Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF I and IGF II) in primary cultures of chicken embryonic cells

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Cells prepared from the body walls of chicken embryos were plated in the absence of serum. Insulin-like growth factors (IGFs) barely stimulated cell replication, but preferentially enhanced the differentiation of muscle cells. Myoblast fusion was favoured in the presence of IGF (or insulin). Concomitantly, acetylcholinesterase activity increased. IGF I and IGF II were equipotent and active in low physiological concentrations, in contrast to insulin, which was known for a long time to exert such effects at pharmacological concentrations.

Chicken embryonic cell *Myoblast differentiation* *Acetylcholinesterase* *Insulin-like growth factor* *Insulin*

1. INTRODUCTION

Skeletal muscle cell differentiation in vitro is favoured by supraphysiological insulin-concentrations [1]. Therefore, insulin is usually added in large amounts to defined media for muscle cell cultures [2]. Differentiation of embryonic muscle cells is associated with several changes such as myoblast fusion, synthesis of contractile proteins and of specific enzymes. We compared the effects of insulin and of IGF I and II in an in vitro model similar to that originally used by authors in [1] who were the first to describe insulin effects on myogenic cells. In addition to the microscopic examination of the cultures, we measured the activity of acetylcholinesterase (AChE) (EC 3.1.1.7), an enzyme specific for muscle cells. Florini [3] has already shown that somatomedins in physiological concentrations promote differentiation of muscle cells as documented by an increased fusion rate and a rise in creatine kinase activity in cells cloned from Yaffe's L6 line. We here report that IGF I and IGF II enhance, in a dose-dependent (equipotent) manner, the differentiation of chicken skeletal muscle cells in primary culture.

2. MATERIALS AND METHODS

Cells were obtained from the body walls of 10-day-old chicken embryos by fractionated trypsinization as in [4] and collected by centrifugation. Enzyme action was stopped by the addition of soybean trypsin inhibitor (Sigma) and the cells were washed 3-times in minimal essential medium (MEM Eagle, Eagle's balanced salt solution, Difco). They were plated at a density of 1.4×10^6 cells/dish (Falcon, 35 mm diameter) and kept in a humid atmosphere of 95% air and 5% CO_2. The culture medium (MEM) contained 2 g/l glucose, 100 units/ml penicillin and 50 μg/ml streptomycin. Non-essential amino acids (1 ml/100 ml), glutamine (2 mM) (both from Difco), and human serum albumin (HSA, 65 mg/l) (supplied by the Swiss Red Cross, Bern, dialyzed against H_2O and containing 15 μmol free fatty acids/g albumin) were routinely added. Pure IGF I (prep. I/3), IGF II (10 SE IV) and whale insulin (identical amino acid sequence to porcine insulin, specific biological activity of 24 units/mg protein) were kindly provided by Dr Humbel (Zürich).

Different treatments (1.5 ml test medium/cul-

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medium/culture dish) began at the time the primary cultures were seeded (day 0). The medium was changed 2, 4, 6, and 8 days after plating.

To determine acetylcholinesterase (AChE) activities, the cell cultures were washed 2-times with Dulbecco's buffer, (pH 7.35, 25°C), then covered with 1.2 ml of Dulbecco's buffer containing 0.2 μCi (118 nmol) of [*acetyl*-1-^{14}C] acetylcholine iodide (purchased from NEN) as substrate. Incubations were carried out at room temperature with cells attached to culture dishes, and after 30 min 200 μl aliquots of the medium were transferred to scintillation vials containing 2 ml of 50 mM glycine–HCl/1 M NaCl buffer (pH 2.5) to stop the reaction. The reaction product, [^{14}C]acetate, was protonated to acetic acid and counted after extraction into 5 ml of scintillation cocktail. This procedure was performed according to the two-phase AChE assay as adapted for the measurement of surface AChE in [5]. Culture dishes without cells handled in the same manner served as blanks.

Parallel dishes used for the determination of the cell number were rinsed as in the enzyme assay. The cells were detached with trypsin–EDTA and counted in a haemocytometer.

3. RESULTS

Cells prepared from the body walls of 10-day old chicken embryos grow well in the presence of serum. This widely used method yields cultures consisting mainly of fibroblasts and some myotubes. When the cells are seeded in the absence of serum, only a minor part of them settle down on the plastic surface of the dish. Whereas the cell number increases rapidly in the cultures treated with serum, cells do not proliferate in the absence of growth factors, but remain viable for several days of culture, as judged by the onset of cell replication upon addition of serum. Control cultures contain quiescent fibroblasts, mitoses are not observed, and some of the myoblasts fuse to form myotubes. The appearance of the latter is favoured by insulin or IGF (fig.1).

