Effect of spermine synthase deficiency on polyamine biosynthesis and content in mice and embryonic fibroblasts, and the sensitivity of fibroblasts to 1,3-bis-(2-chloroethyl)-*N*-nitrosourea

Caroline A. MACKINTOSH[1] and Anthony E. PEGG

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.

Mutant Gy male mice, which have previously been described as having disruption of the phosphate-regulating *Phex* gene and a spermine synthase gene [Meyer, Henley, Meyer, Morgan, McDonald, Mills and Price (1998) Genomics, **48**, 289–295; Lorenz, Francis, Gempel, Böddrich, Josten, Schmahl and Schmidt (1998) Hum. Mol. Genet. **7**, 541–547], as well as mutant Hyp male mice, which have disruption of the *Phex* gene only, were examined along with their respective normal male littermates. Biochemical analyses of extracts of brains, hearts and livers of 5-week-old mice showed that Gy males lacked any significant spermine synthase activity as well as spermine content. Organs of Gy males had a higher spermidine content. This was caused not only by the lack of conversion of spermidine into spermine, but also because of compensatory increases in the activities of other polyamine biosynthetic enzymes. Gy males were half the body weight of their normal male littermates at weaning age. Hyp males, however, were no different in size when compared with their controls. High mortality of Gy males occurs by weaning age and this mortality was shown to be largely post-natal. Embryonic fibroblasts were isolated from Gy males and their normal male littermates and were similarly shown to lack any significant spermine synthase activity as well as spermine content. The lack of spermine, however, had no significant effect on the growth of immortalized fibroblasts or of primary fibroblast cultures. Similarly, there was no difference in the time of senescence of primary fibroblast cultures from Gy males compared with cultures derived from normal male littermates. However, the lack of spermine did increase the sensitivity of immortalized fibroblasts to killing by the chloroethylating agent 1,3-bis(2-chloroethyl)-*N*-nitrosourea. Therefore both the Gy male mice and derived embryonic fibroblasts provide valuable models to study the importance of spermine and spermine synthase, without the use of inhibitors which may have additional side effects.

Key words: 1,3-bis-(2-chloroethyl)-*N*-nitrosourea (BCNU), Gy, gyro, putrescine, spermidine.

INTRODUCTION

Polyamines are ubiquitous cellular components and have long been known to be essential for cell growth. Many studies using inhibitors of the various polyamine biosynthetic enzymes have indicated that the ability to synthesize polyamines is essential for cell growth [1–3]. If synthesis is prevented through the use of such inhibitors, or by mutants causing a loss of enzymic activity, an active-transport system allowing the cell to take up polyamines from its environment becomes critical for normal growth [4,5]. In mammalian cells, the major polyamines are spermidine and spermine, with smaller amounts of their precursor putrescine. Spermine is formed by the action of an aminopropyltransferase called spermine synthase, which adds an aminopropyl group, donated by decarboxylated *S*-adenosylmethionine (dcAdoMet), to a spermidine acceptor. This enzyme is distinct from the related spermidine synthase, which uses putrescine as an acceptor for the aminopropyltransferase reaction forming spermidine (reviewed in [2]). Many micro-organisms do not contain a spermine synthase, and the predominant polyamines in such species are putrescine and spermidine [6]. However, spermine synthases have been isolated and cloned from multiple sources [7–9]. There are no definitive experiments that indicate a specific physiological role for spermine. Many studies in which potentially biologically relevant effects of polyamines have been reported have found that either spermidine or spermine will suffice, although the dose required may be different (reviewed in [3]). Yeast mutants that lack spermine synthase are viable and do not require exogenous spermine for growth [8]. Treatment of rodents with inhibitors of spermine synthase did not produce any striking effects [10], and studies in which mammalian cells in culture have been exposed to inhibitors of spermine synthase have given variable results [11–15]. Serious problems in the interpretation of these experiments are caused by: (a) the possible lack of specificity of the inhibitors; (b) the possibility that the inhibitors might substitute for spermine; and (c) the difficulty in obtaining a major reduction in spermine levels in both cultured cells and in tissues of rodents treated with the maximal tolerated dose of the inhibitors.

Another approach to examine the function of spermine is therefore clearly required. An opportunity to develop such an approach is provided by recent studies that indicate that the only known human spermine synthase gene is located on the X chromosome at a location close to that of a gene termed *Phex* whose inactivation causes hypophosphataemia [16]. Two mouse mutations, hypophosphatemia (Hyp) and gyro (Gy), have been described extensively as models for human X-linked hypophosphatemia [17,18]. Both mutations involve partial deletion of the phosphate-regulating *Phex* gene: the Hyp-associated deletion occurring at the 3′ end of the gene, whereas the Gy-associated

Abbreviations used: ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; CDAP, *N*-cyclohexyl-1,3-diaminopropane; dcAdoMet, decarboxylated *S*-adenosylmethionine; AdoDato, *S*-adenosyl-1,8-diamino-3-thio-octane; BCNU, 1,3-bis-(2-chloroethyl)-*N*-nitrosourea; DMEM, Dulbecco's modified Eagle's medium.

