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Inhibition of myoblast differentiation by lack of zinc

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The impact of restricted zinc availability on myoblast differentiation was investigated. Lack of zinc prevented myoblast fusion and the increase in muscle-specific creatine kinase activity. The depression of activity of creatine kinase in the zinc-deficient cultures was accompanied by a similar decrease in the concentration of creatine kinase mRNA and was apparent even when fusion of the myoblasts was inhibited by cytochalasin B. Thus zinc appears to be necessary for the expression of creatine kinase during myoblast differentiation.

INTRODUCTION

Entry of non-malignant cells into the S phase of the cell cycle is preceded by the induction of a number of the enzymes involved with DNA synthesis, and zinc deficiency has repeatedly been observed to inhibit both the synthesis of DNA and the associated increase in thymidine kinase activity (Dreosti & Hurley, 1975; Duncan & Hurley, 1978; Record & Dreosti, 1979). The impairment of DNA synthesis caused by lack of zinc may therefore result from an inability to induce thymidine kinase and other enzymes involved with DNA synthesis. Indeed, recent studies have indicated that in 3T3 cells the decrease in thymidine kinase activity caused by lack of Zn^{2+} was associated with a comparable decrease in the concentration of the mRNA for the enzyme (Chesters *et al.*, 1990). This decrease in mRNA was consistent with an effect of Zn^{2+} on the induction of the enzyme rather than as an activator of the enzyme protein and provided support for the hypothesis previously advanced that Zn^{2+} is required to facilitate certain changes in gene expression (Chesters, 1978, 1982; Vallee & Falchuk, 1981). This hypothesis was based on studies of the effects of inadequate zinc supply both in animals offered Zn-deficient diets and in cell cultures treated with chelators. However, mostly the observations related to events associated with DNA synthesis. If it has a wider validity, then the altered gene expression necessary for cell differentiation might also be sensitive to lack of zinc even in situations unrelated to DNA synthesis. The differentiation of myoblasts into myotubes represents just such a system since, if anything, inhibition of DNA synthesis facilitates this process (Linkhart *et al.*, 1981).

Myoblast differentiation results in fusion of the cells to form myotubes and in the expression of a range of muscle-specific proteins, of which creatine kinase is a typical example. The present report indicates that induction of creatine kinase and myoblast fusion were dependent on adequate availability of Zn^{2+}. Furthermore, in these investigations, the effects on creatine kinase activity were independent of those on fusion, but were associated with a comparable decrease in creatine kinase mRNA concentration in the Zn-deficient cells.

EXPERIMENTAL

Embryonic-chick muscle cells

Cultures were prepared from the leg muscles of 11-day chick embryos by a modification of the original methods of Yaffe (1973) and Bischoff (1974). The muscle was placed in Dulbecco's Modified Eagle Medium (MEM) supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml) and fungizone (2.5 μg/ml) and chopped until pulped. Trypsin (0.25%, w/v) was then added, and the tissue was incubated at 37 °C in an atmosphere of air/CO_2 (19:1) to release single cells. The cell suspension was filtered through a double layer of lens paper, centrifuged (500 $g_{av.}$ for 5 min) and the cell pellet was resuspended in Dulbecco's MEM containing 10% (v/v) horse serum (Sigma Chemical Co., Poole, Dorset, U.K.)+2% (v/v) chick-embryo extract (Paul, 1975). The cells were then plated directly on to gelatine-coated 12-well plates at a cell density of 3.8×10^5 cells per well and incubated at 37 °C in an atmosphere of air/CO_2 (19:1). On day 3 of culture, the medium was replaced by fresh medium, and diethylenetriaminepenta-acetic acid (DTPA) and bivalent cations were added as required. Low availability of Zn^{2+} was induced by addition to the cultures of DTPA at a final concentration of 600 μM. Cultures supplemented with Zn^{2+} (400 μM final concn.) received appropriate amounts of $ZnSO_4$. The cultures were harvested on day 7 if they were to be used for RNA extraction and Northern blotting, or on day 8 for measurement of creatine kinase activity.

