.5	n.d.
	SPMKO + DENSPM	n.d.	222.9 ± 21.3	n.d.

n.d., not detectable.

TABLE 6
DENSPM concentration of control RW-4 and mutated SPMKO cells grown in the presence of 50 μM DENSPM for time periods indicated. The values are means ± S.E.M. of triplicate cultures

Cell line	DENSPM concentration		
	6 h	24 h	48 h
	pmol / 10^6 cells		
RW-4	977.3 ± 29.7	2395.7 ± 45.5	2069.7 ± 143.6
SPMKO	2157.0 ± 169.3***	2457.7 ± 367.6	1591.0 ± 14.2

n.d., not detectable.
*** $p < 0.001$ statistical significance between drug-exposed parental and targeted cells.

fragments was clearly detected in spermine synthase-deficient cells, but not in the wild-type cells, after 24-h treatment with 10 or 100 μM etoposide (data not shown).

Discussion

The present results indicate that mammalian cells (embryonic stem cells, at least) can proliferate in the absence of spermine and do not show any overt morphological cell or organelle abnormalities. This may be related to the compensatory expansion of the spermidine pool in spermine synthase-deficient cells. The substantial elevation of spermidine content in spermine-deficient cells is in agreement with other studies in which specific inhibitors of spermine synthase were employed to deplete this polyamine (Baillon et al., 1989). Similarly, spermine synthase-deficient *Saccharomyces cerevisiae* had elevated spermidine content because of a 8-fold increase in spermidine synthase activity (Hamasaki-Katagiri et al., 1998). The increase in spermidine pools under conditions in which spermine synthase is inhibited or totally lacking could be related to the apparent regulation of the constitutively expressed spermidine and spermine synthase activities by the availability of their common substrate, decarboxylated AdoMet. Thus, in the absence of spermine synthase activity, all the decarboxylated AdoMet is used for the synthesis of spermidine. However, despite their apparently near-normal growth rate under standard cell culture conditions, the spermine-deficient cells were distinctly more sensitive to inhibitors of polyamine biosynthesis and to polyamine analog. Both MGBG, an inhibitor of AdoMetDC structurally related to the higher polyamines, and DENSPM, a polyamine analog, exerted cytotoxic growth inhibitory effect on the spermine-deficient cells, whereas their antiproliferative action on wild-type cells was only cytostatic. Interestingly, this more profound growth inhibition of the mutated cells did not seem to be directly related to the extent of polyamine (spermidine and spermine) depletion. It seems, then, that spermidine and spermine are not fully exchangeable with regard to their requirements for growth, or spermine may be specifically required for the integrity of some cell organelles, such as mitochondria, under the stress of antiproliferative agents. The uptake rate of MGBG in targeted cells was much higher than that in control cells, which certainly contributed to more rapid response to the drug. It has been shown previously that MGBG is taken up by the cells through the carrier system used by the natural polyamines, spermidine, and spermine (Seppänen, 1981). Hence, the enhanced uptake rate of MGBG may indicate that polyamine transport is up-regulated in spermine synthase-deficient cells. Although, the initial uptake of DENSPM was faster in targeted cells, it does not explain the difference seen in cell

