

Influence of zinc deficiency on cell-membrane fluidity in Jurkat, 3T3 and IMR-32 cells

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We investigated whether zinc deficiency can affect plasma membrane rheology. Three cell lines, human leukaemia T-cells (Jurkat), rat fibroblasts (3T3) and human neuroblastoma cells (IMR-32), were cultured for 48 h in control medium, in zinc-deficient medium (1.5 μM zinc; 1.5 Zn), or in the zinc-deficient medium supplemented with 15 μM zinc (15 Zn). The number of viable cells was lower in the 1.5 Zn group than in the control and 15 Zn groups. The frequency of apoptosis was higher in the 1.5 Zn group than in the control and 15 Zn groups. Membrane fluidity was evaluated using the 6-(9-anthroyloxy)stearic acid and 16-(9-anthroyloxy)palmitic acid probes. Membrane fluidity was higher in 1.5 Zn cells than in the control cells; no differences

were observed between control cells and 15 Zn cells. The effect of zinc deficiency on membrane fluidity at the water/lipid interface was associated with a higher phosphatidylserine externalization. The higher membrane fluidity in the hydrophobic region of the bilayer was correlated with a lower content of arachidonic acid. We suggest that the increased fluidity of the membrane secondary to zinc deficiency is in part due to a decrease in arachidonic acid content and the apoptosis-related changes in phosphatidylserine distribution.

Key words: apoptosis, membrane fluidity, phosphatidylserine, zinc, zinc deficiency.

INTRODUCTION

Subtle changes in cellular zinc content and localization have been reported to have profound effects on cell metabolism and function [1–4]. In humans, zinc deficiency negatively affects the epidermal, central nervous, immune, gastrointestinal, skeletal and reproductive systems. Although many of the signs of zinc deficiency occur only after a prolonged deficit of the mineral, other signs can occur within a remarkably short period of time. For example, in rats, anorexia can be observed within 3 days after the introduction of a zinc-deficient diet, and, if the animal is pregnant, the risk of developmental abnormalities is sharply increased within 4 days [5]. Importantly, the above effects can be noted even when the deficiency is associated with only small changes in tissue zinc concentrations. The above observations suggest that small changes in certain zinc pools can have significant ramifications.

It has been proposed that zinc participates in the maintenance of the normal function and structure of membranes [6]. In red blood cells, the concentration of zinc at the plasma membrane decreases with zinc deficiency in association with increased cell osmotic fragility (see [7] for a review). Zinc deficiency induces alterations in platelet adhesion and impairs macrophage function (see [7] for a review). Different mechanisms could be involved in the observed membrane alterations. A decrease in membrane zinc content could cause subtle changes in membrane rheology, and leave vacant potential binding sites for redox-active metals such as iron and copper [8] that can promote membrane-lipid oxidation. Zinc deficiency can also cause alterations in membrane-

lipid composition [9,10] that can lead to alterations in the physical properties of bilayers.

In both cell cultures and whole animal models, the induction of zinc deficiency can rapidly trigger apoptosis [1,2,11,12]. One of the morphological changes observed during apoptosis is the loss of plasma-membrane asymmetry, associated with phosphatidylserine translocation from the inner to the outer monolayer of the membrane [13]. The exposure of phosphatidylserine perturbs the normal lipid packing of the bilayer, an effect that could alter the function of the plasma membrane [14–17]. In the present study, we examined the effects of zinc deficiency on plasma-membrane fluidity in three cell lines. The lines were chosen based on three relevant targets of zinc deficiency, the immune (Jurkat), nervous (IMR-32) and epidermal (3T3) systems.

After exposing cells to zinc-deficient or zinc-adequate media for 48 h, we characterized (i) intracellular zinc content, (ii) plasma-membrane fluidity, (iii) membrane fatty-acid profiles, (iv) the presence of phosphatidylserine in the outer leaflet of the bilayer, and (v) parameters of cell death by apoptosis (DNA fragmentation and caspase-3 activity). Results indicate that, after 48 h of exposure to a zinc-deficient medium, cell plasma membranes become more fluid in both the interfacial region and the hydrophobic core of the membrane. At the surface level, the change in membrane fluidity could not be attributed to a change in fatty-acid profiles. However, the deficiency did result in a higher exposure of phosphatidylserine, a change that could be associated with the induction of apoptosis. In contrast, a higher fluidity in the hydrophobic region of the membrane in IMR-32 and

Abbreviations used: 16-AP, 16-(9-anthroyloxy)palmitic acid; 6-AS, 6-(9-anthroyloxy)stearic acid; C, control (non-chelated) medium; DMEM, Dulbecco's modified Eagle's medium; ECL[®], enhanced chemiluminescence; FBS, foetal bovine serum; MC, merocyanine 540; α -MEM: minimum essential α -medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; TPEN, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine; TSQ, *N*-6-(6-methoxy-8-quinolyl)-*p*-toluenesulphonamide; UI, unsaturation index; 1.5 Zn, chelated medium containing 1.5 μM zinc; 15 Zn, chelated medium containing 15 μM zinc.

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3T3 cells was correlated with a decrease in the content of arachidonic acid.

EXPERIMENTAL

Materials

Human neuroblastoma cells (IMR-32), human leukaemia T-cells (Jurkat), and Swiss albino fibroblast cells (3T3) were obtained from the American Type Culture Collection (A.T.C.C., Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM, high glucose), F-12 nutrient mixture (Ham's), minimum essential α -medium (α -MEM), RPMI 1640 medium and foetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, U.S.A.). 6-(9-Anthroyloxy)stearic acid (6-AS), 16-(9-anthroyloxy)palmitic acid (16-AP), propidium iodide (PI), merocyanine 540 (MC), and *N*-6-(6-methoxy-8-quinoly)-*p*-toluenesulphonamide (TSQ) were from Molecular Probes (Eugene, OR, U.S.A.). Zinc (II) sulphate and Igepal were from Sigma (St. Louis, MO, U.S.A.). Cell Death Detection ELISA^{PLUS} was obtained from Roche Diagnostics (Indianapolis, IN, U.S.A.). CellTiter 96[®] Non-Radioactive Cell Proliferation assay was obtained from Promega (Madison, WI, U.S.A.). Antibodies against poly(ADP-ribose) polymerase (PARP) and β -tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). PVDF membranes and enhanced chemiluminescence (ECL[®]) Western blotting system were from Amersham Biosciences (Piscataway, NJ, U.S.A.).

