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ZINC INCREASES EGF-STIMULATED DNA SYNTHESIS IN PRIMARY MOUSE HEPATOCYTES

STUDIES IN TUMOR PROMOTER-TREATED CELL CULTURES

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Abstract—To investigate factors influencing cell proliferation, cells are often cultured in serum-free medium. In the present study it is shown that addition of zinc chloride (40 μM) to primary mouse hepatocytes, cultured in Dulbecco's minimal essential medium, markedly enhanced epidermal growth factor (EGF)-stimulated [^{3}H]thymidine incorporation into DNA. Treatment of cell cultures with phenobarbital or 3,4,3′,4′-tetrachlorobiphenyl (enzyme inducers and tumor promoters *in vivo*) or with 12-*O*-tetradecanoylphorbol-13-acetate (the classical skin tumor promoter) further increased EGF-stimulated DNA synthesis. The results emphasize the need to adequately substitute zinc in serum-free cultured cells.

Focal proliferation of putative preneoplastic hepatocytes appears to play a critical role at the stage of tumor promotion in experimental hepatocarcinogenesis [1, 2]. Indeed, enhanced DNA synthesis following various tumor promoting agents has been observed *in vivo* [1–3] and *in vitro* [4–6]. To study cell proliferation *in vitro*, primary hepatocyte culture experiments are generally carried out in serum-free medium containing no zinc. However, zinc is known to be essential for the biosynthesis of nucleic acids, mainly through its involvement in the normal functioning of thymidine kinase and DNA polymerase [7–9] and zinc deficiency *in vivo* is associated with a reduced growth rate of normal and neoplastic tissues [10, 11].

From studies of zinc kinetics and zinc metabolism in cultured hepatocytes, an ongoing exchange between intra- and extracellular zinc has been determined [12] and total zinc turnover was estimated to occur within 30 hr. Furthermore, a functional zinc deficiency was found by Guzelian *et al.* [13] in hepatocytes cultured in zinc deficient medium.

In the present study it was demonstrated that addition of zinc to the culture medium of primary hepatocytes increases the rate of EGF-stimulated DNA synthesis and therefore improves culture conditions for the study of factors influencing cell proliferation *in vitro* like phenobarbital (PB†), 3,3′, 4,4′-tetrachlorobiphenyl (TCB) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). PB and TCB were studied because they represent classical liver tumor promoters and enzyme inducers of the PB- and 3-methylcholanthrene-type [3, 14]. TPA was chosen as the classical tumor promoting agent in skin [15, 16].

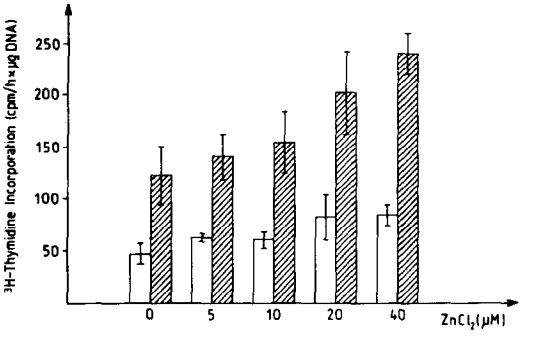

Fig. 1. Dose-dependent increase of [^{3}H]thymidine incorporation into DNA of primary mouse hepatocytes cultured in the presence of various concentrations of zinc. Hepatocytes were harvested after 48 hr. Open bars represent cultures in the absence of, and hatched bars cultures in presence of, EGF. Means ± SD of N = 5 culture plates.

MATERIALS AND METHODS

Dulbecco's minimal essential medium (DMEM), penicillin G and streptomycin were obtained from Biochrom (Berlin, F.R.G.). Fetal calf serum was purchased at Gibco/BRL (Eggenstein, F.R.G.), insulin, dexamethasone, proline and 12-*O*-tetradecanoylphorbol-13-acetate at Sigma Chemical Co. (Deisenhofen, F.R.G.). EGF was obtained from Collaborative Research Inc. (Waltham, U.S.A.), TCB from Riedel de Haen (Seelze, F.R.G.) and [^{3}H]thymidine from Amersham-Buchler (Braunschweig, F.R.G.).

