Targeted Disruption of Spermidine/Spermine N\(^1\)-Acetyltransferase Gene in Mouse Embryonic Stem Cells

EFFECTS ON POLYAMINE HOMEOSTASIS AND SENSITIVITY TO POLYAMINE ANALOGUES\(^*\)

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We have generated mouse embryonic stem cells with targeted disruption of spermidine/spermine N\(^1\)-acetyltransferase (SSAT) gene. The targeted cells did not contain any inducible SSAT activity, and the SSAT protein was not present. The SSAT-deficient cells proliferated normally and appeared to maintain otherwise similar polyamine pools as did the wild-type cells, with the possible exception of constantly elevated (about 30%) cellular spermidine. As expected, the mutated cells were significantly more resistant toward the growth-inhibitory action of polyamine analogues, such as N\(^1\),N\(^11\)-diethyl-norspermine. However, this resistance was not directly attributable to cellular depletion of the higher polyamines spermidine and spermine, as the analogue depleted the polyamine pools almost equally effectively in both wild-type and SSAT-deficient cells. Tracer experiments with [C\(^{14}\)]-labeled spermidine revealed that SSAT activity is essential for the back-conversion of spermidine to putrescine as radioactive N\(^1\)-acetylspermidine and putrescine were readily detectable in N\(^1\),N\(^11\)-diethyl-norspermine-exposed wild-type cells but not in SSAT-deficient cells. Similar experiments with [C\(^{14}\)]-spermine indicated that the latter polyamine was converted to spermidine in both cell lines and, unexpectedly, more effectively in the targeted cells than in the parental cells. This back-conversion was only partly inhibited by MDL72527, an inhibitor of polyamine oxidase. These results indicated that SSAT does not play a major role in the maintenance of polyamine homeostasis, and the toxicity exerted by polyamine analogues is largely not based on SSAT-induced depletion of the natural polyamines. Moreover, embryonic stem cells appear to operate an SSAT-independent system for the back-conversion of spermine to spermidine.

The oxidative catabolism of the higher polyamines spermidine and spermine is accomplished by the concerted action of two different enzymes, namely spermidine/spermine N\(^1\)-acetyltransferase (SSAT)\(^1\) and polyamine oxidase (PAO). Cytosolic SSAT N\(^1\) acetylates both spermidine and spermine whereafter they serve as substrates for peroxisomal PAO (1). As PAO strongly prefers acetylated polyamines to the unmodified polyamines as its substrates, SSAT is generally considered as the rate-controlling enzyme in the back-conversion of spermidine and spermine (2). The final product of the catabolism of spermidine is putrescine whereas the oxidation of spermine or N\(^1\)-acetylspermine yields spermine. In addition to the polyamines, PAO action also generates acetamido propanal and hydrogen peroxide.

During recent years considerable attention has been paid to SSAT as a target for cancer chemotherapy. A number of compounds, among them natural polyamines and their alkylated derivatives, strikingly induce SSAT and subsequently deplete cellular polyamine pools resulting in overt cytotoxicity (2). A large number of studies suggest that depletion of polyamines and growth inhibition by polyamine analogues are closely related to the extent of SSAT induction (3–7). These relationships, however, are complicated by the comparison of paired cell lines with different genetic backgrounds. To make more direct comparisons in this sense, we recently isolated fetal fibroblasts from transgenic mice overexpressing SSAT (8) and their syngenic littermates. We thus established paired cell lines with identical genetic background except for SSAT gene copy number (9). An exposure of these cell lines to a polyamine analogue revealed that the transgenic fibroblasts were much more sensitive to the growth inhibition exerted by the drug than their non-transgenic counterparts (9). The more intense growth inhibition of the transgenic fibroblasts was associated with a distinctly faster depletion of cellular spermidine and spermine pools (9).

McCloskey and Pegg (10) showed recently that altered SSAT activity and/or regulation because of point mutation rendered a Chinese hamster ovary cell line remarkably resistant to a polyamine analogue. The sensitivity to the drug was again restored through an expression of wild-type SSAT in the resistant cells (10).

We have now generated a mouse embryonic stem cell line with targeted disruption of SSAT gene. As SSAT gene is located at the X chromosome, and the embryonic stem cells used were of the XY karyotype, a single homologous recombination event resulted in a null phenotype. The targeted cells did not contain any SSAT activity or enzyme protein even when exposed to N\(^1\),N\(^11\)-diethyl-norspermine (DENSPM), one of the most powerful inducers of SSAT. The SSAT-deficient cells were much more resistant to the growth inhibition by the latter drug than norspermine; DMEM, Dulbecco’s modified Eagle’s medium; PAO, polyamine oxidase; PBS, phosphate-buffered saline.

