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Labile intracellular zinc is associated with 3T3 cell growth

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Abstract

Increasing evidence shows that labile intracellular zinc is metabolically important. Depletion of labile intracellular zinc using chelators suppresses DNA synthesis. In this study, we tested the hypothesis that labile intracellular zinc could be modulated *via* varying zinc nutrition. This could result in an altered availability of labile intracellular zinc, which, in turn, could influence zinc-dependent cellular events involved in cell proliferation and ultimately suppress growth. Labile intracellular zinc was detected by using N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ), a membrane-permeable fluorescence probe. After 48 h culture in a zinc-depleted medium, labile intracellular zinc in 3T3 cells was diminished along with a suppressed DNA synthesis and cell proliferation. In contrast, supplementation of zinc to the zinc-depleted medium increased the labile intracellular zinc and promoted DNA synthesis and cell proliferation. Furthermore, growth factor-dependent stimulation of DNA synthesis and cell proliferation was also accompanied by increased labile intracellular zinc. Together, our data showed an association between the labile intracellular zinc, detected using TSQ, and 3T3 cell growth, suggesting that labile intracellular zinc could be an important cellular link between zinc nutrition and growth.

Keywords: Zinc; Labile intracellular zinc; 3T3 cells; Cell proliferation; DNA synthesis

1. Introduction

Zinc is an essential trace-element required for growth. Growth retardation is a principal sign of zinc deficiency in humans and animals [1], but the underlying mechanisms remain to be elucidated. In rats, severe zinc deficiency retards growth while tissue zinc concentration often remains unaffected [2–3]. In cultured cells, zinc deprivation induced by extracellular cation chelators suppresses the proliferation of numerous types of cells [4–7]. 3T3 cells are the most frequently used model for understanding the influence of zinc on growth. In 3T3 cells, zinc depletion using diethylenetrinitrilopenta acetate (DTPA), an extracellular divalent cation chelator, nearly completely suppressed DNA synthesis [8]. This suppressive effect of DTPA on DNA synthesis was reversed by adding zinc, but not other metals such as calcium or iron, indicating that this DTPA-induced suppression of DNA synthesis was zinc specific. It is interesting to note that while DNA synthesis was suppressed by DTPA, total cell zinc concentration determined using atomic absorption spectrophotometer, remained unchanged [8]. Therefore, it is possible that the effect of zinc deprivation, whether induced by dietary zinc deficiency or by chelators, on growth are mediated *via* a change in intracellular zinc and this change is too small to be detected by conventional determination of total zinc concentration.

The labile intracellular zinc appears to be metabolically important. Labile intracellular zinc can be visualized microscopically using membrane-permeable fluorescent probes such as N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ) [9] and Zinquin [10]. These fluorescent probes are highly selective for zinc in biological systems. The brightness of the fluorescence is a measure of zinc concentration [9–13]. Adding NNN′N′-tetrakis-(2-pyridylmethyl)ethlenediamine (TPEN), a membrane-permeable zinc chelator, to the culture media results in nearly total inhibition of DNA synthesis and a TPEN concentration-dependent reduction of TSQ fluorescence in 3T3 cells and Ha-*ras*$^{val\text{-}12}$-expressing 3T3 cells [14]. If zinc is added together with TPEN, DNA synthesis and TSQ fluorescence are restored. Clearly, labile intracellular zinc is important to DNA synthesis, but manipulating intracellular zinc using chelator is by no means physiological. Therefore, it becomes important to know whether altering zinc nutrition has an influence on the labile intracellular zinc and the related physiological events.

In this study, we tested the hypothesis that labile intra-

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cellular zinc could be modulated *via* varying zinc nutrition. This would result in an altered availability of the labile intracellular zinc, which, in turn, could consequently influence zinc-dependent cellular events involved in cell proliferation and ultimately influence growth. Labile intracellular zinc was detected and visualized using TSQ. The relative brightness of TSQ fluorescence in cells was used as an indication of the effect of zinc nutrition on labile intracellular zinc. Results reported herein showed that zinc depletion suppressed DNA synthesis and cell proliferation in 3T3 cells along with a diminished TSQ fluorescence. In contrast, zinc supplementation resulted in an increased DNA synthesis and cell proliferation in 3T3 cells along with much brighter TSQ fluorescence. We further demonstrated that growth-factor dependent stimulation of DNA synthesis and cell proliferation were also accompanied by an increased TSQ fluorescence. Together, our data showed an association between the labile intracellular zinc, detected using TSQ, and 3T3 cell growth, suggesting that the labile intracellular zinc could be an important cellular link between zinc nutrition and growth.

