Effect of spermine synthase on the sensitivity of cells to anti-tumour agents

Yoshihiko Ikeguchi, Caroline A. Mackintosh, Diane E. McCloskey and Anthony E. Pegg¹

Department of Cellular and Molecular Physiology (H166), Room C4737, Pennsylvania State University College of Medicine, 500 University Drive, P.O. Box 850, Hershey, PA 17033, U.S.A.

The role of spermine in the sensitivity of cells to various established and experimental anti-tumour agents was examined, using paired cell lines that possess or lack spermine synthase. All spermine-synthase-deficient cells had no detectable spermine, and elevated spermidine, content. Spermine content did not alter the cell growth rate. There was little or no difference in sensitivity of immortalized mouse embryonic fibroblasts to doxorubicin, etoposide, cisplatin, methylglyoxal bis(guanyldihydrazone) or H₂O₂ and only a slight increase in sensitivity to vinblastine and nocodazole. However, the absence of spermine clearly increased the sensitivity to 1,3-bis(2-chloroethyl)-*N*-nitrosourea, suggesting that depletion of spermine may be a useful way to increase the anti-neoplastic effects of anti-tumour agents that form chloroethyl-mediated interstrand DNA cross-links. The effects of spermine on the response to polyamine analogues (which have been proposed to be useful anti-neoplastic agents) were complex, and depended on the compound examined

and on the cells tested. Sensitivity to CHENSpm {*N*¹-ethyl-*N*¹¹-[(cycloheptyl)methyl]-4,8-diazaundecane} was substantially greater in immortalized fibroblasts that lack spermine. In contrast, BE-3-4-3 [*N*¹,*N*¹²-bis(ethyl)spermine] and BE-3-3-3 [*N*¹,*N*¹¹-bis(ethyl)norspermine] were more active against cells that contained spermine. The presence of spermine correlated with a greater induction of spermidine/spermine-*N*¹-acetyltransferase by BE-3-3-3, which is consistent with suggestions that this induction is important for the response to this drug. These findings support the concepts that different polyamine analogues have different sites of action and that CHENSpm has a different site of action from BE-3-3-3.

Key words: 1,3-bis(2-chloroethyl)-*N*-nitrosourea, *N*¹,*N*¹¹-bis(ethyl)norspermine, *N*¹-ethyl-*N*¹¹-[(cycloheptyl)methyl]-4,8-diazaundecane, polyamine analogues, spermine.

INTRODUCTION

Polyamines are ubiquitous cellular cations that play an important role in many cellular processes, including cell growth, differentiation and survival [1–3]. Polyamine content and synthesis are enhanced during tumour promotion and progression, and in neoplastic growth [4–7]. Overexpression of several of the enzymes involved in polyamine biosynthesis initiates or promotes neoplastic growth, and several known oncogenes increase the activity of these enzymes [8–12]. There is, therefore, considerable interest in the polyamine pathway as a target for the design of anti-neoplastic and chemopreventive agents [13–17].

The natural polyamines present in mammalian cells are putrescine, spermidine and spermine. Spermine, which is the largest polyamine and is the final product of the pathway, is formed by the action of spermine synthase (SS), which transfers an aminopropyl group donated by decarboxylated *S*-adenosylmethionine (AdoMet) to a spermidine acceptor [18–20]. Its function is unclear, since many of the effects that have been demonstrated in the laboratory can be mimicked by its precursors putrescine and spermidine.

Many micro-organisms do not have SS and spermine. Yeast mutants that lack SS do not require exogenous spermine for growth and viability [21], and mammalian cells can be grown

in culture in the presence of inhibitors of SS [18,22,23]. Furthermore, it is possible to establish fibroblast cell cultures from male embryos [24] or skin [25] of Gy mice. These mice have a deletion of the part of the X chromosome that encodes SS. Embryonic stem cells with targeted deletions of the SS gene have also been grown [26]. It is not necessary to add exogenous spermine to these cultures, and thus it is fully established that some isolated mammalian cells can be grown in the absence of spermine.

However, it is likely that spermine does play a key role in some cells at some stage of mammalian development. Male Gy mice, which can only be propagated in the B6C3H background [27], are sterile and have a shorter life span and a much smaller size than unaffected littermates. They also have inner ear abnormalities, deafness, hyperactivity and exhibit circling behaviour [27–29]. The functions of spermine, whose loss is related to this phenotype, are unknown. It must also be noted that the X-chromosomal deletion in the Gy mice is not limited to the SS gene, but includes an adjacent region which contains at least one other gene called *phex*, and the loss of this region may also contribute to the phenotype in the Gy mice.