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\(^1\) The abbreviations used are: SSAT, spermidine/spermine N\(^1\)-acetyltransferase; ANOVA, analysis of variance; DENSPM, N\(^1\),N\(^11\)-diethyl-norspermine; DMEM, Dulbecco’s modified Eagle’s medium; PAO, polyamine oxidase; PBS, phosphate-buffered saline.
the wild-type cells. However, the analogue-induced depletion of cellular spermidine and spermine pools occurred at a very much similar rate in both parental and targeted cell lines. Unlike the parental cells, the SSAT-deficient cells were unable to convert spermidine to putrescine but converted spermine to spermidine even at an enhanced rate in comparison with the wild-type cells.

EXPERIMENTAL PROCEDURES

Construction of the Targeting Vector—An 18-kb fragment of mouse SSAT gene was isolated from a mouse strain 129/SvJ genomic library (Stratagene, La Jolla, CA). The library was screened by a probe amplified from reverse-transcribed mouse brain RNA. The probe covered nucleotides 137–744 of mouse (Mus domesticus) SSAT cDNA. For the construction of the targeting vector, an EcoRI and BamHI fragment containing all six exons of SSAT gene was inserted into pTV-O vector. A SmaI fragment containing part of exon 1 was deleted and replaced by neomycin phosphotransferase from pMC1Neo (Stratagene). The structure of the targeting vector is depicted in Fig. 1A.

Targeting the Mouse SSAT Gene in Mouse Embryonic Stem Cells—Mouse embryonic stem cell line RW-4 (Genome Systems Inc., St Louis, MO) was grown at the undifferentiated state on mitomycin-inactivated mouse fetal fibroblasts (feeder fibroblasts) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 2 mM glutamine, murine leukemia inhibitory factor (1000 units/ml) (ESGRO; Invitrogen), and 15% fetal bovine serum. Targeting vector was linearized with EcoRI and purified by phenol extraction and ethanol precipitation. Thirty μg of linearized DNA was introduced into RW-4 embryonic stem cells by electroporation (Gene Pulser; Bio-Rad). Clones that survived from G-418 and ganciclovir (Syntex Research) selection were analyzed by PCR using primers 5'-ATACAAGAAATGCAGCTGTGG-3' and 5'-AATTGGCCGACTGACGAGGAGC-3'. The correct targeting of PCR-positive clones was confirmed by Southern blot analyses. For Southern blot analysis, 10 μg of DraI-digested DNA was subjected to electrophoresis and transferred onto nylon membranes. Blots were hybridized with digoxigenin-labeled probe external to the targeting vector (see A). Clones 5, 6, and 7 were targeted properly showing only the mutated allele. MW, molecular weight.

with a mixture of primers annealing sequences for evolutionary conserved 16 S rRNA of nine different mycoplasma species including those most commonly found in cell cultures, Mycoplasma hominis, Mycoplasma hyorhinis, Mycoplasma arginini, Mycoplasma pneumoniae, Mycoplasma fermentans, Mycoplasma orale, Mycoplasma pirum, Acholeplasma laidlawii, and Spirroplasma mirum.

Determination of the SSAT Protein, SSAT Activity, PAO Activity, and Polyamines—Western blot analysis of the SSAT protein was carried out using the C-terminal antibody to SSAT as described earlier (9). SSAT activity was assayed as described (12). PAO activity was assayed essentially as described by Kumazawa et al. (13) except using radioactive diacetylspermine, instead of acetylspermine, as the substrate. Concentrations of polyamines and their acetylated derivatives were determined with the aid of high performance liquid chromatography essentially as described by Hyvönen et al. (14). The concentration of DENSPM was determined as described (15).

Tracer Studies—Parental and SSAT-deficient cells were plated in six-well culture plates in DMEM supplemented with heat-inactivated 10% fetal bovine serum and gentamycin (50 μg/ml). The cells were let to adhere for 24 h before drug exposure. The cells were then exposed to 50 μM DENSPM for 48 h whereafter the inhibitors of amine oxidases were added for 24 h with 50 μM DENSPM. Before pulse labeling with [14C]spermidine (specific radioactivity 112 mCi/mmol; Amersham Biosciences) or with [14C]spermine (specific radioactivity 110 mCi/mmol; Amersham Biosciences) the growth medium was removed, and the cells were washed twice with PBS, replenished with DMEM without serum, and incubated with the tracers for 3 h. After the incubation, the cells were washed with PBS, detached with trypsin, counted, and subjected to further analyses. The polyamines were determined after sulfosalicylic acid precipitation from the supernatant fractions. A sample of 20 μl of the supernatant fraction was injected into high performance liquid chromatography, and fractions were collected and counted for radioactivity.