2. Methods and materials

2.1. Cell culture system and treatments

3T3 Swiss murine fibroblasts (CCL-92; ATCC, Manassas, Virginia) were maintained at 37°C in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS), 584 mg/L L-glutamine, 4.5 g/L glucose, 110 mg/L sodium pyruvate and pyridoxine hydrochloride, 1.5 g/L sodium bicarbonate, 50 μg/ml streptomycin, 50 units/ml penicillin, and 2.5 μg/ml Fungizone in an atmosphere containing 5% CO_2.

DMEM contains no zinc while FBS does. DMEM is usually supplemented with 10% FBS providing a final zinc concentration of 4–5 μmol/L zinc [15,16]. To prepare a zinc-depleted medium, FBS was batch-treated with Chelex-100 (Bio-Rad, Montreal, Quebec; 50 g Chelex-100/500 ml FBS) in an ice-bath for 24 h followed by passing the batch-treated FBS through a Chelex-100 column (60 g Chelex-100, 1 cm diameter). Finally, the Chelex-100 treated FBS was sterilized immediately by membrane filtration (NALGENE PES low-protein binding filter; 0.2 μm). Then the Chelex-100-treated FBS was added to DMEM to formulate the zinc-depleted medium with a final zinc concentration of 0.1 μmol/L zinc. To assess the modulating effects of zinc nutrition on labile intracellular zinc and the subsequent effects on cell growth, the zinc-depleted medium was supplemented with 5 μmol/L zinc as $ZnSO_4$ to mimic the zinc concentration presented in most of the regular media, or with 10 or 20 μmol/L zinc as $ZnSO_4$ to form the high zinc groups.

Cells (passage 121 or 122) were cultured in DMEM supplemented with 10% FBS for 72 h at an initial seeding density of 1×10^5 cells per T75 flask followed by the induction of quiescence in low-serum DMEM (1% FBS) for 48 h. Upon induction of quiescence, cells were cultured in the zinc-depleted medium supplemented with 0, 5, 10, or 20 μmol/L zinc for 48 h followed by biochemical analyses. At the time of harvesting, cells did not reach total confluency.

To further assess the relationship between labile intracellular zinc and cell growth, cells were cultured in the zinc-supplemented medium (20 μmol/L zinc) with or without the presence of a combination of growth factors (platelet-derived growth factor (PDGF), 100 ng/ml; epidermal growth factor (EGF), 50 ng/ml; and insulin-line growth factor-I (IGF-1), 20 ng/ml). The same culture system and experimental protocol described above were used in this series of experiment except the initial seeding density, which were 1×10^4 cells per T25 flask.

2.2. Labile intracellular zinc assay

TSQ (Molecular Probes, Eugene, Oregon) stock solution (5 mmol/L) was prepared by dissolving TSQ (25 mg) in 100% hot ethanol and stored at 4°C till use. The TSQ working solution (80 μmol/L) was prepared by diluting the stock solution with phosphate-buffered saline (PBS; pH 7.4) immediately prior to use.

For assessing the labile intracellular zinc using fluorescence microscopy, cells were seeded on a histological slide in a 100-mm Petri dish. The culture system and experimental protocol were the same as described above. After culturing in the treatment media for 48 h, the media was completely removed and the slide was rinsed 3 times with PBS (pH 7.4) to remove traces of the media. Subsequently, the cells were stained with TSQ working solution for 15 min at room temperature. After staining, the cells were carefully rinsed 3 times with PBS (pH 7.4) to remove excess TSQ solution. The fluorescence images of the cells were obtained by passing the fluorescence emissions through a long pass emission filter (cut-off: 400 nm; Omega Optical, Brattleboro, Vermont) to exclude scattered UV rays and studied using a UV fluorescent microscope (Axiophot, Carl Zeiss, Don Mills, Ontario). Camera settings (shutter speed and aperture) were the same for all the photomicrographs. The relative brightness of the fluorescence images among the treatments was used as an indication of the labile intracellular zinc.