It is likely that spermine, which is more highly charged than putrescine and spermidine, is bound more tightly to cellular components including proteins and nucleic acids. Since these

Abbreviations used: SS, spermine synthase; AdoMet, *S*-adenosylmethionine; AdoMetDC, *S*-adenosylmethionine decarboxylase; BCNU, 1,3-bis(2-chloroethyl)-*N*-nitrosourea; ODC, ornithine decarboxylase; SSAT, spermidine/spermine *N*¹-acetyltransferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; CHENSpm, *N*¹-ethyl-*N*¹¹-[(cycloheptyl)methyl]-4,8-diazaundecane; CBENSpm, *N*¹-ethyl-*N*¹¹-[(cyclobutyl)methyl]-4,8-diazaundecane; CPENSpm, *N*¹-ethyl-*N*¹¹-[(cyclopropyl)methyl]-4,8-diazaundecane; BE-3-4-3, *N*¹,*N*¹²-bis(ethyl)spermine; BE-3-3-3, *N*¹,*N*¹¹-bis(ethyl)norspermine; BE-4-4-4-4, 1,19-di-(ethylamino)-5,10,15-triazanonadecane; MGBG, methylglyoxal bis(guanyldihydrazone); EF-SS[−] cells, primary cultures of mouse embryonic fibroblasts having a deletion of the SS gene; EF-SS⁺ cells, primary cultures of mouse embryonic fibroblasts from normal littermates having an active SS gene; IEF-SS[−] cells, immortalized EF-SS[−] cells; IEF-SS⁺ cells, immortalized EF-SS⁺ cells; IEF-SS[−]/pSS cells, IEF-SS[−] cells that express SS after stable transfection with plasmid pZeo-Spmsyn; IEF-SS[−]/pZeo cells, IEF-SS[−] cells stably transfected with the empty plasmid vector pZeoCMV.

¹ To whom correspondence should be addressed (e-mail apegg@psu.edu).

interactions are likely to mediate the biological effects of polyamines, including those needed for growth and survival of neoplastic cells, it is possible that the spermine content might influence the response to certain toxic agents, including anti-neoplastic drugs, particularly those that have some direct interaction with nucleic acids or with the polyamine pathway. Such a difference may be exploitable for therapeutic purposes since effective inhibitors of SS are available and can be administered without major toxicity [18]. The availability of cultured cells derived from male Gy mice provides a useful system to test the hypothesis that spermine content influences response to exogenous agents. Preliminary studies indicated that the cells from Gy mice lacking spermine were more easily killed by the chloroethylating agent 1,3-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) and UV light [25,30] than cells from control mice [24]. In the present study, we have extended these observations to a variety of other anti-proliferative agents, including some polyamine analogues that are currently undergoing laboratory and clinical trials [17,31–35]. We also used cells in which SS activity had been restored, by stable transfection, to provide a more definitive control than the comparison with normal cells, which differ from Gy in other ways, as described above. The results indicate that the effects of spermine on the development of cytotoxicity are dependent on the agent employed, with both protective effects and sensitization being observed.

MATERIALS AND METHODS

Materials

All chemicals, unless noted, were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Heterozygous Gy female mice were a gift from Dr R. A. Meyer, Jr (Department of Orthopaedic Surgery, Carolinas Medical Center, Charlotte, NC, U.S.A.).

Polyamine and enzyme analysis

Polyamine content was determined by HPLC using an ion-pair reversed-phase HPLC separation method with post-column derivatization using *o*-phthalaldehyde, as described previously [36].

All enzyme assays were carried out as described previously. Ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC) were assayed by measuring the release of $^{14}\text{C}_2$ from [1- ^{14}C]ornithine or *S*-adenosyl-L-[carboxyl- ^{14}C]methionine (radiolabelled AdoMet) respectively [37]. Spermidine synthase and SS assays were carried out by measuring the production of [^{35}S]methylthioadenosine from [^{35}S]decarboxylated AdoMet in the presence of the appropriate amine acceptor as described previously [24,38]. Spermidine/spermine *N*¹-acetyltransferase (SSAT) was measured by following the transfer of [^{14}C]acetyl groups from [^{14}C]acetyl-CoA to spermine [39].

Cell culture

Primary cultures of embryonic fibroblasts from Gy mutant male mice, which have a deletion of the SS gene (EF-SS⁻ cells), and from normal littermates (EF-SS⁺ cells) were isolated from 14-day-old embryos and cultured in a humidified incubator (37 °C, 5 % CO₂) essentially as described previously [24]. Immortalized mouse embryonic fibroblasts were produced by taking the EF-SS⁻ cells and EF-SS⁺ cells at passage 3 and transfecting them with a plasmid expressing SV40 large- and small-T antigens [40] using LIPOFECTAMINE plus (Gibco BRL Life Technologies,

Burlington, ON, Canada). Transfected cells were selected by their resistance to 500 µg/ml G418 (Gibco BRL Life Technologies), and surviving cells were isolated and expanded to give immortal IEF-SS⁻ and IEF-SS⁺ cells [24,30].