Cell culture

Jurkat, 3T3, and IMR-32 cells were cultured at 37 °C in the following media containing 50 units/ml penicillin and streptomycin. (i) Jurkat cells: RPMI 1640 medium, supplemented with 2 mM L-glutamine and 10% (v/v) FBS; (ii) 3T3 cells: DMEM supplemented with 7.5% (v/v) FBS; and (iii) IMR-32 cells: complex medium (55% DMEM, 30% Ham's F-12, 5% α -MEM), supplemented with 10% (v/v) FBS. Zinc-deficient FBS was prepared as previously described [18]. 3T3 and IMR-32 cells (at 80% confluence), and Jurkat cells (1×10^6 cells/ml) were washed with DMEM, and cultured for 48 h in control non-chelated medium (C), or in chelated medium containing either 1.5 or 15 μ M zinc (1.5 Zn and 15 Zn respectively). Cell viability was measured by evaluating the exclusion of the dye Trypan Blue and by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Cell Titer 96[®] Non-Radioactive Cell Proliferation assay).

Determination of TSQ-reactive zinc

The content of TSQ-reactive zinc was evaluated as previously described [19]. Cells (1.2×10^6) were incubated in the corresponding medium for 48 h. After incubation, the medium was decanted and cells were rinsed with warm DMEM and 1 ml of DMEM containing 25 μ M TSQ was added. Cells were dispersed and incubated at 37 °C in the dark for 15 min, transferred to 1.5 ml conical tubes and centrifuged at 800 g for 10 min. The cell pellet was rinsed twice with warm PBS, and finally resuspended in 0.2 ml of PBS containing 0.1% Igepal. After a brief sonication, the fluorescence at 480 nm ($\lambda_{\text{excitation}}$, 365 nm) was measured in a Kontron SFM-25 spectrofluorimeter (Kontron Instruments SPA, Milan, Italy). To evaluate DNA content, samples were subsequently treated with 50 μ M PI, incubated for 20 min at room temperature (25 °C), and the fluorescence emission at 590 nm ($\lambda_{\text{excitation}}$, 538 nm) was registered. Results are expressed as the TSQ/PI fluorescence ratio.

Evaluation of membrane-lipid packing

Jurkat (6×10^4 cells), 3T3 (1×10^4 cells) and IMR-32 (2×10^4 cells) cells, suspended in 0.3 ml of 50 mM Hepes buffer (pH 7.4) containing 125 mM KCl, were added to 50 μ M fluorescent probe, 6-AS or 16-AP. Cells were incubated at 37 °C for 15 min to allow the complete incorporation of the probe into the plasma membrane (results not shown). Membrane fluidity was evaluated at 37 °C from the fluorescence polarization at 435 nm ($\lambda_{\text{excitation}}$, 384 nm).

Evaluation of phosphatidylserine translocation

The presence of phosphatidylserine in the outer layer of the plasma membrane was evaluated measuring the binding of MC to the cells [20]. Jurkat (6×10^4 cells), 3T3 (1×10^4 cells) and IMR-32 (1×10^4 cells) cells suspended in 0.3 ml of 50 mM Hepes buffer (pH 7.4) containing 125 mM KCl, were added to 10 μ M MC, and incubated at room temperature for 10 min in the darkness. MC binding to phosphatidylserine was evaluated from the increase in the fluorescence emission at 578 nm ($\lambda_{\text{excitation}}$, 555 nm). Afterwards, cells were disrupted by a 30 min incubation in the presence of 0.1% (v/v) Igepal, and treated with 50 μ M PI. DNA content was evaluated after incubating samples with 50 μ M PI for 20 min ($\lambda_{\text{excitation}}$, 538 nm; $\lambda_{\text{emission}}$, 590 nm). Results are expressed as the MC/PI fluorescence ratio.

Evaluation of apoptosis

Cell death by apoptosis was evaluated measuring DNA fragmentation and PARP cleavage by caspase 3.

Cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were quantified using the Cell Death Detection ELISA^{PLUS} kit, following the manufacturer's protocol. The amount of DNA fragments (measured from the absorbance at 405 nm) was expressed relative to cell viability determined simultaneously for each group. Cell viability was measured by the MTT assay. Results are expressed as the nucleosome/MTT absorbance ratio.

For PARP cleavage determination, cells (1×10^4) were washed in PBS and centrifuged for 20 min at 800 g. Cell pellet was re-suspended in 50 mM Hepes (pH 7.4), containing 150 mM NaCl, 0.5 mM EDTA, 2% (v/v) Igepal, phosphatase inhibitors (1 mM sodium pervanadate and 100 mM NaF), and protease inhibitors (1 mM PMSF, 5 mg/ml leupeptin, 1 mg/ml pepstatin and 10 mg/ml aprotinin). Samples were exposed to one cycle of freezing and thawing, incubated for 30 min on ice and centrifuged at 15 000 g for 30 min. Proteins (50 μ g/lane) were resolved by SDS/PAGE, and transferred on to PVDF membranes. Membranes were immunoblotted with rabbit polyclonal anti-PARP or anti- β -tubulin antibodies (1:1000 dilution) for 90 min at 37 °C, and incubated for a further 90 min in the presence of the secondary antibody conjugated to horseradish peroxidase (1:10 000 dilution). Conjugates were detected and quantified by ECL[®] in a Phosphorimager 640 (Amersham Biosciences). Results are expressed as the ratio of PARP (116 kDa)/ β -tubulin content.

