Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 1629–1637
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Polyamine modulation of iron uptake in CHO cells

François Gaboriau[a,b,*], Anne Kreder[a], Nicolas Clavreul[a], Jacques-Philippe Moulinoux[a], Jean-Guy Delcros[a], Gérard Lescoat[b]

[a]*Groupe de Recherche en Thérapeutique Anticancéreuse, CNRS FRE 2261, Faculté de Médecine, 35043 Rennes Cedex, France*
[b]*Inserm U522, Régulations des Équilibres Fonctionnels du Foie Normal et Pathologique, CHU de Rennes, Hôpital Pontchaillou, 35033 Rennes Cedex, France*

Received 28 July 2003; accepted 16 December 2003

Abstract

Polyamines are ubiquitous molecules, which, like iron, are essential for cell growth. All eukaryotic cells are equipped with a specific polyamine transport system (PTS). Polyamines have primary and secondary amino groups which chelate bivalent metal cations such as Fe and Cu. In the present study, we investigated the potential contribution of naturally occurring polyamines and their active transport system to iron uptake. In presence of subtoxic Fe(III) (10 μM), treatment of CHO cells with spermine, and to a lesser extent with spermidine (10–100 μM), resulted in a marked cytotoxic effect. This cytotoxicity was prevented by the addition of an iron-chelator, deferioxamine, and was not observed in CHO-MG cells, a mutant cell line devoid of polyamine transport activity. Experiments using ^{14}C-polyamines and ^{55}Fe(III) revealed that these toxic effects were related to polyamine-modulation of iron uptake, and were dependent on the presence of the active PTS. These results demonstrated active uptake of polyamine–iron complexes via the PTS. The number of amino groups affected the efficacy of the studied natural polyamines to transport iron via the PTS. Spermine, a tetramine, was more efficient than the triamine spermidine. Co-transport of iron by the diamine putrescine was not observed. These results demonstrate that the cell polyamine transport system is a potential cell entry pathway for iron. The studied polyamines, spermine and spermidine, may be components of the pool of transferrin-independent iron-chelating vectors, which have recently attracted the attention of many investigators.

Keywords: Iron overload; Non-transferin-bound-iron; Polyamine transport system; Iron cytotoxicity

1. Introduction

Polyamines such as putrescine, spermidine and spermine (Fig. 1) are ubiquitous cell components, essential for cell growth and differentiation (for a review [1]). Exchange between the intracellular polyamine pools and the cellular environment occurs via an active transport system. Although the polyamine transport system (PTS) remains to be characterized at the molecular level, its biochemical properties are well established (for a review [2,3]). The PTS provides a saturable and energy-dependent uptake mechanism for exogenous polyamines. It is noteworthy that the low stringency of PTS for structural features authorizes the uptake of various synthetic polyamine derivatives and analogues [4,5]. This is particularly of interest for the development of pharmacological strategies for cancer therapy. PTS activity is regulated by intracellular levels of polyamines [6]. Polyamine depletion (e.g. with α-difluoromethylornithine, DFMO) greatly stimulates this activity [7–9].

Due to their amino groups, polyamines are able to chelate metallic cations [10]. The stability constants of these chelates increase with the number of amino groups (Fig. 1) and with the chain length of the polyamine [11]. This chelating efficiency, that remains to be fully investigated, might be a basis of their antioxidant and anti-inflammatory activities [10,12,13].

Iron is vital for all living organisms. Mammalian cells use the iron-binding protein transferrin for their iron uptake (for a review [14]). Iron is also provided by low molecular

Abbreviations: DAPI, 4′,6′-diamino-2-phenylindole; DFMO, α-difluoromethylornithine; HVA, homovanillic acid; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NTBI, non-transferrin-bound iron; PTS, polyamine transport system; TBARS, thiobarbituric acid-reactive substances.

* Corresponding author. Tel.: +33-2-99-54-74-02; fax: +33-2-99-54-01-37.
E-mail address: francois.gaboriau@univ-rennes1.fr (F. Gaboriau).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.12.033

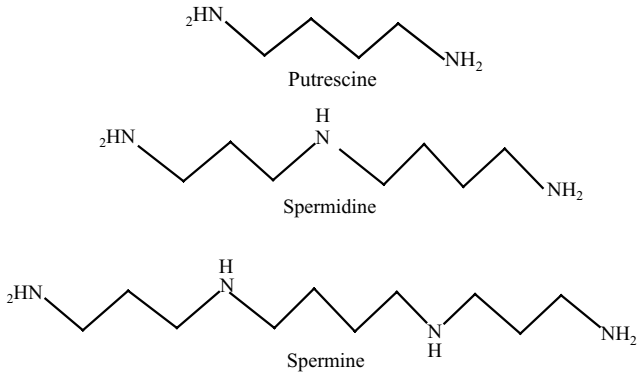

Fig. 1. Chemical structures of natural polyamines.

weight iron chelates included in the pool of non-transferrin-bound iron (NTBI), taken up by saturable and temperature-dependent transport systems [15–18]. In the absence of transferrin, the iron delivered from NTBI, including ferric-citrate, supports the growth of various normal and neoplastic cell lines [19,20]. In iron overload diseases, such as hemochromatosis, the increase in plasma NTBI leads to iron deposition in various tissues including liver and triggers biological damages through free radical formation [21]. Normal serum concentration of NTBI is below 1 μM while it can reach 15 μM or even higher levels in iron overload diseases such as hemochromatosis [21]. The composition of NTBI, a heterogeneous mixture of iron–anion complexes, and the mechanisms responsible for its uptake are poorly characterized. Due to their metal-chelating capacity, the naturally occurring polyamines, spermine, spermidine and putrescine (Fig. 1), were assumed to be potential NTBI components.

In the present study, we analyzed the ability of these polyamines to deliver iron into eukaryotic cells via the PTS, in iron overload conditions. Two Chinese hamster ovary cell lines, CHO (cells equipped with an active PTS) and the mutant CHO-MG (cells devoid of PTS activity) [22] provide a useful model to study the involvement of polyamines and their active transport in transferrin-independent iron uptake. Cytotoxic effects of the co-transport of iron by polyamines in conditions where the PTS is activated (DFMO-treated cells) or reduced (confluent cells) as well as cellular uptake of both radiolabeled polyamines and ferric chloride were evaluated. Membrane damages and the effect of the co-transport of iron by polyamines on the cell cycle are also reported.

