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Development and Postnatal Regulation of Adult Myoblasts

Zipora Yablonka-Reuveni

Department of Biological Structure, School of Medicine, University of Washington, Seattle, Washington 98195

Abstract

The myogenic precursor cells of postnatal and adult skeletal muscle are situated underneath the basement membrane of the myofibers. It is because of their unique positions that these precursor cells are often referred to as satellite cells. Such defined satellite cells can first be detected following the formation of a distinct basement membrane around the fiber, which takes place in late stages of embryogenesis. Like myoblasts found during development, satellite cells can proliferate, differentiate, and fuse into myofibers. However, in the normal, uninjured adult muscle, satellite cells are mitotically quiescent. In recent years several important questions concerning the biology of satellite cells have been asked. One aspect has been the relationship between satellite cells and myoblasts found in the developing muscle: are these myogenic populations identical or different? Another aspect has been the physiological cues that control the quiescent, proliferative, and differentiative states of these myogenic precursors: what are the growth regulators and how do they function? These issues are discussed, referring to previous work by others and further emphasizing our own studies on avian and rodent satellite cells. Collectively, the studies presented indicate that satellite cells represent a distinct myogenic population that becomes dominant in late stages of embryogenesis. Moreover, although satellite cells are already destined to be myogenic precursors, they do not express any of the four known myogenic regulatory genes unless their activation is induced in the animal or in culture. Furthermore, multiple growth factors are important regulators of satellite cell proliferation and differentiation. Our work on the role of one of these growth factors [platelet-derived growth factor (PDGF)] during proliferation of adult myoblasts is further discussed with greater detail and the possibility that PDGF is involved in the transition from fetal to adult myoblasts in late embryogenesis is brought forward.

Keywords

Myogenesis; Myosin; MyoD; Myogenin; PDGF; FGF; Transferrin; Chicken; Rat; C2 cells

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Address reprint requests to Dr. Zipora Yablonka-Reuveni, Department of Biological Structure SM-20, School of Medicine, University of Washington, Seattle, WA 98195.

NOTE ADDED IN PROOF

Many important studies concerning muscle development and satellite cell regulation have been published since this manuscript has been submitted for publication. The following reviews and original articles are especially pertaining to the major aspects discussed in the present paper:

INTRODUCTION

Histogenesis of skeletal muscle begins early in embryogenesis and continues through adulthood. Early in development, progenitor cells undergo myogenic determination, giving rise to myoblasts. These myoblasts first proliferate, then withdraw from the cell cycle, and eventually fuse into multinucleated fibers. In many species, the total number of adult muscle fibers in each muscle is already present at the time of birth. However, addition of nuclei into existing muscle fibers can still occur during the postembryonic phase by proliferation of muscle precursor cells and fusion of their progeny into the fiber. This process slows as the animal grows, so that little or no myoblast proliferation or fusion into myofibers occurs in the adult. Myoblast proliferation and fusion into myofibers can still occur in the adult following injury or other more subtle stresses (reviewed in Campion, 1984; Grounds and Yablonka-Reuveni, 1993; White and Esser, 1989).

Satellite cells are thought to be the primary source of myogenic cells during postnatal growth and regeneration of skeletal muscle (Campion, 1984; Grounds and Yablonka-Reuveni, 1993; Mauro, 1961, 1979). These cells are situated adjacent to the myofiber underneath the basement membrane of the fiber (Mauro, 1961). Individual myofibers become encased by a basement membrane during late embryogenesis and it is at that stage that distinction of satellite cells by their morphology and location is first possible (reviewed in Grounds and Yablonka-Reuveni, 1993). Figures 1 and 2 demonstrate the localization of the basement membrane around the myofibers in developing and adult chicken muscle and the location of the satellite cells underneath the basement membrane of the fiber. The original satellite cell culture described by Bischoff (1974) demonstrated that cells that eventually gave rise to myotubes in culture (i.e., myogenic precursors) could be effectively liberated from adult rat skeletal muscle by proteinases that were shown to attack the basement membrane. Consequently, myogenic cells isolated from normal juvenile or adult postnatal muscle by using such proteinases are commonly presumed to be satellite cells (Allen et al., 1991; Yablonka-Reuveni et al., 1987). Such enzymatically liberated cells, similar to myoblasts found during embryogenesis, will proliferate, differentiate, and fuse into myotubes expressing muscle-specific proteins (Allen et al., 1991; Düsterhöft et al., 1990; Hartley et al., 1991; Yablonka-Reuveni et al., 1987). Perhaps the most convincing evidence that muscle fibers contain myogenic precursors was the work of Bischoff (1975) demonstrating that cultured single fibers isolated from adult rat can give rise to colonies with multinucleated myotubes. In vivo studies analyzing the incorporation of ^3H -thymidine into satellite cell nuclei and subsequently into fiber nuclei indicated that at least some of the satellite cells are proliferative in young animals, contributing additional nuclei to the fibers (Moss and LeBlond, 1971). In the adult, satellite cells are mitotically quiescent but can reinitiate proliferative activity in response to injury (Carlson and Faulkner, 1983; Schultz et al., 1985; Snow, 1977a,b, as well as in response to more subtle stresses such as stretch, exercise, denervation, or mild compression (for summary, see Bischoff, 1989; White and Esser, 1989). Depending on the level of injury, the resulting progeny either fuse into preexisting fibers or form new myofibers.¹

SATELLITE CELLS DIFFER FROM MYOBLASTS PRESENT DURING THE FETAL STAGE OF MUSCLE DEVELOPMENT

Evidence from different laboratories studying avian, rodent, and human myoblasts has indicated that developing muscles consist of multiple myogenic populations (or lineages) (Rutz and Hauschka, 1975; Seed and Hauschka, 1984, 1988; Stockdale, 1992; Stockdale and Miller, 1987; White et al., 1975). At first, this was primarily based on the observations that myoblasts from earlier and later stages of development exhibit different characteristics in culture, such as media requirements for their growth and morphology of myotubes they form (Rutz and Hauschka, 1975; Seed and Hauschka, 1984, 1988; White et al., 1975). By such criteria, and with correspondence to the embryonic (earlier) and fetal (later) phases of muscle embryogenesis, the early and late myoblasts were named by Stockdale and colleagues embryonic and fetal myoblasts, respectively (Stockdale, 1992; Stockdale and Miller, 1987). Further discrete populations of myoblasts (which are often referred to as lineages) have been identified within these embryonic and fetal myoblasts by the type of myosins expressed within the myotubes formed in culture (reviewed in Stockdale, 1992; Stockdale and Miller, 1987). In the chick limb, for example, the early (embryonic) period ends and the late (fetal) period begins near the first week of avian embryogenesis (Stockdale, 1992; Stockdale and Miller 1987). Embryonic myoblasts are most abundant on day 5, while fetal myoblasts are most abundant between days 8 and 12 (Stockdale, 1992; Stockdale and Miller, 1987). It has been suggested that early myoblasts are not likely to be the precursors of late myoblasts, since the migration of these two populations to the limb buds is temporally separated (Seed and Hauschka, 1984).