Fatty-acid analysis

Membrane lipids were esterified according to the method described by Lepage and Roy [21]. Briefly, cells were suspended in 0.1 ml of 50 mM Hepes buffer (pH 7.4), containing 125 mM KCl, and 2 ml of methanol/benzene (4:1, v/v) and 0.2 ml of acetyl chloride were added. Methanolysis was achieved after 1 h of incubation at 100 °C. The reaction was stopped by the addition of 5 ml of 6% (w/v) potassium carbonate, and after centrifugation at 800 g for 10 min, the benzene layer was carefully separated and kept at -20 °C for further fatty-acid determination.

Fatty-acid analysis was performed by GLC (Shimadzu GC-8A gas chromatograph) on a DB-23 column (J&W Scientific, Folsom, CA, U.S.A.) with temperature programming at 3 °C/min between 175 °C and 220 °C. Fatty acids were identified by retention time and co-chromatography with commercial standards. The unsaturation index (UI) was calculated using the following equation:

$$UI = \frac{\sum \text{UFA} (\%) \times \text{NU}}{\sum \text{SFA} (\%)}$$

where UFA (%) and SFA (%) are the relative content of unsaturated and saturated fatty acids respectively, and NU is the number of unsaturations in a given fatty acid.

Statistics

One-way ANOVA test, followed by Fisher's PLSD (protected least-squares difference) test and correlations, were performed using the routines available in StatView 5.0 (SAS Institute, Cary, NC, U.S.A.). $P < 0.05$ was considered as statistically significant.

RESULTS

Cell viability

The number of viable cells after 48 h of incubation in the different media was evaluated for Jurkat, 3T3 and IMR-32 cells by the Trypan Blue dye-exclusion method. For Jurkat cells, zinc-deficient cell viability was 41 % and 23 % lower than in the C and 15 Zn cells ($P < 0.0001$) respectively (Figure 1). With 3T3 and IMR-32 cells, a lower number of viable cells (35 % and 27 % respectively) was observed in the 1.5 Zn group than in C group (Figure 1). In both cell types, the supplementation of the zinc-deficient medium with 15 μM zinc prevented the zinc-deficiency-mediated decrease in the number of viable cells.

To evaluate the possibility that the lower cell viability found in the 1.5 Zn group in the three cell lines was due to a higher number of dead cells, the incorporation of PI was tested both before and after membrane disruption, and results were expressed in arbitrary units. In Jurkat cells, the ratio between PI incorporation, in the absence (dead cells) and in the presence (total cells) of 1 % (v/v) Igepal, it was 35 % higher in the 1.5 Zn group ($P < 0.001$) than in the C group (0.78 ± 0.02 and 0.57 ± 0.02 respectively), whereas in the 15 Zn group, it was 16% higher (0.66 ± 0.02) than in the C group. In 3T3 cells, the ratio was 14% higher in the 1.5 Zn group (0.90 ± 0.02 ; $P < 0.005$) than in the C and 15 Zn groups (0.79 ± 0.02 and 0.80 ± 0.02 respectively). Finally, in IMR-32 cells, PI incorporation was 7 % higher in the 1.5 Zn group (0.77 ± 0.02 ; $P < 0.01$) than in the C group (0.72 ± 0.01). Contrary to the findings with Jurkat and 3T3 cells, in IMR-32 cells PI incorporation in the 15 Zn group was 7 % lower than in the C group (0.67 ± 0.01 ; $P < 0.02$).

TSQ-reactive zinc

TSQ can bind to membrane-bound and loosely bound zinc: pools of zinc that are rapidly available for cellular requirements. After a 48 h incubation in the respective media, the TSQ-reactive zinc content was significantly lower in the 1.5 Zn cells than in the C cells (39 %, 31 % and 52 % for Jurkat, 3T3 and IMR-32 cells respectively) (Figure 1). In Jurkat cells, a trend ($P = 0.10$) towards lower TSQ-reactive zinc was observed in the 15 Zn group compared with the C group. The concentration of TSQ-reactive zinc in the 15 Zn and C cells was similar in 3T3 and IMR-32 cells (Figure 1). Significant correlations were found between the amount

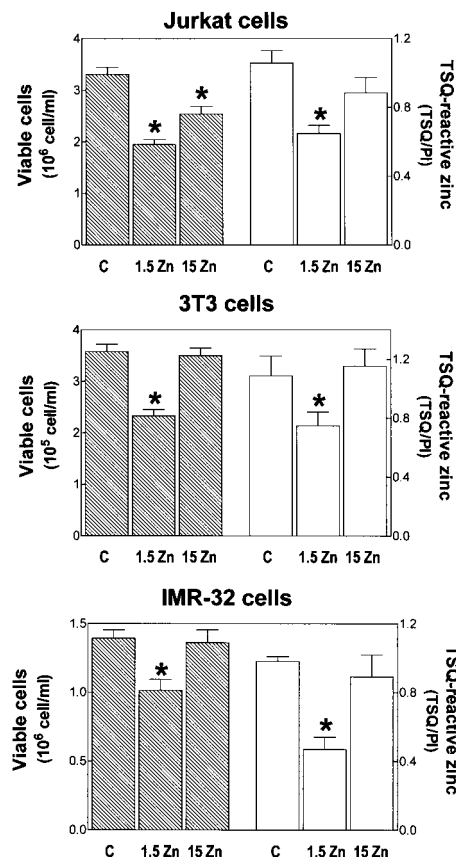


Figure 1 Evaluation of cell viability and TSQ-reactive zinc

Jurkat, 3T3 and IMR-32 cells were incubated for 48 h in control medium (C), or in chelated medium containing 1.5 or 15 μM zinc (1.5 Zn and 15 Zn respectively). Cell viability (Trypan Blue dye exclusion; hatched bars) and TSQ-reactive zinc (open bars) were evaluated. The TSQ/PI ratio was determined as described in the Experimental section. Results are means \pm S.E.M. ($n = 5$). * indicates results significantly different from control values; $P < 0.05$ (one-way ANOVA).

of intracellular zinc and the number of viable cells ($r = 0.99, 0.98$ and 0.99 , for Jurkat, 3T3 and IMR-32 cells respectively).