Re-expression of SS in the IEF-SS⁻ cells was accomplished by isolation of stable transfectants after transfection with plasmid pZeo-Spmsyn (see below) to form cell lines IEF-SS⁻/pSS. Control cell lines (IEF-SS⁻/pZeo) were obtained using the plasmid vector without an insert.

Construction and use of pZeo-Spmsyn plasmids

A plasmid vector (pQE-hSpS) for the expression of recombinant human SS in *Escherichia coli* was constructed as follows. A sense primer (5'-CACCATGGATCCGCGAGCAGCACGGCAC-3') containing a *Bam*HI restriction site (underlined) before the codon for the second amino acid (alanine), and an anti-sense primer (5'-CGTACGGGTACCTCAGGGTTTAGC-3') containing a *Kpn*I restriction site (underlined) after the stop codon, were synthesized based on the cDNA sequence of the human SS. These were used for amplification of SS DNA sequence from a human liver cDNA expression library (Clontech, Palo Alto, CA, U.S.A.). The PCR was carried out in a 100 µl reaction volume containing 2.5 units of *Pfu* polymerase, 50 ng of human liver cDNA expression library DNA as template, and 25 pmol of each of the sense and anti-sense primers. The conditions used were: an initial denaturation for 2 min at 92 °C, followed by 25 cycles of denaturation (92 °C for 30 s), annealing (54 °C for 30 s) and extension (72 °C for 1 min), with a final extension at 72 °C for 5 min. The 1125 bp PCR product was digested with *Bam*HI and *Kpn*I restriction enzymes, purified on a 1 % agarose gel using the Qiagen gel extraction kit (Qiagen, Chatsworth, CA, U.S.A.), ligated into pQE-30 vector, digested with the same enzymes and transformed into *E. coli* XL1-Blue cells. The SS DNA insert was sequenced to ensure that no secondary mutations were introduced during plasmid construction. The proteins expressed from pQE-30 plasmids carry a 12-amino-acid (Met-Arg-Gly-Ser-His₆-Gly-Ser-) tag at their N-terminal end. The *Bam* HI (GGATCC) site codes for the amino acids Gly-Ser that follow the six histidine residues and is the site of ligation for the human SS gene. Recombinant human SS was purified by immobilized metal affinity chromatography using TALON[®] affinity resin (Clontech Laboratories, Palo Alto, CA, U.S.A.), in accordance with manufacturer's instructions.

In order to construct a vector, pZeo-hSpS, for expression of SS in mammalian cells, a sense primer (5'-TCTGCA-GAATTCATGGCAGCAGCACGGCAC-3') containing an *Eco*RI restriction site (underlined), and an anti-sense primer (5'-AAGCTTGGTACCTCAGGGTTTAGC-3') containing a *Kpn*I restriction site (underlined) after the stop codon, were synthesized and used for amplification of the pQE-hSpS plasmid. The PCR was carried out using the conditions described above. The PCR product was digested with *Eco*RI and *Kpn*I restriction enzymes, purified on a 1 % agarose gel using a Qiagen gel extraction kit, ligated into the pZeoCMV vector (Invitrogen, Carlsbad, CA, U.S.A.) digested with the same enzymes and transformed into *E. coli* XL1-Blue cells, forming pZeo-hSpS. All plasmids were purified using Qiagen plasmid purification columns.

For transfection, IEF-SS⁻ cells were plated in triplicate using 2×10^5 cells per 60-mm-diameter tissue culture dish and allowed to adhere to the plates for 12–18 h. The cells were incubated with LIPOFECTAMINE (20 µg/plate) and 2 µg of plasmid DNA (pZeo-hSpS or pZeoCMV) for 5 h at 37 °C in a humidified 5 % CO₂ atmosphere. After 5 h, the medium was aspirated and 5 ml of fresh Dulbecco's modified Eagle's medium (Gibco BRL Life

Technologies) supplemented with 10% ES cell-qualified fetal bovine serum (Gibco BRL Life Technologies), 2 mM glutamine, 100 units/ml penicillin and 100 units/ml streptomycin was added. The cultures were incubated for an additional 48 h. Cells were replated at low density in selective medium with 375 $\mu\text{g/ml}$ zeocin (Invitrogen) and incubated for 8–11 days, at which time individual colonies were selected and transferred to individual wells of a 24-well plate. The selected clones were screened for SS activity levels. Stably transfected cells were maintained in 375 $\mu\text{g/ml}$ zeocin (Invitrogen) throughout the experiments.