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

deletion occurs at the 5′ end [19]. The mutations are X-linked and dominant with affected males and females showing symptoms of hypophosphataemia including renal phosphate wasting, impaired mineralization and growth retardation [17,18]. Analysis of the Gy-associated *Phex* deletion found the deletion to extend upstream of the 5′-untranslated region of *Phex* [19] and to include disruption of the spermine synthase gene [20,21]. Spermine deficiency has been reported in various organs of Gy males [20,21], but to date no studies on the activity of spermine synthase in tissues from these mice have been reported.

Although both Gy males and Hyp males have hypophosphataemia, the former have additional symptoms, including sterility, smaller size, inner-ear abnormalities, deafness, hyperactivity and circling behaviour [18,22–24]. The extent to which these features may be related to the deletion of part of the spermine synthase gene on the X chromosome remains to be determined, but the Gy-mutant mouse may provide an excellent model to investigate the function of spermine.

In the present studies, we have confirmed by direct assay that extracts from tissues of Gy mice do lack virtually all spermine synthase activity and that the tissues contain very little spermine. However, there is a compensatory increase in total polyamine content due to increased activities of other enzymes in the polyamine biosynthetic pathway. Primary cultures and immortalized embryonic fibroblast cell lines were derived from Gy males and also found to lack spermine synthase and spermine without any significant effect on the growth of these cells. However, the sensitivity of fibroblasts to killing by the chloroethylating agent 1,3-bis-(2-chloroethyl)-*N*-nitrosourea (BCNU) was increased by the lack of spermine synthase and spermine.

MATERIALS AND METHODS

Materials

All chemicals unless otherwise stated were purchased from Sigma.

Production of mutant Gy and Hyp mice

Heterozygous Gy and Hyp females, as well as B6C3H and C57BL/6J males, were kindly provided by Dr R. A. Meyer, Jr, Department of Orthopaedic Surgery, Carolinas Medical Center, Charlotte, NC, U.S.A. Further animals were purchased from The Jackson Laboratory (Bar Harbor, ME, U.S.A.). Gy females were bred with B6C3H males, and Hyp females were bred with C57BL/6J males. Mice were weaned at 3 weeks of age and genomic DNA was isolated from the tails of male mice (DNeasy tissue kit; Qiagen, Inc., Valencia, CA, U.S.A.). Female offspring were retained and the heterozygous females required for breeding distinguished from homozygous females by phosphate analyses of blood plasma [25]. Two different PCR analyses were used to identify the mutant male mice of each species. For Gy mice, the primers used amplified intron 3 of the spermine synthase gene [21] and exon 3 of the *Phex* gene [19], whereas for the Hyp mice, the primers used amplified exons 19 and 21 of the *Phex* gene [19]. In each PCR, the absence of an amplified product indicated the mouse to be a mutant. However, in one of the reactions for the Gy mouse strain, the primers also amplified a pseudogene for spermine synthase [21], which enabled us to confirm that the PCR had been successful and that the absence of an amplified product indicated a mutant mouse. Primers were synthesized in the Macromolecular Core Facility, Pennsylvania State University College of Medicine, Hershey, PA, U.S.A. PCR reagents including *Taq* DNA polymerase and an Ultrapure dNTP Set were purchased from Promega (Madison, WI, U.S.A.) and Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.) respectively.

Characterization of mutant Gy and Hyp mice

Mutant mice and their male littermates were weighed at weaning (3 weeks old). At 5 weeks of age, the mice were killed using an inhalation anaesthetic, methoxyflurane ('Metofane'; Schering-Plough Animal Health, Union, NJ, U.S.A.), followed by cervical dislocation. Three organs were identified as prudent for examination of spermine synthase activity and spermine content: the brain, heart and liver. Those organs taken from adult rats had previously been shown to have high spermine synthase activity [26]. Organs were removed from experimental animals immediately and divided in half, snap-frozen in liquid nitrogen, and stored at −80 °C until required for analysis.

Polyamine content was determined using samples corresponding to half of the organs. Polyamines were acid extracted using 10% trichloroacetic acid (Fisher Scientific, Fair Lawn, NJ, U.S.A.), and aliquots analysed using an ion-pair reversed-phase HPLC separation method with post-column derivatization using *o*-phthalaldehyde, as described previously [15].

The remaining half of each organ was divided in two. Half was placed in 1 ml of ice-cold ornithine decarboxylase (ODC) assay buffer [25 mM Tris/HCl, pH 7.5, 2.5 mM dithiothreitol, 0.1 mM EDTA, 1 × protease inhibitors (Protease Inhibitor Cocktail Set I; Calbiochem–Novabiochem, La Jolla, CA, U.S.A.), 0.01% Tween 80 (Fisher Scientific)], and homogenized on ice using a Polytron for 15 s on/15 s off/15 s on. Centrifugation was carried out at 4 °C for 30 min at 20000 ***g***, and the cytosolic fraction used to assay ODC and *S*-adenosylmethionine decarboxylase (AdoMetDC) activities. The remainder of each organ was placed in ice-cold spermidine/spermine synthase assay buffer (50 mM sodium phosphate, pH 7.2, 0.3 mM EDTA, 10 mM 2-mercaptoethanol), and homogenized as described above. Centrifugation was carried out at 4 °C for 20 min at 12000 ***g***, and the cytosolic fraction used to assay spermidine and spermine synthase activities. Protein measurements were carried out using Bio-Rad reagent with BSA as a standard [27].