Evaluation of plasma-membrane fluidity

Plasma-membrane fluidity was evaluated in Jurkat, 3T3 and IMR-32 cells using two fluorescent probes, 6-AS and 16-AP, that sense lipid packing at two different depths in the bilayer. In Jurkat cells, a low 6-AS fluorescence polarization value was observed in the 1.5 Zn group, indicating a higher membrane fluidity in the interfacial region of the bilayer (Figure 2). This effect, a 10 % change in 6-AS fluorescence polarization, was lower than the value obtained for the C and 15 Zn cells ($P < 0.0001$). In Jurkat cells, membrane fluidity in the hydrophobic region of the bilayer was similar in the three groups as assessed with the probe 16-AP (Figure 2).

In 3T3 cells, the 6-AS fluorescence polarization was 16 % lower in the 1.5 Zn cells than in the C cells ($P < 0.0001$); no significant differences were observed between the C and 15 Zn cells (Figure 2). Using the probe 16-AP, membrane fluidity was also higher in the 1.5 Zn group, with a 37 % decrease in the fluorescence polarization with respect to the C group (Figure 2). Differences were not observed between the C and 15 Zn groups.

In the IMR-32 cells, zinc deficiency resulted in an 11 % higher fluorescence polarization of 6-AS compared with the values found in the C cells ($P < 0.005$). Membrane fluidity was similar in the

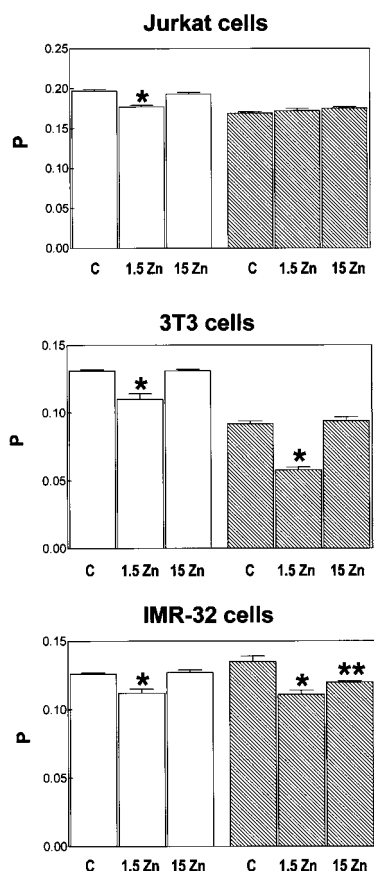


Figure 2 Evaluation of membrane fluidity

Jurkat, 3T3 and IMR-32 cells were incubated for 48 h in control medium (C), or in chelated medium containing 1.5 or 15 μM zinc (1.5 Zn and 15 Zn respectively). Membrane fluidity was evaluated from the changes in fluorescence polarization (P) of the probes 6-AS (open bars) and 16-AP (hatched bars). Results are means \pm S.E.M. ($n=8$). * and ** indicate results significantly different from the values obtained for the C or 1.5 Zn groups respectively; $P < 0.05$ (one-way ANOVA).

15 Zn and C groups. Similar results were observed with the probe 16-AP (Figure 2). This effect, which corresponded to an 11% change in fluorescence polarization, was significantly different

with respect to C cells ($P < 0.005$), and was partially prevented in the 15 Zn group (Figure 2).

To evaluate whether or not the higher plasma membrane fluidity found in the zinc-deficient cells could be associated with changes in fatty-acid profiles, the relative amounts of the individual acyl chains were measured. In the Jurkat cells, a 48 h incubation in zinc-deficient medium did not affect the proportions of the tested fatty acids (Table 1). In 3T3 cells, a 28% decrease ($P < 0.005$) in arachidonic acid ($\text{C}_{20:4}$) was observed in the 1.5 Zn cells compared with the C cells (Table 1). The reduction in arachidonic acid was partially compensated for by a 10% increase in stearic acid ($\text{C}_{18:0}$). Fatty-acid profiles were similar in the 15 Zn cells and the C cells. A trend ($P = 0.21$) towards lower values in the content of arachidonic acid was observed in the 1.5 Zn group of the IMR-32 cells (Table 1). A positive correlation was observed between the amount of arachidonic acid ($\text{C}_{20:4}$) and membrane fluidity as evaluated with 16-AP in the 3T3 ($r = 0.85$; $P < 0.001$) and IMR-32 ($r = 0.61$; $P < 0.05$) cells.

The UI, a parameter that evaluates the proportion of unsaturated fatty acids in the membrane, was similar in both Jurkat and IMR-32 cells, among the groups (Table 1). In 1.5 Zn 3T3 cells, the lower amount of arachidonic acid associated with a higher content of stearic acid resulted in a lower UI (Table 1), indicating that the decrease in the relative proportion of this particular fatty acid was not compensated for by the increase of another unsaturated species.

Evaluation of phosphatidylserine exposure

The possibility that the higher membrane fluidity found in the zinc-deficient cells could be associated with a higher phosphatidylserine exposure in the outer leaflet of the bilayer was investigated. Cells were incubated in the presence of MC, a probe that changes its fluorescent properties in the presence of phosphatidylserine [20]. To correct the results for differences in cell numbers, MC fluorescence was expressed relative to PI fluorescence.

In Jurkat cells, a significantly higher (47%) binding of MC to the plasma membrane was observed in 1.5 Zn cells compared with the C cells ($P < 0.0001$) (Figure 3). The repletion of the zinc-deficient medium with 15 μM zinc partially prevented phosphatidylserine exposure (18% higher values than in C cells). Similarly, in 3T3 and IMR-32 cells, the binding of MC was higher (10% and 11% respectively) in the 1.5 Zn cells than in the C

Table 1 Fatty-acid composition in Jurkat, 3T3 and IMR-32 cells after a 48 h incubation in C, 1.5 Zn or 15 Zn medium

Jurkat, 3T3 and IMR-32 cells were incubated for 48 h in C, 1.5 Zn or 15 Zn medium. After incubation, cells were harvested, and fatty acids were quantified as described in the Experimental section. Results are means \pm S.E.M. ($n=8$).