Growth-inhibition assays

Studies with polyamine analogues and other agents (except BCNU) were carried out using a standard assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] staining for cytotoxicity. Exponentially growing cells were plated in triplicate at $2.5\text{--}3 \times 10^3$ cells/cm² in 100 μl medium/well in 96-well plates. After a 12–18 h period for the cells to attach, 200 μl medium containing $1.5\times$ the desired final drug concentration was added. Cells were incubated in the absence or presence of at least six drug concentrations for 144 h, at which time the medium was aspirated and 100 μl of 5 mg/ml MTT in Optimum (Life Technologies, Inc., Rockville, MD, U.S.A.) was added. The cells were incubated for an additional 4–6 h at 37 °C, after which the fluid and MTT were removed by aspiration and 100 μl of 50% ethanol in DMSO was added to each well. After 20 min, the A_{570} (a value directly proportional to the number of viable cells [26]) was determined using a BioRad plate reader. IC₅₀ values were determined from plots of percentage of untreated control cell number versus the logarithm of the drug concentration.

Sensitivity to BCNU was determined using a colony-forming assay [24] which has been used extensively for this agent in the past. Briefly, cells (2.5×10^5) were plated in triplicate in 60-mm-diameter dishes and cultured for 24 h in a humidified incubator at 37 °C and 5% CO₂. Various concentrations of BCNU were then added and the cells were incubated for a further 2 h before the medium was replaced with fresh medium. After 16–18 h, cells were re-plated into 25 cm² flasks at a density of 200 cells/flask. Cells were cultured for 7 days, after which the cells were washed once with 0.9% saline, stained with 0.5% Crystal Violet in ethanol, and the colonies were counted.

Western-blot analysis

Proteins present in cell extracts were resolved by SDS/PAGE using a 12% gel. Electrotransfer to PVDF membrane (Micron Separations, Waterborough, MA, U.S.A.) was followed by hybridization with crude polyclonal rabbit antiserum to human SS and detection using the Vistra Western blot detection kit (Amersham Biosciences, Piscataway, NJ, U.S.A.). A Molecular Dynamics FluorImager model 595 and Imagequant application software were used for visualization and quantification.

RESULTS

Preliminary studies with IEF-SS⁻ and EF-SS⁺ cells

Previous studies [24] have reported the properties of immortalized embryonic fibroblast cell lines established from normal and mutant Gy male 14-day-old embryos by transfection with a plasmid which expresses SV40 large- and small-T antigens. Activity of SS and spermine were undetectable in the cells derived from the Gy mutants (IEF-SS⁻ cells). The spermidine content was

Table 1 Sensitivity of IEF-SS⁻ and IEF-SS⁺ cell lines to polyamine analogues, anti-cancer drugs and other agents

Experiments were carried out in duplicate using two IEF-SS⁻ cell lines and two IEF-SS⁺ lines. Cells were treated with drugs over an appropriate concentration range, which varied from at least 5-fold below and 5-fold above the IC₅₀ values, and cell viability was measured using the MTT assay method described in the Materials and methods section. The results were expressed as IC₅₀ values representing the amount of drug needed to inhibit growth of the cells by 50%. The ratio of the mean of the values for IEF-SS⁻ cells to the mean of the control IEF-SS⁺ lines is shown.

Agent	Ratio of IC ₅₀ values (IEF-SS ⁻ /IEF-SS ⁺)
CHENSpm	0.25
BE-3-3-3	3.26
BE-3-4-3	2.01
BE-4-4-4-4	1.26
CBENSpm	1.04
CPENSpm	1.22
Cisplatin	0.90
Doxorubicin	0.93
Etoposide	0.83
Nocodazole	0.51
Vinblastine	0.66
MGBG	1.13
H ₂ O ₂	1.07