All enzyme assays were carried out as reported previously. Briefly, for ODC activity, ODC assay mix (50 μl) [12.5 μl of 1 M Tris/HCl, pH 7.5, 5 μl of 2 mM pyridoxal 5′-phosphate, 2.5 μl of 250 mM dithiothreitol, 1 μl of L-[1-^{14}C]ornithine (specific radioactivity 47.70 mCi/mmol; NEN Life Science Products, Boston, MA, U.S.A.) and 29 μl of deionized water] was added to organ extract (200 μl), and the ODC activity determined by measuring the release of $^{14}CO_2$ over a 1 h incubation period at 37 °C [28]. For AdoMetDC activity, AdoMetDC assay mix (150 μl) [25 μl of 0.5 M sodium phosphate, pH 6.8, 12.5 μl of 25 mM dithiothreitol, 15 μl of 50 mM putrescine, 12.5 μl of 2.7 mM *S*-adenosylmethionine, 5 μl of *S*-adenosyl-L-[*carboxy*-^{14}C]methionine (specific radioactivity 50 mCi/mmol, NEN Life Science Products) and 80 μl of deionized water] was added to organ extract (100 μl), and the AdoMetDC activity determined by measuring the release of $^{14}CO_2$ over a 1 h incubation period at 37 °C [29].

Spermidine and spermine synthase assays were carried out using the method reported previously [30]. For spermidine synthase assays, 10 μl of organ extract was added to 50 μl of assay mix (40 μl of 0.5 M sodium phosphate, pH 7.5, [^{35}S]dcAdoMet and deionized water to total a volume of 10 μl; 40000 c.p.m. of radioactivity per 50 μl of assay mix was used). A 10 μl aliquot of 10 mM putrescine was included in the reaction, which took place in a total volume of 200 μl, the volume made up with deionized water. To prevent the measurement of enzymic activity resulting from the presence of endogenous spermidine, the reaction took place in the presence of 20 μM *N*-cyclohexyl-1,3-diaminopropane (CDAP), a potent inhibitor of spermine

synthase [31]. As a method of measuring spermidine synthase activity, 20 μM *S*-adenosyl-1,8-diamino-3-thio-octane (AdoDato), a transition-state-analogue inactivator of spermidine synthase [32], was added to an additional two assay reactions. The enzymic activity remaining after treatment with CDAP and then inhibitable by AdoDato was taken to be the activity of spermidine synthase. Reactions were carried out at 37 °C for 30 min.

Spermine synthase assays were carried out similarly. Organ extract (120 μl) was used and reactions occurred in the presence of 10 μl of 10 mM spermidine. To prevent the measurement of enzymic activity resulting from the presence of endogenous putrescine, 20 μM AdoDato was added, and as a method of measuring spermine synthase, 20 μM CDAP was used in an additional two assay reactions. The CDAP inhibitable enzymic activity remaining after treatment with AdoDato was taken to be the activity of spermine synthase. Reactions were carried out at 37 °C for 1 h.

Isolation of mouse embryonic fibroblasts from Gy and normal male littermate embryos

Mouse embryonic fibroblasts were derived from 14-day-old embryos. Embryos were rinsed in PBS supplemented with 100 units/ml penicillin and 100 units/ml streptomycin. After removal of internal organs, the embryos were minced using scalpels and digested with 10 ml of trypsin for 10 min at 37 °C with agitation. Trypsin was made up as follows: NaCl (137 mM), KCl (5.4 mM), $NaHCO_3$ (4.2 mM), dextrose (5.6 mM) and EDTA (free acid form, 0.7 mM) were dissolved in 500 ml of deionized water, and the solution was allowed to stir overnight before adjusting the pH to 7.0. Solution (420 ml) and 0.25 % trypsin (80 ml) (Gibco BRL Life Technologies, Grand Isalnd, NY, U.S.A.) were filter sterilized together and used thereafter as the trypsin solution. Following agitation, samples were pipetted vigorously, the tissue allowed to settle, and approx. 7 ml of trypsin/cell suspension was removed. Trypsin was inactivated by adding the trypsin/cell suspension to 20 ml of DMEM [500 ml of 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, 100 units/ml streptomycin and 0.1 mM 2-mercaptoethanol]. Trypsin (approx. 7 ml) was added to the remaining undigested tissue and the resulting suspension incubated for 10 min at 37 °C with agitation. The trypsin/cell suspension was pipetted vigorously, the tissue allowed to settle, and as much of the trypsin/cell suspension as possible transferred to the DMEM already containing trypsin/cell suspension. The DMEM/trypsin/cell suspension mixture was then centrifuged using a clinical centrifuge for 10 min at 250 ***g***. The supernatant was removed and the cell pellet resuspended in fresh DMEM (10 ml) before plating on to a 100-mm tissue culture dish, and culturing in a humidified incubator at 37 °C, in air/CO_2 (19:1).

The following day, the cells were confluent and one 100-mm dish was plated using a 1:20 dilution and the remaining cells frozen. Once the plated cells were confluent (approx. 3–4 days later), they were harvested and DNA extracted (DNeasy tissue kit; Qiagen). Cells were genotyped using the same primers as were used for identification of the mice. In addition, cells were sexed by PCR using a sense primer 5′-CCG CTG CCA AAT TCT TTG G-3′ and an antisense primer 5′-TGA AGC TTT TGG CTT TGA G-3′, which gave two products for a male embryo and a single product for cells derived from a female embryo [33]. Thus cells derived from mutant embryos and their normal male littermates were identified.