Fatty acid	Content (%)								
	Jurkat			3T3			IMR-32		
	C	1.5 Zn	15 Zn	C	1.5 Zn	15 Zn	C	1.5 Zn	15 Zn
14:0	2.1 \pm 0.1	2.2 \pm 0.2	2.4 \pm 0.2	2.4 \pm 0.3	2.3 \pm 0.1	2.8 \pm 0.2	3.4 \pm 0.1	3.2 \pm 0.1	3.1 \pm 0.2
16:0	31 \pm 2	30 \pm 1	32 \pm 1	31 \pm 1	32 \pm 1	32 \pm 1	23 \pm 1	25 \pm 1	26 \pm 1
18:0	23.8 \pm 0.8	25.1 \pm 0.3	22.1 \pm 0.2	30 \pm 1	33 \pm 1	30 \pm 1	33 \pm 1	33 \pm 1	32 \pm 1
18:1	19.1 \pm 0.3	19 \pm 1	19.2 \pm 0.8	19 \pm 1	18 \pm 2	19 \pm 1	15 \pm 1	15 \pm 1	15 \pm 1
20:0	2.7 \pm 0.2	3.0 \pm 0.6	3.3 \pm 0.1	1.5 \pm 0.3	1.2 \pm 0.1	1.1 \pm 0.1	3.2 \pm 0.2	2.8 \pm 0.3	3.0 \pm 0.3
20:4	11.2 \pm 0.9	11.1 \pm 0.3	10.9 \pm 0.2	10.9 \pm 0.2	7.8 \pm 0.2*	10.1 \pm 0.3	10.1 \pm 0.3	9.3 \pm 0.4	10.1 \pm 0.4
22:0	3.2 \pm 0.6	2.6 \pm 0.3	3.1 \pm 0.2	1.8 \pm 0.3	2.0 \pm 0.4	1.2 \pm 0.1	3.5 \pm 0.1	3.7 \pm 0.8	3.5 \pm 0.2
22:4	3.0 \pm 0.5	3.0 \pm 0.2	2.9 \pm 0.2	1.3 \pm 0.3	1.9 \pm 0.4	1.4 \pm 0.3	3.7 \pm 0.1	3.5 \pm 0.1	3.2 \pm 0.2
22:6	4.0 \pm 0.2	3.8 \pm 0.2	4.2 \pm 0.1	1.3 \pm 0.2	1.7 \pm 0.2	1.8 \pm 0.2	5.1 \pm 0.3	5.1 \pm 0.3	4.7 \pm 0.4
UI	1.59 \pm 0.07	1.57 \pm 0.06	1.53 \pm 0.06	1.14 \pm 0.06	0.96 \pm 0.07*	1.14 \pm 0.04	1.53 \pm 0.06	1.43 \pm 0.03	1.45 \pm 0.07

* Significantly different from control; $P < 0.05$ (one-way ANOVA).

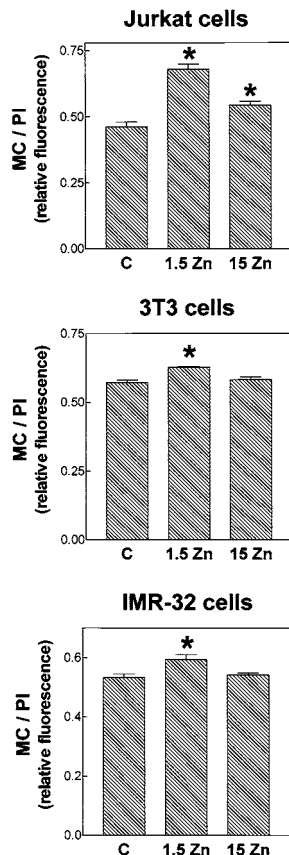


Figure 3 Evaluation of phosphatidylserine exposure to the outer leaflet of the membrane

Jurkat, 3T3 and IMR-32 cells were incubated for 48 h in control medium (C), or in chelated medium containing 1.5 or 15 μM zinc (1.5 Zn and 15 Zn respectively). The amount of phosphatidylserine in the outer layer of cell membrane was evaluated fluorimetrically measuring MC binding, and is expressed relative to the total amount of DNA measured as PI fluorescence. Results are means \pm S.E.M. ($n=8$). * indicates results significantly different from control values; $P < 0.05$ (one-way ANOVA).

cells ($P < 0.005$) (Figure 3). Values for the C and 15 Zn cells were similar.

The MC binding to phosphatidylserine was correlated with the change in plasma membrane fluidity as evaluated with the probe 6-AS. As depicted in Figure 4, the higher contents of phosphatidylserine were associated with higher membrane fluidity in the Jurkat ($r=0.75$; $P < 0.005$), 3T3 ($r=0.84$; $P < 0.001$) and IMR-32 ($r=0.70$; $P < 0.01$) cells.

To determine if the observed correlation between MC binding and membrane fluidity was specific for zinc, Jurkat cells incubated in the zinc-deficient medium were treated with three other bivalent cations (Cu^{2+} , Fe^{2+} and Cd^{2+}) and were incubated for 48 h. At concentrations of 5–30 μM , Cu^{2+} , Fe^{2+} and Cd^{2+} did not reverse the increased MC binding, and decreased 6-AS polarization, induced by zinc deficiency (Table 2).

Evaluation of cell apoptosis

To investigate whether a 48 h incubation in a zinc-deficient medium could lead to cell death by apoptosis, two events were evaluated: DNA fragmentation (as the presence in the cytoplasm of mono- and oligo-nucleosomes) and PARP cleavage by caspase-3. Zinc deficiency resulted in a marked increase in the nucleosome compared with C cells (10-, 13- and 11-fold higher

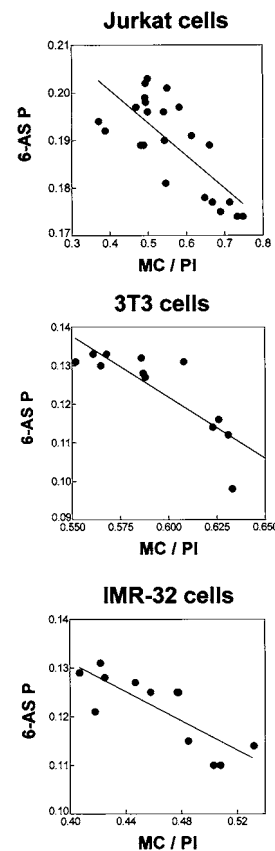


Figure 4 Correlation between MC binding to phosphatidylserine and membrane fluidity

Jurkat, 3T3 and IMR-32 cells were incubated for 48 h in control medium (C), or in chelated medium containing 1.5 or 15 μM zinc (1.5 Zn and 15 Zn respectively). Membrane fluidity in the interfacial region of the membrane was evaluated with the fluorescent probe 6-AS, as described in the legend to Figure 2, and the exposure of phosphatidylserine was evaluated by MC binding, as described in the legend to Figure 3. P, fluorescence polarization.