Cells for creatine kinase assay were collected in microcentrifuge tubes and disrupted by ultrasonication at 0 °C. After centrifugation for 30 min at 11000 g at 4 °C, the creatine kinase activity of the supernatant fraction was assayed by using a UV-45 kit (Sigma) and its protein content was estimated by the method of Lowry *et al.* (1951).

RNA extraction and blotting

RNA was extracted from the cells by the method of Chomczynski & Sacchi (1987) and was subjected to Northern-blot analysis as previously described (Chesters *et al.*, 1990). The blots were probed for muscle creatine kinase mRNA with a 5′ fragment of the mouse creatine kinase cDNA which had been excised from the plasmid pMCK-X3 (Ordahl *et al.*, 1984) with the restriction enzymes *Sma*I and *Hin*dIII. Successive probes were removed from the blots by treatment with 0.1% (w/v) SDS at 90 °C for 7 min, and the blots were re-probed for S6-ribosomal-protein mRNA (pHS6-2B; Lott & Mackie, 1988) and also for 18 S rRNA (Erickson *et al.*, 1981). The quantities of mRNA present were estimated as previously described (Chesters *et al.*, 1990) and were then expressed as ratios of the corresponding amounts of 18 S rRNA to compensate for any variation in quantities of total RNA loaded from each sample. Standard

Abbreviation used: DTPA, diethylenetriaminepenta-acetic acid.
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errors were based on variation between blots. Statistical significance was tested at the $P = 0.05$ probability level.

RESULTS AND DISCUSSION

In control cultures of chick-embryo myoblasts, approx. 80% of the cells fused. When DTPA was added to the cultures on day 3 just as the cells lined up before fusing, fusion and creatine kinase activity were severely inhibited, but this effect was almost completely reversible by adding Zn^{2+} (Table 1). The inclusion of Cu^{2+}, Co^{2+} or Ca^{2+} in place of Zn^{2+} had no effect on the creatine kinase activity of DTPA-treated cells (results not shown) and, although Mn^{2+}, Fe^{2+} and Ni^{2+} produced some activation, this was always substantially less than that caused by Zn^{2+} (Table 1). Although fusion can be inhibited by lowering the availability of Ca^{2+} (Merlie & Gros, 1976), the lack of response of the DTPA-treated cells to additional Ca^{2+} was not surprising, since the medium already contained Ca^{2+} at 3 times the molar concentration of the DTPA. It is probable that the three metals producing restricted activation all had an affinity for DTPA substantially greater than that of Zn^{2+} (Koltry & Sucha, 1985). This conclusion is based on the assumption that iron and manganese added as the bivalent cations would have been converted into the tervalent form by the aerobic conditions of culture and by the presence of the chelator disturbing the redox equilibrium in favour of the much more firmly bound tervalent ion. Thus the effects of these metals on creatine kinase activity probably reflected partial re-activation of differentiation by endogenous Zn^{2+} displaced from the chelator by preferential binding of the added metal.

From the above results and from similar evidence obtained in preliminary studies with rat satellite cells (not shown), it was clear that Zn^{2+} was specifically required when proliferating myoblasts ceased to replicate and started to fuse together into multinucleated myotubes capable of producing proteins specific to muscle. What was not clear was whether Zn^{2+} was promoting

Table 1. Effects of DTPA and bivalent cations on the creatine kinase activity of fusing chick-embryo myoblasts in culture

Results are means ± S.E.M. for groups of four cultures. All additions of DTPA were at 600 μM and of metal ions at 400 μM.