Cells from Gy embryos and normal male littermate embryos were thawed and cultured in a humidified incubator [(37 °C, air/CO_2 (19:1)]. At passage 3, the cells were immortalized by transfection using LIPOFECTAMINE™ plus (Gibco BRL Life Technologies), with a plasmid expressing SV40 large and small T antigens [34]. Transfected cells were selected by their resistance to 500 μg/ml G418 (Gibco BRL Life Technologies), and surviving cells isolated and expanded. Surviving cells were not necessarily clonal, and those cells isolated and expanded are referred to hereafter as individual 'populations'.

Cell growth studies were carried out by plating 2000 cells per well of a 24-well cell culture plate and counting the cells daily in triplicate. For ODC and AdoMetDC enzyme activity measurements, cells were plated in triplicate using 2.5×10^5 cells per 60-mm tissue culture dish. Cells were harvested 48 h later using ODC assay buffer (200 μl), and cell extracts prepared by snap-freezing and thawing three times. Centrifugation was carried out at 4 °C for 20 min at 20000 ***g***, and ODC and AdoMetDC assays were carried out as described above using 150 μl and 100 μl of cytosolic cell extract respectively. Assays were carried out at 37 °C for 30 min. Similarly, for polyamine content analyses, cells were plated in triplicate using 2.5×10^5 cells per 60-mm dish and cultured for 48 h. Cells were harvested using 10 % trichloroacetic acid (200 μl) and polyamine analyses carried out as described above. More cells were required to assay spermidine and spermine synthase. Cells were plated on to eight 100-mm dishes using 5×10^5 cells per dish and cultured for 48 h. Cells were harvested using spermidine/spermine synthase assay buffer (200 μl) and subjected to a snap-freeze–thaw procedure three times. Cell extracts were then centrifuged at 4 °C for 20 min at 12000 ***g*** before pooling cytosolic extracts together. Assays were carried out in duplicate as described above using 10 μl of cell extract for spermidine synthase activity measurements and 120 μl for spermine synthase assays. Spermidine synthase assays were carried out for 30 min, whereas spermine synthase assays were carried out for 1 h (both at 37 °C).

For primary cultures, cells from Gy embryos and normal male littermates embryos were thawed and cultured in a humidified incubator at 37 °C and air/CO_2 (10.1:1). Cells were grown until they were confluent, and the next day split 1:3 on to their next passage. Thereafter, cells were cultured following the same procedure until they senesced. Growth curves were studied at passages 3, 6 and 9, where 20000 cells were plated per 60-mm dish and cells counted daily in duplicate.

Effect of spermine on killing of fibroblasts by the chloroethylating agent BCNU

Immortalized cells (2.5×10^5) were plated on to 60-mm dishes and cultured for 24 h in a humidified incubator at 37 °C and air/CO_2 (19:1). Cells were then treated with 100 μM O^6-benzylguanine for 2 h. Various concentrations of BCNU (0, 5, 10, 15, 20, 30 and 40 μM) were then added and the cells incubated for a further 2 h before the medium was replaced with fresh medium, including 100 μM O^6-benzylguanine. After 16–18 h, cells were re-plated into 25 cm^2 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 solution, stained with 0.5 % Crystal Violet in ethanol, and colonies counted.

Statistical analysis

Statistical analysis of all results was carried out using two-tailed Student's *t* tests.

RESULTS

Polyamine biosynthetic enzymes in Gy mice

The activities of the four enzymes involved in polyamine biosynthesis were assayed in tissue extracts from 5-week-old Gy and Hyp mice and normal male littermates (Table 1). Spermine synthase activity was calculated by measuring the formation of radiolabelled 5′-methylthioadenosine from radiolabelled dcAdoMet in the presence of spermidine as an aminopropyl acceptor [30]. In order to ensure that only spermine synthase activity was measured, additional assays were conducted in the presence of 20 μM CDAP to inhibit spermine synthase activity. Spermine synthase activity was measured in extracts from Gy normal male littermates, Hyp mutants and Hyp normal male littermates (results not shown), but was completely inactivated by the presence of CDAP (Table 1). Spermine synthase activity was virtually undetectable in all organs analysed from Gy mutant mice (Table 1). This is the first direct measurement of spermine synthase activity in Gy mice and confirms the reports of extensive disruption of the spermine synthase gene [20,21]. It also provides strong evidence that there is only one spermine synthase gene in mice. There was no difference in the spermine synthase activities

Table 1 Activities of enzymes involved in polyamine biosynthesis in extracts from organs taken from 5-week-old Gy, Hyp and normal male littermate mice

Values are given as means ± S.E.M. Numbers of mice are shown in parentheses. Significant differences, comparing Gy mutants with normal littermates, or Hyp mutants with normal littermates, are shown as: *$0.01 < P \leqslant 0.05$; **$0.001 < P \leqslant 0.01$; ***$P \leqslant 0.001$; ns, no significant difference.