Hepatocytes from male C57BL/6 mice were isolated by collagenase perfusion *in situ* [17]. Viability as determined by trypan blue dye exclusion was around 80%. Cells were cultured in a medium containing Dulbecco's minimal essential medium supplemented with 0.1 μM insulin, 0.1 μM dexamethasone, proline (20 μg/mL), 10% fetal calf serum, penicillin G (100 units/mL) and streptomycin (100 μg/mL) on collagen-coated 60-mm Petri dishes at a density of 2×10^5 viable cells per plate. After

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† Abbreviations: EGF, epidermal growth factor; PB, phenobarbital; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TCB, 3,3′,4,4′-tetrachlorbiphenyl.

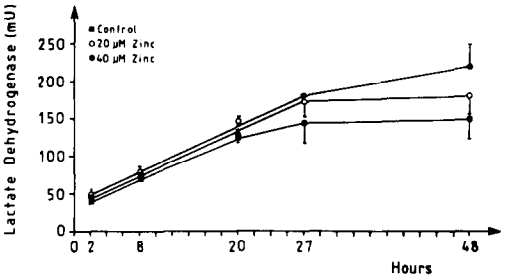

Fig. 2. Cumulative LDH-leakage of primary mouse hepatocytes in presence of 20 μM and 40 μM of zinc. Means ± SD of N = 3 culture plates.

2 hr in culture, the medium was changed to remove fetal calf serum and at this time PB (0.5 mM), TCB (1 nM) or TPA (1.6 μM) was added to respective plates. Concomittantly, the medium was also supplemented with various concentrations of zinc chloride. Six hours later, EGF (30 ng/mL) was added to the cultures. Medium was changed after 12 hr. Hepatocytes were harvested at various times between 42 and 68 hr of culture.

To determine DNA synthesis, 0.5 μCi of [^{3}H]thymidine (81 Ci/mmol) per plate was added 2 hr before cell harvest. [^{3}H]Thymidine incorporation was determined in the presence and absence of hydroxyurea (10 μM) in order to distinguish between replicative DNA synthesis and DNA repair [18]. Trichloroacetic acid precipitable material was hydrolysed to determine both [^{3}H]thymidine incorporation and DNA content [19].

The activity of lactate dehydrogenase (LDH) was determined photometrically using a commercial test kit (Boehringer Mannheim, F.R.G.).

RESULTS

Addition of zinc to the culture medium led to a dose-dependent increase of [^{3}H]thymidine incorporation in presence and absence of EGF (Fig. 1). At 40 μM zinc, DNA synthesis was about double that of controls. With 10 μM hydroxyurea in the culture medium, DNA synthesis was reduced to 2–5% of the original. The increase in DNA synthesis following zinc supplementation could be abolished by addition of equimolar concentrations of EDTA to the medium (not shown).

Addition of high concentrations of zinc were cytotoxic. Cumulative LDH-leakage was determined in cultures containing 20, 40 and 60 μM of zinc at hour 2, 8, 20, 27 and 48 of cultivation. Cytosolic LDH was determined after 48 hr. In the presence of 20 and 40 μM of zinc, cumulative LDH-leakage over 48 hr in plates containing 2×10^5 hepatocytes was 180 ± 31 mUnits (11% of total LDH) and 219 ± 39 mUnits (13%) compared to 149 ± 35 mUnits (9%) in controls (Fig. 2). In these cultures, the rate of LDH-leakage diminished with time. However, in cultures of the same cell density exposed to 60 μM of zinc, cumulative LDH-leakage rose dramatically following the first 8 hr. After 27 hr it reached a plateau of 1670 mUnits of LDH (which was practically 100% of the LDH leakage determined after 48 hr). At this time, most of the hepatocytes were dead. Toxicity of 60 μM zinc could be completely abolished by adding 60 μM EDTA to the medium.

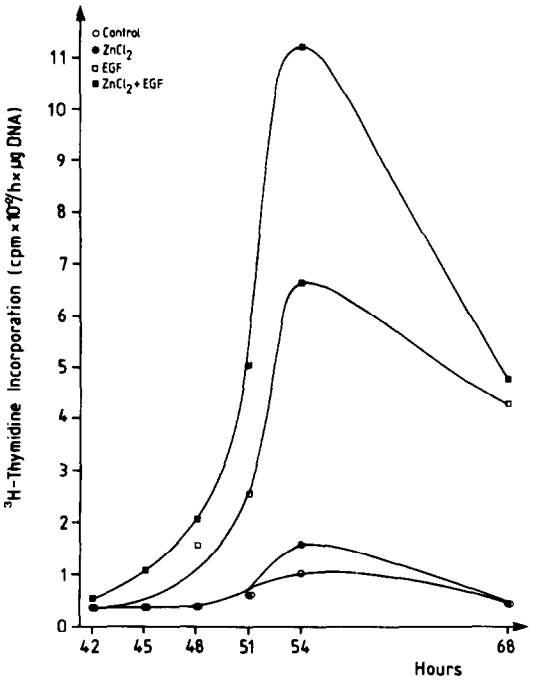

Fig. 3. Time course of [^{3}H]thymidine incorporation into DNA by mouse primary hepatocytes cultured in presence and absence of zinc and EGF. Means ± SD of N = 5 culture plates did not exceed 15%.