Chemicals—N7,N11-Diethylornospermine was synthesized as described (16). N7,N11-Bis[2,3-butadienyl]-1,4-butanediamine (MDL72527) was a generous gift from Hoechst-Roussel. Semicarbazide and aminoguanidine were purchased from Sigma.

Statistical Analyses—Two-way and one-way (with Dunnett's post hoc multiple comparison test) analysis of variance (ANOVA) or Student's two-tailed t test, when appropriate, were used for statistical analyses with the aid of a software package, GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Targeted Disruption of SSAT Gene in Mouse Embryonic Stem Cells—The wild-type allele of SSAT, the targeting vector,
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and the targeted allele are presented in Fig. 1A. Altogether 16 clones resistant both to G-418 and ganciclovir were isolated from the selection plates. From these clones, seven appeared to be correctly targeted and contained no additional integrations. Southern blot analysis of some of the clones is presented in Fig. 2. As indicated, clones 5, 6, and 7 were targeted correctly and showed only the mutated allele (5.4 kb). Clone 3 in Fig. 1A, Western blot analysis of wild-type and SSAT-deficient (SSAT KO) cells. Means ± S.D. were obtained from triplicate cultures. The cultures were exposed to 100 µM polyamines for 24 h. put, putrescine; spd, spermidine; spm, spermine. Table I lists the cellular concentrations of the polyamines after the exposure. As indicated in the table, both cell lines accumulated exogenous polyamines in a very similar fashion. The basal levels of putrescine and spermidine were significantly higher and that of spermine significantly lower in the SSAT-deficient cells (Table I).

**Effect of the Polyamine Analogue, DENSPM—**An induction of SSAT by polyamine analogues and the subsequent depletion of cellular spermidine and spermine pools are supposed to be the major mediators of the cytotoxicity exerted by the latter drugs. Fig. 3 depicts an experiment where wild-type cells and SSAT knock-out cells were exposed to DENSPM, one of the most potent inducers of SSAT. As indicated in the figure, SSAT-deficient cells grew at a somewhat slower rate (p < 0.001; ANOVA) than the parental cells in the absence of the drug, yet they were significantly more resistant (p < 0.001; ANOVA) to the analogue in comparison with their parental counterparts, whose proliferation practically was halted already at 24 h. The same phenomenon is likewise obvious in the dose-response curve presented in Fig. 4. In fact, IC50 of DENSPM for the SSAT-deficient cells was at least 50 times higher (p < 0.001; ANOVA) than for the wild-type cells. To exclude the possibility that the analogue was differently taken up by the two cell lines, we measured the intracellular concentrations of DENSPM over a period of 5 days. Even though the levels of the drug fluctuated, there was no significant difference between the two curves (data not shown). Fig. 5 depicts the DENSPM-induced depletion of cellular spermidine (Fig. 5A) and spermine (Fig. 5B) pools. As shown, the SSAT-deficient cells appeared to maintain higher (p < 0.001; ANOVA) spermidine levels (Fig. 5A) than their parental counterparts in the absence of the drug. Exposure to DENSPM resulted in a rapid and near complete depletion of spermidine in both cell lines though in the parental cells the spermidine pool appeared to be correctly targeted and contained no additional integrations. The positive control (+) was from liver of a DENSPM-treated transgenic mouse overexpressing SSAT. A further piece of evidence indicating that the targeted cells were also devoid of SSAT activity is presented in Fig. 2B. In this experiment, both wild-type and targeted cells were exposed to DENSPM for 5 days. As seen in the figure, the parental cells responded to the drug with immense induction of SSAT activity whereas the targeted cells did not show virtually any activity during the whole period of observation.

**Effect of SSAT Deficiency on Polyamine Homeostasis—**As SSAT is believed to function as an integral part of the machinery maintaining polyamine homeostasis and possibly also protect the cells from the toxicity of excess polyamines, we exposed wild-type and SSAT-deficient cells to exogenous polyamines. The cells were grown in the presence of 100 µM putrescine, spermidine, or spermine for 24 h. The exposure to the polyamines did not have any effect on the viability of any of the cell lines. Table I lists the concentrations of the polyamines after the exposure. As indicated in the table, both cell lines accumulated exogenous polyamines in a very similar fashion. The basal levels of putrescine and spermidine were significantly higher and that of spermine significantly lower in the SSAT-deficient cells (Table I).