2.3. ^{3}H-thymidine incorporation assay and cell count

^{3}H-thymidine incorporation was assessed by using an established procedure [17] with some modifications. After a 44 h culture in the assigned treatment medium, cells were labeled with 2 μCi ^{3}H-thymidine for 4 h at 37°C. The media was then removed and the cells were rinsed twice with 2 ml of ice-cold PBS (pH 7.4). Subsequently, the cells were washed with 2 ml of 5% ice-cold TCA for 10 min followed by a second TCA

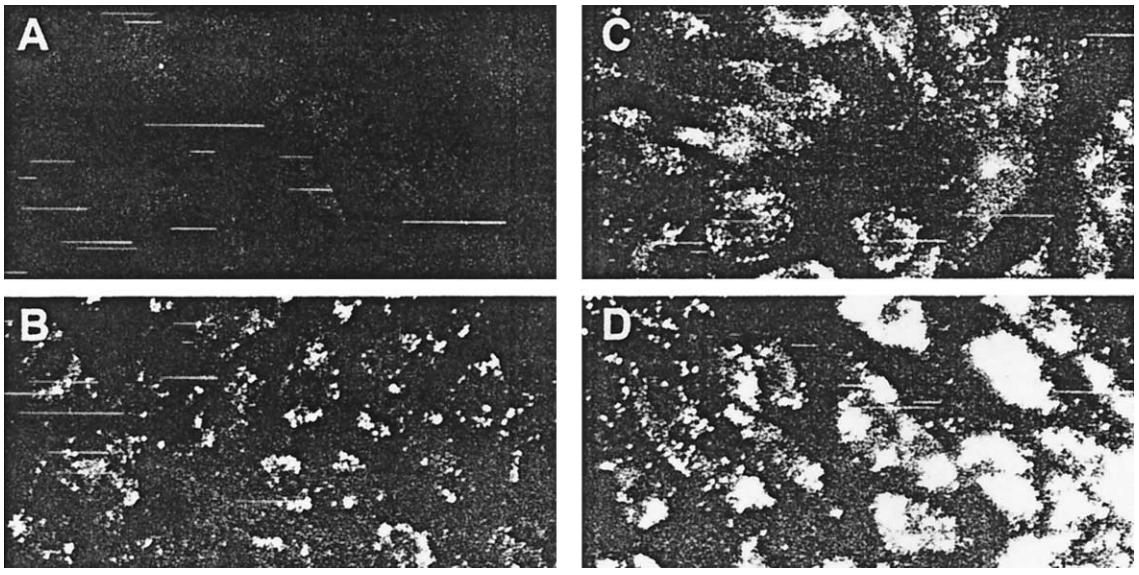

Fig. 1. Zinc-dependent TSQ fluorescence in 3T3 cells. After the induction of quiescence, TSQ fluorescence in 3T3 cells cultured in zinc-depleted medium (A), or zinc-depleted medium supplemented with 5 (B), 10 (C), or 20 (D) μmol/L zinc for 48 h was photographed (×200).

wash for 5 min. After washing the cells twice with 95% ethanol (5 min/wash), the cells were lysed in 1 ml of 1 mol/L NaOH. Half of the lysate (0.5 ml) was transferred to a liquid scintillation counting vial and neutralized with an equal volume of 1 mol/L HCl. The radioactivity in the lysate was determined by liquid scintillation counting (Beckman LS-6500 Multi-purpose Scintillation Counter, Fullerton, CA). The remaining half of the lysate (0.5 ml) was used for protein determination using the Lowry's procedure [18]. The amount of radioactivity was normalized on a per mg protein basis and used as a measure of ^{3}H-thymidine incorporation. Total cell numbers were counted using a hematocytometer to assess cell proliferation.

2.4. Statistical analysis

The significant differences among the treatment means were analyzed using ANOVA followed by Turkey's honest significant difference procedure ($p < 0.05$) (The SAS System for Windows Release 6.12).

3. Results

3.1. Zinc-nutrition affected the labile intracellular zinc detected using TSQ

TSQ fluorescence was visible in the cytoplasm, but much less visible in the nucleus of the cells regardless of the medium zinc concentration (Fig. 1). Compared to the cells cultured in the zinc-depleted medium (Fig. 1A), cells cultured in the zinc-supplemented media were much brighter in TSQ fluorescence (Fig. 1B–1D), indicating zinc-dependent changes in the labile intracellular zinc. Among the zinc-supplemented groups, an increase of zinc supplementation from 5 to 20 μmol/L zinc resulted in increased labile intracellular zinc.