In order to avoid toxicity, a concentration of 30 μM zinc was chosen for further experiments.

The time course of DNA synthesis stimulation was determined in presence and absence of 30 μM zinc and 30 ng/mL EGF (Fig. 3). While addition of EGF to the medium increased [^{3}H]thymidine incorporation from 105 cpm/hr × μg DNA to 670 cpm/hr × μg DNA after 54 hr of culture, supplementation of the medium with 30 μM zinc chloride increased [^{3}H]thymidine incorporation from 160 cpm/hr × μg DNA to 1125 cpm/hr × μg DNA at this time.

The effect of PB, TCB or TPA on EGF-stimulated DNA synthesis was determined after 54 hr of culture in the presence and absence of 30 μM zinc (Table 1). Both PB and TPA significantly increased [^{3}H]thymidine incorporation in hepatocytes cultured without zinc. Addition of very low concentrations of TCB also resulted in a small and reproducible increase of DNA synthesis which however did not reach statistical significance. Interestingly, higher concentrations of TCB reduced [^{3}H]thymidine incorporation into DNA, although no increased LDH-leakage was determined at these higher concentrations (results not shown).

The stimulation of DNA synthesis following PB, TCB or TPA was much more marked after further addition of 30 μM zinc to the medium (Table 1). Under these culture conditions, many mitotic figures could be seen (Fig. 4).

DISCUSSION

The results of this study show that EGF-stimulated [^{3}H]thymidine incorporation into DNA occurs at a

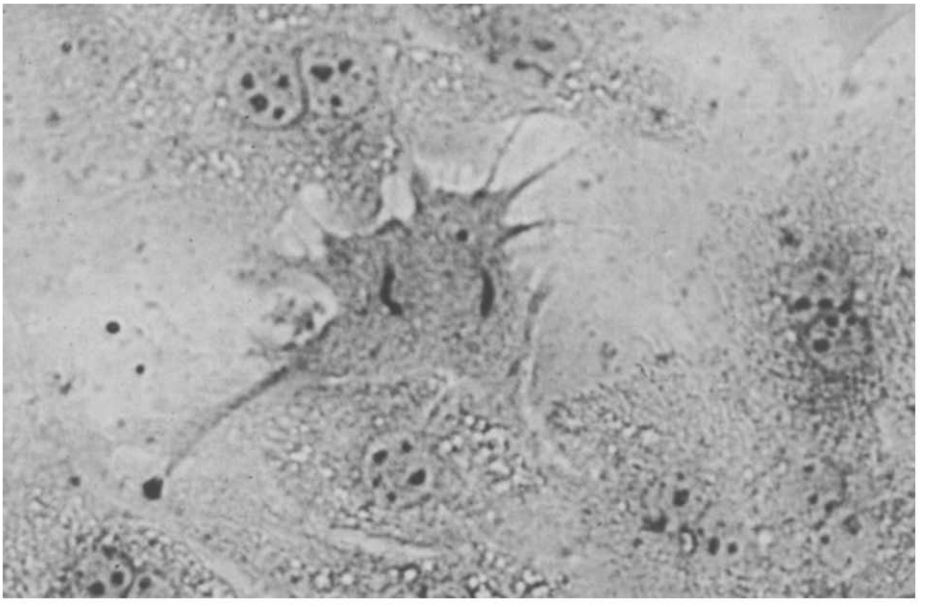

Fig. 4. Mouse hepatocyte in mitosis. Hepatocytes were stained with hematoxylin and eosin.

Table 1. Effect of zinc on the stimulation of [^{3}H]thymidine incorporation by phenobarbital (PB), 3,3′,4,4′-tetrachlorobiphenyl (TCB) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the presence of EGF*

Treatment	cpm/hr × μg DNA
$-Zn^{2+}$	
Controls	116 ± 9†
PB (0.5 mM)	145 ± 16‡
TCB (1 nM)	138 ± 13
TPA (1.6 μM)	228 ± 24‡
$+Zn^{2+}$	
Controls	156 ± 19
PB (0.5 mM)	285 ± 34‡
TCB (1 nM)	214 ± 24‡
TPA (1.6 μM)	582 ± 53‡

* Primary mouse hepatocytes were harvested after 54 hr.