2. Experimental procedures

2.1. Chemicals

[^{14}C]-Putrescine dihydrochloride (107 mCi/mmol), [^{14}C]-spermidine trihydrochloride (112 mCi/mmol), [^{14}C]-spermine tetrahydrochloride (110 mCi/mmol) and [^{55}Fe]-iron chloride (1.7 Ci/μmol) were provided by Amersham (Les Ulis, France) and Microscint^{TM} 20 by Packard (IL, USA).

Unless stated otherwise, all other chemicals were purchased from Sigma Chemical Co. (La Verpillière, France) or Merck (Darmstadt, Germany). DFMO was obtained from ILEX Oncology (San Antonio, TX). All chemicals were of first-grade purity and were used without further purification.

2.2. Cells

2.2.1. Cell culture

The mutant cell line, deficient for PTS (CHO-MG) was previously selected by Mandel and Flintoff [22] for resistance to the cytotoxicity of methylglyoxal-*bis*-guanylhydrazone, an inhibitor of *S*-adenosyl-L-methionine decarboxylase. This resistance has been shown to result from a decrease in the activity of the PTS that is responsible for uptake of this inhibitor. Comparison of cytotoxicity and iron uptake in these two cell lines provides a useful test to demonstrate the involvement of the PTS in iron co-transport by polyamines. CHO and CHO-MG cells were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum (Eurobio, Les Ulis, France), 2 mM glutamine (Bio Media, Boussens, France), 100 units/ml penicillin and 50 μg/ml streptomycin, at 37 °C in 5% humidified CO_2. L-Proline (2 μg/ml) was added to the culture medium for CHO-MG cells.

2.2.2. Cell treatment

For experiments, cells were harvested with trypsin and seeded 48 h before the polyamine treatment in 96-well microplates (Becton Dickinson, Oxnard, CA) at a density of 10^3 cells per well. In these conditions, cells reached confluency in 4–5 days. To test the effect of cell growth status on cytotoxicity, the 96-well microplates were seeded at various cell densities (250, 500, 1000, 1500, 2000 cells per well). For cell cycle analysis by flow cytometry and for peroxidative index measurements (TBARS), the cells were seeded in 25 cm^2 culture flasks at a density of 7.5×10^4 cells in 5 ml of complete medium.

Cytotoxicity of the treatment with iron in the presence or in the absence of polyamines was also tested in the presence of DFMO (5 mM), a selective inhibitor of ornithine decarboxylase (ODC) which induced a depletion of intracellular putrescine and spermidine and leads to an activation of the PTS. In this assay, the cells were seeded at 2×10^3 cells per well in the presence of the inhibitor. Aminoguanidine (1 mM) was added to the medium to prevent polyamine oxidation by serum amine oxidase present in fetal calf serum.

Cells were treated at 37 °C for 48 h with the required concentrations of polyamines (1–200 μM) with or without 10 μM ferric chloride ($FeCl_3$). Each concentration was tested in triplicate. Supernatants were then collected for lactate dehydrogenase (LDH) release or TBARS assay and

cells were rinsed twice with 100 μl of culture medium before the viability assay or the protein determination.

2.2.3. In vitro evaluation of drug cytotoxicity (MTT assay)

Effect of treatment on cell growth rate was determined in 96-well plates by measuring formazan formation from 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [23]. Absorbance readings at 540 nm were performed on a Multiscan RC microreader (Labsystem, Les Ulis, France).

2.2.4. Lactate dehydrogenase (LDH) analysis

LDH release is a permeability assay providing a non-invasive means of documenting membrane damage and cell integrity. The LDH activity released in supernatants was determined by measuring the rate of NADH disappearance during LDH-catalyzed conversion of pyruvate to lactate [24]. The various effectors added to the supernatants were checked in preliminary tests and found to have no effect on NADH measurements. Kinetic data, which were recorded with a microreader, were deduced from the linear part of the curves for times less than 3 min. Data, expressed as micromoles of NADH oxidized per minute, are the mean of three independent measurements. They are reported as a percentage of released activity with respect to the control value.

2.2.5. Thiobarbituric acid-reactive substances (TBARS) assay

Peroxidation of membrane polyunsaturated fatty acids was assayed by measuring the amount of TBARS released into the supernatant and in the cell extracts. Folin phenol reagent was used to determine cell protein content [25]. Quantity of TBARS, expressed in malondialdehyde (MDA) equivalents can be considered as a reliable evaluation of lipid peroxidation [26,27].

Immediately after supernatant collection, 10% (v/v) butylated hydroxytoluene (2%, w/v in ethanol) was added and samples were kept frozen (−20 °C) until TBARS measurements. Cells were washed twice with ice-cold NaCl solution (9 g/l) and scraped in 500 μl of water. Five hundred microliters of sodium dodecyl sulfate (SDS 1%, w/v in water) were added to this solution, which was used for protein determination [25]. These cell samples were kept frozen (−20 °C) until TBARS measurements.

Defrosted samples were fluorometrically assayed for TBARS according to a previously described procedure [28]. Briefly, to 500 μl of the defrosted sample were added 50 μl of butylated hydroxytoluene (2%, w/w in ethanol). Then 1 ml of a 0.375% (w/v) thiobarbituric acid solution in 0.25 M HCl containing 15% (w/v) trichloroacetic acid was added. The mixture was heated at 80 °C for 15 min, cooled on ice and extracted with 2 volumes of *n*-butanol. The organic phase was collected for fluorescence analysis ($\lambda_{exc} = 515$ nm; $\lambda_{em} = 550$ nm) with a SPEX 112 spectrofluorometer (Jobi-Yvon, Longjumeau, France).

In the acidic conditions of the TBARS test, tetraethoxypropane was quantitatively transformed into malondialdehyde that reacted with the thiobarbituric acid [28]. Malondialdehyde was used for calibration. TBARS values were normalized to cell protein content.