		[^{35}S]5′-Methylthioadenosine formed (c.p.m.)/30 min per μg of protein			
Enzyme	Mice…	Gy mutant (4)	Gy normal (6)	Hyp mutant (6)	Hyp normal (4)
Spermine synthase					
Brain		0.5 ± 0.2*	56 ± 15	63 ± 11^{ns}	54 ± 10
Liver		0.2 ± 0.2***	2.6 ± 0.1	2.8 ± 0.4^{ns}	2.9 ± 0.2
Heart		0.5 ± 0.4**	13 ± 2	10 ± 2^{ns}	10 ± 1
Spermidine synthase					
Brain		714 ± 181^{ns}	1122 ± 222	1087 ± 94^{ns}	1313 ± 393
Liver		209 ± 81^{ns}	241 ± 35	297 ± 45^{ns}	254 ± 33
Heart		1304 ± 316*	670 ± 58	790 ± 84*	423 ± 83
		pmol of CO_2 released/30 min per mg of protein			
Enzyme	Mice…	Gy mutant (4)	Gy normal (6)	Hyp mutant (6)	Hyp normal (4)
ODC					
Brain		13.0 ± 3.8**	1.7 ± 0.7	1.8 ± 0.6^{ns}	2.3 ± 0.6
Liver		2.9 ± 0.2*	2.2 ± 0.1	2.5 ± 0.1^{ns}	3.2 ± 0.5
Heart		16.6 ± 2.2**	4.8 ± 0.7	4.1 ± 0.7^{ns}	4.0 ± 1.4
AdoMetDC					
Brain		700 ± 59***	218 ± 10	178 ± 19^{ns}	200 ± 2
Liver		338 ± 20*	212 ± 36	167 ± 24*	265 ± 31
Heart		1033 ± 169***	54 ± 6	58 ± 4^{ns}	57 ± 11

Table 2 Polyamine analyses of organs taken from 5-week-old Gy, Hyp and normal male littermate mice

Numbers of mice are shown in parentheses. Significant differences, comparing Gy mutants with normal littermates, or Hyp mutants with normal littermates, are shown as: ** $0.001 < P \leqslant 0.01$; ***$P \leqslant 0.001$; ns, no significant difference.

		Polyamine levels (nmol/g of tissue) ± S.E.M.			
Polyamine	Mice…	Gy mutant (4)	Gy normal (7)	Hyp mutant (10)	Hyp normal (4)
Putrescine					
Brain		49 ± 9.4***	4 ± 0.5	5 ± 0.8^{ns}	4 ± 0.3
Liver		3 ± 1.4^{ns}	4 ± 0.3	9 ± 2.6^{ns}	3 ± 0.2
Heart		2 ± 0.4**	5 ± 0.5	10 ± 2.6^{ns}	4 ± 0.3
Spermidine					
Brain		424 ± 57.0***	176 ± 12.4	172 ± 9.4^{ns}	179 ± 27.3
Liver		1977 ± 172.5***	585 ± 46.5	655 ± 43.8^{ns}	707 ± 52.6
Heart		1032 ± 82.1***	192 ± 23.6	166 ± 13.4^{ns}	129 ± 8.7
Spermine					
Brain		< 3***	172 ± 5.7	170 ± 8.8^{ns}	163 ± 14.8
Liver		< 3***	621 ± 35.8	492 ± 65.1^{ns}	553 ± 36.9
Heart		< 5***	157 ± 7.7	235 ± 20.1^{ns}	217 ± 18.3

Table 3 Mean weight ± S.E.M. of 3-week-old Gy, Hyp and normal male littermate mice

Numbers of mice are shown in parentheses. Significant differences, comparing Gy mutants with normal littermates, or Hyp mutants with normal littermates, are shown as: $^{***}P \leqslant 0.001$; ns, no significant difference.

Mice	Weight (g)
Gy males (12)	$6.7 \pm 0.2^{***}$
Normal littermates (20)	11.7 ± 0.4
Hyp males (5)	10.0 ± 0.7^{ns}
Normal littermates (8)	9.8 ± 0.6

of the mutant and normal Hyp mice. Also, despite the difference in genetic backgrounds between the Gy (B6C3H) and Hyp (C57BL/6J) mice, the spermine synthase activities in the control littermates were not different.

Spermidine synthase activity was measured using a similar assay, in which the formation of 5′-methylthioadenosine from dcAdoMet in the presence of putrescine was determined [30]. This activity (results not shown) was totally inactivated by 20 μM AdoDato, and was largely unaffected when comparing all four groups of mice (Table 1). However, the spermidine synthase activity in the hearts of affected Gy males was increased by 1.9-fold over the Gy normal males (Table 1). Little difference was observed in the activities of spermidine synthase in the Hyp males and their normal control littermates; however, enzymic activity in the heart was significantly higher in the Hyp mutants compared with the normal males (Table 1). Therefore it cannot be ruled out that the small elevation in cardiac spermidine synthase in Gy and Hyp mutant mice is related to the changes in *Phex* expression. There was also a small apparent decrease in spermidine synthase activity in the brain of the Gy mice, but this difference was not statistically significant (Table 1).

Both ODC and AdoMetDC activities were increased in brains, livers and hearts from the Gy mice compared with their normal male littermates. The increase in ODC activity was largest in the brain (7.6-fold), whereas in the heart it was increased by 3.5-fold and there was only a slight increase in the liver (Table 1). AdoMetDC activities were increased by 3.2-fold in the brain, with only a slight increase in the liver, and an increase of 18.9-fold in the heart (Table 1). Little difference was apparent in ODC or AdoMetDC activity measurements between Hyp mutants or their normal control littermates, although AdoMetDC activity in the liver was slightly lower in the mutant Hyp mice (Table 1).