Chick/quail grafting experiments have indicated that, like myoblasts of the developing muscle, cells in the satellite cell position in postnatal muscle are derived from the somites (Armand et al., 1983). However, cultured satellite cells isolated from postnatal muscle have different properties than cultured fetal myoblasts that occupy the muscle during development, prior to the emergence of adult myoblasts. Studies on mammalian myoblasts (reviewed by Cossu and Molinaro, 1987) have shown differences between fetal and adult myoblasts in their sensitivity to a tumor promoter (Cossu et al., 1983, 1985), in expression of acetylcholine receptors (Cossu et al., 1987), and in the form of acetylcholine esterase expressed (Senni et al., 1987). Studies of avian myoblasts have also identified differences between cultured satellite cells and cultured fetal myoblasts; compared with fetal myoblasts, cultured satellite cells express desmin as cycling cells more frequently (Yablonka-Reuveni and Nameroff, 1990), fuse into myotubes later (Yablonka-Reuveni et al., 1987), have a different sensitivity to merocyanine 540 (Nameroff and Rhodes, 1989), have specific patterns of myosin heavy chain isoform expression (Feldman and Stockdale, 1992; Hartley et al., 1991, 1992; further discussion below), and express more binding sites for platelet-derived growth factor (PDGK) (Yablonka-Reuveni et al., 1990c; Yablonka-Reuveni and

¹The term *adult myoblasts* is often used in the paper to describe myoblasts present in the muscle from late stages of development throughout adulthood (i.e., satellite cells). This term has been adapted because, as we show below, adult myoblasts emerge in late embryogenesis. In contrast to the term *satellite cells*, which in dependent on the in vivo location of the cells underneath the basement membrane, the term *adult myoblasts* creates a distinction between myoblasts present in postnatal and adult muscle and myoblasts dominant during embryonic and fetal stages of development.

Seifert, 1993; further discussion below). Recent studies on the expression of myogenic regulatory genes by cultured myoblasts from different stages of mouse and chicken development also identified a distinct expression pattern for satellite cells (further discussion below). Many of these studies suggest that myogenic precursors with the characteristics of adult myoblasts become dominant in late stages of embryonic development. As in the case of embryonic and fetal myoblasts, it is not clear whether adult myoblasts or precursors for adult myoblasts are destined as such during early embryogenesis or whether the distinctions of the adult cells are acquired when development progresses.

MYOBLASTS FROM FETAL AND ADULT CHICKEN SKELETAL MUSCLE REGULATE MYOSIN EXPRESSION DIFFERENTLY

One of the best studied muscle proteins is skeletal muscle myosin, which consists of two heavy chains and several light chains. There are numerous myosin heavy chain isoforms in vertebrates coded by different genes (Moore et al., 1993). These isoforms are progressively expressed during muscle development and regeneration and are differentially expressed in fast and slow muscle (Bandman et al., 1990, 1992). In the chicken pectoralis muscle, embryonic fast myosin heavy chain (MHO was considered to be an early developmental form of MHC expressed during embryogenesis and regeneration of both fast and slow muscles. This isoform is sequentially replaced with other developmental isoforms of MHC including neonatal and adult fast MHC; similar transitions take place following injury (Bader et al., 1982; Bandman et al., 1990). More recent studies have shown that a ventricular MHC (which is the major myosin isoform in adult chicken ventricle) is also expressed during earlier stages of development and during regeneration of all skeletal muscles (Kennedy et al., 1989; Sweeny et al., 1989).

In view of the distinct developmental program of myosin isoform expression, we proposed that myotubes from fetal and adult myoblasts could express different patterns of MHC isoforms. To test this hypothesis we compared the expression of several MHCs by myogenic cultures from the pectoralis muscle of fetal (E10) and adult (12–16 week posthatch) chickens² with isoform-specific monoclonal antibodies and immunofluorescence assays (Hartley and Yablonka-Reuveni, 1990; Hartley et al., 1991, 1992). During this investigation we made an unexpected observation: double immunolabeling with anti-ventricular MHC (mAb HV11) and anti-embryonic MHC (mAb EB165) showed that the vast majority of fetal myocytes (differentiated mononucleated myoblasts) and myotubes coexpressed ventricular and embryonic MHCs in both primary mass cultures and clonal cultures derived from individual myoblasts. In contrast, all adult myocytes and newly formed adult myotubes expressed just the ventricular MHC, whether plated at mass or clonal densities. Eventually, 12–24 hours after the onset of fusion, adult myotubes began to express embryonic MHC as well. The delay in the expression of embryonic MHC until after fusion was also seen in passaged adult myoblasts (which proliferate at a rate similar to that of E10 myoblasts) and in myoblasts isolated from regenerating adult muscle (which begin proliferating in vivo in response to the injury). These data indicated that the distinct MHC expression of adult

²Chickens used for all studies described here are White Leghorn.

myoblasts is not simply a result of culturing myoblasts that were mitotically quiescent initially. Furthermore, the expression of embryonic myosin could be abolished in adult but not E10 cultures by inhibiting fusion with EGTA. We concluded that in culture, both fetal (E10) and adult myotubes can express ventricular and embryonic MHCs but that *only* fetal myocytes express the embryonic isoform prior to fusion. This difference between fetal and adult myoblasts in the regulation of the expression of embryonic myosin supports the hypothesis that these cells may represent two distinct population of myogenic precursors. A model describing the phenotypes of fetal (E10) and adult myoblasts with respect to the ventricular and embryonic MHCs expression is shown in Figure 3.