2.2.6. Nuclear fragmentation visualized by DAPI staining

This detection is based on DNA staining by 4′,6′-diamino-2-phenylindole (DAPI), a double-strand DNA intercalating fluorescent probe. Cells were seeded in 2-well Lab-Tek® chamber slides (Nunc Inc, Naperville, IL) at a cell seeding density of 2000 cells/cm^2 in complete culture medium. After 2 days incubation, cells were treated at 37 °C for 48 h with 10 μM ferric iron in the absence or presence of 100 μM spermine. Supernatants were discarded and cells were washed three times with ice-cold NaCl solution (9 g/l) and fixed for 25 min with 3% paraformaldehyde. After washing in PBS, the cells were incubated at room temperature for 15 min with a 0.5 μg/ml DAPI solution in 10 mM Tris–HCl, 10 mM NaCl and 100 mM EDTA (pH 7.4). Cells were washed twice with PBS and covered with a drop of DAKO® fluorescent mounting medium (Glostrup, Denmark), containing 15 mM NaN_3. Cells were analyzed by phase contrast microscopy and nuclei were visualized under UV light ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 460$ nm) with a Zeiss microscope.

2.2.7. Flow cytometry analysis of DNA content

The cells were seeded in 25 cm^2 culture flasks at a density of 7.5×10^4 cells in 5 ml of complete medium and treated for 48 h with 10 μM ferric iron in the absence or presence of 100 μM spermine. At the end of the incubation period, the medium was discarded and the culture dishes were washed three times with ice-cold PBS and scraped in 4 ml of PBS. After centrifugation, ethanol (70%) was added to the cell pellets, and the cells were kept at 4 °C until flow cytometry analysis. DNA was stained by adding 500 μl of propidium iodide in PBS (50 μg/ml) after cell digestion in 50 μg/ml of RNase A. DNA content was analyzed using an EPICS Elite flow cytometer (Coultronics, Hialeah, FL) equipped with an argon laser (488 nm). Data analysis was carried out using Multicycle software (Phœnix Flow Systems, San Diego, CA).

2.2.8. [^{55}Fe] and [^{14}C]-polyamines uptake by CHO cultures

CHO and CHO-MG cells were plated in 24-well microplates (Becton Dickinson, Oxnard, CA) with initial densities of 2×10^4 cells per well with 1 mM aminoguanidine in the absence or presence of 5 mM DFMO.

The cells were pulsed for 3 h with 10 μM [^{55}Fe]-iron chloride in the presence or absence of 100 μM unlabeled polyamines (Put, Spd or Spm) as well as with 100 μM [^{14}C]-polyamine in the presence or absence of unlabeled iron chloride (10 μM). At the end of the incubation period, the medium was discarded and the culture dishes were

washed three times with ice-cold NaCl solution (9 g/l) to count intracellular radioactivity. The cells were dissolved in 100 μl of 0.1N sodium hydroxide under vigorous shaking for 30 min. Aliquots (20 μl) of this cell extract were transferred to 96-well microplates for protein determination [25]. Eighty microliters of the cell extract were added to 1.25 ml of Microscint^{TM} 20 scintillator (Packard) in 24-well microplates and the radioactivity was measured on a Topcount NXT microplate scintillation counter (Packard Instrument Co., Meriden, USA). Quantities of transported polyamines and iron were deduced from calibration curves and corrected for protein content.

3. Results

3.1. Iron(III)-induced decrease in viable cell number in the presence of polyamines

The effect of 10 μM ferric chloride in the absence or in the presence of spermine on the CHO and CHO-MG cell density was evaluated using the MTT assay. Incubation of CHO and CHO-MG cells for 48 h, in the presence of spermine alone (0 up to 100 μM) did not significantly affect the number of living cells (Fig. 2). Treatment of CHO cells with 10 μM ferric iron plus increasing concentrations of spermine (0 up to 100 μM) decreased the number of viable cells in a dose-dependent manner (Fig. 2). However, treatment of CHO-MG cells with 10 μM ferric iron in the presence of spermine had no effect on cell viability.

The doubling times of CHO and CHO-MG cells were similar ($t = 23$ h, data not shown). After 96 h of culture both control CHO and CHO-MG cells reached confluency when they were seeded at an initial cell density above 1500–2000 cells per well while they were exponentially growing for lower initial cell density (data not shown). After 48 h culture, they were treated for 48 h with 10 μM ferric chloride in the presence or absence of 100 μM spermine. The MTT values were expressed as percentage of the values obtained with spermine alone. The decrease in the viable cell number after incubation with iron and spermine was high in exponentially growing CHO cultures, while it was low in CHO cultures reaching confluency, which were practically growth arrested (Fig. 3). In CHO-MG cells, deficient in polyamine transport activity, the treatment with iron and spermine did not reduce the cell number whatever the growth conditions (Fig. 3).

In the absence of polyamine, treatment with 10 μM iron induced a 9% decrease in the viable cell number (Table 1). The decrease in the viable cell number induced by the treatment with 10 μM iron in the presence of 100 μM polyamines, was shown to be slightly higher for spermine (65% MTT), than for spermidine (78%); putrescine did not modify significantly this iron effect. Depletion of intracellular putrescine and spermidine by pre-treatment with 5 mM DFMO for 48 h, enhances polyamine uptake [6]. The DFMO pre-treatment amplified the decrease in the viable cell number induced by iron in the presence of spermine (32%) and spermidine (53%) in CHO cells while it remained inefficient in the presence of putrescine (89%). In the CHO cultures, the decrease in the viable cell number