Table 2 Effects of other bivalent metals (Cu^{2+} , Fe^{2+} and Cd^{2+}) on membrane fluidity, and phosphatidylserine exposure in zinc-deficient Jurkat cells

Jurkat cells were incubated for 48 h in the presence of control medium (C) or chelated medium containing 15 μM zinc (15 Zn) or 1.5 μM zinc (1.5 Zn). At the initiation of the incubation in medium containing 1.5 μM zinc, some of the cells were supplemented with 5–30 μM Cu^{2+} , Cd^{2+} or Fe^{2+} . After 48 h of incubation, phosphatidylserine exposure (MC/PI) and membrane fluidity [changes in membrane polarization (P) of the probes 6-AS or 16-AP] were evaluated as described in the Experimental section. Results are means \pm S.E.M. for three independent experiments.

Treatment	MC/PI	6-AS (P)	16-AP (P)
C	0.462 \pm 0.007	0.197 \pm 0.001	0.169 \pm 0.002
15 Zn	0.544 \pm 0.008	0.193 \pm 0.002	0.175 \pm 0.002
1.5 Zn	0.680 \pm 0.003*	0.177 \pm 0.002*	0.170 \pm 0.003
+ 5 μM Cu^{2+}	0.671 \pm 0.005*	0.169 \pm 0.004*	0.170 \pm 0.001
+ 10 μM Cu^{2+}	0.676 \pm 0.010*	0.171 \pm 0.004*	0.169 \pm 0.002
+ 15 μM Cu^{2+}	0.656 \pm 0.010*	0.173 \pm 0.004*	0.166 \pm 0.003
+ 10 μM Fe^{2+}	0.699 \pm 0.007*	0.172 \pm 0.001*	0.170 \pm 0.003
+ 15 μM Fe^{2+}	0.681 \pm 0.006*	0.174 \pm 0.001*	0.169 \pm 0.001
+ 30 μM Fe^{2+}	0.687 \pm 0.005*	0.175 \pm 0.002*	0.171 \pm 0.002
+ 5 μM Cd^{2+}	0.691 \pm 0.004*	0.173 \pm 0.002*	0.169 \pm 0.001
+ 10 μM Cd^{2+}	0.681 \pm 0.005*	0.174 \pm 0.001*	0.171 \pm 0.002
+ 15 μM Cd^{2+}	0.712 \pm 0.006*	0.173 \pm 0.002*	0.168 \pm 0.002

* Significantly different from control; $P < 0.05$ (one-way ANOVA).

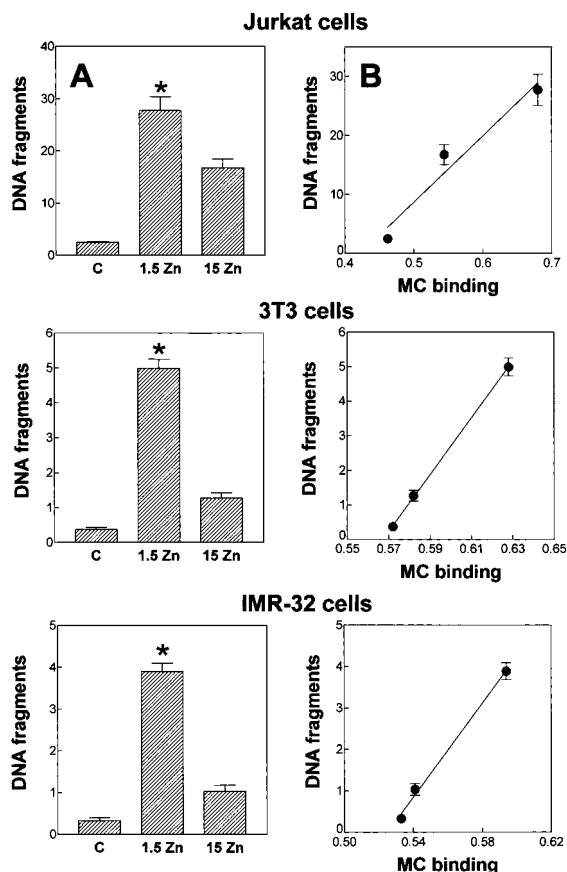


Figure 5 Evaluation of DNA fragment content

Jurkat, 3T3 and IMR-32 cells were incubated for 48 h in control medium (C), or in chelated medium containing 1.5 or 15 μ M zinc (1.5 Zn and 15 Zn respectively). (A) DNA fragment content was evaluated by measuring the amount of cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) using a commercial kit, and expressed relative to the amount of viable cells determined by MTT reduction. Results are means \pm S.E.M. ($n=4$). * indicates results significantly different from control values; $P < 0.05$ (one-way ANOVA). (B) Correlation between DNA fragment content and phosphatidylserine exposure.

for Jurkat, 3T3 and IMR-32 cells respectively) ($P < 0.0001$) (Figure 5A). In the three cell lines, a higher level of DNA fragmentation was observed in the 15 Zn cells than in the C cells. However, the values obtained were significantly lower than those observed in 1.5 Zn cells ($P < 0.005$). Significant correlations were found between the extent of DNA fragmentation and phosphatidylserine exposure in Jurkat ($r = 0.98$; $P < 0.05$), 3T3 ($r = 0.99$; $P < 0.05$) and IMR-32 cells ($r = 0.99$; $P < 0.05$) (Figure 5B).