3.2. Zinc supplementation increased the abundance and brightness of TSQ fluorescent 'spots'

Highly intense TSQ fluorescent 'spots' were clearly visible in the cytoplasm of the cells cultured in zinc-supplemented media (Fig. 1B–1D), but were absent in the cells cultured in the zinc-depleted medium (Fig. 1A). These TSQ fluorescent 'spots' appeared to be more abundant in cells cultured in medium supplemented with 20 μmol/L zinc than in cells cultured in medium supplemented with 5 or 10 μmol/L zinc. These observations suggest that the presence and abundance of these TSQ fluorescent 'spots' are a function of media zinc concentration.

3.3. Zinc-dependent stimulation of DNA synthesis

^{3}H-thymidine incorporation assay was performed to assess the influence of zinc on DNA synthesis (Fig. 2). Compared to cells cultured in the zinc-depleted medium, the cells cultured in the media supplemented with 5, 10 and 20 μmol/L zinc showed increased ^{3}H-thymidine incorporation by 5, 25, and 34%, respectively. This zinc-dependent stimulation of ^{3}H-thymidine incorporation reached a significant level when the zinc-depleted medium was supplemented with 20 μmol/L zinc ($p < 0.05$), indicating a zinc-dependent promotion of DNA synthesis.

3.4. Zinc-dependent stimulation of cell proliferation

The effects of varying zinc nutrition on cell number were used as a measure of cell proliferation (Fig. 3), Compared to

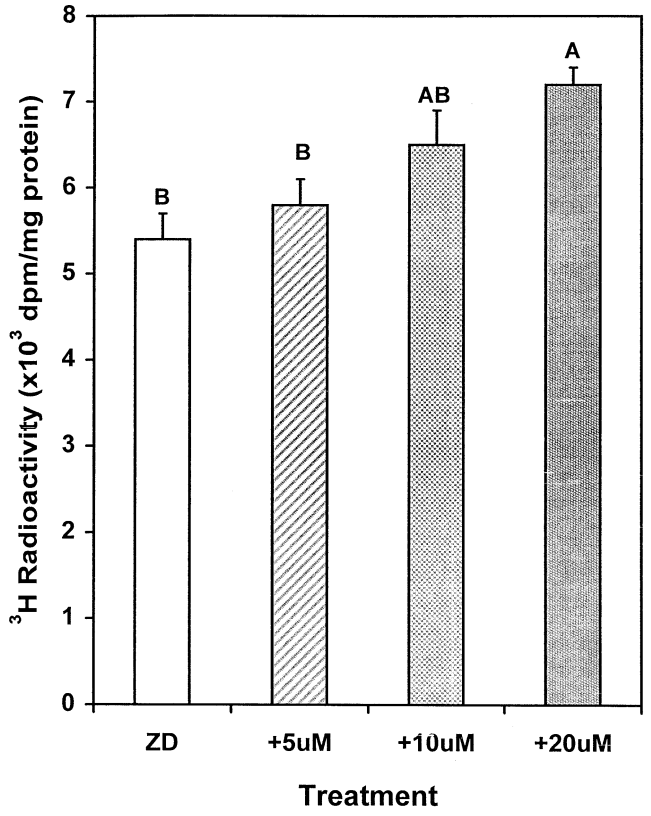

Fig. 2. Zinc-dependent stimulation of ^{3}H-thymidine incorporation in 3T3 cells. 3T3 cells were cultured in the zinc-depleted medium (ZD) or the zinc-depleted medium supplemented with 5 (+5), 10 (+10), or 20 (+20) μmol/L zinc for 44 h following the induction of quiescence. Subsequently, the cells were labeled with ^{3}H-thymidine (2 μCi/flask) for 4 h at 37°C. Values are means ± SEM (n = 6, except ZD where n = 5). Means with different superscripts are statistically significantly different ($p < 0.05$).