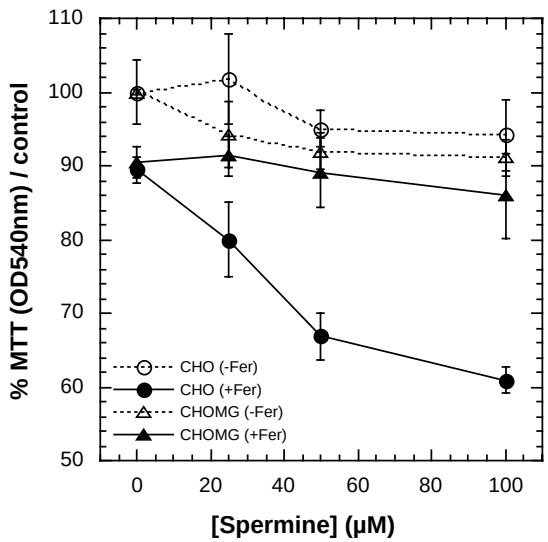

Fig. 2. Decrease in the viable cell number induced by iron(III) in the presence of spermine, in CHO cells and in polyamine transport-deficient CHO-MG cells. Cells were treated for 48 h, in the presence (plain lines) or absence (dotted lines) of sub-toxic ferric chloride concentrations (10 μM), with various spermine concentrations. Cell viability was determined by MTT assay. Data expressed as a percent of the control (untreated cells) are the mean ± S.D. of three independent measurements.

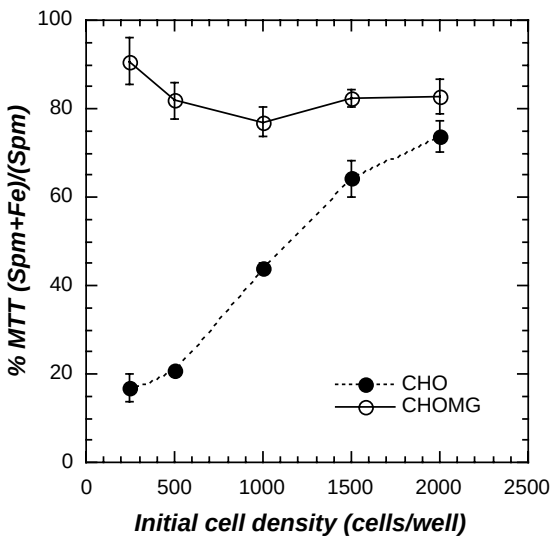

Fig. 3. Effect of growth phase on decrease in viable cell number induced by iron(III) in the presence of spermine. CHO and CHO-MG cells were seeded at an initial cell density ranging from 250 to 2000 cells per well and were grown in complete RPMI medium (10% FCS) for 48 h. They were treated for 48 h with 100 μM spermine in the presence or absence of 10 μM ferric chloride. The MTT values observed in the presence of both iron(III) and spermine were expressed as percentages of the values obtained with spermine alone.

Table 1
Increase in the iron toxicity induced by spermine, spermidine and putrescine in CHO cells and in its mutant cell line CHO-MG, deficient for the polyamine transport system

	No polyamine	Spermine 100 μM	Spermidine 100 μM	Putrescine 100 μM
CHO				
Fe 10 μM	91 ± 3	65 ± 2[a]	78 ± 2[a]	92 ± 6
Fe 10 μM + DFMO 5 μM	85 ± 5	32 ± 6[a]	53 ± 1[a]	89 ± 3
Fe 10 μM + DFO 25 μM	88 ± 5	85 ± 1	89 ± 4	84 ± 3
CHO-MG®				
Fe 10 μM	90 ± 7	94 ± 6	91 ± 2	92 ± 4

After 48 h growth, cells were treated for 48 h, in the presence or absence of sub-toxic ferric chloride concentrations (10 μM), with various polyamines (100 μM). Depletion of intracellular putrescine and spermidine as well as activation of the polyamine transport system were obtained when cells were seeded in the presence of 5 mM DFMO, a selective inhibitor of ODC. The protective effect of the iron chelator, deferrioxamine (DFO, 25 μM), was also investigated in the presence or absence of 10 μM Fe(III). Cell viability was determined by MTT assay. Data from triplicate runs were expressed as percentage with respect to the MTT values measured in the presence of the polyamine alone (100 μM) or compared to the control without polyamine.

[a] Significantly different from the cells incubated in absence of polyamine, according to the non-parametric Mann–Whitney test ($P \leq 0.01$).

induced by iron plus spermine or spermidine was inhibited by 25 μM of the metal chelator desferrioxamine (Table 1). The MTT values that were close to that obtained with iron alone (88%) suggested this chelator provided efficient protection against the decrease in viable cell number induced by the treatment with iron in the presence of spermine or spermidine. Competition between desferrioxamine and spermine (or spermidine) to form iron-chelates was probably the mechanism of this inhibitory effect. In the CHO-MG cells, no decrease of the viable cell number was observed with 10 μM iron in the presence of 100 μM of any polyamines (Table 1).

3.2. Morphological analysis of cells treated with iron(III) and spermine

Cytological analysis after cell treatment was performed by phase contrast microscopy and nuclei were visualized under UV light after DNA staining with DAPI. Treatment with 10 μM $FeCl_3$ or 100 μM spermine alone did not change the morphology of CHO and CHO-MG cells (data not shown). Treatment of CHO cells with iron and spermine (Fig. 4b) reduced the cell density in comparison to the control (Fig. 4a). It led to membrane damage (blebbing) and to an increase in the nucleo-cytoplasmic ratio without any nuclear fragmentation. In contrast, CHO-MG cells treated with 10 μM $FeCl_3$ and 100 μM spermine (Fig. 4d) remained quite similar to the control cells (Fig. 4c).

3.3. Cell membrane damage induced by iron(III) and spermine treatment

Treatment of CHO cells with 10 μM $FeCl_3$ in the presence of 100 μM spermine led to a significant increase in TBARS formation in cells extracts which reflects lipid peroxidation (Fig. 5A). In contrast, the same treatment did not amplify TBARS formation in CHO-MG cells.

As deduced from LDH release in cell supernatants (Fig. 5B), treatment of CHO and CHO-MG cells with iron alone induced low-level membrane damages (20% increase over controls) while no effect was observed with 100 μM spermine alone. In contrast, a 90% increase in LDH leakage was observed in CHO cells incubated with iron in the presence of spermine whereas this treatment did not damage the CHO-MG cell membranes.