Generally, the muscle cells begin to contract after 4–5 days of culture and then detach. In contrast to cultures without serum, cells detach by day 7–8 from serum-treated dishes due to overcrowding and are removed. The time course of

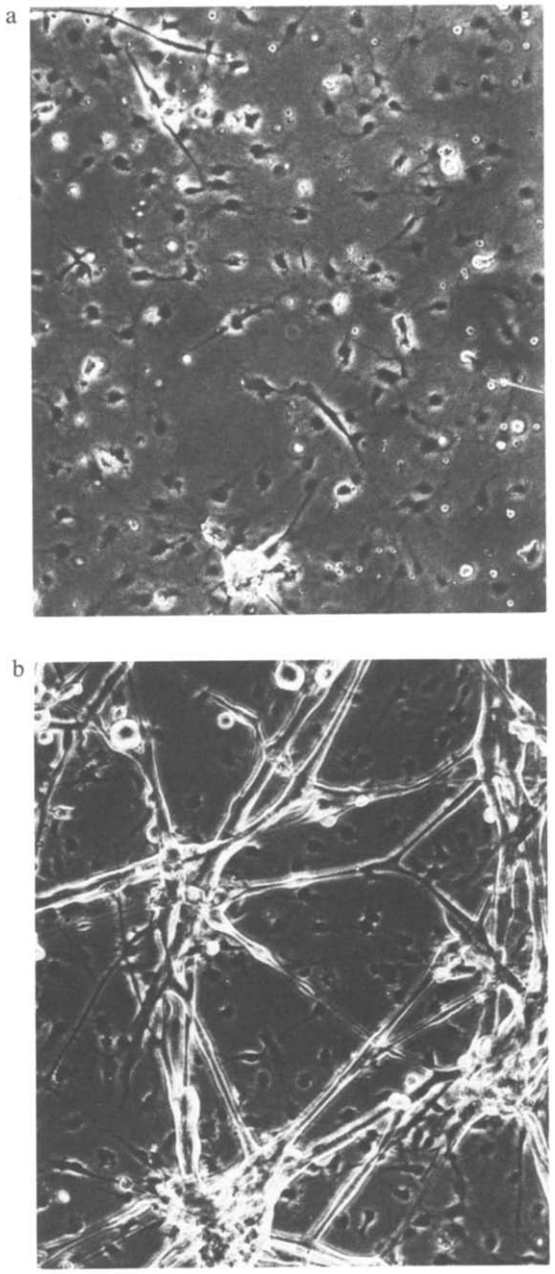
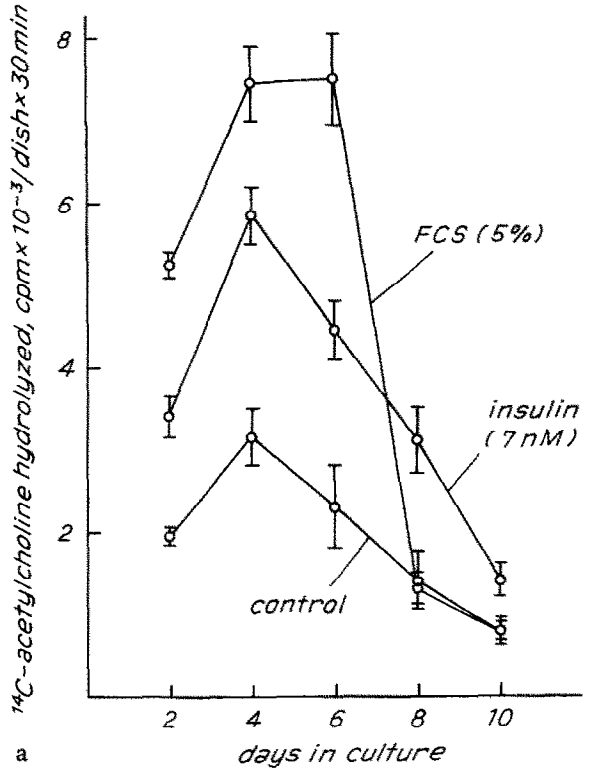


Fig.1. Chicken embryonic cells (phase contrast photomicrographs) after 3 days of culture in the absence (a: control) and in the presence (b: IGF I, 13 nM) of insulin-like growth factor I. Cells were cultured as described in section 2.

