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INHIBITION OF CHICKEN MYOGENESIS IN VITRO BY PARTIALLY PURIFIED INTERFERON

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Continuous treatment of cultured chicken myoblasts with partially purified chicken interferon (IFN) inhibited myotube formation and the expression of MM-creatine kinase (MM-CK) at day 3, followed by continued MM-CK inhibition and concomitant stimulation of BB-creatine kinase (BB-CK) at day 4. Inhibition of MM-CK was also seen in IFN-treated cells prevented from fusing with EGTA. The degree of inhibition at day 3 depended on IFN dose over a range of 2.5-250 international units (IU)/ml; there was no evidence of cytotoxicity. Thus, IFN appears to inhibit the expression of muscle-specific traits during myogenesis.

Although noted primarily for its antiviral activity, the glycoprotein interferon (IFN) also has various effects on cellular functions (1), including cytodifferentiation (2-5). To determine whether IFN affects the processes of differentiation in non-transformed primary cells, we have studied its effects on the differentiation of cultured chicken myoblasts. In this system, undifferentiated myoblasts cease dividing, fuse to form multinucleate myotubes and synthesize muscle-specific isoproteins, such as the M subunit of creatine kinase (CK) (6). As differentiation ensues, dramatic increases in synthesis of the M subunit relative to the embryonic B subunit of the CK dimer result in an isozyme transition, thought to reflect differential gene expression (7), from BB homodimer to MB heterodimer and MM homodimer (8). Our results indicate that IFN treatment can inhibit myogenic events during the differentiation of skeletal muscle in vitro.

MATERIALS AND METHODS

Myoblasts derived from breast muscle of 12-day-old chicken embryos (SPAFAS, Inc., Roanoke, IL) were plated as previously described, except

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that the concentration of embryo extract was 5% (9). Culture medium, with or without IFN or mock IFN, was changed daily.

Chicken IFN was induced by WS influenza virus infection in the allantois of 9-day-old White Leghorn embryos as previously described (10). The allantoic fluid was clarified by centrifugation, proteins were precipitated by trichloroacetic acid (TCA) 7% final concentration, resuspended in 0.1 M KCl-HCl, pH 2, dialyzed against the same, and fractionated by Sephadex G-100 chromatography at pH 2. The column fractions containing peak antiviral activity (MW $\sim$ 25,000) were precipitated with TCA, resuspended in buffer, dialyzed, and samples were taken for protein concentration (11) and antiviral assay. Mock IFN was identically prepared from allantoic fluid of uninoculated embryos. The antiviral activity of IFN was determined by a Semliki Forest virus hemagglutinin yield-reduction bioassay in primary chicken embryo fibroblast cultures and expressed in international units (IU)/ml; this assay measures three times (2.41 $\log_{10} \pm 0.21$) the number of units (1.90 $\log_{10}$) assigned to the international chicken IFN standard, British Medical Research Council 67/18 (12). This partially purified IFN had a specific activity of 4×10^5 IU/mg protein. The IFN preparation was further characterized as follows: (i) it was shown to be a 20,000 Dalton protein, determined by SDS-PAGE; (ii) it had several isoelectric species between pH 6.25 and 7.7, determined by focusing in polyacrylamide gels (Pharmacia) with major peaks at 7.6, 7.3 and 6.8, consistent with published data (13); (iii) it was neutralized by rabbit antiserum that also neutralizes activity of the international chicken IFN standard, British Medical Council 67/18; and (iv) it was stable to prolonged exposure to pH 2 and relatively stable to heating, such that 21% of antiviral activity and 28% of antidifferentiation activity remained after 23 h at 56°C.

CK isozymes were fractionated on minicolumns of DEAE-A50 Sephadex by stepwise salt elution as previously described (9). Activity of separated isozymes (milliunits per 35 mm dish) was assessed by the method of Rosalki (14) using reaction mixture UV-340 (Sigma). DNA and protein were estimated by the diphenylamine reaction (15) and the G250 Coomassie blue micromethod (11), respectively.

RESULTS

Fig. 1 shows the morphological effects of different IFN doses on myogenic cultures treated for 65 h from the time of plating. Whereas large, branching myotubes formed in the absence of IFN (panel a), cultures treated with increasing amounts [2, 20, and 100 IU/ml] of IFN (panels b, c, and d) displayed diminished myotube formation in dose-dependent fashion. Similar inhibition was observed at 89 h *in vitro*. Mock-IFN-treated cultures were indistinguishable from controls. No evidence of cytotoxicity in IFN-treated cultures was noted at any time, even in cultures treated daily with 1000 IU/ml of IFN. Moreover, this did not appear to result from an effect on cell proliferation inasmuch as DNA content of IFN-treated cultures was similar to that of controls (Table 1).