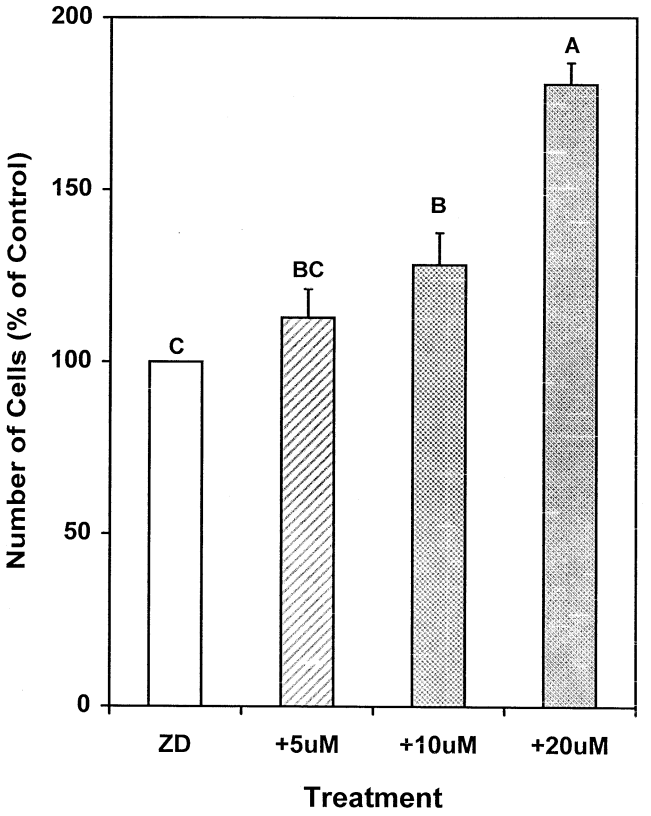

Fig. 3. Zinc-dependent stimulation of 3T3 cell proliferation. 3T3 cells were cultured in the zinc-depleted medium (ZD) or zinc-depleted medium supplemented with 5 (+5), 10 (+10), or 20 (+20) μmol/L zinc for 48 h following the induction of quiescence. Values are means ± SEM (n = 6). Number of cells in the zinc-depleted medium group was 1.1 × 10^5 cells/ml. Means with different superscripts are statistically significantly different ($p < 0.05$).

the cells cultured in the zinc-depleted medium, a supplementation of 5 μmol/L zinc resulted in a moderate increase in cell number (13%). With further increase of zinc supplementation to 10 and 20 μmol/L, cell number was increased by 28 and 81% ($p < 0.05$), respectively, over the cells cultured in the zinc-depleted medium.

3.5. Growth factor-dependent stimulation of DNA synthesis and cell proliferation, and increase in the labile intracellular zinc

In the first series of experiments, we observed a zinc-dependent increase in the brightness of TSQ fluorescence along with an increased DNA synthesis and cell proliferation, indicating an association between the labile intracellular zinc and cell growth. To further test this association, a combination of PDGF, EGF, and IGF-I was used to stimulate cell growth with the presence of 20 μmol/L zinc. In response to growth factor treatment, ^{3}H-thymidine incorporation and cell numbers were increased by 40% (Fig. 4) and 50% (Fig. 5), respectively, compared to the non-growth factor control ($p < 0.05$). This demonstrated a growth factor-dependent stimulation of DNA synthesis and cell proliferation. Along with this increased DNA synthesis and cell proliferation, growth factor treatment also clearly increased labile intracellular zinc as shown by a brighter TSQ fluorescence when compared to the control (Fig. 6).

4. Discussion

In biological systems, TSQ fluorescence is zinc specific [9,11,12] and the brightness of the TSQ fluorescence is a measure of labile intracellular zinc [9,14]. Depleting labile intracellular zinc using intracellular chelators TPEN and D-penicillamine diminishes TSQ fluorescence in 3T3 cells [14] and hamster sperm [12], respectively. Similarly, TPEN also diminishes the fluorescence of zinquin, which is also a membrane-permeable zinc-specific fluorescent probe, in lymphocytes [10]. This chelator-induced abolishment of fluorescence can only be reversed by adding zinc ions, but not calcium or magnesium ions, back to the system [12]. Similar to the chemical manipulation of labile intracellular zinc, the data reported herein showed that altering the zinc