Polyamine content in Gy mice

Spermine was undetectable by HPLC in all organs analysed from 5-week-old Gy mutant mice (Table 2). Conversely, spermidine was increased in all organs analysed from Gy mutant mice compared with their normal male littermate mice by 2.4-fold in the brain, 3.4-fold in the liver and 5.4-fold in the heart (Table 2). Little change in putrescine was seen in Gy mutant mice with the exception of the brain, where levels were increased 12-fold (Table 2). The Hyp mutant mice did not differ from the Hyp controls or the Gy normals in their organ content of polyamines.

In all three organs examined, the total polyamine content was increased in Gy males compared with normal male littermates. In the brain, total polyamine content was increased from 352 nmol/g of tissue to 473 nmol/g of tissue, in the liver the increase was from 1210 nmol/g of tissue to 1980 nmol/g of tissue, and in the heart from 354 nmol/g of tissue to 1034 nmol/g of tissue.

Physical characterization of Gy mice

The Gy males used in our experiments were observed to be approximately half the weight of their normal male littermates at 3 weeks of age (Table 3), and this difference was maintained at older ages (results not shown). The body weights of Hyp males, however, were observed to be no different from their normal male littermates at that same age (Table 3). The incidence of viable Hyp mutant males at weaning (3 weeks of age) was 20%, close to the expected ratio of 25% (total of 114 animals in analysis). In contrast, the incidence of viable Gy mutant males was only 11% of the total surviving mice at 3 weeks (total of 194 animals in analysis). The incidence of Gy males found when isolating 14-day-old embryos to prepare embryonic fibroblasts was 32% (22 embryos analysed from three females), suggesting that there is considerable mortality of the Gy males during the last 7 days of pregnancy or during the first 3 weeks of life. A total of 28 embryos from five females were examined 1 day before birth and the incidence of Gy males was established as 39%. It would therefore appear that the mortality of Gy males is largely post-natal.

Table 4 Activities of enzymes involved in polyamine biosynthesis in extracts from immortalized embryonic fibroblast cells from Gy male embryos or normal male littermate embryos

Values are given as means ± S.E.M. Numbers of cell populations are shown in parentheses. Significant differences are shown as: $^{*}P < 0.05$; $^{***}P \leqslant 0.001$; ns, no significant difference.

		[^{35}S]5′-Methylthioadenosine formed (c.p.m.)/30 min per μg of protein	
Enzyme	Embryonic fibroblasts…	Gy cell populations (5)	Normal cell populations (3)
Spermine synthase		$0.5 \pm 0.4^{***}$	15.0 ± 1.2
Spermidine synthase		129 ± 9^{ns}	117 ± 16
		pmol of CO_2 released/30 min per mg of protein	
Enzyme	Embryonic fibroblasts…	Gy cell populations (5)	Normal cell populations (3)
ODC activity		966 ± 221^{ns}	957 ± 69
AdoMetDC activity		$1605 \pm 236^{*}$	765 ± 49

Table 5 Polyamine analyses of immortalized embryonic fibroblast cells from Gy male embryos or normal male littermate embryos, 48 h after plating

Numbers of cell populations are shown in parentheses. Significant differences are shown as: $^{**}0.001 < P \leqslant 0.01$; $^{***}P \leqslant 0.001$; ns, no significant difference.

	Mean (nmol/mg of protein) ± S.E.M.	
Polyamine	Gy cell populations (5)	Normal cell populations (3)
Putrescine	$1.5 \pm 0.18^{**}$	5.2 ± 1.19
Spermidine	74.3 ± 12.43^{ns}	37.4 ± 4.12
Spermine	$< 2.5^{***}$	12.5 ± 0.63

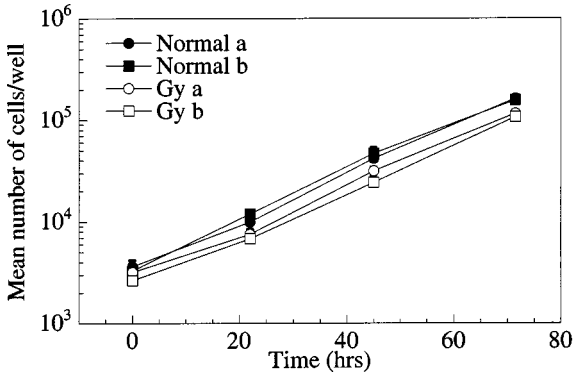

Figure 1 Growth of two immortalized cell populations derived from Gy males ('a' and 'b') and two immortalized cell lines derived from normal male littermates ('a' and 'b')

Each point represents the mean of triplicate measurements ± S.E.M. Cells were plated and counted the following day as the zero time point.

Properties of embryonic fibroblasts derived from Gy and normal male littermates

Immortalized embryonic fibroblast cell lines were established from normal and mutant Gy male 14-day-old embryos by transfection with a plasmid which expresses SV40 large and small T antigens. Spermine synthase activity was virtually undetectable in the cells derived from the Gy mutants (Table 4), whereas spermidine synthase activity was unchanged and ODC activity also unaltered. AdoMetDC activity was increased in the Gy cells compared with cells derived from normal male littermates (Table 4).