Sample	Creatine kinase activity (units/mg of protein)
Control	2.41 ± 0.10
DTPA	0.27 ± 0.07
DTPA + Zn^{2+}	2.33 ± 0.16
DTPA + Fe^{2+}	0.73 ± 0.10
DTPA + Mn^{2+}	1.33 ± 0.19
DTPA + Ni^{2+}	0.81 ± 0.12

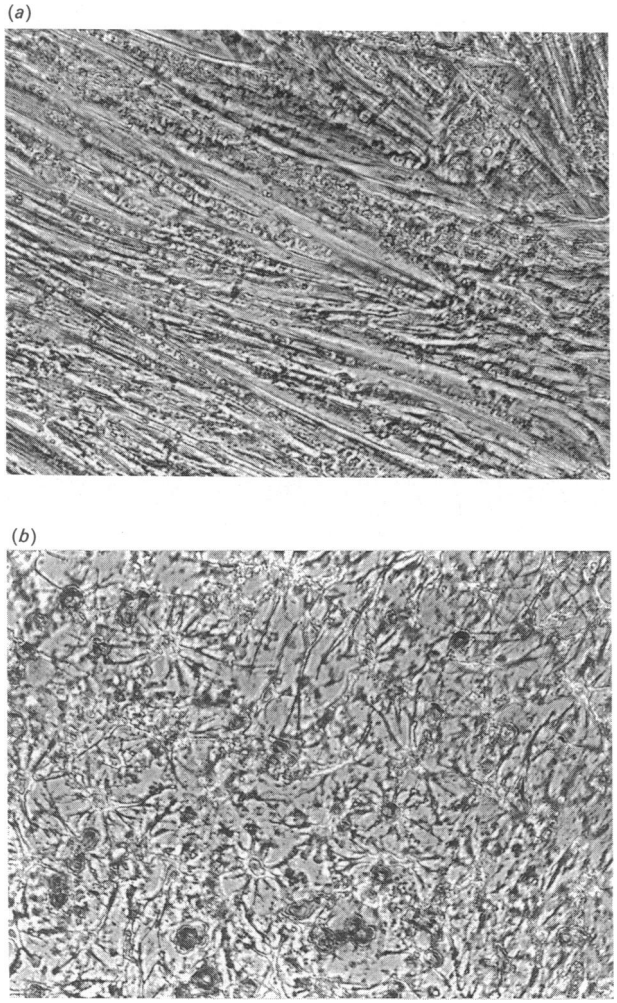

Fig. 1. Appearance of chick myoblasts after culture for 8 days (*a*) in control medium or (*b*) in medium containing cytochalasin B (5 μg/ml)

Both photographs were taken at the same magnification (approx. × 140).

Table 2. Effects of cytochalasin B, DTPA and Zn^{2+} on creatine kinase activities in cultures of chick-embryo myoblasts

Results are means ± S.E.M. for groups of four cultures. All additions of DTPA were at 600 μM and of metal ions at 400 μM.

Treatment	Creatine kinase activity (units/mg of protein)	
	Control	+ Cytochalasin B (5 μg/ml)
Control	1.42 ± 0.09	1.34 ± 0.06
DTPA	0.13 ± 0.02	0.14 ± 0.02
DTPA + Zn^{2+}	1.20 ± 0.12	1.07 ± 0.11

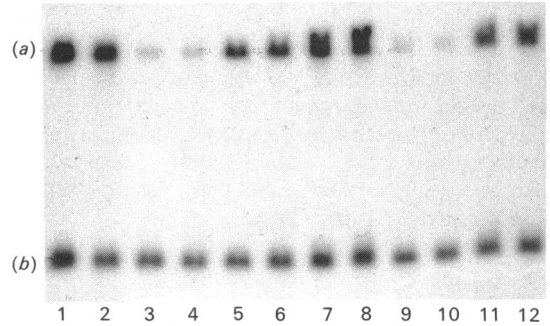

Fig. 2. Northern-blot analysis of (*a*) creatine kinase mRNA and (*b*) S6-ribosomal-protein mRNA in cultures of chick-embryo myoblasts with and without treatment with DTPA and Zn^{2+}

Both autoradiographs are of the same blot probed (*a*) for creatine kinase mRNA and (*b*) for S6-ribosomal-protein mRNA. From left to right, lanes 1, 2, 7 and 8 contained RNA from control cultures, 3, 4, 9 and 10 RNA from DTPA-treated cultures, and 5, 6, 11 and 12 RNA from cultures which received both DTPA and Zn^{2+}.