3.4. Cellular uptake of polyamines and iron(III)

Polyamine transport into CHO and CHO-MG cells was analyzed after a 3 h incubation with radiolabeled spermine, spermidine or putrescine. In the absence of iron, spermine, spermidine and putrescine uptake was respectively 13, 7 and 18 times higher in CHO than in CHO-MG cells (Table 2). This confirmed the deficient polyamine transport

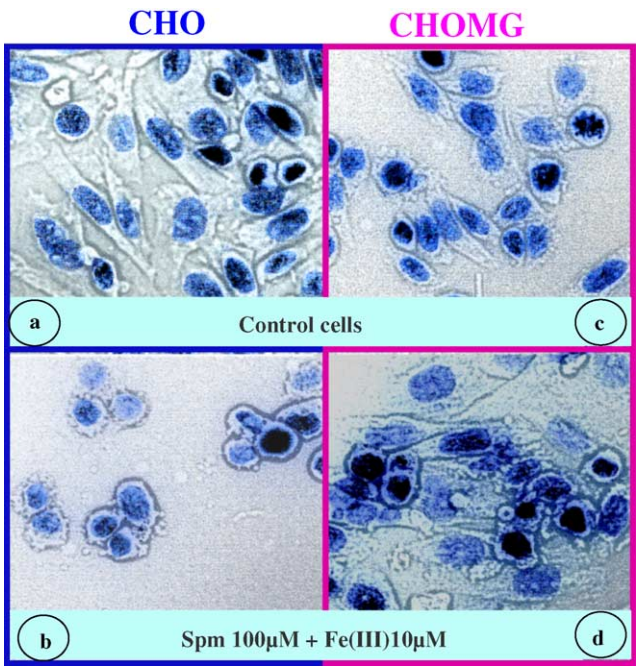

Fig. 4. Effect of treatment with spermine 100 μM + Fe(III) 10 μM in CHO and CHO-MG cells. After 48 h growth, cells were treated at 37 °C for 48 h. Cytological analysis was performed by phase contrast microscopy and nuclei were visualized under UV light after double-strand DNA staining by DAPI.

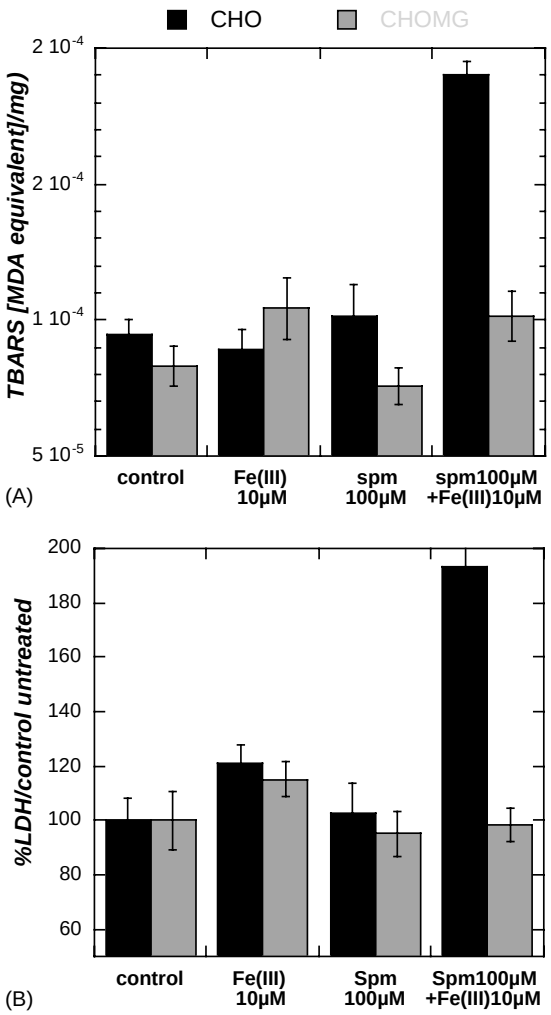

Fig. 5. CHO and CHO-MG cell membrane damage induced by treatment with iron(III) and spermine. After 48 h growth, cells were treated at 37 °C for 48 h. (A) Quantities of thiobarbituric acid-reactive substances (TBARS) released into supernatants and cell extracts, evaluated as an index of lipid peroxidation, were corrected for protein content. (B) Lactate dehydrogenase (LDH) leakage into supernatants as an index of cell membrane damage. Data, expressed as a percent of the control (untreated cells) are the mean ± S.D. of three independent measurements.

in CHO-MG cells and validated our experimental models. The presence of 10 μM iron(III) did not affect [^{14}C]-polyamine uptake in these cells.

Iron uptake into CHO and CHO-MG cells was analyzed after a 3 h incubation with ^{55}Fe(III) in the absence of polyamine or in the presence of 100 μM spermine, spermidine or putrescine. In the absence of polyamine, low iron uptake was observed in the CHO and CHO-MG cells (Table 2). Incubation of the cells in the presence of spermine or spermidine induced respectively a 4.6- and 1.5-fold increase in iron uptake in CHO cells but not in CHO-MG cells. Putrescine, the most efficiently transported polyamine in CHO cells, had no effect in the two cell models (Table 2).

In CHO cells, spermine transport-induced iron uptake was temperature-dependent. At 4 °C, [^{14}C]-spermine uptake was reduced by 78% (data not shown) and the

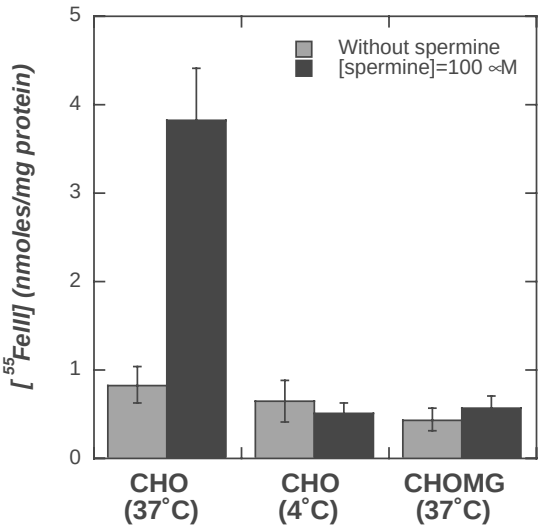

Fig. 6. Involvement of spermine and the polyamine transport system in the transport of ^{55}Fe(III) into CHO cells. After 48 h growth in 24-well microplates, cells were treated for 3 h at 37 °C or 4 °C in the presence of 10 μM ^{55}Fe(III) with or without 100 μM unlabeled spermine. ^{55}Fe(III) radioactivity was counted in cell extracts and Fe(III) uptake, which was deduced from calibration curves, was corrected for cell protein content. Data are the mean ± S.D. of three independent measurements.