**TABLE I**

| Treatment    | Polyamine pools |
|--------------|-----------------|
|              | Putrescine      | Spermidine     | Spermine      |
| Wt           | 25 ± 21         | 4200 ± 100     | 3280 ± 68     |
| Wt + put     | 102 ± 8         | 4970 ± 88      | 3120 ± 45     |
| Wt + spd     | 0 ± 0           | 4510 ± 230     | 2980 ± 117    |
| Wt + spm     | 0 ± 0           | 1041 ± 33      | 5010 ± 180    |
| SSAT KO      | 188 ± 12°       | 5480 ± 67°     | 2990 ± 21°    |
| SSAT KO + put| 522 ± 23        | 5970 ± 35      | 3040 ± 90     |
| SSAT KO + spd| 129 ± 14        | 6450 ± 25      | 3510 ± 82     |
| SSAT KO + spm| 30 ± 26         | 2540 ± 6       | 4980 ± 172    |

° p < 0.001; † p < 0.01; comparison between wild-type and SSAT KO cells.
lular polyamines, but it rather represents a replacement of the natural polyamines from their intracellular binding sites by the analogue.

Effect of SSAT Deficiency on the Back-conversion of Spermidine and Spermine—We next carried out a series of tracer experiments to follow the back-conversion of spermidine to putrescine and that of spermine to spermidine. The cell were grown for 3 h in the presence of labeled polyamine in a serum-free (to avoid extracellular amine oxidases) medium whereafter the formation of radioactive $N^1$-acetylspermidine and putrescine (from spermidine) and radioactive spermidine (from spermine) was measured. The back-conversion of spermidine to $N^1$-acetylspermidine and putrescine in the absence or presence of DENSPM, an inducer of SSAT, and MDL72527, a potent inhibitor of polyamine oxidase, is depicted in Fig. 6. In the absence of the analogue, the back-conversion rate of spermidine was very low in the wild-type cells and at the background level in the SSAT-deficient cells. An exposure to DENSPM enhanced dramatically the formation of $N^1$-acetylspermidine and putrescine in the parental cells whereas the products of spermidine remained at the background level in the mutated cells. An inclusion of MDL72527 prevented any putrescine formation and more than tripled the level of $N^1$-acetylspermidine in the wild-type cells, which is in full agreement with a total inhibition of PAO by the latter drug. In the SSAT-deficient cells, the products remained at hardly detectable levels (Fig. 6). It thus appears that SSAT is indispensable for the back-conversion of spermidine to putrescine (via the formation of $N^1$-acetylspermidine) and that PAO cannot convert spermidine to putrescine without a prior acetylation.

Fig. 7 depicts the results of a similar tracer study with radioactive spermine. The situation now strikingly differed from that found for spermidine. As indicated in the figure, spermine was converted readily to spermidine (but not to acetylspermine) in both cell types. In fact, the SSAT-deficient cells converted spermine to spermidine about four times ($p < 0.001$) more efficiently than did the parental cells. The enhanced conversion of spermine to spermidine in SSAT-deficient cells was not attributable to stimulated PAO activity, as in the wild-type cells PAO activity was 273, and in the targeted cells it was 316 pmol/10⁶ cells per 60 min. The apparently slower conversion of spermine to spermidine in the wild-type cells was not because of a further metabolism of spermidine to acetylspermidine and putrescine, as all samples were monitored for the whole range of metabolites, and no detectable amounts of labeled acetylspermidine or putrescine were found. In striking contrast to the spermidine back-conversion, the inclusion of DENSPM did not change the rate of spermine conversion in the parental cells but significantly ($p < 0.01$) inhibited the conversion or facilitated the efflux of spermidine in the mutated cells (Fig. 7). DENSPM has no effect, whatsoever, on PAO activity in either of the cell lines, MDL72527, which completely prevented the conversion of acetylspermidine to putrescine (Fig. 6), only partially (40 to 50%) inhibited the conversion of spermine to spermidine (Fig. 7). Inclusion of the known amine oxidase.
represents the mean S.D. obtained from triplicate cultures. The difference between DENSPM-treated and DENSPM-exposed SSAT KO cultures (wild-type and SSAT KO cultures) and that between untreated and DENSPM-exposed wild-type and SSAT-deficient (SSAT KO) cells. The cells were exposed to 50 μM DENSPM (DEN) for 48 h, whereafter 20 μM MDL72527 (MDL) was added for a further 24 h. Before the addition of 10 μM [14C]spermidine (specific activity 30.9 mCi/mmol), medium was removed, and the cells were washed twice with PBS, replenished with DMEM without serum, and incubated for 3 h in the presence of the tracer. Each column represents the mean ± S.D. obtained from triplicate cultures.