increased by approx. 2-fold compared with the IEF-SS⁺ cells, which have endogenous SS. Despite these changes, there was only a minimal and statistically insignificant difference in growth rate between IEF-SS⁺ cells (average doubling time of 12.8 h) compared with IEF-SS⁻ cells (average doubling time of 12.5 h). These cells were used for preliminary experiments to examine whether the absence of spermine led to a difference in response to agents that are either established anti-tumour agents or under investigation for this purpose. The sensitivity to H₂O₂ was also tested, since spermine has been reported to be important as a means for cells to resist oxidative damage. Assays were carried out using a number of such compounds with two IEF-SS⁻ cell lines and two IEF-SS⁺ lines as described in the Materials and methods section. The results were expressed as IC₅₀ values representing the amount of drug needed to reduce the viability of the cells by 50%. The ratio of the mean of the values for IEF-SS⁻ cells to the mean of the control IEF-SS⁺ lines for each agent is shown in Table 1. The sensitivity to CHENSpm {N¹-ethyl-N¹¹-[(cycloheptyl)methyl]-4,8-diazaundecane} was clearly greater in these IEF-SS⁻ cells that lack spermine. In contrast, BE-3-4-3 [N¹,N¹²-bis(ethyl)spermine], and, to an even greater extent BE-3-3-3 [N¹,N¹¹-bis(ethyl)norspermine], were more active against the IEF-SS⁺ cells that contain spermine (Table 1). There was no difference in sensitivity to doxorubicin, etoposide, cisplatin, MGBG [methylglyoxal bis(guanyldrazone)], H₂O₂, CBENSpm {N¹-ethyl-N¹¹-[(cyclobutyl)methyl]-4,8-diazaundecane}, CPENSpm {N¹-ethyl-N¹¹-[(cyclopropyl)methyl]-4,8-diazaundecane} or BE-4-4-4-4 [1,19-di-(ethylamino)-5,10,15-triazanonadecane]. Previous studies using these cells have shown that there was a small but clear increase in sensitivity to killing by BCNU in the IEF-SS⁻ cells lacking spermine [24]. In the current study, small differences were also seen in the responses to vinblastine and nocodazole, which were slightly more active in IEF-SS⁻ cells.

Effect of transfection of IEF-SS⁻ cells with SS cDNA

A serious disadvantage with the cells used for these preliminary experiments [24] is that the Gy mutation, leading to the loss of SS activity, is actually a deletion of a chromosomal region on the X

Figure 1 Detection of SS protein in extracts of transfected IEF-SS⁻/pSS cells

Western-blot analysis was carried out using cell extracts from IEF-SS⁻/pSS (-1 or -2) cells and IEF-SS⁻/pZeo cells. After separation of 50 µg of protein from cell extracts by SDS/PAGE, the proteins were transferred to PVDF membrane and hybridized with a crude polyclonal rabbit antiserum to human SS. A marker lane (Histag SS) loaded with purified recombinant human SS with a 12-amino-acid extension containing the His₆ tag is also shown.

chromosome. This includes not only part of the SS gene but also some other DNA [27,41]. Also, the Gy mutation is maintained on the B6C3H hybrid background. Therefore, the normal male littermates that were used for the production of the IEF-SS⁺ cell lines do not differ from their Gy siblings only in the lack of SS. In order to make a more closely matched pair of cell lines, the IEF-SS⁻ cells were transfected with a construct in which the human SS cDNA was under the control of a cytomegalovirus promoter. Two of the resulting cell lines (IEF-SS⁻/pSS-1 and -2) had SS activities of 24.5 and 22.1 units per mg of protein respectively, which were equivalent to the SS activities in the IEF-SS⁺ cell lines. A control cell line (IEF-SS⁻/pZeo), which was transfected with the plasmid vector only, maintained undetectable SS activity.

Western-blot analysis of cell extracts from IEF-SS⁻/pSS-1 and -2 and IEF-SS⁻/pZeo cells developed with an antiserum to human SS confirmed the presence of SS protein in the former cell lines (Figure 1). The only available antiserum to SS is not totally specific for the enzyme, but, as shown in Figure 1, there was an immunoreactive band of 41 kDa present only in the cells transfected with SS cDNA. This band is slightly smaller than the purified SS marker, since this protein has a 12-amino-acid extension due to a histidine tag. Since there were no significant differences in SS activity or protein expression between IEF-SS⁻/pSS-1 and IEF-SS⁻/pSS-2, the IEF-SS⁻/pSS-1 line was chosen for further studies.

Determination of intracellular polyamine pools indicated that IEF-SS⁻/pSS-1 cells have a significant level of spermine, whereas IEF-SS⁻/pZeo cells have no detectable spermine (Table 2). The level of spermidine in the IEF-SS⁻/pSS-1 cells was lower than that in the IEF-SS⁻/pZeo, reflecting the fact that some of the spermidine can be converted into spermine by SS. The activities of other enzymes in the polyamine biosynthetic pathway were also examined in the transfected IEF-SS⁻/pSS-1 and IEF-SS⁻/pZeo cells. There was no difference in the activities of spermidine synthase and ODC, but AdoMetDC was decreased significantly in the IEF-SS⁻/pSS-1 cells (Table 2).

Comparison of the role of spermine in determination of sensitivity to drugs using IEF-SS⁻/pSS-1 and IEF-SS⁻/pZeo cells

The paired cell lines of IEF-SS⁻/pSS-1 and IEF-SS⁻/pZeo were used to follow up on the preliminary information of sensitivity

Table 2 Polyamine content and activity of polyamine biosynthetic enzymes in transfected IEF-SS⁻ cells

Polyamines and enzyme activities were measured as described in the Materials and methods section. Values are given as means ± S.E.M. for at least four determinations, in cells grown for 40 h. Differences in spermidine and spermine were significant with $P < 0.001$. Values are given as means ± S.E.M. * $P < 0.001$ significantly different from IEF-SS⁻/pZeo, † $P < 0.03$ significantly different from IEF-SS⁻/pZeo. N. D., not detectable.