^{55}Fe(III) uptake in the presence of 100 μM spermine was decreased by 85% (Fig. 6). Iron uptake was inefficient in CHO-MG cells lacking active transport system or in polyamine transport competent cells whose PTS was inhibited at low temperature.

4. Discussion

In iron-overloaded patients, the iron-binding capacity of plasma transferrin is often exceeded, leading to an increase in plasma NTBI. This favors iron deposition in various tissues including the liver and triggers biological damage through free radical formation [21]. An understanding of the chemical nature of NTBI appears to be essential for preventing its occurrence. Its composition probably depends on the severity and on the type of iron overload. The role of polyamines, as potential NTBI components, was investigated in the present study.

Treatment of CHO cell cultures with spermine and to a lesser extent with spermidine (10–100 μM) in the presence of a subtoxic concentration of iron (10 μM) resulted in membrane peroxidation and to a marked increase in cytotoxicity resulting from necrosis rather than apoptosis. It was prevented by the iron chelator desferrioxamine and was not observed in PTS-deficient CHO-MG cells. Further evidence for the involvement of the PTS in the cytotoxicity of the spermidine– and spermine–iron complexes was deduced from its increase after PTS activation by DFMO and from its decrease in cells reaching confluency (confluent cells are known to exhibit reduced polyamine uptake capacity).

Table 2
Involvement of the polyamine transport system in ^{14}C-polyamine (A) and ^{55}Fe(III) (B) uptake

	(A) ^{14}C-Polyamine uptake (nmol/mg protein)		
	[Spermine]	[Spermidine]	[Putrescine]
CHO			
^{14}C-PA	6.33 ± 0.31	7.28 ± 0.47	12.72 ± 073
^{14}C-PA + Fe 10 μM	5.73 ± 1.92	7.14 ± 0.56	12.95 ± 1.42
CHO-MG®			
^{14}C-PA	0.46 ± 0.02	1.00 ± 0.13	0.71 ± 0.03
^{14}C-PA + Fe 10 μM	0.69 ± 0.24	0.91 ± 0.13	0.77 ± 0.11

	(B) ^{55}Fe(III) uptake (nmol/mg protein)			
	No polyamine	Spermine 100 μM	Spermidine 100 μM	Putrescine 100 μM
CHO	0.59 ± 0.27	3.82 ± 0.59[a]	0.97 ± 0.21[a]	0.33 ± 0.04
CHO-MG®	0.36 ± 0.15	0.57 ± 0.14	0.43 ± 0.12	0.27 ± 0.13

After 48 h growth in 24-well microplates, cells were incubated for 3 h at 37 °C, in the presence of 100 μM ^{14}C-polyamine with or without 10 μM of unlabeled Fe(III) (A) as well as in the presence of 10 μM ^{55}Fe(III) with or without 100 μM of unlabeled polyamine (B). Data from triplicate runs are expressed in nanomoles of Fe(III) or polyamine with respect to the protein content.

[a] Significantly different from the cells incubated in presence of Fe(III) alone, according to the non-parametric Mann–Whitney test ($P \leq 0.01$).

Double labeling experiments ([^{14}C]-polyamines and [^{55}Fe(III)]) revealed that these toxic effects were related to the modulation of cellular iron uptake by polyamines, itself PTS-dependent. Iron uptake by spermine– and spermidine–iron complexes and PTS was not observed in CHO-MG cells lacking PTS and was inhibited in CHO cells at 4 °C. The efficacy of the different polyamines to transport iron via the PTS was dependent on their number of amino groups, with spermine being more effective than spermidine. Iron transport by diamine putrescine was not observed.

The relative efficiency of polyamines to co-transport iron could be related to their metal chelating ability. Polyamines are able to form complexes with metal ions, including Zn^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} [11,29,30]. It is generally assumed that their antioxidative and anti-inflammatory actions are mainly due to their metal-chelating properties that depend on the number of nitrogen atoms and on their position along the molecule [10]. As deduced from X-ray crystallography analysis [31], the structure of a Cu(II)–spermine complex resembles that of iron–porphyrin complexes. Metal binding involves charge transfer with the nitrogen donor atoms positioned in a square plane around the central copper ion.

A correlation has been reported between the binding constant for divalent metal ions such as Cu^{2+}, the antioxidative efficacy of polyamines [10,12] and their inhibiting effect on Cu^{2+}-induced HVA autoxidation [32]. The metal dependence of the HVA autoxidation reaction, which was also previously shown to be activated by ferrous ions [32] and to a lesser extent by ferric ions, was used to compare the chelating efficiency of natural polyamines. A 50% inhibition of HVA autoxidation rate has been observed to occur in the presence of micromolar spermine and with about 100-fold higher spermidine concentrations (Gaboriau, unpublished data). While no affinity constant data have been reported in the literature, our data suggest that the binding constant of spermine with ferrous ions is higher than that of spermidine and that these two polyamines weakly interact with ferric ions and probably modify their solubility (data not shown). Studies on the interaction of polyamines with iron(III) are under progress in our laboratory.

Further indirect evidence for the interaction of spermine with iron comes from the reports of Tadolini and coworkers [33,34]. Binding of spermine to liposomes decreased the reactivity of both ferrous and ferric ions and inhibited lipid peroxidation whereas free spermine had no effect. This inhibition was suggested to be due to the ability of spermine to form a ternary complex with the phospholipid polar head and the iron ion. This type of complex could be recognized by the PTS.