In all three cell lines, a significantly lower intensity in the band that corresponds to the full-length protein PARP (116 kDa) was observed in the 1.5 Zn cells (Figure 6). In the 1.5 Zn group, the intensity of the band normalized by tubulin content was 87% ($P < 0.001$), 85% ($P < 0.005$) and 23% ($P < 0.02$) lower than that in C group, for Jurkat, 3T3 and IMR-32 cells respectively. In Jurkat and 3T3 cells, the 15 Zn group had showed a PARP content that was 78% and 46% lower than that in the C cells respectively, whereas in IMR-32 cells, the values were similar to those observed in the C group (Figure 6).

DISCUSSION

A decrease in plasma-membrane-bound zinc can lead to alterations in membrane-associated processes, such as the correct ex-

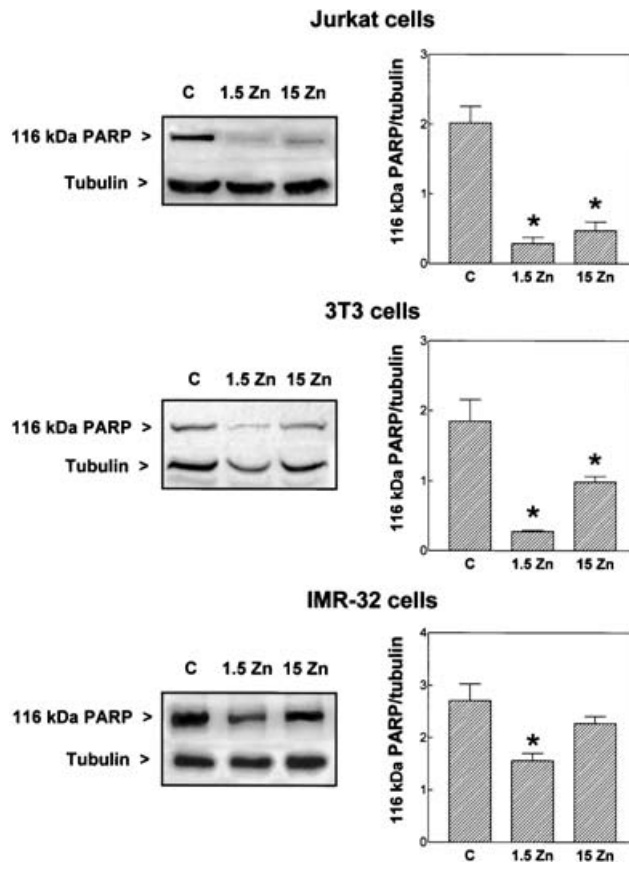


Figure 6 Evaluation of PARP cleavage

Jurkat, 3T3 and IMR-32 cells were incubated for 48 h in control medium (C), or in chelated medium containing 1.5 or 15 μ M zinc (1.5 Zn and 15 Zn respectively). Western blots show the bands corresponding to intact PARP (116 kDa) and β -tubulin from one representative experiment. Graphs show the amount of PARP remaining after a 48 h incubation, corrected by the content of β -tubulin. Results are means \pm S.E.M. ($n=3$). * indicates results significantly different from control values; $P < 0.05$ (one-way ANOVA).

pression of surface receptors, the function of water and ion channels, enzyme activities, and cell signalling [6,22]. These changes may not only affect plasma-membrane-related events, but they can also propagate the effect to the inside of the cell, affecting the concentrations of second messengers and ions [22]. Zinc deficiency can also affect the plasma membrane secondary to the generation of an oxidative stress condition, which can lead to the oxidation of membrane components, and to alterations in membrane lipid composition [18,23].

In the present study, we evaluated, using cultured cells, the hypothesis that zinc deficiency can lead to changes in membrane fluidity and the possible mechanisms involved. With this goal in mind, we incubated Jurkat, 3T3 and IMR-32 cells in media containing variable concentrations of zinc, and evaluated changes in cell viability, intracellular zinc content, membrane fluidity, fatty-acid composition and phosphatidylserine exposure.

After 48 h of culture in a zinc-deficient medium, cell viability was lower than that observed for cells grown in a non-chelated control medium. The repletion of the zinc-deficient medium with 15 μ M zinc partially (Jurkat cells), or completely (3T3 and IMR-32 cells), prevented the loss of viable cells. The lower number of viable cells observed in the 1.5 Zn group could be due to both a lower cell proliferation rate [22] and cell death by apoptosis or necrosis. The relative amount of dead cells in both the 3T3 and IMR-32 groups was lower than in the Jurkat group. This difference

could be due to the fact that 3T3 and IMR-32 cells grow attached to the culture dish; dead cells lose their adhesive properties and are removed along with the culture medium. In contrast, Jurkat cells are grown in suspension, and therefore live and dead cells coexist in the samples.

In the three cell lines tested, the amount of viable cells was significantly correlated with the intracellular concentration of zinc. These results are in accordance with recent work from our laboratory [19], demonstrating that a low concentration of zinc in the incubation medium can result in a decrease in the intracellular zinc content, even in cells incubated for time periods as short as 3 h.

Plasma-membrane fluidity was evaluated using two fluorescent probes, 6-AS and 16-AP. These molecules, when inserted between membrane lipids, polarize the fluorescence in accordance with the fluidity of their environment [24]. The difference between the two probes resides in the location of the anthroyloxy moiety that acts as a sensor. In 6-AS, the anthroyloxy group is situated near to the membrane water/lipid interface that contains the phospholipid headgroup, whereas in 16-AP, it is located in a deeper region of the bilayer, containing the hydrocarbon chains of phospholipids.

Using 6-AS, a low plasma-membrane fluidity was observed in the zinc-deficient cells relative to the control cells; this effect did not depend on the nature of the cell line investigated. The average change in membrane fluidity in the cells was approx. 10%. Although this change in membrane fluidity might be considered to be small, several previous reports have demonstrated that minute variations in membrane fluidity can affect the activity of membrane-associated enzymes [15,25,26], intracellular transport [27,28], the functionality of receptors [16,17,29,30] and signal transduction [31].