In order to determine that the adult pattern of ventricular/embryonic MHC expression is not just a peculiarity of adult myoblasts from the adult pectoralis muscle but a common trait of myoblasts from other adult skeletal muscles, we examined expression of embryonic and ventricular MHCs in cultures of adult chicken anterior latissimus dorsi, a slow tonic muscle, and posterior latissimus dorsi, a fast twitch muscle. We also examined E10 hindlimb muscles, which consist of fast and fast/slow fibers, to determine if the fetal pattern of ventricular/embryonic MHC coexpression is present in other fetal muscles. We found that the adult pattern is present in cultures from both fast and slow adult muscles, as is the fetal pattern in cultures from mixed fast/slow fetal muscles (Hartley et al., 1992). This study demonstrates again the consistency of the adult pattern of MHC expression as shown in Figure 3.

MYOBLASTS WITH THE ADULT PATTERN OF MHC EXPRESSION BECOME DOMINANT IN LATE STAGES OF CHICKEN EMBRYOGENESIS

The specific MHC expression by adult myocytes in culture (expression of ventricular but not embryonic MHC) was used to determine when adult myoblasts first emerge during chicken development. We analyzed mass and clonal cultures from the pectoralis muscle of fetal (E14, E18) and posthatch (1-day, 3-week) chickens, using the same double-labeled, immunohistochemical approach described above for the identification of the adult and fetal (E10) patterns of ventricular/embryonic MHC expression. We showed that adult myoblasts comprise the large majority of myocytes from late fetal development (E18) and are the only kind present at hatching and beyond. Adult myoblasts are a minor population at E14, contributing no more than 20–25% of the myocytes. In E10 cultures, about 6% of the myocytes are of the adult type. These results suggest that adult-type myoblasts are present by mid-fetal development (E14) but predominant only at late fetal development (E18) (Hartley et al., 1992). A model describing the emergence of adult myoblasts, based on analysis of the various prehatch and posthatch stages, is summarized in Figure 4. An unanswered question is: when do satellite cells first appear during development? Our preliminary studies on cultured myoblasts isolated from somites and limb buds of E3–E4 chicken embryos identified two populations of myocytes. One population expresses ventricular MHC only and the second population expresses both ventricular and embryonic MHC. Under our experimental conditions these myocytes did not fuse into myotubes (Hartley and Yablonka-Reuveni, unpublished data). Further experiments are required to

determine the relationships between these two myogenic populations in the early developing muscle and adult/fetal myoblasts.

ACTIVATED BUT NOT QUIESCENT SATELLITE CELLS EXPRESS MYOGENIC REGULATORY FACTORS

Myogenic regulatory factors (which contain DNA binding domains and are thought to function as muscle-specific transcription factors) have been implicated in inducing myogenic determination during embryogenesis and in regulating the balance between proliferation and differentiation during myogenesis (reviewed in Olson, 1992; Sassoon, 1993; Weintraub et al., 1991). It has been further suggested that interactions between growth factors and these myogenic regulatory factors are likely to be involved in the proliferation-differentiation interplay (Olson, 1992). The members of this regulatory factor family include MyoD, myogenin, myf-5, and MRF4 (also known as myf-6 and herculin). Northern blot analyses of RNA from total muscle tissues of rodent species have established that mRNA levels for MyoD, myogenin, and myf-5, which are high in developing muscle, are drastically reduced in postnatal muscle and are present only at low levels in uninjured adult muscles (Beilhartz et al., 1992; Buonanno et al., 1992; Eftimie et al., 1991). There is, however, high expression of MRF4 mRNA in such adult muscles (Buonanno et al., 1992; Hinterberger et al., 1991). Janney et al. (1992) reported that none of the four known myogenic regulatory genes are expressed, at the RNA level, in myogenic precursors of adult rat muscle, but their expression is initiated following culturing. Therefore, the low levels of MyoD, myogenin, and myf-5 and the high level of MRF4 are probably due to mRNA expression in the multinucleated fibers and not in the quiescent satellite cells. In situ hybridization studies on tissue sections from adult mouse muscle did not detect mRNA for MyoD or myogenin, but expression of these mRNA in cells (presumably satellite cells) is detected 6 hours after muscle injury (Grounds et al., 1992). Transcription peaks at 24 to 48 hours postinjury and declines to preinjury levels by about 8 days (Grounds et al., 1992). Analysis of cross sections from adult mouse muscle by immunohistochemistry did not detect expression of MyoD or myogenin at the protein level unless the muscle was subjected to injury; it has been suggested that this expression is due to activated satellite cells (Füchtbauer and Westphal, 1992).

The identification of satellite cells in the latter two studies is not straightforward due to the complexity of the tissue; the positive structures are considered to be satellite cells (or myogenic precursors) because it is assumed that these are the only cells in adult muscle that can express MyoD or myogenin. Our laboratory has analyzed myogenesis of satellite cells on individual fibers isolated from the flexor digitorum brevis (FDB) muscle of the hind foot from postnatal and adult rats; fiber isolation is according to Bischoff (1986a). The fibers obtained are covered with a basal lamina, retain normal morphological characteristics, and contain two to three satellite cells and about 125 myonuclei (Bischoff, 1989). This system has allowed us to determine expression of myogenic regulatory factors by satellite cells without the ambiguity of cross sections. Immunohistochemical analysis of the fibers employing specific antibodies against MyoD and myogenin has revealed that satellite cells on fibers from 2.5–3-month-old rats (adults) do not express myogenin or MyoD. Upon

culturing, however, satellite cells on the intact fiber enter the cell cycle and begin expressing MyoD after 1 day in culture. Myogenin expression follows after 2 days in culture, and the number of positive cells peaks during the third day when the myogenic precursors differentiate. This is followed by a decline in myogenin expression, although expression of differentiation markers such as embryonic myosin persists (Yablonka-Reuveni and Rivera, 1994). Satellite cells on fibers from younger rats (3-week and 6-week) demonstrate a myogenin expression pattern similar to that of adult fibers. It is important to note that for all ages tested, we could not detect reactivity of the myonuclei with the antibodies. Smith et al. (1993) compared the expression of the four known myogenic regulatory proteins by cultured myoblasts from embryonic, fetal, and newborn muscles via immunohistochemistry. Expression of myogenic regulatory proteins by newborn myoblasts (satellite cells) lagged by at least 1 day behind the expression by embryonic and fetal myoblasts. For example, MyoD was expressed 1 day after initial culturing of embryonic and fetal myoblasts, but 2 days after culturing of newborn myoblasts. We obtained similar results with cultured chicken satellite cells regarding the expression of mRNA for chicken MyoD; fetal myoblasts expressed MyoD mRNA within the first day of culture, but expression in adult myoblasts began a day later. Correlated with the lag in MyoD expression, cultured adult chicken myoblasts expressed mRNA for differentiation-specific myosin several days after cultured fetal myoblasts (Hartley, Camoretti-Nercado, Paterson, and Yablonka-Reuveni, unpublished data).