In the absence of experimental data clearly showing the formation of spermine–iron complexes, one cannot exclude the involvement of a spermine–Fe(II) complex in the PTS-dependent increase in iron uptake. The reduction of ferric ions by an active system present at the plasma membrane surface of CHO and CHO-MG cells could favor the formation of such spermine–Fe(II) complexes. Dcytb, a cytochrome *b* highly expressed in duodenal brush-border membrane, was previously shown to be implicated in dietary iron absorption by reducing dietary ferric iron to the ferrous form for transport via the divalent metal-transporter 1 (DMT1) [35]. This ferric reductase is also expressed in other tissues [36] and may be present at the plasma membrane of CHO and CHO-MG cells.

Finally, other mechanisms independent of the chelating properties of polyamines have to be considered. In particular, the endocytosis pathway, which was suggested to be an integral part of the PTS in CHO cells [37], could play an important role in this iron uptake. The internalization of fluorescent-spermidine analogues in granular structures

within the cytoplasm, by a mechanism involving receptor-mediated endocytosis, was inhibited in the transport-deficient CHO-MG cell line [38,39]. A further experimental argument dealing with the decrease in the endocytosis efficiency of these cells can also be deduced from our own unpublished observations. The fluorescent hydrophobic plasma membrane probe, trimethylamino-diphenylhexatriene (TMA-DPH) was previously shown to follow the plasma membrane throughout its internalization and recycling process and thus to behave as a marker for endocytosis in living cells [40]. Our comparison of the TMA-DPH internalization in CHO and CHO-MG cell lines showed that the fluid phase endocytosis of CHO-MG cells was lower than that of CHO cells (data not shown). Such deficiency of CHO-MG cells could also explain their inability to internalize the iron(III)–spermine complexes. Further experiments to test this hypothesis are now in progress.

The apparent Michaelis–Menten constants (K_m) for spermidine and spermine uptake by the polyamine transport system in CHO cells were 3.7 and 1.8 μM, respectively [6]. We observed in the present study that the co-transport of iron through the PTS requires higher spermine and spermidine levels. Assuming that polyamine–Fe(III) complexes are formed, this suggests that high spermidine or spermine concentration are required for their formation. In CHO cells, the ratios of co-transported iron (corrected from the iron uptake in the absence of polyamine) versus polyamine uptake, provides an estimation of these cells molecular iron/polyamine uptake ratios. These ratios were 0.50 and 0.045 for spermine and spermidine, respectively, suggesting that the stoichiometries of the iron–spermine and iron–spermidine interactions were 1/2 and 1/20, respectively.

Our study was conducted with relatively high concentrations of both polyamines (10–100 μM) and iron (10 μM). High iron concentration may be encountered in iron overload diseases such as hemochromatosis [21]. Polyamine concentrations in easily accessible biological fluids like blood, cerebrospinal fluid, exocrine glandular secretions or urine, are generally lower than that used in the present study; however, we have no accurate data related to interstitial fluid. Some exocrine glands produce large quantities of polyamines and millimolar concentrations of polyamines have been reported in human seminal plasma [41]. In exocrine glands as well as in the intestinal lumen, cells are exposed to polyamine concentrations higher than those used in this work [1]. Moreover, in cancer tissues, cell necrosis may lead to high local polyamine levels and an interstitial fluid exceptionally rich in polyamines surrounding cancer cells would be a reasonable postulate.

The present study demonstrates that polyamines, such as spermine and spermidine, and their active transport system could participate in transferrin-independent iron(III) uptake. These polyamines may be components of the pool of transferrin-independent iron-chelating vectors, which have recently attracted the attention of many investigators.

Uptake of polyamine–iron(III) complexes appears to be an important mechanism of iron accumulation and resulting toxicity in cultured CHO cells. In the present study, this uptake was documented for high concentrations of polyamines (10–100 μM) and iron (10 μM) similar to those observed in pathological iron- and polyamine-overload conditions. The biological relevance of polyamine–iron-(III) complex uptake in physiological situations requires further investigation.

Acknowledgments

We are indebted to Pr. W. Flintoff (University of Western Ontario) for providing the CHO-MG cells. We thank C. Leduc for technical assistance. We acknowledge the Ligue Nationale Contre le Cancer (LNCC-Comité d'Ille-et-Vilaine) for financial support.

References

[1] Cohen SS. Polyamines in the animal cell. In: Cohen SS, editor. A guide to the polyamines. Oxford: Oxford University Press; 1998. p. 184–207.
[2] Seiler N, Delcros JG, Moulinoux J-P. Polyamine transport in mammalian cells. An update. Int J Biochem Cell Biol 1996;28:843–61.
[3] Seiler N, Dezeure F. Polyamines transport in mammalian cells. Int J Biochem 1990;22:211–8.
[4] Seiler N, Douaud F, Havouis R, LeRoch N, Renault J, Vaultier M, et al. Dimethylsilane polyamines, a new class of potential anticancer drugs: effects on the growth of Lewis lung carcinoma in mice. Int J Oncol 1997;11:835–41.
[5] Martin B, Posseme F, Le Barbier C, Carreaux F, Carboni B, Seiler N, et al. *N*-Benzylpolyamines as vectors of boron and fluorine for cancer therapy and imaging: synthesis and biological evaluation. J Med Chem 2001;44:3653–64.
[6] Byers TL, Pegg AE. Properties and physiological function of the polyamine transport system. Am J Physiol 1989;257 [Cell Physiol 26]: C545–53.
[7] Alhonen-Hongisto L, Seppanen P, Janne J. Intracellular putrescine and spermidine deprivation induces increased uptake of the natural polyamines and methylglyoxal bis(guanylhydrazone). Biochem J 1980; 192:941–5.
[8] Porter CV, Ganis B, Vinson T, Marton LJ, Kramer DL, Bergeron RJ. Comparison and characterization of growth inhibition in L1210 cells by alpha-difluoromethylornithine, an inhibitor of ornithine decarboxylase, and *N*1,*N*8-bis(ethyl)spermidine, an apparent regulator of the enzyme. Cancer Res 1986;46:6279–85.
[9] Rinehart CAJ, Chen KY. Characterization of the polyamine transport system in mouse neuroblastoma cells. Effects of sodium and system A amino acids. J Biol Chem 1984;259:4750–6.
[10] Løvaas E. Antioxidative and metal-chelating effects of polyamines. In: Sies H, editor. Antioxidants in disease mechanisms and therapy, vol. 38. New York: Academic Press; 1997. p. 116–49.
[11] Palmer BN, Powell HKJ. Polyamine complexes with seven-membered chelate rings: complex formation of 3-azaheptane-1,7-diamine, 4-azaoctane-1,8-diamine (spermidine), and 4,9-diazadodecane-1,12-diamine (spermine) with copper(II) and hydrogen ions in aqueous solution. JCS Dalton 1974:2089–92.