AChE activity parallels these morphological observations (fig.2). Dishes covered with monolayers of passaged fibroblasts are not different from empty



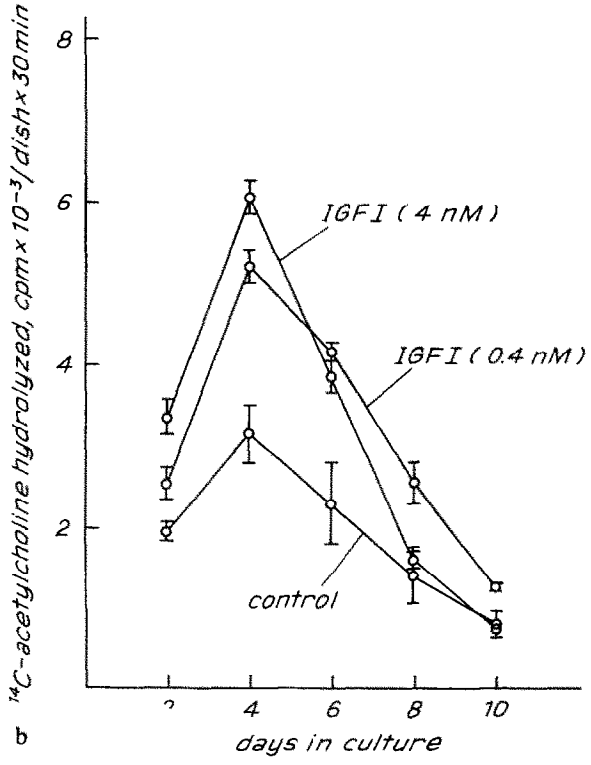


Fig.2. Acetylcholine cleavage by chicken embryonic cells after 2, 4, 6, 8, and 10 days of culture in the presence of insulin and of fetal calf serum (2a) and of IGF I (2b). Cells were cultured as described in section 2. Results represent the mean ± SEM of 4 different experiments which were performed in duplicate; i.e., $n = 8$ dishes.

dishes with regard to acetylcholine cleavage; there is no detectable formation of the reaction product during 30 min of incubation at 25°C. The hydrolysis of [^{14}C]acetylcholine is mainly catalyzed by enzyme molecules that have active sites towards the outside of intact muscle cells [5]. Fig.2a shows that the enzyme activity per culture dish is highest when the cells are grown in serum; i.e., when the dishes are full of cells. The cell number in dishes with defined peptide hormones or no addition (control) is much smaller. Insulin has a marked effect on AChE activity of the cultures (fig.2a), and cultures (fig.2a), and so does IGF I (fig.2b). In the presence of 7 nmol/l of insulin the enzyme activity shows a similar time course as with IGF I and IGF II in low doses. 0.7 nM insulin is about equivalent to 0.4 nM IGF (fig.3; time course not shown). Higher concentrations of insulin (70 nM) result in a more rapid accumulation of myotubes and in an

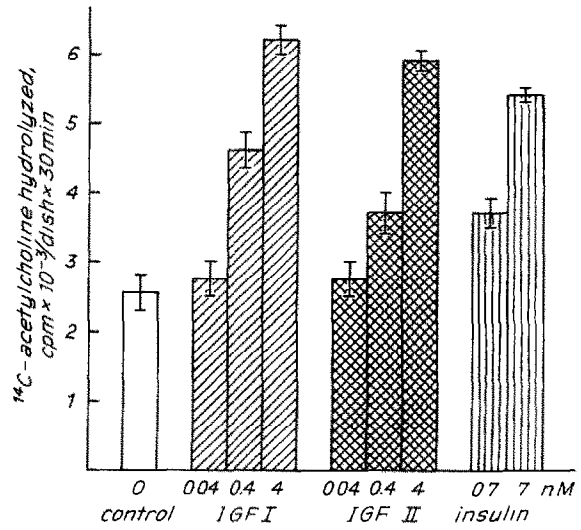


Fig.3. Acetylcholine cleavage by chicken embryonic cells after 4 days of culture in medium containing IGF I, IGF II, or insulin. Cells were handled as described in section 2. Values represent the mean ± SEM of 10 dishes, 4 experiments.