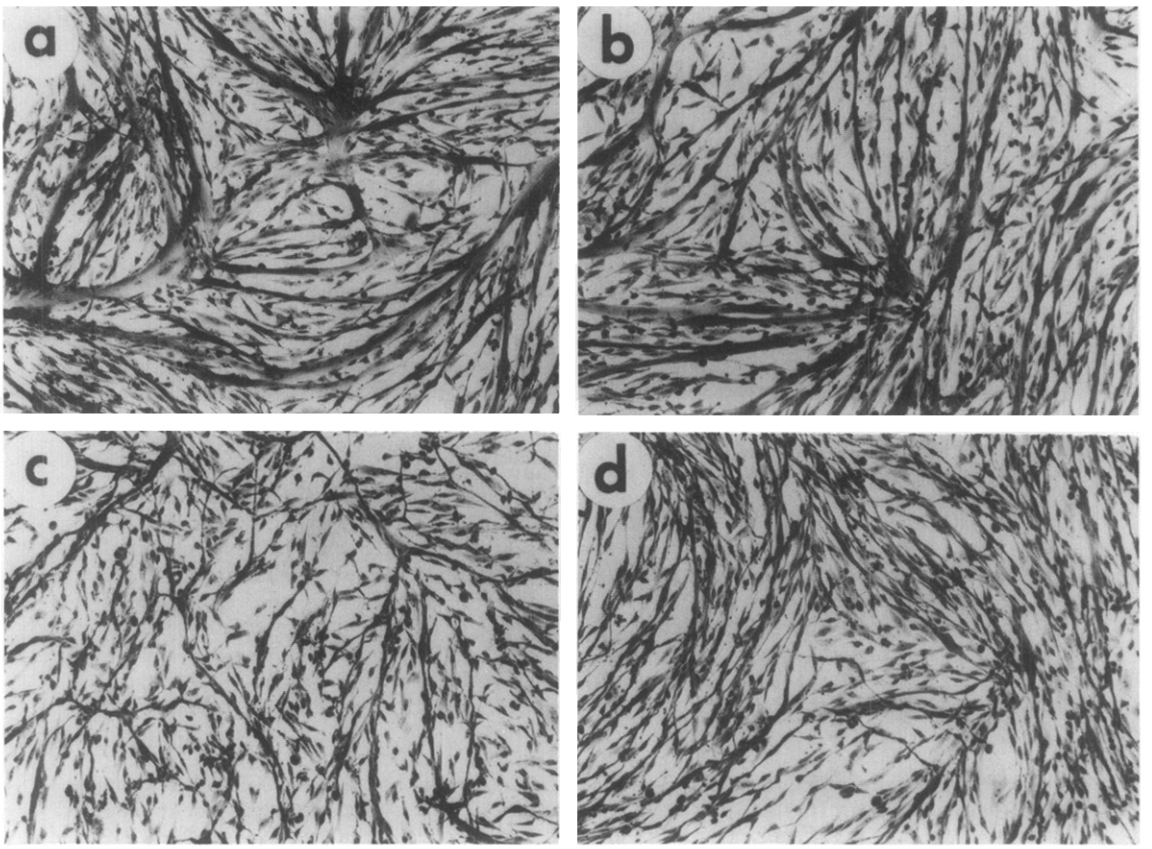

Fig. 1. Myogenic cultures fixed (ethanol:formalin:acetic acid, 20:2:1) and hematoxylin-stained 65 h after plating (a) without IFN and (b, c, d) with 2, 20, and 100 IU/ml of IFN, respectively. The formation of large, branching myotubes in cultures treated with little or no IFN (b, a) is not observed in cultures treated with 20 (c) or 100 (d) IU/ml of IFN. Magnification, 94x.

To determine whether the morphological effects of IFN were accompanied by changes in biochemical markers of differentiation, the CK isozyme pattern was examined in cultures exposed to 100 IU/ml IFN for 4 days from the time of plating. Figure 2 shows that whereas no effects on biochemical differentiation were detected at day 2, the interference with myotube formation at day 3 (fig. 1) was accompanied by inhibition of total and MM-CK, the latter being reduced to 43% of control value. No effects on BB-CK were seen at day 3. By day 4, however, total and BB-CK were increased 30 and 65%, respectively, whereas MM-CK was still inhibited 24%. Recently, extension of these observations to day 5 revealed an even more pronounced

TABLE 1. DNA CONTENT OF IFN-TREATED CULTURES[a]

	μg DNA per 35mm dish			
	Day 3		Day 4	
Experiment	Control	Treated	Control	Treated
I	14	15	11	9
	18	17	11	8
II	15	19	32	33
	17	17	41	28
III	15	16	16	27
	24	16	31	26

[a] Cultures were treated with 100 IU/ml IFN from the time of plating. DNA content was estimated using the diphenylamine reaction (15). Performance of the two-way analysis of variance did not reveal significantly altered DNA content in IFN-treated cultures at either day ($P > 0.1$).

differential effect on these isozymes (not shown), suggesting that IFN may enhance expression of embryonic (B-CK) genes while inhibiting mature (M-CK) genes.