The alteration of membrane fluidity in the polar region of the membrane in the zinc-deficient cells could be a consequence of the higher translocation of phosphatidylserine towards the outer monolayer of the cell. Using liposomes composed of phosphatidylcholine, we have observed that the membrane becomes more fluid as the percentage of phosphatidylserine increases (S. V. Verstraeten and P. I. Oteiza, unpublished work). For example, when phosphatidylserine was present in the outer monolayer in a 0.2 molar fraction, the change in the fluorescence polarization of the probe 6-AS corresponded to a 10% decrease. The present results are in agreement with those reported by Jessel et al. [32], who, using EL 4 cells incubated in the presence of staurosporine to induce cell apoptosis, observed that a higher membrane fluidity was associated with a higher phosphatidylserine translocation, identified through the annexin V-fluorescein assay.

Zinc deficiency affected membrane fluidity not only at the water/lipid level, but also in the hydrophobic portion of the membrane. Interestingly, this effect was only observed in two of the three cell lines assessed (3T3 and IMR-32). In 3T3 cells, in which zinc deficiency had the greatest impact on fatty-acid composition, with a lower (28%) content of arachidonic acid, and higher (10%) content of stearic acid (18:0), the largest increase of fluidity in the hydrophobic part of the membrane was observed. In this cell line, a significant correlation was found between the content of arachidonic acid and membrane fluidity as evaluated with 16-AP, suggesting that the changes in the ordering of lipids in the hydrophobic portion of the bilayer could be due to alterations in fatty-acid composition. Although the decrease in arachidonic acid content in IMR-32 cells was less dramatic than in 3T3 cells, a significant correlation between arachidonic acid content and membrane fluidity was observed. The lower content of arachidonic acid observed in the 3T3 and IMR-32 cells is in accordance with previous reports [33,34] that zinc deficiency results in decreased activities of $\Delta 5$ and $\Delta 6$ desaturases. These enzymes, involved in the synthesis of long-chain unsaturated fatty

acids, including 20:4 and 22:5 ω 6, are particularly relevant in the maintenance of membrane homeostasis [33,34]. It was reported that the decrease in these fatty acids was partially compensated for by an increase in the total content of 22:6 ω 3. In 3T3 cells, a trend towards higher values of 22:6 was observed in the content of the fatty acid.

It is known that zinc deficiency can induce apoptosis in cell and animal models [11,12]. The higher exposure of phosphatidylserine in the outer monolayer of the plasma membrane observed in the zinc-deficient cells may be related to the oxidative stress condition that can be triggered by zinc deficiency [23,35–43]. In a previous study, where 3T3 cells were submitted to similar experimental conditions to those used in the present study, we observed a significantly higher content of oxidants in the zinc-deficient cells with respect to control cells [18]. It has been demonstrated that, as a consequence of the increase in the intracellular levels of oxidants, plasma membrane phosphatidylserine can become particularly oxidized [44]. Oxidized phosphatidylserine can be externalized either spontaneously or via the enzymic action of the scramblase, and acts as a recognition signal for the macrophage scavenger receptor [44–47], resulting in a rapid engulfment of the apoptotic cell [48].

Although it has been proposed that phosphatidylserine exposure is an early event in the apoptotic pathway, preceding cell shrinkage and nuclear condensation [46], the present results indicate that, in zinc deficiency, it occurs at a later stage of apoptosis, when DNA damage has already begun. For example, in IMR-32 cells, a 24 h zinc deficiency produced a significant increase in caspase-3 activity, while phosphatidylserine externalization was still undetectable (results not shown). In accordance, after a 48 h incubation in zinc-deficient medium, the nucleosome content was 11 times higher than in control cells, whereas the MC binding was only 11% higher. The fact that, in these cells, phosphatidylserine externalization is not an early event in apoptosis could be related to a longer period of zinc deficiency being required to obtain a significant accumulation of intracellular oxidant species [18,49].

Caspase-3 activity in the three cell lines was also evaluated by measuring PARP cleavage, a nuclear enzyme involved in DNA repair that participates in the early events of apoptosis [50]. Working with lymphocytes in culture, Marini et al. [50] reported that a mild zinc deficiency induced by a 4 h cell treatment with the metal chelator TPEN [*N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine] does not induce PARP cleavage. However, when cells were previously incubated with 50 μ M H₂O₂, and afterwards treated with 3 μ M TPEN, a significant decrease in the full-length PARP was observed, indicating that both zinc deficiency and oxidative stress are necessary for PARP cleavage [50]. In our experimental model, a marked decrease in full-length PARP was observed in the three cell lines assessed after a 48 h zinc deficiency, an effect that was partially (Jurkat and 3T3) or totally (IMR-32) prevented by repletion of the medium with 15 μ M zinc. The low intracellular levels of zinc, or a higher production of H₂O₂ [49], could be involved in the triggering of apoptosis in the zinc-deficient cells.

The observation that the other bivalent cations (Cu²⁺, Fe²⁺ and Cd²⁺) did not reverse either the increased phosphatidylserine exposure or the increased membrane fluidity, associated with a decrease in intracellular zinc, underscores the fact that zinc has specific effects in membranes. This specificity should not be too surprising, given the observation that, despite the fact that the essential metals Cu²⁺, Fe²⁺ and Zn²⁺ have very similar atomic masses, which result in them being able to bind to similar ligands and share some similar transport mechanisms, they exert very different effects in membranes as well as in the cytoskeleton.

In summary, the present results indicate that zinc deficiency induces a decrease in the lipid ordering of the membrane. Membrane fluidification could be mediated by two different mechanisms. First, zinc deficiency leads to a higher exposure of phosphatidylserine in the outer leaflet of the membrane. Secondly, zinc deficiency causes a decrease in the content of polyunsaturated fatty acids. While the former mainly acts on membrane fluidity at the water/lipid interface, the latter alters the fluidity of the hydrophobic core of the bilayer. Taken together, both phosphatidylserine externalization and a decrease in arachidonic acid content contribute to the fluidification of membranes in zinc deficiency.

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