	Polyamine content (nmol/mg of protein) or enzyme activity (pmol/min per mg of protein)	
	IEF-SS ⁻ /pSS-1	IEF-SS ⁻ /pZeo
Putrescine	< 0.1	< 0.1
Spermidine	33.8 ± 3.7*	46.8 ± 8.6
Spermine	1.9 ± 0.2*	< 0.2
Ornithine decarboxylase	530 ± 84	568 ± 48
AdoMet decarboxylase	97 ± 28†	200 ± 28
Spermidine synthase	655 ± 49	689 ± 58
Spermine synthase	24 ± 5	N. D.

Figure 2 Effect of BCNU on IEF-SS⁻/pSS-1 and IEF-SS⁻/pZeo cells

Cells were treated with BCNU at the levels shown and cell survival was measured by the colony-forming assay described in the Materials and methods section. Results are shown as means with bars indicating S.E.M.

to drugs shown above in Table 1. Even though the cells lacking spermine had a higher level of AdoMetDC, the sensitivity to the AdoMetDC inhibitor MGBG was not different between the cell lines (results not shown). This result is consistent with the lack of difference in response to MGBG seen in the preliminary experiments with IEF-SS⁻ and IEF-SS⁺ cells.

As shown in Figure 2, the IEF-SS⁻/pZeo cells (IC₅₀ 3.6 µM) were two-fold more sensitive to killing by BCNU than the IEF-SS⁻/pSS-1 (IC₅₀ 7.6 µM), confirming, with this more rigorous test, that spermine levels do protect against the toxicity of the chloroethylating agent. Similarly, the increased sensitivity of spermine-synthase deficient cells to CHENSp_m was also confirmed using the IEF-SS⁻/pZeo and IEF-SS⁻/pSS-1 cells which had IC₅₀ values of 3.2 µM and 9.4 µM respectively (Figure 3A). The contrasting effect of resistance to the polyamine analogue BE-3-3-3, produced by the absence of spermine, was also confirmed. The IC₅₀ value for IEF-SS⁻/pSS-1 was 1.6 µM, whereas that for IEF-SS⁻/pZeo cells was 3.7 µM (Figure 3B). Polyamine analogue concentrations in the two cell lines were not significantly different at any time points, indicating that the

Figure 3 Sensitivity of IEF-SS⁻/pSS-1 and IEF-SS⁻/pZeo cells to CHENSpm and BE-3-3-3

Immortalized embryonic fibroblasts were treated with CHENSpm (A) or BE-3-3-3 (B) at the levels shown, and cell viability was measured using the MTT assay method described in the Materials and methods section. Results are shown as means with bars indicating S.E.M. Cells having spermine synthase activity and, therefore, containing spermine are shown as open symbols; cells lacking spermine are shown as filled symbols.

introduced SS activity did not alter either transport or accumulation of the analogues (Table 3).

Many polyamine analogues, including BE-3-3-3, are known to induce SSAT activity, and this induction has been correlated with sensitivity to BE-3-3-3 [14,33,42]. SSAT activity was induced by BE-3-3-3, and the increase was higher in IEF-SS⁻/pSS-1 than in IEF-SS⁻/pZeo cells (Table 4). This is consistent with a role for SSAT induction in the toxicity of BE-3-3-3. CHENSpm is known to be an ineffective inducer of SSAT [43,44]. Both the low basal SSAT levels and SSAT levels in the presence of CHENSpm were not significantly different in IEF-SS⁻/pSS-1 and IEF-SS⁻/pZeo cells (Table 4). The results of analysis of the cellular polyamines were consistent with these results. Exposure to BE-3-3-3 depleted spermine in IEF-SS⁻/pSS-1 cells and decreased spermidine in both cell lines. CHENSpm did not affect significantly the polyamine content in either cell line (Table 4).

Possible role of the immortalization procedure

The response to drugs in the cell lines used in the experiments described above may be affected by the use of the SV40 genes for immortalization. Therefore, the sensitivity to polyamine

Table 3 Accumulation of BE-3-3-3 and CHENSpm in transfected IEF-SS⁻ cells

The content of the polyamine analogues was measured as described in Materials and methods. Values are given as means $\pm$ S.E.M. for three or four estimations.