Collectively, the above in vivo and cell culture studies have indicated that satellite cells do not express the four known myogenic regulatory factors, but once the cells become activated, the expression patterns of the regulatory factors are somehow similar to those of embryonic and fetal myoblasts. Whether adult myoblasts ever expressed any of the known myogenic regulatory genes as part of their developmental program, prior to quiescence, is unknown. It is intriguing to speculate that satellite cells represent a population of cells that have not yet acquired the expression of the four known myogenic factors. If so, satellite cells may resemble the early myoblasts in the limb buds, which do not express the four known myogenic regulatory factors when first arriving from the somites (Sassoon et al., 1989; reviewed in Sassoon, 1993).

MYOGENESIS OF SATELLITE CELLS IS REGULATED BY GROWTH FACTORS

Observations from cell culture studies support the notion that multiple physiological cues control satellite cell activation, proliferation, and differentiation during growth and regeneration (reviewed in Florini and Magri, 1989; Grounds and Yablonka-Reuveni, 1993). These studies have primarily focused on three families of growth factors and demonstrated that: 1) fibroblast growth factors (FGFs) can stimulate proliferation and depress differentiation of isolated satellite cells (Allen et al., 1984) and of satellite cell-derived cell lines (Clegg et al., 1987); furthermore, basic FGF (bFGF) can promote proliferation of satellite cells situated in their native position underneath the basal lamina of cultured rat myofibers (Bischoff, 1986a; Yablonka-Reuveni and Rivera, 1994); 2) insulin and the insulin-like growth factor family (IGFs) can stimulate the proliferation (and possible

differentiation) of isolated satellite cells (Duclos et al., 1990); and 3) transforming growth factor β (TGF β) can depress proliferation and differentiation of rat satellite cells, including those on isolated rat fibers (Allen and Boxhorn, 1987; Bischoff, 1990a). The quiescent, proliferative, and differentiative phases of cultured rat satellite cells could be manipulated by altering the combination and concentrations of FGF, IGF-I, and TGF β (Allen and Boxhorn, 1989) suggesting that in vivo, the three growth factors may work in concert to regulate the physiological state of satellite cells. It is important to note that proliferation and differentiation of cultured myoblasts isolated from developing muscle are also modulated by FGF, IGFs, and TGF β (reviewed in Florini and Magri, 1989), indicating that the effect of those agents is not restricted to certain stages of development. Studies on the immunolocalization of growth factors in the muscle indicate that FGF (acidic and basic) is present in the fiber extracellular matrix and within nuclei of myofibers (Anderson et al., 1991; DiMario et al., 1989; Oliver et al., 1992) and that IGF-I is expressed in many mononucleated cells in the muscle following injury (Jennische et al., 1987).

Bischoff's studies of soluble material released from crushed rodent muscles further demonstrated that skeletal muscle contains a factor that promotes satellite cell activation and proliferation (Bischoff, 1986b). About half of the mitogenic activity in this released material was shown to be due to transferrin (Chen and Quinn, 1992). However, it has been suggested first by Bischoff (1986b, 1989) and more recently by others (Chen and Quinn, 1992; Johnson and Allen, 1993) that the material released from crushed muscle contains an additional muscle-specific mitogenic activity that acts differently than purified bFGF or transferrin. Since purification of this unknown material has not been achieved, it is still possible that the unique mitogenic behavior of the soluble material is due to the additive effect of several known growth factors rather than a special unknown factor. It has also been shown by Bischoff that the proliferation of satellite cells can be suppressed by association of these cells with the muscle fiber itself (Bischoff, 1990b). Such a negative signal could potentially contribute to the maintenance of the quiescent state of satellite cells in vivo, but the nature of the signal is unclear.

Cell culture studies by myself and colleagues indicate that PDGF can promote proliferation of adult mouse and chicken myoblasts; the same studies suggest that PDGF might be important for proliferation of adult but not fetal myoblasts (Yablonka-Reuveni et al., 1990a, c; Yablonka-Reuveni and Seifert, 1993; see discussion below). Evidence for a PDGF role during myogenesis has been provided by a study with a rat myogenic cell line (Jin et al., 1990, 1991). Our current research on cultured adult rat myoblasts has indicated that PDGF has a role during myogenesis of rat satellite cells as well (work in progress).

SPECIFIC ISOFORMS OF PDGF PROMOTE PROLIFERATION AND DELAY DIFFERENTIATION OF MYOBLASTS DERIVED FROM ADULT MOUSE SKELETAL MUSCLE

PDGF is a major mitogen and a potent chemoattractant for many connective tissue cell types maintained in culture, including fibroblasts and smooth muscle cells (Ross et al., 1986; Westermarck and Sorg, 1993). PDGF, a dimeric protein with a molecular weight of about 30

kD, assembles by disulfide bonds from two distinct but highly homologous polypeptide chains (PDGF-A and PDGF-B). All three possible homo- and heterodimer combinations of PDGF (AA, AB, BB) have been isolated from natural sources. PDGF-AB was initially isolated from human platelets but it is now clear that other cell types can also produce this growth factor, and different sources may have different amounts of the three PDGF isoforms (Ross et al., 1986; Singh et al., 1982; Westermarck and Sorg, 1993). It is now believed that there are two PDGF receptor subunits, each with a molecular weight of about 170–180 kDa: the α -subunit (PDGFR α), which can bind both PDGF A-chain and B-chain, and the β -subunit (PDGFR β), which can bind only PDGF B-chain (Seifert et al., 1989; Westermarck and Sorg, 1993). These receptor subunits dimerize noncovalently in response to PDGF to form three distinct forms of high-affinity binding sites for the dimeric PDGF ligand ($\alpha\alpha$, $\alpha\beta$, $\beta\beta$). PDGF-AA can only bind to the $\alpha\alpha$ receptor, PDGF-AB can bind to both the $\alpha\alpha$ and $\alpha\beta$ receptors (with much lower affinity to the $\beta\beta$ receptor), and PDGF-BB can bind to all three receptor subunit combinations (Seifert et al., 1989, 1993). The binding of PDGF to its receptor activates a cascade of signal transduction events and metabolic activities leading to DNA synthesis (Westermarck and Sorg, 1993; Williams, 1989). PDGF and PDGFR have been detected by a variety of approaches in the intact tissue (Orr-Urtreger and Lonai, 1992; Palmieri et al., 1992; Schattman et al., 1992; Westermarck and Sorg, 1993; Yeh et al., 1991). Taking together the in vitro and in vivo observations, many investigators have proposed roles for the PDGF/PDGFR system in various physiological, pathological, and developmental processes (Ross et al., 1986; Westermarck and Sorg, 1993; Wolswijk and Noble, 1992).

