Formation of striated muscle from myoblasts in vitro: inhibition of myotube formation by cis-4-hydroxy-L-proline and its reversal by native or denatured collagen (gelatin)

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Summary

Previous efforts to define the nature of the complex requirements for the development of striated muscle in vitro led us to the finding that the presence of collagen in the extracellular environment is essential for the formation from myoblasts of the multinucleated myotube (1). In the present report we demonstrate that the proline analog, cis-4-hydroxy-L-proline, will inhibit myotube formation in vitro without affecting the aggregation of cells (fusion?). The presence of collagen or gelatin as a culture substratum overcomes the action of the analog. The role of collagen in the development of the myotube is discussed.

Introduction

The development of myotubes from chick embryonic myoblasts during culture requires a complex environment: a defined nutritive medium (e.g., Eagle's), animal serum (e.g., horse), and chick embryo extract. Over the past years we have endeavored to identify the essential components of these complex substances. We believe that there exist external and as yet undefined biochemical 'signals' that are required for the development of muscle in vitro and therefore possibly also in vivo, and that these 'signals' may be provided by extramuscular tissues of the developing embryo in vivo, and serum and embryo extract in vitro.

We have previously reported that the serum requirement for myotube formation from myoblasts in vitro can be satisfied by insulin (2). Embryo extract yielded upon purification a low molecular weight fraction that promotes myotube formation in the presence of collagen and insulin (1). In the absence of collagen globular structures appeared; these globular structures are probably syncytia as previously reported (3). Therefore, the processes of myoblast fusion and myotube formation are experimentally separable in vitro (3).

Mayne and Strahs have shown that myotubes in culture synthesize and secrete procollagen into the culture medium (4). It would appear likely that myoblasts also secrete (pro)collagen. Therefore, it became pertinent to ask if an inhibitor of collagen production would interfere with myotube formation. Uitto and Prockop reported that cis-4-hydroxy-L-proline inhibits the production of extracellular procollagen by chick embryo tendon cells (5). We therefore tested the effect of this analog of proline on myotube formation.

Materials and methods

The medium employed consists of Eagle's minimum essential medium (MEM, powdered containing glutamine, from Grand Island Biological Co., (GIBCO) containing by volume, 10% horse serum and 10% chick embryo extract (11–12-day-old embryos used to make the extract). The antibiotic-antimycotic solution of penicillin, streptomycin, and fungizone (100X, GIBCO) was added to the medium at 1% v/v.

The isolation of cells from breast muscle of 12-day-old White Leghorn chick embryos (Shaw Hatcheries, West Chester, PA) with highly purified collagenase (CLSPA, Worthington Biochemical Corp., Freehold, N. J.) has been described previously (2); however, in this report we have reduced the concentration of collagenase to 0.1 mg/ml, and the incubation time to 60 min.

Cultures were initiated with 10^6 cells in Leighton tubes containing 1.0 ml of culture medium with a glass cover slip as the culture surface (Bellco Glass Inc., Vineland, N.J., Leighton tube #1903, cover slip #1916–10522, 10.5 × 22 mm). The cover slips were coated either with rat tail collagen prepared as previously described (1), or with a 1% solution of gelatin. The collagen or gelatin solutions applied to the cover slips were allowed to evaporate to dryness under ultraviolet light. Cultures were incubated at 37°C. The cis-4-hydroxy-L-proline was kindly supplied by Dr. D. J. Prockop and used at a concentration of 32 μg/ml of culture medium. It is essential that the concentration of cis-hydroxyproline for optimum effect be determined periodically because we have found it to vary, and suspect a variation in the sensitivity of the embryonic muscle cells possibly due to a variability in the strain of chickens used by the breeder.

Results

As discussed above, our previous studies have implicated collagen as an essential element in the formation of myotubes during muscle development in culture (1). One would therefore predict that if the synthesis or production of collagen by myogenic cultures were inhibited, myotube formation should also be inhibited. This is precisely what we have observed: if myoblasts are cultured in the presence of cis-hydroxyproline cells are seen to aggregate to form globular structures, but myotubes fail to develop, as shown in Fig. 1b. However, if the culture substratum is coated with collagen, or as in this experiment gelatin, myotube formation occurs to an extent comparable to that observed in control cultures (Fig. 1c and 1a respectively).

In this experiment the complex culture medium containing serum and embryo extract was employed. However, identical results are obtained if the partially defined medium consisting of purified factors from embryo extract, insulin, and somatotropin are used (3).

Reversibility of the cis-hydroxyproline effect on myogenesis:

The inhibition of myotube formation by cis-hydroxyproline is reversible: if globular structures formed in response to cis-hydroxyproline (as in Fig. 1b but after 72 h) are then fed with medium lacking cis-hydroxyproline, one observes after 72 h the formation of myotubes projecting from the globular structures. As we have previously reported, globular structures similar to those shown in Fig. 1b will also transform into myotubes in the presence of a factor purified from embryo extract (3).

Fig. 1. Effects of cis-hydroxyproline on myogenesis. a) Control culture incubated for 48 h on a cover slip coated with 340 μg of gelatin. b) As in (a) but in the presence of cis-hydroxyproline (32 μg/ml of medium) in the absence of gelatin. c) As in (b) but with the cover slip coated with gelatin as in (a). Magnification X50.