Treatment	Time	Analyte (nmol/mg protein)	
		IEF-SS ⁻ /pSS-1	IEF-SS ⁻ /pZeo
5 μ M BE-3-3-3	15 min	2.4 $\pm$ 0.4	2.3 $\pm$ 0.3
5 μ M BE-3-3-3	30 min	3.4 $\pm$ 0.5	3.1 $\pm$ 0.4
5 μ M BE-3-3-3	24 h	26.6 $\pm$ 2.7	21.5 $\pm$ 2.9
10 μ M CHENSpm	15 min	0.9 $\pm$ 0.3	0.8 $\pm$ 0.3
10 μ M CHENSpm	30 min	1.2 $\pm$ 0.4	1.1 $\pm$ 0.4
10 μ M CHENSpm	48 h	1.9 $\pm$ 0.5	1.7 $\pm$ 0.4

Figure 4 Sensitivity of EF-SS⁺ and EF-SS⁻ cells to CHENSpm and BE-3-3-3

Primary fibroblasts were treated with CHENSpm (A) or BE-3-3-3 (B), and cell viability was measured using the MTT assay method described in the Materials and methods section. Results are shown as means with bars indicating S.E.M. Cells having spermine synthase activity and, therefore, containing spermine are shown as open symbols; cells lacking spermine are shown as filled symbols.

analogues was also examined in primary fibroblast cultures derived from normal (EF-SS⁺ cells) and Gy mice (EF-SS⁻ cells) (Figure 4). Much larger amounts of BE-3-3-3 were needed to kill these cells, as they have a much slower growth rate. This observation is consistent with the recent report that growth status dramatically affects the response of human lung cancer cells to

Table 4 Effect of BE-3-3-3 and CHENSpm on polyamine content and SSAT activity in transfected IEF-SS⁻ cells

Polyamine levels were measured in cells treated with BE-3-3-3 or CHENSpm for 48 h as described in the Materials and methods section. Values are given as means $\pm$ S.E.M. for three or four estimations. The putrescine values were in all cases < 0.1 nmol/mg of protein. * $P < 0.001$ significantly different from untreated; † $P < 0.01$ significantly different from IEF-SS⁻/pSS-1.

Cells	Treatment	Polyamines (nmol/mg of protein)		SSAT (pmol/min per mg of protein)
		Spermidine	Spermine	
IEF-SS ⁻ /pSS-1	None	21.1 $\pm$ 2.1	2.6 $\pm$ 0.3	9.4 $\pm$ 3.5
IEF-SS ⁻ /pSS-1	5 μ M BE-3-3-3, 48 h	5.7 $\pm$ 0.3*	< 0.2 †	2026 $\pm$ 71*
IEF-SS ⁻ /pSS-1	10 μ M CHENSpm, 48 h	24.0 $\pm$ 3.6	3.9 $\pm$ 0.5	14.5 $\pm$ 3.5
IEF-SS ⁻ /pZeo	None	27.9 $\pm$ 2.6	< 0.2	19.2 $\pm$ 2.9
IEF-SS ⁻ /pZeo	5 μ M BE-3-3-3	8.3 $\pm$ 0.2*†	< 0.2	852 $\pm$ 36*†
IEF-SS ⁻ /pZeo	10 μ M CHENSpm, 48 h	25.7 $\pm$ 2.0	< 0.2	19.5 $\pm$ 5.5

polyamine analogues [43]. However, the response of primary fibroblasts to BE-3-3-3 (Figure 4B) was consistent with the results obtained using the immortalized cell line (Figure 3B), showing that lack of SS activity imparts resistance to this drug. The EF-SS⁻ cells with undetectable SS activity had an IC₅₀ of 114 μ M in response to BE-3-3-3. This is approximately three times greater than the IC₅₀ of 34.1 μ M for the EF-SS⁺ cells with SS activity (Figure 4B). Exposure to 25 μ M BE-3-3-3 for 48 h induced SSAT activity to a greater extent in EF-SS⁺ cells (from 13.5 $\pm$ 6.5 to 1767 $\pm$ 61 pmol/min per mg) compared with EF-SS⁻ cells (from 10.0 $\pm$ 5.0 to 1213 $\pm$ 34 pmol/min per mg).

However, the toxic response to CHENSpm was not the same when primary cultures were examined (Figure 4A). In this case, the EF-SS⁻ cells lacking spermine (IC₅₀ 5.3 μ M) were more resistant than primary EF-SS⁺ cells (IC₅₀ 2.1 μ M). This is the opposite result from that obtained using immortalized cell lines (Figure 3A).