A preliminary observation obtained by us was that primary cultures of chicken satellite cells (isolated from adult pectoralis) can bind low levels of PDGF-AB but that cultured fetal (E10) myoblasts bind very little, if any (Yablonka-Reuveni et al., 1988, 1990c). Our earlier studies also indicated that, in contrast to E10 myoblasts, fibroblast-like cells isolated directly from E10 breast muscle bound all PDGF isoforms (Yablonka-Reuveni and Seifert, 1993; Yablonka-Reuveni et al., 1988). To avoid the complexity introduced by fibroblast-like cells, which could be present in adult primary myogenic cultures and are capable of binding PDGF, we continued the PDGF/PDGFR studies using a myogenic cell line. The C2 cell line was chosen because it was originally derived from normal skeletal muscle of adult mouse, probably from a satellite cell (Yaffe and Saxel, 1977; C2 cells were provided by Dr. D. Yaffe, Weizmann Institute of Science, Israel). We showed that proliferating C2 cells bind higher levels of PDGF-BB (165×10^3 molecules/cell at saturation) and much lower levels of PDGF-AA and PDGF-AB ($6\text{--}12 \times 10^3$ molecules/cell at saturation). This indicated that C2 myoblasts express high levels of PDGF receptor β and low levels of PDGF receptor α . In order to analyze the effect of PDGF on cell proliferation, C2 cells were maintained in low serum medium [MEM containing 2% fetal calf serum (FCS)]; incorporation of ^3H -thymidine following a 2 hour pulse was determined in the presence and absence of PDGFs. PDGF-BB proved a potent mitogen for C2 cells (enhanced ^3H -thymidine incorporation by about fivefold). PDGF-AB had a moderate effect on DNA synthesis (less than twofold), and PDGF-AA had no effect (Yablonka-Reuveni et al., 1990a). The effect of PDGF-BB³ on proliferation of C2 myoblasts is demonstrated in Figure 5. The mitogenic effect of PDGF-BB can be abolished in the presence of an antibody that recognizes all three forms of PDGF.

“Baseline proliferation” in the low serum medium in the absence of added PDGF-BB is not reduced when the anti-PDGF is added. This suggests that baseline proliferation is not dependent on PDGF, which might be produced by the C2 cells or might be present in the medium (In addition to recognizing human PDGFs, the antibody used can recognize both bovine and rodent PDGFs according to company specifications.) In contrast to the anti-PDGF antibody, control IgG fraction does not have a significant effect on C2 proliferation in the absence or presence of PDGF-BB, indicating that the effect of the anti-PDGF is specific.

We further analyzed the effect of PDGF-BB on differentiation decision and terminal differentiation by analyzing the expression of the myogenic regulatory gene myogenin and of sarcomeric myosin. In many myogenic cell systems, expression of myogenin begins after the onset of MyoD expression and is noted just prior to the expression of sarcomeric myosin or fusion into myotubes. This phase of myogenin expression prior to full terminal differentiation (i.e., myosin expression) was named by Wright and colleagues *mid-decision* (Wright et al., 1989). We found that C2 cells also follow this pattern of myogenin expression just prior to myosin expression. We tested myogenin and myosin expression by the cultured cells using specific antibodies in an indirect immunofluorescence assay and quantitatively measured the numbers of positive cells at different time points. A monoclonal antibody against myogenin (mAb F5D, kindly provided by Dr. W. Wright; for antibody description see Cusella-De Angelis et al., 1992) and a monoclonal antibody against sarcomeric myosin heavy chain (MF20, originally developed in Dr. Fischman’s laboratory; Badar et al., 1982) were used. We found in a variety of C2 experiments that when PDGF is present, the frequency of myogenin-positive cells (and eventually myosin-positive cells) is drastically reduced, but that once a certain cell density is achieved, PDGF is no longer effective in suppressing expression of myogenin, or eventually, of myosin. Collectively, our studies suggest that myoblasts are capable of responding to PDGF only before entering differentiation decision (or mid-decision as termed by Wright) and that differentiation decision can be delayed in the presence of PDGF. In parallel experiments, we determined that the delay in differentiation decision imposed by PDGF-BB correlates with an effect of PDGF-BB on increasing the number of proliferating cells as detected by indirect immunofluorescence of cells that incorporated bromodeoxyuridine (Yablonka-Reuveni and Rivera, in preparation).

Collectively, based on these C2 experiments, we propose that PDGF increases the number of proliferating myoblasts required for regeneration by stimulating proliferation and inhibiting differentiation.

PROLIFERATION OF ADULT CHICKEN MYOBLASTS IS REGULATED BY SPECIFIC ISOFORMS OF PDGF

Once we established that PDGF would regulate proliferation of a murine myogenic cell line, we went on to investigate whether PDGF also regulates chicken myogenesis. As we were interested in the transition from fetal to adult myoblasts, we analyzed PDGF-receptor

³Highly purified PDGF-BB (human recombinant; produced in yeast) used for the studies described here was kindly provided by Dr. C.E. Hart (ZymoGenetics Incorporation, Seattle, WA).

expression by primary myogenic cultures from late stages of embryogenesis (E19 breast muscle, adult myoblasts) and from mid-embryogenesis (E10, fetal myoblasts). The most striking result was the high-level binding of PDGF-BB by E19 cells ($40\text{--}45 \times 10^3$ molecules/cell at saturation) compared with the lower level of PDGF-BB binding by E10 cells ($1\text{--}3 \times 10^3$ molecules/cell at saturation), a reflection of many more PDGFR β subunits in E19 cells than in E10 cells (Fig. 6). In accordance with the model shown in Figure 4, E19 myoblasts are representative of adult myoblasts. Furthermore, the results regarding the level of PDGF-AB binding by E19 compared with E10 myoblasts are in agreement with our previous observations that E10 myoblasts binds very little, if any, PDGF-AB compared with myoblasts from adult chickens (Yablonka-Reuveni et al., 1990c). Binding of PDGF-AA was not detected for either ages. Fibroblasts were capable of binding PDGF-AA, but neither myogenic clones nor myogenic mass cultures bind PDGF-AA (Yablonka-Reuveni and Seifert, 1993). This result indicated that myoblasts, and not contaminating fibroblasts, were binding PDGF-AB and -BB in cultures of E19 myoblasts. In accordance with our model of the appearance of adult myoblasts in late embryogenesis (Fig. 4), we concluded that adult myoblasts express many more receptors for PDGF than fetal myoblasts, and that the receptor is predominantly the β -type.