To determine whether the degree of inhibition correlated with IFN antiviral activity, the experiment described in Figure 3 was performed. Cultures treated with IF for 65h from the time of plating displayed a dose-dependent inhibition of total CK (fig. 3A) and isozymes (fig. 3B) at concentrations ranging from 2.5-250 IU/ml. Treatment with mock-IFN did not reduce CK levels or isozyme patterns (not shown). Inhibition of total CK ranged from 21% at 2.5 IU/ml to 44% at 250 IU/ml (fig. 3A). Figure 3B shows that 100 IU/ml of IFN reduced activity of MM-CK by 73%, suggesting that IFN inhibits expression of muscle-specific traits. In this interpretation, the slight reduction of BB-CK, which is not usually seen, may in this instance reflect inhibition of the small increase in this isozyme that normally occurs in early myotubes (6,9,16,17). In three additional experiments, 100 IU/ml of IFN inhibited MM-CK 69%, 63% and 52%, respectively, in 65 h cultures. In the last instance, BB-CK was actually increased 26% over control values.

To determine whether IFN affects differentiation by interfering with the fusion of myoblasts to form multinuclear myotubes, a range of IFN

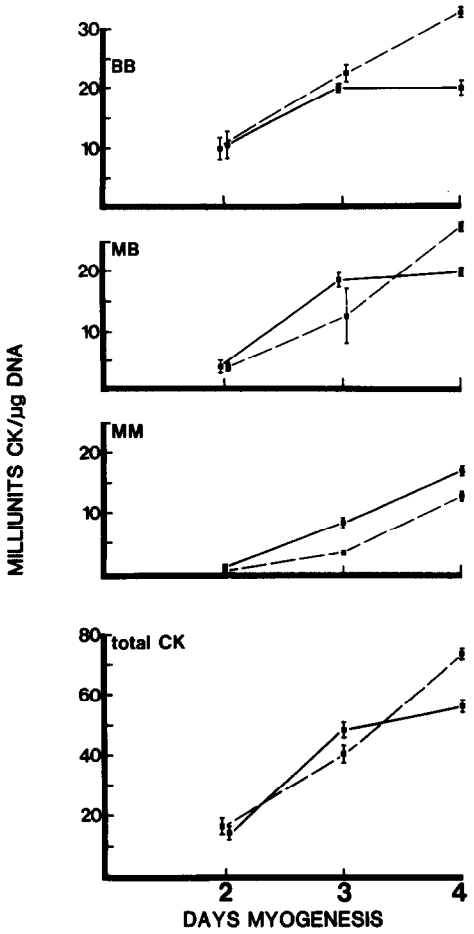

Fig. 2. Effects of IFN on the CK isozyme transition. Solid lines, control; broken lines, IFN-treated. Cells were plated in 100 U/ml IFN, which was also included in daily medium changes. Vertical bars indicate the range of duplicate values; absence of a bar indicates identity of values. This effect has been documented in four separate experiments; assessment at day 5 (not shown) reveals a more pronounced effect on MM and BB-CK. Levels of total protein synthesis in the same cultures showed a slight (∿10%) decline at day 3; at days 4 and 5, no effect of IFN on total protein synthesis was observed (not shown).

dosages was applied to cells cultured in the presence of 1.85 mM EGTA, which prevents fusion by chelating calcium in the medium. By day 4, the appearance of control (EGTA only) and IFN-treated cells was identical; no myotubes were observed. Nonetheless, IFN inhibited the CK isozyme transition characteristic of biochemical differentiation. These results, presented in Figure 4, suggest that IFN does not inhibit myogenesis at the cell membrane level, implying an intracellular site of antidifferentiative action.