Spermine in cell populations derived from Gy males was undetectable by HPLC (Table 5). The spermidine content was increased by approx. 2-fold, whereas putrescine content was decreased (Table 5). Despite these changes, the immortalized cells from Gy mice grew only very slightly more slowly (average doubling time of 12.8 h), compared with those cells from the normal littermate controls (average doubling time of 12.5 h) (Figure 1). The difference in growth rate was not statistically significant, suggesting that the absence of spermine is not a critical factor in the growth of transformed cells.

A similar lack of effect of spermine deficiency on cell growth rate was seen when primary cultures of mouse embryonic fibroblasts were examined (Figure 2) as opposed to transformed cells. However, at all three time points examined (passages 3, 6 and 9) little difference was found in the growth of primary cultures derived from Gy males compared with those derived

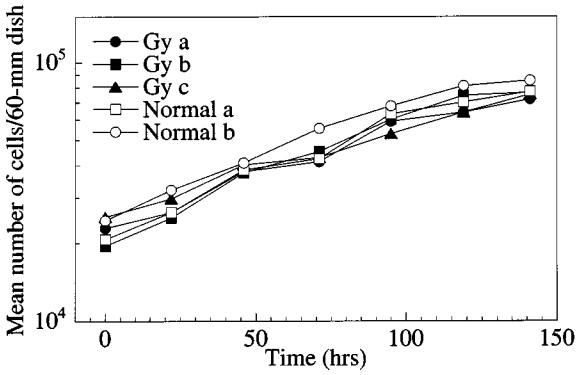

Figure 2 Growth of primary cultures (passage 9) derived from Gy males ('a'–'c') or from normal male littermates ('a' and 'b')

Each point represents the mean of duplicate measurements. Cells were plated and counted the following day as the zero time point.

from normal male littermates (Figure 2). At passage 9, primary cultures derived from Gy males had an average doubling time of 78 h, whereas those cultures derived from normal male littermates had an average doubling time of 70 h. Also, no difference was observed in the senescence of those primary cultures with both the cultures from Gy males and those from normal male littermates senescing at passages 13–14 (results not shown).

Effect of lack of spermine on killing by the chloroethylating agent BCNU

It is well established that the therapeutic chloroethylating agent BCNU acts as a cytotoxic agent by generating an O^6-chloroethylguanine–DNA adduct, which then rearranges to form a highly toxic interstrand cross-link [35]. It has been suggested on the basis of studies with the spermine synthase inhibitor, *N*-butyl-1,3-diaminopropane, that the presence of spermine may reduce the ability of BCNU to interact with cellular DNA [13]. This hypothesis is strongly supported by the results of the experiment shown in Figure 3 in which it was found that the immortalized fibroblasts derived from Gy males, lacking sperm-

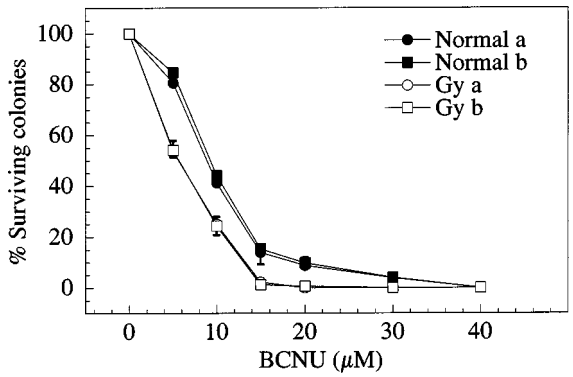

Figure 3 Effect of spermine content on killing of immortalized fibroblasts by the chloroethylating agent BCNU

Each point represents the mean of four measurements ± S.E.M. Results are expressed as the percentage of colonies present when no chloroethylating agent was used. 'a' and 'b' represent different cell populations.

ine, were more sensitive to killing by the chloroethylating agent BCNU than those immortalized fibroblasts derived from normal males containing spermine. The possibility that this response is due to an effect of spermine on the enzymic repair of the initial O^6-chloroethylguanine–DNA adduct, which is brought about by O^6-alkylguanine-DNA alkyltransferase, was ruled out by conducting these experiments in the presence of O^6-benzylguanine, which inactivates the alkyltransferase repair protein [36].

DISCUSSION

Our results confirm that the affected Gy mouse lacks significant spermine synthase activity. The absence of spermine synthase was previously reported on the basis of the loss of part of the X chromosome containing a spermine synthase gene and a decline in tissue spermine content [20,21]. Our results are the first direct measurement of spermine synthase activity *in vitro* in extracts derived from Gy mice and show that there is little detectable enzyme activity, indicating that mammals have only one active spermine synthase gene. Our finding that spermine levels are very low in brains, livers and hearts of 5-week-old Gy mice is also in agreement with the reported findings of barely detectable spermine in the pancreas and livers of 12-day-old Gy mice [20] and in the brains, kidneys and testes of Gy mice over 2 months old [21]. The increase in spermidine in these tissues is also consistent with the findings in older Gy mice [21]; however, an increase in spermidine content was not observed in the pancreas or livers of 12-day-old Gy males [20]. Increased putrescine content of organs was also not seen in younger animals [20].