We also analyzed ^{125}I -PDGF binding and ^3H -thymidine incorporation in response to PDGF using clonally derived chicken myoblasts from E10 and E19 muscle; clonally derived myoblasts were used in order to ensure purity of the myoblast preparation and to study potential heterogeneity between myogenic precursors within the same developmental age. Analyzing multiple clones from both stages, we observed that clonally derived myoblasts were capable of binding PDGF-AB and PDGF-BB, and their proliferation is significantly enhanced in the presence of these PDGF isoforms, whereas PDGF-AA had no effect. All clones from E19 were responsive to PDGF, as were the clones derived from E10 that expressed receptors to PDGF (but some E10 clones expressed very few PDGF receptors). Given the low level of PDGF binding to primary myogenic cultures from E10 detected by us, and a previously observed low frequency of expandable clones in E10 cultures detected by others (Quinn et al., 1984), we suggested that the procedure of clonal expansion resulted in amplification of E10 myoblasts that are already of the adult type. Collectively, the PDGF studies, like the myosin isoform studies, suggest that as early as E10, a small number of myogenic cells expresses an adult myoblast phenotype.

PDGF MIGHT BE INVOLVED IN THE TRANSITION FROM FETAL TO ADULT MYOBLASTS DURING LATE CHICKEN EMBRYOGENESIS

Our working assumption is that adult-type myocytes (myocytes expressing ventricular but not embryonic MHC) are derived from adult myoblasts and that myoblasts expressing PDGF receptors belong to the adult myoblast population. The myosin isoform studies and the PDGF receptor studies have demonstrated that cells with the adult myoblast phenotype are present in the chick muscle, although in small numbers, as early as mid-embryogenesis (E10). Such a low number of adult myoblasts (or their precursors) could be set aside early in development and maintained at low frequency until a later period of development when they can proliferate extensively and become dominant. At that time, the environment might not

be ideal to support proliferation of fetal myoblasts, and the existing fetal myoblasts could be depleted by fusing into myotubes. Adult myoblasts would eventually become quiescent but would resume mitotic activity under the appropriate cues. Because our results suggest that adult, but not fetal, myoblasts express receptors to PDGF, it is possible that the availability of PDGF in appropriate biological form in late embryogenesis may facilitate the transition to predominantly adult myoblasts. These eventually become quiescent in the adult stage but can be recruited as a result of injury. We further favor the possibility that fetal and adult myoblasts share a common precursor that is set aside early in development; this precursor is separated from the precursor of embryonic myoblasts during early embryogenesis, as was suggested by Seed and Hauschka (1988). Our clonal analysis of cultured chicken myoblasts has demonstrated that some myogenic clones from E14 are mixed clones: they contain myocytes that express ventricular myosin only and myocytes that express both ventricular and embryonic myosins. Moreover, the number of ventricular-only myocytes in such clones increased with time in culture (Hartley et al., 1992). Mixed clones were not detected in clones from postnatal muscle. Given our operational definition of adult myoblasts, the finding of mixed clones supports the notion that there is a myogenic precursor capable of producing both fetal and adult myoblasts; in posthatch animals the fetal/adult precursor completed its developmental program and can no longer give rise to fetal myoblasts. In vivo, the type of progeny the fetal/adult precursor give rise to can be developmentally regulated and the transition from predominantly fetal to predominantly adult myoblasts could in this instant also be facilitated by the presence of bioavailable PDGF(s) during late stages of muscle development. It is noteworthy that the idea that PDGF can indeed influence the fate of a cell within a specific lineage has been demonstrated in studies on the proliferation and differentiation of oligodendrocytes (Hart et al., 1989; Wolswijk and Noble, 1992).

SEARCH FOR PHYSIOLOGICAL OR CELLULAR AGENTS THAT MAY ACT TOGETHER WITH PDGF TO MODULATE MYOGENESIS

Multiple agents have been implicated as regulators of myogenesis of satellite cells in vivo. We were interested in identifying potential physiological agents that might modulate the effect of PDGF on proliferation of satellite cells. We have approached the issue in cell culture conducting the experiments only with PDGF-BB—the isoform that can interact with all three PDGF receptor combinations.

Transferrin

Transferrin, an iron-binding protein, has been considered an important regulator of myoblast proliferation. It has been shown to stimulate myogenesis when added to myogenic cell culture and a large portion of the activity in chicken embryo extract and muscle extract, which support myogenesis in vitro, has been attributed to transferrin (Chen and Quinn, 1992; Li et al., 1982; Kardami et al., 1985a). Species specificity has been established for transferrin effect during myogenesis (Beach et al., 1985; Li et al., 1982; Kardami et al., 1985a). Transferrin was shown to act synergistically with FGF during chick myogenesis in culture (Kardami et al., 1985a, b). Furthermore, transferrin is present in skeletal muscle in vivo and thus its effect can be of physiological relevancy (Kardami et al., 1985a; Matsuda et al., 1984; Seed et al., 1988). We therefore examined whether transferrin would enhance the

ability of PDGF to promote DNA synthesis as measured by ^3H -thymidine incorporation assays. The study described in this paper was conducted using chicken myoblasts prepared by expansion of one myogenic clone from the breast muscle of E19 embryo (as discussed above, an E19 clone is presumably a descendent of an adult myoblast). As shown in Figure 7, treating the cultures with both PDGF-BB and highly purified chicken transferrin (fortified with iron) led to an increase in cell proliferation that is higher than the effect of either agent alone. PDGF-BB alone increased ^3H -thymidine incorporation by about 2.5-fold; transferrin also promoted proliferation, although to a lesser level than PDGF-BB. Hence, the two agents have a concerted effect on chicken myoblast proliferation, and the effect of PDGF can be augmented in the presence of transferrin.