DISCUSSION

Our results, using a comparison between two cell lines that differ only in SS activity, confirm and extend previous less-rigorous studies which suggest that spermine content may influence response to BCNU [24]. Similar results (not shown) were obtained with two similar drugs, ACNU [1-(4-amino-2-methylpyrimidin-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea] and CCNU [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea]. This finding may be exploited to enhance the activity of these agents, since several inhibitors of spermine synthase, such as substituted 1,3-diaminopropanes, are available and have been shown to be well tolerated when administered to experimental animals [18,19,45]. The effectiveness of spermine in modulating the toxicity of these chloroethylating agents may be due to its ability to reduce the degree to which this agent forms interstrand cross-links in the DNA, or to its ability to influence response to this form of DNA damage [46]. The presence of unrepaired interstrand cross-links during cell replication causes apoptosis, particularly in the absence of cell-cycle checkpoint controls [47,48]. Spermine depletion did not generally increase cellular responses to DNA damaging agents. There was no significant effect on response to cisplatin, which forms mainly intrastrand cross-links, or on response to H₂O₂, which causes single-strand breaks and oxidative DNA-base damage. Although BCNU-mediated cross-links induce apoptosis [47,48], as do polyamine analogues [34,44,49,50] it is unlikely that spermine depletion has any general effect on the induction of apoptosis. Polyamines have been implicated in caspase activation [51], but the etoposide-mediated

expression of Bcl-2, and caspase-3 and caspase-9 occurred in IEF-SS⁻ cells to the same extent as in IEF-SS⁺ cells [30].

Our finding of elevated AdoMetDC in cells lacking spermine (Table 2) is consistent with other studies showing that spermine is more active than spermidine in repression of AdoMetDC activity (reviewed [37]). Alteration in AdoMetDC did not correspond with a change in cellular sensitivity to MGBG, which is an inhibitor of AdoMetDC *in vitro* [52]. This supports much other data, showing that the toxicity of MGBG is not related to reduction in polyamine synthesis [13,53]. Our results are not in agreement with those of Korhonen et al. [26], who reported that embryonic stem cells with a disruption of the SS gene were more sensitive to MGBG. Their conclusion was based only on a single growth curve over a period of 72 h in the presence of 10 μ M MGBG, whereas our studies were carried out with a more detailed investigation using the MTT-based cytotoxicity assays more commonly used for testing response to anti-tumour agents. Our results are also the opposite of Korhonen et al. [26] with respect to the greater resistance of our SS-deficient cells to BE-3-3-3 (Table 1, Figures 4 and 5). We have no explanation for this difference, except for the different response assays, since they also carried out their studies on BE-3-3-3 by comparing growth curves, with only a single concentration of BE-3-3-3 (50 μ M) being tested.

Exposure to BE-3-3-3 caused a profound induction of SSAT, and this induction was greater in the cells expressing SS (Table 4). The increase was not due to a difference in uptake of BE-3-3-3 (Table 3), but it is possible that the presence of spermine alters the subcellular distribution of the polyamine analogue, and it may potentiate its ability to induce SSAT in this way. Studies with CHO cells selected for resistance to BE-3-3-3 [42] and with ES cells from SSAT knockout mice [54] have indicated that the induction of SSAT is an important mediator of response to this drug. This may be due to the need for SSAT-mediated reduction of cellular polyamines to allow BE-3-3-3 to interact with critical cellular targets. There was slightly more residual spermidine in the IEF-SS⁻/pZeo cells compared with the IEF-SS⁻/pSS-1 cells (Table 4). Increased oxidative damage resulting from polyamine oxidase acting upon the N¹-acetyl polyamines generated from the SSAT reaction may also be a relevant factor.

It is not possible to generalize the findings with BE-3-3-3 to apply to other terminally alkylated polyamine analogues. Several analogues such as BE-4-4-4-4, CBENSpm and CPENSpm showed little difference in response in cells lacking SS (Table 1), and the absence of spermine clearly increased sensitivity to CHENSpm in IEF cells. This result adds to an increasing and convincing body of evidence suggesting that there are different cellular targets for the different polyamine analogues [31,33–35, 42–44,55]. CHO cells selected for resistance to BE-3-3-3 are

not cross-resistant to CHENSpM [42]. CHENSpM and BE-3-3-3 differ dramatically in their abilities to induce SSAT [33,43,44]. CHENSpM has been shown to cause tubulin polymerization, and this has been implicated in its ability to induce apoptosis in a manner not dependent on SSAT [33,56]. It is possible that spermine could compete with CHENSpM for the interaction with tubulin and that this would account for its ability to protect against this drug. However, the increased sensitivity to CHENSpM seen in immortalized cells lacking spermine was not seen when primary fibroblast cultures were used. The explanation for this difference may relate to the much slower growth rate of these cells or to the presence of active p53, which would be inactivated in the immortalized cells, since these were transformed with SV40 genes. The possibility that CHENSpM acts in a p53-dependent manner should be investigated, since it would have important connotations for chemotherapy with this agent.

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