[12] Løvaas E, Carlin G. Spermine: an antioxidant and anti-inflammatory agent. Free Radic Biol Med 1991;11:455–61.
[13] Løvaas E. Hypothesis: spermine may be an important epidermal antioxidant. Med Hypotheses 1995;45:59–67.
[14] Richardson DR, Ponka P. The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells. Biochim Biophys Acta 1997;1331:1–40.
[15] Basset P, Quesneau Y, Zwiller J. Iron-induced L1210 cell growth: evidence of a transferrin-independent iron transport. Cancer Res 1986; 46:1644–7.
[16] Brissot P, Wright TL, Ma WL, Weisiger R. Efficient clearance of non-transferrin-bound iron by rat liver. Implications for hepatic iron loading in iron overload states. J Clin Invest 1985;76:1463–70.
[17] Kaplan J, Jordan I, Sturrock A. Regulation of the transferrin-independent iron transport system in cultured cells. J Biol Chem 1991;266: 2997–3004.
[18] Sturrock A, Alexander J, Lamb J, Craven CM, Kaplan J. Characterization of a transferrin-independent uptake system for iron in HeLa cells. J Biol Chem 1990;265:3139–45.
[19] Neumannova V, Richardson DR, Kriegerbeckova K, Kovar J. Growth of human tumor cell lines in transferrin-free, low-iron medium. In Vitro Cell Dev Biol Anim 1995;31:625–32.
[20] Richardson DR, Chua AC, Baker E. Activation of an iron uptake mechanism from transferrin in hepatocytes by small-molecular-weight iron complexes: implications for the pathogenesis of iron-overload disease. J Lab Clin Med 1999;133:144–51.
[21] Gutteridge JM, Rowley DA, Griffiths E, Halliwell B. Low-molecular-weight iron complexes and oxygen radical reactions in idiopathic haemochromatosis. Clin Sci (Lond) 1985;68:463–7.
[22] Mandel JL, Flintoff WF. Isolation of mutant mammalian cells altered in polyamine transport. J Cell Physiol 1978;97:335–44.
[23] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[24] Bergmeyer HU, Bernt E, Hess B. Lactic dehydrogenase. In: Bergmeyer HU, editor. Methods of enzymatic analysis. New York: Academic Press; 1963. p. 736–41.
[25] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[26] Jentzsch AM, Bachmann H, Fürst P, Biesalski HK. Improved analysis of malondialdehyde in human body fluids. Free Radic Biol Med 1995; 20:251–6.
[27] Kosugi H, Kikugawa K. Potential thiobarbituric acid-reactive substances in peroxidized lipids. Free Radic Biol Med 1989;7:205–7.
[28] Aust SD. Lipid peroxidation. In: Greewald RA, editor. Handbook of methods for oxygen radical research. Boca Raton, FL: CRC Press; 1986. p. 203–7.
[29] Hares GB, Fernelius WC, Douglas BE. Equilibrium constants for the formation of complexes between metal ions and polyamines. J Am Chem Soc 1956;78:1816–8.
[30] Bertsch CR, Fernelius WC, Block BP. A thermodynamic study of some complexes of metal ions with polyamines. J Phys Chem 1958; 62:444–50.
[31] Boggs R, Donohue J. Spermine copper(II) perchlorate. Acta Cryst 1975:B31.
[32] Gaboriau F, Delcros JG, Moulinoux J-P. A simple assay for the measurement of plasma antioxidant status using spontaneous autoxidation of homovanillic acid. J Pharmacol Toxicol Methods 2002;47: 33–43.
[33] Tadolini B, Cabrini L, Varani E, Sechi AM. Spermine binding and aggregation of vesicles of different phospholipid composition. Biogenic Amines 1985;3:87–96.
[34] Tadolini B. Polyamine inhibition of lipoperoxidation. Biochem J 1988;249:33–6.
[35] McKie AT, Barrow D, Latunde-Dada GO, Rolfs A, Sager G, Mudaly E, et al. An iron-regulated ferric reductase associated with the absorption of dietary iron. Science 2001;291:1755–9.
[36] McKie AT, Latunde-Dada GO, Miret S, McGregor JA, Anderson GJ, Vulpe CD, et al. Molecular evidence for the role of a ferric reductase in iron transport. Biochem Soc Trans 2002;30:722–4.
[37] Soulet D, Covassin L, Kaouass M, Charest-Gaudreault R, Audette M, Poulin R. Role of endocytosis in the internalization of spermidine-C(2)-BODIPY, a highly fluorescent probe of polyamine transport. Biochem J 2002;367:347–57.
[38] Cullis PM, Green RE, Merson-Davies L, Travis N. Probing the mechanism of transport and compartmentalisation of polyamines in mammalian cells. Chem Biol 1999;6:717–29.
[39] Wang C, Delcros JG, Biggerstaff J, Phanstiel O. Molecular requirements for targeting the polyamine transport system. Synthesis and biological evaluation of polyamine–anthracene conjugates. J Med Chem 2003;46:2672–82.
[40] Illinger D, Poindron P, Fonteneau P, Modollel M, Kuhry JG. Internalization of the lipophilic fluorescent probe trimethylamino-diphenylhexatriene follows the endocytosis and recycling of the plasma membrane in cells. Biochim Biophys Acta 1990;1030:73–81.
[41] Fair WR, Clark RB, Wehner N. A correlation of seminal polyamine levels and semen analysis in the human. Fert Steril 1972;23:38–42.