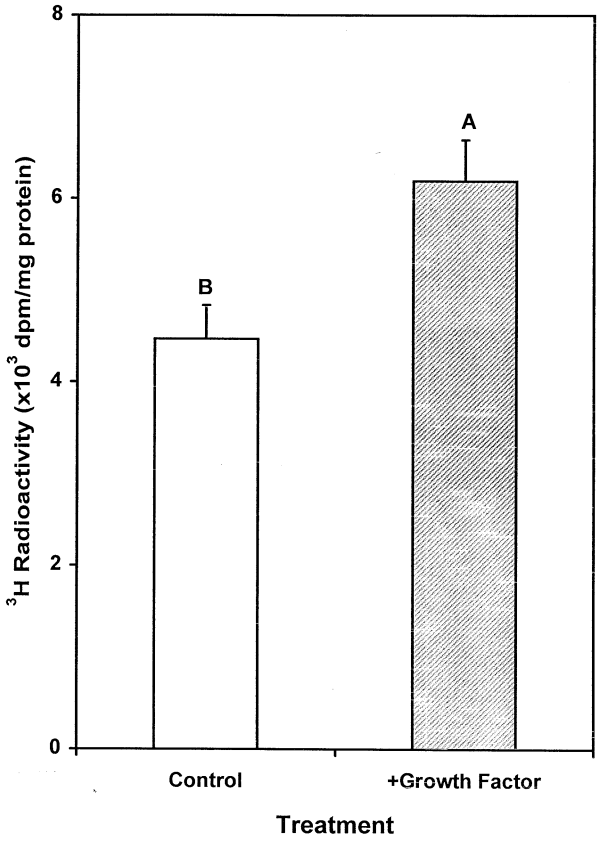

Fig. 4. Growth factor-dependent stimulation of ^{3}H-thymidine incorporation in 3T3 cells. After the induction of quiescence, 3T3 cells were cultured in the zinc-depleted medium supplemented with 20 μmol/L zinc and with or without the presence of growth factors (PDGF, EGF, and IGF-1) for 44 h. Subsequently, the cells were labeled with ^{3}H-thymidine (2 μCi/flask) for 4 h at 37°C. Values are means ± SEM ($n = 6$). Means with different superscripts are statistically significantly different ($p < 0.05$).

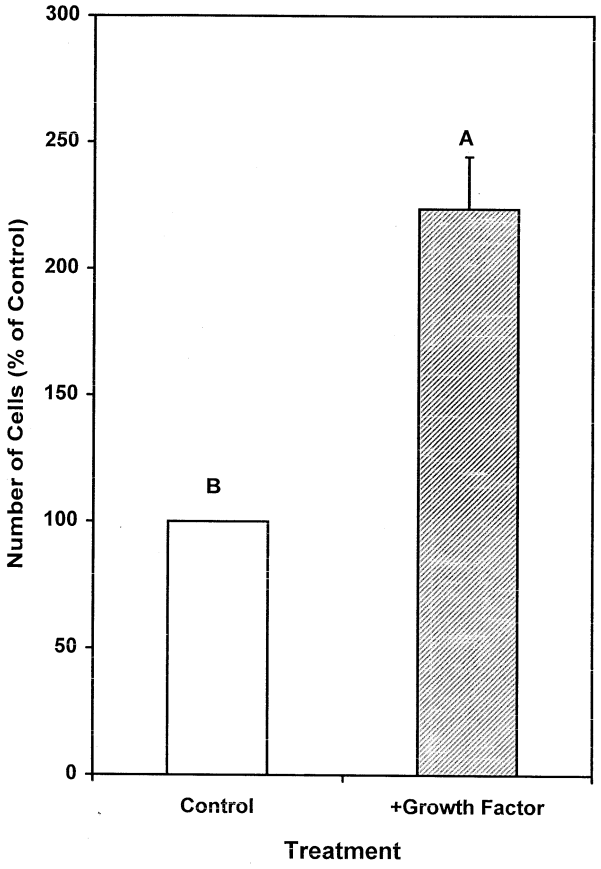

Fig. 5. Growth factor-dependent stimulation of 3T3 cell proliferation. After the induction of quiescence, 3T3 cells were cultured in the zinc-depleted medium supplemented with 20 μmol/L zinc and with or without the presence of growth factors (PDGF, EGF and IGF-1) for 48 h. Values are means ± SEM ($n = 6$). Number of cells in the control group was 2.3×10^5 cells/ml. Means with different superscripts are statistically significantly different ($p < 0.05$).

concentration in culture media also resulted in corresponding changes in the labile intracellular zinc in 3T3 cells. That is: zinc-depletion diminished the labile intracellular zinc while zinc supplement increased labile intracellular zinc. These observations suggest that zinc nutrition have a direct influence on labile intracellular zinc detected using TSQ in 3T3 cells.