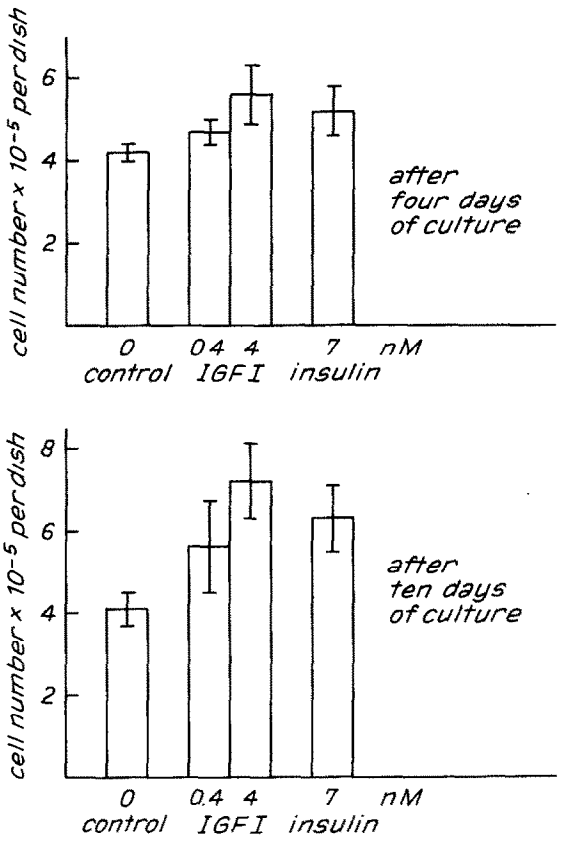


Fig.4. Number of chicken embryonic cells after 4 and 10 days of culture, respectively, in medium supplemented with IGF I and with insulin. The data are derived from the same (4 different) experiments as those shown in fig.2 and represent the mean ± SEM, $n = 4$. Cell numbers in the dishes treated with FCS, 5%, were $18.0 \pm 2.1 \times 10^5$ after 4 days and $3.2 \pm 0.8 \times 10^5$ after 10 days.

earlier detachment from the culture dishes. The dose–response curves of the enzyme activity after 4 days (fig.3) show that IGF I and IGF II are equipotent, whereas insulin is somewhat less effective. The total number of cells is not markedly influenced by IGF I and by insulin after 4 days, and it increases only slightly when culture periods are continued over 10 days (fig.4).

4. DISCUSSION

By using cell clones and low plating densities, cell-to-cell interactions are kept to a minimum, and the study of the mechanism of action of growth factors and hormones is facilitated. A disadvantage is the fact that transformed cells may have lost sensitivity to physiologically relevant regulatory mechanisms. The high plating cell density (1.4 m.o.i./35 mm diameter dish) is required so that besides a great number of matrix forming fibroblasts enough myoblasts capable to differentiate to myotubes are present. Detachment of contracting muscle cells is not prevented by prior coating of the dishes with fibronectin (as in section 2). Our results do not exclude the possibility that insulin and IGFs could stimulate fibroblasts to synthesize proteins beneficial for myoblast differentiation.

The MEM medium may not be suited for long-term growth of cells in the absence of serum, since it lacks some essential nutrients. The culture medium does not only lack substrates but also growth factors and hormones for optimal fibroblast proliferation, and myoblast differentiation. Besides IGF, platelet-derived growth factor (PDGF) seems to be an especially important constituent of serum that contributes to growth of cells in culture. PDGF and IGFs act synergistically to promote growth of cultured cells [6]. The data presented here (fig.4) demonstrate that IGF I alone has only minor effects on chicken fibroblast replication.

In [3] it was shown that somatomedins are likely to promote differentiation independently of the stimulation of growth of myogenic precursors. IGFs markedly favour myoblast differentiation, an effect which does not require the presence of other hormones. IGF I and IGF II are equally potent, as was previously found with respect to replication of chicken embryo fibroblasts [4]. It was recently reported that IGF I stimulates the late stages of erythroid differentiation [7]. This is another example where IGF, but not insulin, promotes the differentiation of a cell type of mesodermal origin in concentrations in which it circulates in plasma. Both, myogenesis and erythropoiesis, can be studied in culture models where precursors stop dividing and other genes are activated, leading to the synthesis of specific, fairly well characterized proteins. In the case of chondrocytes, multiplication stimulating activity (another member of the family of IGFs) supports both proliferation and differentiation [8]. Other growth factors, such as fibroblast growth factor and

PDGF, stimulate mostly DNA-synthesis.

IGF I mediates proliferation-promoting effects of growth hormone in vivo [9]. In addition, IGFs may contribute to the differentiation of several cell types of mesodermal origin, such as erythrocytes, skeletal muscle cells, and chondrocytes.

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