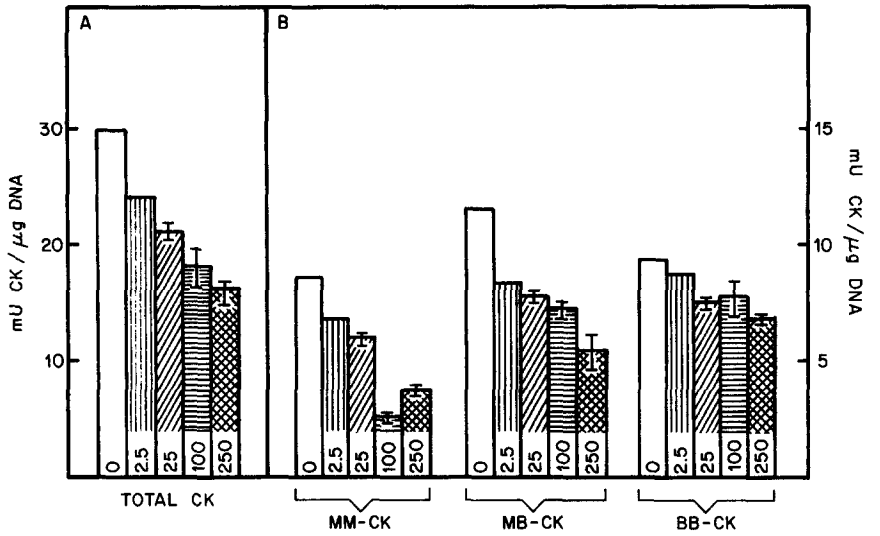

Fig. 3. Creatine kinase (CK) isozyme transition in myoblasts treated with different doses of IFN. (A), total CK; (B), CK isozymes. After 65 h at the indicated IFN doses, total CK activity was assessed followed by fractionation and quantitation of isozymes. Vertical bars indicate the range of duplicate determinations; absence of a bar indicates identity of values.

Finally, to determine whether various myogenic stages are equally IFN-sensitive, 200 IU per milliliter were added to cultures at intervals during differentiation (not shown). Inhibition of isozyme transition was observed only when IFN was added relatively early, i.e., at the time of plating or

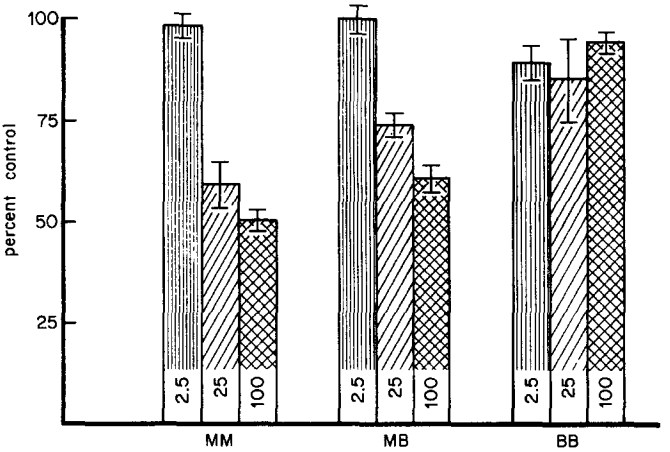

Fig. 4. Effects of IFN in cells prevented from fusing with EGTA. Control and IFN-treated cells were provided with 1.85 mM EGTA from 24-96 h in vitro. No myotubes were observed in the cultures. Control values (mU/μgDNA) were as follows: MM, 6.2; MB, 10.5; BB, 17.2; these low values reflect both decreased CK and increased DNA levels caused by EGTA.

at 24 hours after plating. Addition to cultures after the commencement of myotube formation had no effect on the CK isozyme transition. This suggests that only dividing myoblasts are sensitive to the antidifferentiative effects of IFN.

DISCUSSION

Interferon has been shown to inhibit the insulin-induced conversion of mouse 3T3-L1 fibroblasts to adipose cells (2,3) and the hydrocortisone-induced appearance of glutamine synthetase activity in embryonic chicken neural retina cells (18), without affecting cell proliferation. In contradistinction, IFN inhibits both proliferation and differentiation in erythroleukemia cells which are virus-transformed (4,5). The mechanism by which IFN regulates differentiation is unknown. The inhibition of myogenesis reported here describes the effects of IFN on the differentiation of non-transformed cells in primary culture. Since the inhibition of myotube formation and CK isozyme transition is not accompanied by decreases in DNA content or numbers of nuclei, it is unlikely that such inhibition reflects decreased cell proliferation. Also, no cytotoxicity was observed even at very high IFN doses. The pronounced inhibition of MM-CK relative to BB-CK suggests that IFN selectively inhibits the appearance of muscle-specific traits during myogenesis, possibly by altering the expression of genes directing the differentiation of skeletal muscle. Since Ca^{++}-deprived, unfused, IFN-treated myoblasts display inhibition of MM-CK as compared to Ca^{++}-deprived, unfused controls, it is unlikely that this IFN effect is membrane-mediated. IFN inhibition of myogenesis may provide a model to further elucidate IFN's mechanism of action, its possible role in regulating eukaryotic gene expression, as well as its potential effect on embryonic development (19).

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