Table 3. Effects of DTPA and Zn^{2+} on mRNA concentrations for creatine kinase and S6 ribosomal protein in fusing chick-embryo myoblasts in culture

Individual values for creatine kinase (CK) and ribosomal-protein-S6 (S6) mRNAs were corrected for variations in loading of the samples by using the ratios of the appropriate mRNAs to the corresponding values for 18 S rRNA (18 S). S.E.D. means S.E. of differences between means within a column.

	mRNA ratios		
Treatment	CK/18 S	S6/18 S	CK/S6
Control	1.62	1.16	1.40
DTPA	0.09	0.63	0.15
DTPA + Zn^{2+}	1.13	0.93	1.20
S.E.D.	0.29	0.19	0.19

fusion which in turn caused the cells to produce muscle-specific proteins, or whether the Zn^{2+} was needed for the changes in the gene expression resulting in production of the muscle proteins. With chick-embryo myoblasts it is possible to separate these two processes by addition of cytochalasin B (Sanger & Holtzer, 1972), and its effects on the Zn^{2+} response have been investigated.

When cytochalasin B (5 μg/ml) was used to block cell fusion, after 24 h the cultures contained numerous arborized cells of the type reported by Sanger & Holtzer (1972), but essentially no normal myotubes (Fig. 1). The cells remained in this state until the end of the experiment, when they were assayed for creatine kinase activity. Despite inhibiting fusion almost totally, the addition of cytochalasin B to the cultures made no difference either to creatine kinase induction or to the effects of DTPA and Zn^{2+} (Table 2). A number of previous reports have suggested that some of the biological effects of zinc deficiency relate to an impairment of membrane stability (Bettger & O'Dell, 1981). However, the present results strongly suggest that the effects of lack of Zn^{2+} on the increase in creatine kinase activity resulted directly from an effect on enzyme expression and not from an inhibition of cell fusion. Since there appeared to be no evidence to suggest that creatine kinase is a zinc-containing or zinc-activated enzyme, it was of interest to determine whether or not the effect of Zn^{2+} deprivation on the enzyme's activity was preceded by a similar effect on the concentration of creatine kinase mRNA. Total RNA was extracted from chick-embryo myoblast cultures at a time, 1 day before creatine kinase activity had been previously measured, when activity of the enzyme was still increasing. The RNA was separated on agarose gels and subjected to Northern-blot analysis using a creatine kinase cDNA probe (Fig. 2). Because of variations in cell number and RNA yield between blots, the results (Table 3) have been expressed relative to the concentration of 18 S rRNA in each preparation. It is clear that, although low Zn^{2+} availability may have produced a slight generalized lowering of mRNA concentrations, as witnessed by the values for ribosomal protein S6, the decrease in creatine kinase mRNA was much greater and was very similar to the previous changes in creatine kinase activity (Table 2).

In conclusion, decreased availability of Zn^{2+} has been shown to prevent the increase in creatine kinase mRNA, and in the activity of this enzyme, normally associated with myoblast differentiation. This action is consistent with the hypothesis previously advanced that Zn^{2+} is required during certain alterations in gene expression. Clearly, further work is necessary to determine whether the inhibition by lack of Zn^{2+} of the increase in expression of specific mRNAs, observed both here with creatine kinase and previously with thymidine kinase in cells preparing for DNA synthesis, relates to an alteration in mRNA stability, or processing, or to a need for Zn^{2+} during changes in gene activation. Nevertheless, the present findings suggest that the known effects of lack of Zn^{2+} on DNA synthesis may represent only one aspect of a more generalized phenomenon in which Zn^{2+} is required to facilitate a variety of alterations in the types of protein expressed by cells.

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