![Diagram](http://www.jbc.org/fig.png)

**FIG. 6.** Conversion of radioactive spermidine to N-acetylrepeatidine and putrescine in wild-type (Wt) and SSAT-deficient (SSAT KO) cells. The cells were exposed to 50 μM DENSPM (DEN) for 48 h, whereafter 20 μM MDL72527 (MDL) was added for a further 24 h. Before the addition of 10 μM [14C]spermidine (specific activity 23 mCi/mmol), medium was removed, and the cells were washed twice with PBS, replenished with DMEM without serum, and incubated for 3 h in the presence of the tracer. Each column represents the mean ± S.D. obtained from triplicate cultures.

![Diagram](http://www.jbc.org/fig.png)

**FIG. 7.** Conversion of radioactive spermidine to spermidine in wild-type and SSAT-deficient (SSAT KO) cells. The cells were exposed to 50 μM DENSPM for 48 h, whereafter 20 μM MDL72527 (MDL) or 1 mM aminoguanidine (AG) or 1 mM semicarbazide (SC) were added for an additional 24 h. Before the addition of 10 μM [14C]spermidine (specific activity 23 mCi/mmol), medium was removed, and the cells were washed twice with PBS, replenished with DMEM without serum, and incubated for 3 h in the presence of the tracer. Each column represents the mean ± S.D. obtained from triplicate cultures. The statistical significances of the difference (p < 0.001) between untreated wild-type and SSAT KO cultures and that between untreated and DENSPM-exposed SSAT KO cultures (p < 0.01) are shown. ***, p < 0.001, refers to the difference between DENSPM-treated and untreated DENSPM-exposed SSAT KO cultures. *, p < 0.05, refers to the difference between DENSPM-treated and DENSPM + aminoguanidine-treated SSAT KO cultures.

inhibitors, aminoguanidine and semicarbazide, only marginally affected the conversion of spermine to spermidine in both cell lines. We also checked whether the cells contained spermine-specific acetylase activity by measuring SSAT activity using spermine as the substrate. Only DENSPM-exposed wild-type cells showed spermine acetylation, which was about one-fourth of spermidine acetylation. It thus appears that spermine can be converted to spermidine by an SSAT-independent system that is not inducible by polyamine analogues and is not particularly sensitive to the PAO inhibitor, MDL72527.

**DISCUSSION**

The acetylation of spermidine and spermine by SSAT is considered to play a central role in the maintenance of polyamine homeostasis in mammalian cells. The reduction of the positive charge of the polyamines through acetylation is believed to facilitate their excretion and hence prevent any toxic overaccumulation of the polyamines. The enzyme likewise facilitates the back-conversion of spermidine and spermine as acetylated polyamines are much better substrates for PAO than unmodified spermidine and spermine (2). A large number of studies has shown convincingly that the cytotoxicity of polyamine analogues is closely correlated with their ability to induce SSAT in a given cell type (3–7). In addition to the studies carried out in cultured cell lines, we have shown that transgenic mice overexpressing SSAT in most of their tissues are much more sensitive to the general toxicity exerted by polyamine analogues than their syngenic littermates (17).