Our findings are consistent with the concept that the increased putrescine and spermidine present in tissues from Gy mice are not only due to their lack of conversion into spermine but also due to compensatory increases in their biosynthesis. Increases in both ODC and AdoMetDC activities were detected in tissues from Gy mice in our experiments. The latter increase is consistent with studies showing that AdoMetDC is negatively regulated by polyamines and that spermine is more effective than spermidine in this regulation [37,38]. An increased supply of dcAdoMet and putrescine is likely to contribute to the increase in total polyamines seen in the Gy mice, which is predominantly due to the fact that spermidine levels increase by more than spermine is reduced. The increase in ODC activity may also be due to a lack of negative feedback from the higher polyamines, but this seems unlikely since spermidine is greatly increased. It is more likely that the rise in ODC activity is a response to the impaired growth of the Gy mice, and, consistent with this suggestion, the rise in ODC activity was not observed in the immortalized cells derived from Gy male embryos.

It is noteworthy that the spermine levels in the tissues of Gy mice are not maintained by uptake of exogenous polyamines. There is an active system for polyamine uptake, which becomes up-regulated and of particular importance when polyamine biosynthesis is blocked by the use of inhibitors of ODC or AdoMetDC [4,5]. All mice in these studies were fed on the Harlan Teklad S-2335 Mouse Breeder Sterilizable Diet, which contains 432 nmol/g putrescine, 598 nmol/g spermidine and 27 nmol/g spermine. The most probable reason that this spermine is not taken up is, therefore, that the compensatory increase in intracellular spermidine down-regulates the transport system.

The greatly reduced growth rate and early mortality of Gy males compared with normal littermate males may be due to the derangement in polyamine metabolism. However, a significantly reduced growth rate or early senescence was not seen in primary cultures of embryonic fibroblasts derived from Gy mice. Previous studies in which cultured tumour cells have been exposed to inhibitors of spermine synthase have given variable results. In some cases, there was no effect on growth [11,13–15], but in others there was a clear detrimental effect [12,13,15]. Our results support the concept that the latter effects may be due to other non-specific actions of the inhibitors. Additionally, our results show that the lack of effect of spermine synthase inhibitors on cell growth in culture is not due to their ability to substitute for spermine itself.

It is quite possible that effects are cell-type specific and that a need for spermine for the growth or development of certain cells in mice does lead to the retarded growth of the Gy mice. It is also possible that the growth retardation in the mice is due to an indirect effect via the paracrine or endocrine system. The involvement of polyamines in the regulation of ion channels [39,40] may also be relevant to the alterations seen in Gy males. It remains to be seen to what extent the specific phenotypic abnormalities associated with Gy males (which are not seen in Hyp males) are actually due to the loss of the ability to synthesize spermine. The replacement of spermine synthase in Gy mice will allow a test of the hypothesis that the absence of spermine cannot be fully compensated for by the increase in spermidine and is responsible for the phenotypic abnormalities observed. Gy males do not survive to weaning age on a C57BL/6J background [41]. In addition to their reduced viability and retarded growth on the B6C3H background, Gy males have additional symptoms including sterility, inner ear abnormalities, deafness, hyperactivity and circling behaviour [18,22–24]. In this context, it is of interest that the total polyamines were changed least in the brain (Table 2). The inability of the brain to raise spermidine levels sufficiently may be a factor in the neurological and hearing defects.

Similarly, the limited lifespan of the Gy mice, which we show to be due to increased post-natal mortality, may be due to the absence of spermine. The post-natal mortality may indicate an ability of Gy embryos to utilize spermine provided via the placenta. However, suckling pups may be unable to take up spermine provided in their mothers' milk, which is known to be a dietary source of polyamines [42,43]. In the present study, sudden death of many Gy males was observed in the weeks following weaning at 3 weeks of age and to facilitate experimentation, animals were killed at 5 weeks of age. Previous workers have also observed that Gy males have a higher propensity to sudden death, and a limited number of animals survive past 3 months of age [18,21,41]. While no histological examination of mouse tissues has been carried out in the present study, Lorenz and co-workers [20] reported that no gross or histological abnormalities were detected in the brains of 12-day-old Gy males. Similarly, the heart, thymus, kidney, liver and spleen all appeared histologically normal [20]. If the poor survival of Gy mice is due to the absence of spermine, it would suggest that spermine plays an important protective role. The presence of spermine bound to nuclear DNA may impart protection from oxidative and radiative damage. This would be consistent with work showing that spermine may be involved in the resistance to oxidative stress [44,45], protection of DNA from damage [46,47] and also in apoptosis [48,49]. In all of these cases, spermine is considerably more potent than spermidine, and the increase in spermidine observed in these mice may be insufficient to maintain normal protective functions of spermine.

Such protection would be consistent with our finding of the increased sensitivity of immortalized fibroblasts lacking spermine to killing by the chloroethylating agent BCNU. It is likely that the lack of interaction between spermine and DNA renders the cellular DNA more reactive with this alkylating agent and/or increases the chances of cell death in response to such chemically induced DNA damage. It is possible that the efficacy of other

cancer chemotherapeutic agents, which act by causing DNA damage in proliferating tumour cells, may also be enhanced by spermine depletion and thus the Gy cells are a good model in which to test this hypothesis. If it is validated, the case for the use of specific spermine synthase inhibitors to enhance chemotherapy by these agents [1] would be strengthened greatly.

It should be noted that the reduction of spermine in the tissue studies in the Gy mice and in the cultured cells was much more complete than that which can be achieved by the use of inhibitors of polyamine biosynthesis. Historically, the pool of spermine has always been the most difficult to perturb in cell systems. Therefore these mice and cells are valuable models for studying the role of spermine in a variety of physiological processes.

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