† Means ± SD are given for N = 5 culture plates.

‡ $P \leq 0.05$ for respective controls.

higher rate when cells are provided with adequate amounts of zinc in the medium. [^{3}H]Thymidine incorporation in the presence and absence of EGF and/or zinc was reduced by 95–98% by hydroxyurea and can therefore be assumed to be due to replicative DNA synthesis under all experimental conditions [18]. Since intra- and extracellular zinc is continuously exchanged in cultured primary hepatocytes [12], with time, most of the intracellular zinc is likely to be lost to the medium if cells are kept in zinc-deficient, serum-free medium. Since zinc has been shown to be extensively involved in DNA synthesis [7–9], intracellular zinc concentrations could become limiting for cell replication. In this context, it is desirable to provide cultured hepatocytes with sufficient amounts of zinc.

Total cellular zinc concentration of hepatocytes is about 0.6 mM (Kleineke, personal communication). The physiologically relevant free zinc concentration is presently unknown. It can however be assumed to be several orders of magnitude lower because of the high affinity of zinc to amino acids (histidine and cysteine) and metallothionein. In the present study, a stimulatory effect on [^{3}H]thymidine incorporation was observed at medium zinc concentrations between 5 and 40 μM.

A change in the intracellular free zinc concentration can be assumed to occur since a dose-dependent increase of DNA synthesis was observed that could be completely blocked by addition of equimolar concentrations of EDTA.

If cells are cultured in the presence of fetal calf serum, supplementation of the medium with zinc had no effect on DNA synthesis. Indeed, in presence of 10% fetal calf serum, Kobayashi *et al.* [20] did not observe any effect on [^{3}H]thymidine incorporation into a human liver cell line (Chang cells) with 19, 38 and 76.5 μM of zinc in the medium. Since total zinc concentration in serum is about 0.45 mM [21], intracellular zinc is not likely to be depleted in the presence of 10% fetal calf serum (45 μM zinc) and therefore the activity of zinc-requiring enzymes is assured.

If hepatocytes are cultured in the presence of zinc in a medium containing no albumin, zinc apparently accumulates intracellularly with time [12]. Zinc uptake is speeded up in presence of dexamethasone [22]. Massive LDH-leakage beginning after 8 hr was observed from hepatocytes cultured in the presence of 60 μM zinc. Hepatocytes cultured in the presence of zinc concentrations of up to 40 μM showed LDH-leakage only during the first hours of cultivation. In these cultures LDH-leakage ceased almost completely after 27 hr. The inhibition of [^{3}H]thymidine incorporation into mouse epithelial cells in presence of 100 μM zinc as observed by Alitalo *et al.* [23], is likely to be due to the toxicity of zinc.

The EGF-receptor is characterized by two cystein-rich sequence repeat regions in its extracellular

domain [24]. EGF-stimulated autophosphorylation of tyrosine residues by the receptor-integrated tyrosine kinase is hypothesized to represent the first intracellular signal triggered by some mitogens [25]. Interestingly, specific inhibition of the phosphotyrosyl-protein phosphatase was observed in the presence of 10 μM zinc [26]. An inhibition of the EGF-receptor dephosphorylation by zinc might result in an increase of the mitogenic stimulus exerted by EGF and might represent a further possibility to explain the results of the present study.

The present findings on the role of zinc have been obtained in an effort to optimize experimental conditions to study the effect of EGF on DNA synthesis in hepatocytes exposed to various tumor promoting agents. In rat hepatocytes it has previously been demonstrated that EGF-stimulated DNA synthesis could be enhanced by PB [4], TCB [5] or TPA exposure [4]. As suggested in the present study, qualitatively similar responses can be obtained with mouse hepatocytes exposed to these agents, In this context, it is noteworthy that in contrast to the rat, TCB only stimulated [^{3}H]thymidine incorporation into DNA at very low concentrations (1 nM). Higher concentrations that were stimulatory in the rat (1 μM) [5] had inhibitory effects on mouse hepatocytes. The reasons for these differences are presently not known. They might however also be linked to the differences observed *in vivo* regarding the effect of TCB-treatment on *N*-nitrosomorpholine-induced enzyme-altered hepatic foci in the mouse [27] and the rat [5].

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