The present results with SSAT-deficient mouse embryonic stem cells raise some reservations as regards the central role of SSAT in polyamine homeostasis and their back-conversion reactions. As shown here, the SSAT-deficient cells appeared to maintain their polyamine homeostasis very much like their wild-type counterparts. Furthermore, the mutated cells did not show any overt toxicity upon exposing them to extracellular polyamines, and the cellular accumulation of external polyamines occurred in a similar fashion in both parental cells and SSAT-deficient cells. However, the targeted cells were distinctly more resistant to the cytotoxic action exerted by the polyamine analogue, DENSPM, as the IC_{50} was increased at least by a factor of 50 (Fig. 4). The cytotoxicity of polyamine analogues is believed to be based mainly on a rapid depletion of intracellular polyamines upon SSAT induction. However, the present result indicated that the contribution of SSAT activity to the depletion of cellular polyamines by DENSPM is rather marginal, as the depletion of spermine was identical in both wild-type and mutated cells, and that of spermidine was only initially slightly slower in the SSAT-deficient cells (Fig. 5). Thus the enhanced resistance of the SSAT-deficient cells toward the polyamine analogue was not attributable to a more efficient polyamine depletion in the parental cells. An alternative possibility to explain the reduced sensitivity of the targeted cells toward the analogue is the lack of an SSAT/PAO system able to generate hydrogen peroxide and potentially cytotoxic aldehydes from acetylated polyamines. In fact, it has been reported that polyamine analogue-induced programmed cell death can be partly prevented by inhibition of PAO and hence the transformation of hydrogen peroxide (18). On the other hand, we found that fetal fibroblasts derived from transgenic mice overexpressing SSAT are remarkably sensitive to DENSPM-induced cytotoxicity, which is not alleviated by PAO inhibitor (9). It is thus possible that the contribution of reactive oxygen species generated by PAO or polyamine depletion to the cytotoxicity exerted by polyamine analogues varies depending on the cell type. Mouse embryonic stem cells may belong to the first category, i.e. to cells in which the generation of reactive oxygen species, and not polyamine depletion, is largely responsible for the observed cytotoxicity. It is also possible that polyamine analogues exert direct cellular toxicity not dependent on SSAT or polyamine depletion.

The contribution of SSAT to the back-conversion reactions of
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spermidine and spermine is an interesting issue. The present results with radiolabeled polyamines showed unambiguously that SSAT-deficient cells, unlike parental cells, failed to form any detectable amounts of radioactive putrescine from labeled spermidine under any experimental conditions. On the other hand, the wild-type cells appeared to operate the classic SSAT/PAO pathway, i.e. the formation of N1-acetylspermidine and putrescine was strikingly induced by the polyamine analogue and an inclusion of the PAO inhibitor, MDL72527, totally abolished the formation putrescine and gave rise to marked accumulation of N1-acetylspermidine (Fig. 6). The conversion of spermine to spermidine, however, seemed to be carried out by an entirely different system, as SSAT-deficient cells converted spermine to spermidine even at an enhanced rate in comparison with the parental cells (Fig. 7). Instead of an induction, DENSPEM appeared to inhibit the conversion of spermine to spermidine in the targeted cells. Moreover, unlike in spermidine back-conversion, equimolar concentrations of the PAO inhibitor only partially blocked the formation of spermidine from spermine in both parental and SSAT-deficient cells (Fig. 7). It is possible that the conversion of spermine to spermidine was catalyzed by PAO, which can use unmodified spermine as a substrate (19). However, this view is not supported by our findings indicating that PAO activity was not enhanced in the SSAT-deficient cells, yet the latter cells converted spermine to spermidine four times faster than the parental cells. Wang et al. (20) reported recently the cloning and partial characterization of a putative human PAO. They confirmed that recombinantly generated human PAO uses unmodified spermine as the substrate with a reasonably low \( K_m \) of 18 \( \mu M \) (20). However, cloned human PAO shows features that are not in line with our present results. The transcription and the activity of the human PAO were induced by polyamine analogue 5- and 3-fold, respectively (20). Moreover, Wang et al. (20) reported virtually a complete inhibition of PAO activity by the polyamine oxidase inhibitor, MDL72527. Unfortunately, the concentration of the inhibitor was not stated nor was the substrate specificity mentioned (20). According to our results, the conversion of spermine to spermidine occurred at an enhanced rate in the SSAT-deficient cells but was not induced by the polyamine analogue in either cell types. The enzyme or system apparently did not use spermidine as a substrate, and the polyamine oxidase inhibitor, MDL72527, at concentrations fully preventing putrescine formation from spermidine, only partially (by 40 to 50%) blocked the conversion of spermine to spermidine in wild-type and SSAT-deficient cells. It thus appears that at least embryonic stem cells operate a back-conversion system for spermine that is independent of SSAT and may even represent a new enzyme system. The significantly higher spermidine concentration in SSAT-deficient cells (see Table I and Fig. 3) may, in fact, reflect both a blockage of spermidine catabolism (Fig. 6) and enhanced conversion of spermine to spermidine (Fig. 7).

Our present results appear to indicate that (i) SSAT may not play that central role in the maintenance of polyamine homeostasis; (ii) cytotoxicity of polyamine analogues may, in certain circumstances, be based on mechanisms other than the depletion of cellular polyamines; and (iii) spermidine and spermine appear to have different pathways for their degradation of which only spermidine catabolism is SSAT-dependent.

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