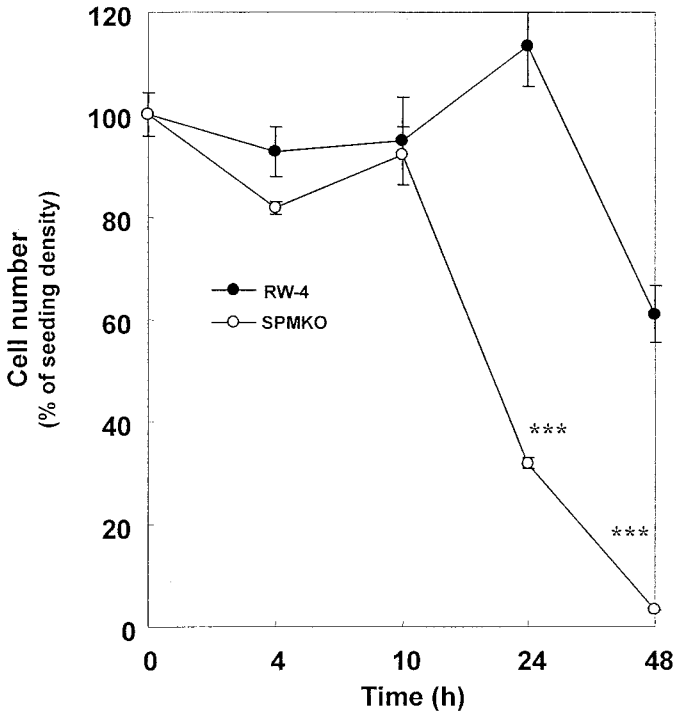

Fig. 8. The effect of etoposide treatment on the growth of parental RW-4 and spermine synthase-deficient SPMKO cells. Cells were grown in the presence of 10 μM etoposide for the time periods indicated. The results are means ± S.E.M. from triplicate cultures. ***$p < 0.001$, statistical significance between drug-exposed parental and targeted cells.

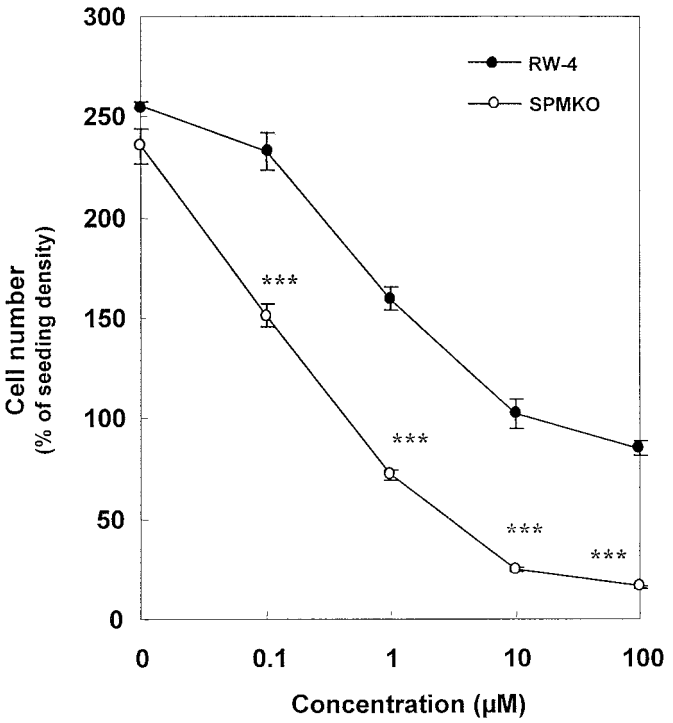

Fig. 9. Etoposide dose-dependent growth inhibition of parental RW-4 and spermine synthase deficient SPMKO cells. Cells were grown in the presence of various concentrations of etoposide for 24 h. The results are means ± S.E.M. from triplicate cultures. ***$p < 0.001$, statistical significance between drug-exposed parental and targeted cells.

growth, because after 24 h, the concentration of the drug was comparable in both cell types (Table 6.).

It is likewise obvious that the spermine-deficient cells were more sensitive to etoposide, an inhibitor of topoisomerase II inducing single and double strand breaks in DNA. It has previously been shown by employing inhibitors of polyamine biosynthesis that polyamine depletion affects topoisomerase II activity in cultured cells by reducing the cytotoxic effect of etoposide (Bakic et al., 1987; Desiderio et al., 1997). Alm et al. (1999) indicated that polyamine depletion with DFMO and CGP 48664 (4-amidinoindan-one-2′-aminohydrazone) influenced the function of topoisomerase II, resulting in a decreased number of DNA strand breaks in etoposide-treated cells, whereas the enzyme activity itself was not directly affected. In our study, however, spermine synthase-deficient cells were more sensitive to etoposide treatment. This is not in line with the findings of Desiderio et al. (1997), who showed that depletion of spermine reduced the cytotoxic effect exerted by etoposide, whereas an addition of spermine restored the cytotoxicity of the topoisomerase II inhibitor. This difference may be related to the fact that a pharmacological inhibition of polyamine biosynthesis reduces cellular growth rate and DNA synthesis, whereas our spermine-deficient cells grew at a normal or nearly normal rate. It has been suggested that the polyamines spermidine and spermine influence topoisomerase II activity by maintaining an optimal chromatin conformation (Alm et al., 1999). In our cells, which have an increased amount of spermidine and are totally devoid of spermine, the chromatin structure may be different from that in wild-type cells.

The present results indicate that although mammalian cells are able to proliferate in the absence spermine, this polyamine seems to protect cells from toxicity exerted not only by inhibitors of polyamine biosynthesis and polyamine analogues but also by drugs that induce DNA damage.

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