Basic Fibroblast Growth Factor

As discussed above, the FGF family of growth factors has been shown to promote proliferation of myoblasts from a variety of species and ages; both basic and acidic FGF were identified in the muscle tissue by immunohistochemical localization. Therefore, it is likely that FGF plays an important role in modulating myogenesis in vivo of both embryonic and adult muscle. FGF and PDGF have been shown to work in concert in a variety of cell culture systems (McKinnon et al., 1990; Schollman et al., 1992; Wolswijk and Noble, 1992). We were therefore interested to determine whether FGF and PDGF exert synergistic effect on adult myoblast proliferation. Specifically, we examined the possible interaction of basic FGF with PDGF during proliferation of C2 myoblasts. The results of this study are shown in Figure 8. Whereas both FGF and PDGF enhanced myoblast proliferation to similar levels, their effects are not additive. PDGF and FGF may thus replace or complement each other to promote proliferation of myoblasts effectively, but their maximal effects do not require the presence of their counterpart factor.

Cell-Secreted Agents

Myoblasts could potentially express agents that work in concert with PDGF to enhance myoblast proliferation. Such agents, which could include other growth factors, might be secreted by the cells and accumulate in the extracellular environment. These same agents secreted in cell culture could potentially be present in the medium in a soluble form and/or bound to the extracellular matrix. For example, autocrine secretion of IGF-II by cultured myoblasts has been described by Florini et al. (1991). IGFs, FGF, and PDGF were localized to the extracellular matrix of a variety of cell types (Jones et al., 1993; Raines and Ross, 1992; Vlodavsky et al., 1991). Also, both basic and acidic FGF were localized in vivo to the extracellular matrix of muscle fibers (DiMario et al., 1989; Oliver et al. 1992). As for the FGF studies, we used the C2 cell culture model to test the possibility that myoblasts may secrete agents that can work in concert with PDGF.

In one set of experiments we tested whether low serum medium (containing 2% FCS) that was conditioned by high-density proliferating C2 cells (which are responsive to PDGF at the time of medium harvest, as determined on parallel cultures) could modulate the effect of PDGF-BB on C2 cells. C2 cells were maintained in conditioned 2% FCS medium ($\pm$ PDGF-BB) or in fresh 2% FCS medium ($\pm$ PDGF-BB) and examined for cell proliferation at daily intervals, as shown in Figure 9. The effect of PDGF-BB on ^3H -thymidine incorporation was

identical whether cells were maintained in conditioned or fresh 2% FCS medium. Thus, cellular agents that might have been secreted to the medium by the C2 cells do not modulate the PDGF effect in our experimental system.

In a second set of experiments we tested whether the C2 cells deposited any components into the extracellular matrix that could modulate PDGF effect. For this purpose, C2 cells cultured at high density into gelatin-coated 16-mm wells were allowed to proliferate while in 2% serum medium, and eventually while still in the proliferative state were removed from the wells by a procedure that lifted off the cells from the well surface but left substrate-associated material behind. Cell lifting was achieved by overlaying the cultures with a physiological buffer containing 0.01 M EGTA for 60 minutes with vigorous agitation. Fresh C2 cells were then cultured into the conditioned wells and maintained in 2% FCS medium $\pm$ PDGF-BB. We could not detect any significant change in the effect of PDGF-BB on the proliferation of fresh C2 cells cultured at either low or high densities into the conditioned wells (Yablonka-Reuveni and Rivera, unpublished data).

Collectively, our search for physiological or cellular agents that could work in concert with PDGF to enhance myoblast proliferation has identified transferrin as an important modulator of PDGF effect. Basic FGF, an important mitogen for myoblasts isolated from many species and ages, enhanced proliferation of C2 cells but did not modulate the effect of PDGF-BB on C2 cell proliferation.

SUMMARY

The morphology of satellite cells in normal, injured, and pathological muscle has been investigated for many years (Mauro, 1979; reviewed in Grounds and Yablonka-Reuveni, 1993). The information gleaned contributed immensely to current concepts and hypotheses in the field. However, the understanding of the mechanisms governing the regulation of satellite cells in postembryonic life, or the appearance of adult myogenic precursors as a distinct myogenic population in late development, is just in its beginning. Work by our group and others suggests that satellite cells represent a unique myogenic population that becomes dominant in late stages of embryogenesis. Additional studies have indicated that satellite cells do not express any of the known myogenic regulatory genes prior to their activation, suggesting that the satellite cells might be kept in an early myogenic determination phase, similar to that of the myogenic precursors that enter the limb buds during early development. Future experiments on the expression of yet unknown myogenic regulatory genes in satellite cells may assist in identifying an earlier phase of myogenic determination. Multiple growth factors seem to be important for adult myoblast proliferation in culture. Future experiments on the effect of growth factors during activation of satellite cells in vivo should assist in resolving the physiological importance of these agents.

We propose that PDGF promotes satellite cell activation and proliferation by directly acting on these cells. To effect satellite cell proliferation, PDGF must be bioavailable in vivo and receptors for PDGF should be expressed by the cells. In injured muscle, PDGF is probably released from platelets, phagocytes, and other inflammatory cells that are present in injured tissues including muscle (Mitchell et al., 1992; Ross et al, 1986). Also, PDGF might be

released from endothelial and smooth muscle cells of the blood vessel wall that express PDGF (Ross et al., 1986, Westermark and Sorg, 1993). Furthermore, we now have results indicating that PDGF-like mitogenic activity is present in rat fibers (work in progress). Our published studies on mouse and chicken myoblasts have demonstrated that adult-derived myoblasts express receptors for PDGF. More recently, we documented by specific antibodies that cultured rat satellite cells express both the α - and β -receptor proteins (work in progress). Immunolocalization of the PDGF receptor in muscle cross sections (Tidball et al., 1992; Yablonka-Reuveni et al., work in progress) provides further support for the physiological importance of PDGF during growth and repair of postnatal muscle. The interpretation of the role of growth factors during muscle growth and regeneration based on cell culture studies is limited because of uncertainties about the effects of culture conditions on the properties of myoblasts. Although this is usually the first approach toward establishing a mitogenic role for a growth factor in a new system, we have recently begun examining the role of PDGF and other growth factors during myogenesis of satellite cells on isolated rat muscle fibers. This system may better preserve the *in vivo* status of satellite cells. We propose that PDGF is also an important regulator of the appearance of adult myoblasts in late embryogenesis. At present, this notion is only based on the observation that adult, but not fetal, myoblasts express receptors to PDGF. Further work on the availability of PDGF at the time that adult chicken myoblasts become dominant, as well as localization of the PDGFs and their receptors in the intact muscle during different stages of development, will shed light on the issue.