In this study, the effect of zinc nutrition on DNA synthesis, a necessary step in cell replication, and cell proliferation appeared to be correlated with the effect of zinc nutrition on the labile intracellular zinc. More importantly, growth factor-dependent stimulation of DNA synthesis and cell proliferation was also accompanied by increased labile intracellular zinc detected using TSQ. In the growth factor study, the media zinc concentration was the same between the groups. Therefore, a co-increase in labile intracellular zinc and cell growth strongly argues for an association between labile intracellular zinc and cell proliferation. The biochemical significance of this association is presently unclear. However, it is known that zinc is required for cell replication. Zinc is a structural component of functional DNA polymerase [19] and RNA polymerase [20], the primary enzymes involved in DNA and RNA synthesis, respectively. Moreover, low zinc availability induced by chelator reduced the thymidine kinase mRNA level [21,22] and its activity [22,23], resulting in a reduced DNA synthesis [24,25]. Furthermore, zinc depletion induced by chelator also causes G_1/S arrest [21,22]. When labile intracellular zinc was depleted with TPEN, which has a high affinity towards zinc ions and readily out-competes TSQ derivatives for zinc [26], DNA synthesis in 3T3 cells and the Ha-*ras*$^{val\text{-}12}$-expressing 3T3 cells was inhibited and TSQ

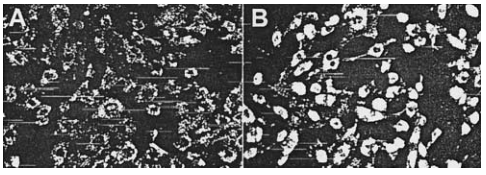

Fig. 6. Increased TSQ fluorescence in growth factor-treated 3T3 cells. After the induction of quiescence, TSQ fluorescence in 3T3 cells cultured in the medium either without (A) or with growth factors (PDGF, EGF and IGF-1) (B) for 48 h was photographed (×100).

fluorescence was abolished [14]. This TPEN induced inhibition of DNA synthesis can be removed by adding zinc back to the system. These observations support the concept that labile intracellular zinc is of vital importance to cellular events involved in cell replication. When cells are stimulated by external signals such as growth factors to undergo division, the demand for zinc to sustain the zinc-dependent cellular events involved in cell proliferation is expected to increase. Since majority of the intracellular zinc is bound to proteins and thus not metabolically available, and free zinc ion concentration is extremely low, an increased demand on zinc to sustain cell proliferation would logically be met by increasing zinc influx. This increase in zinc influx would then result in an increase in labile intracellular zinc, which is detectable using fluorescence probes such as TSQ. Therefore, the corresponding changes between labile intracellular zinc detected using TSQ and DNA synthesis and cell proliferation reported herein provides direct evidence showing an association between labile intracellular zinc and cell growth.

Highly intense TSQ fluorescent 'spots' were clearly visible in cells cultured in zinc supplemented media, but not in the zinc-depleted medium. Since TSQ fluorescence is specific to zinc [9,11], these 'spots' with intense fluorescence represented areas of high zinc concentration. Palmiter et al. [27] reported that when zinc-sensitive BHK cells are transfected with ZnT-2, a zinc transporter localized on vesicles, these cells can tolerate otherwise toxic media zinc concentrations with a 10–20 fold increase in total cell zinc concentration. Studies using Zinquin, another zinc-specific fluorescent probe, revealed an accumulation of zinc in these vesicles [27]. More recently, O'Halloran and co-workers demonstrated that these vesicles are possibly intracellular storage compartments of zinc [28]. In this study, the abundance of these 'spots' with intense TSQ fluorescence seemed to be correlated with the media zinc concentration. Furthermore, zinc supplementation promoted both DNA synthesis and cell proliferation. We postulate that these 'spots' with intense TSQ fluorescence were the sites where zinc accumulate to provide metabolically active zinc for sustaining cell proliferation. Thus, these intensely fluorescent 'spots' could reflect an increased zinc influx in cells undergo replication.

In summary, this study provided evidence that zinc nutrition had a direct influence on labile intracellular zinc detected using TSQ in 3T3 cells. Corresponding with the changes in labile intracellular zinc, altering zinc nutrition also influenced DNA synthesis and cell proliferation, indicating an association between labile intracellular zinc and cell growth. This association was affirmed by the observation that growth factor-dependent stimulation of cell growth was accompanied by an increase in labile intracellular zinc. The apparent association between labile intracellular zinc detected using TSQ and cell growth suggests that labile intracellular zinc could be a cellular link between zinc nutrition and growth.

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