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Fig. 1.

Demonstration by indirect immunofluorescence of the laminin component of the basement membrane surrounding muscle fibers in the pectoralis muscle from late embryonic, young, and mature chickens. Unfixed frozen cross sections were prepared from (A) 19-day-old embryo; (B) 19-day-old chicken; and (C) 2-month-old-chicken. Sections were reacted with a monoclonal antibody against chicken laminin (prepared by Bayne et al., 1984) followed by fluorescein-labeled goat anti-mouse IgG; arrows indicate blood vessels that are also positive for laminin (40 $\times$ objective was used for all micrographs). (Reproduced from Grounds and Yablonka-Reuveni, 1993, with permission of Chapman & Hall.)

Fig. 2.

Electron micrographs of a muscle cross section from the pectoralis muscle of 2-month-old chicken. **A:** Satellite cell lying between the basement membrane and plasma membrane of muscle fiber. **B:** Nuclei within the muscle fibers and other cells outside the fiber that can be easily mistaken for satellite cells at the light microscopy level. Adult chicken muscle was immersion fixed in mixed aldehyde, post-fixed in osmium tetroxide, and embedded in epoxy resin. Sections were stained with lead citrate and examined in a Philips 201 TEM operated at 60 KV. (Yablonka-Reuveni and Anderson, unpublished data). **A:** bar = 1 μ m and magnification = $\times 13,440$; **B:** bar = 1 μ m and magnification = $\times 5,584$. Sat, satellite cell nucleus; f, myofiber; fn, nucleus within myofiber; cap, capillary; e, endothelial cell; p, pericyte; black arrows indicate basement membrane: white arrows indicate the plasma membrane of the myofiber and the plasma membrane of the satellite cell.

Fig. 3.

Schematic of embryonic and ventricular MHC expression patterns in myogenic cultures from skeletal muscle of fetal (E10) and adult (2-month-old) chickens detected by monoclonal antibodies against specific MHC. Monoclonal antibodies against specific isoforms of MHC were originally prepared in the laboratory of Dr. E. Bandman (University of California, Davis) and are described in more details in Hartley et al. (1991). This schematic does not exclude the possibility that other sarcomeric MHC are expressed by the cells as well. (Reproduced from Hartley and Yablonka-Reuveni, 1992, with permission of Cordon and Breach. Model is based on data published in Hartley et al., 1991, 1992.)

Fig. 4.

Schematic of the presence of fetal and adult myoblasts during embryogenesis and posthatch stages of chicken development. E10, E14, and E18 represent days of embryogenesis; PH1 represents 1 day after hatching; 3WK, 8WK, and 16–20 WK represent weeks posthatching. H refers to hatching, which occurs on days 20–21. (Reproduced from Hartley and Yablonka-Reuveni, 1992, with permission of Gordon and Breach. Model is based on data published in Hartley et al., 1991, 1992.)

Fig. 5.

Effect of PDGF-BB on proliferation of adult mouse-derived C2 myoblasts. Cells were plated into gelatin-coated, 24-well trays (well diameter = 16 mm) at a density of 1×10^4 cells/well. Cells were maintained for the initial 24 hours in proliferation medium, which consists of MEM fortified with 20% FCS. Medium was then changed to MEM containing 2% FCS in the absence and presence of PDGF-BB (20 ng/ml) and antibodies. Goat anti-PDGF (IgG fraction) and control goat IgG were added to the wells at 50 μ g/ml. The 2% FCS medium with or without PDGF and antibodies was replaced every 24 hours to ensure ample supply of additives and cells were harvested following 48 hours in the 2% FCS. Two hours before cell harvesting, the cultures were exposed to ³H-thymidine at 1 μ C/ml. and the extent of ³H-thymidine incorporation into TCA-precipitable material was determined as in Yablonka-Reuveni et al. (1990a). Plotted values represent the average of triplicate wells. FCS used for this study was heat inactivated (56°C, 30 minutes) to avoid possible complement reaction in the presence of the antibodies. Note that our original studies on the response of C2 cells to PDGF were conducted with untreated FCS (Yablonka-Reuveni et al., 1990a); in experiments that are not shown here we demonstrated that ³H-thymidine incorporation by C2 cells maintained in 2% FCS medium alone and its enhancement by PDGF-BB is similar whether untreated or heat-inactivated FCS is used. Antibody against PDGF was purchased from Upstate Biotechnology (Lake Placid, NY); this polyclonal antibody is made in goat immunized with human PDGF-AB. Using Western blotting we confirmed that this antibody recognizes all three forms of PDGF of human origin as well as rat PDGF-like proteins. Inhibition of PDGF-BB effect in the presence of anti-PDGF was demonstrated by us for C2

cultures initiated at a lower density as well (2.5×10^3 /16-mm well), (Yablonka-Reuveni and Rivera, unpublished results).

Fig. 6.

Saturation binding of PDGF-AB and PDGF-BB to primary myogenic cultures from the pectoralis muscle of 10-day (E10) and 19-day (E19) chicken embryos. Freshly isolated cells were cultured into gelatin-coated 24-well trays (well diameter = 16 mm) using our standard medium for chicken myogenic cultures (MEM containing 10% horse serum and 5% chick embryo extract) for 24 hours. Medium was then replaced with MEM containing 2% FCS for about 18 hours (to reduce any possible exogenous PDGF) and at the end of this period cultures were subjected to a PDGF binding assay. The plotted values represent the average specific binding to triplicate wells, and standard error was less than 5–10%. Cell numbers were determined on parallel triplicate cultures. (Reproduced from Yablonka-Reuveni and Seifert, 1993, with permission of Academic Press.)

Fig. 7.

PDGF-BB and transferrin have a concerted effect on DNA synthesis by chicken myoblasts. Chicken myoblasts isolated by a clonal expansion of a myogenic clone from the pectoralis muscle of a 19-day-old chicken embryo (clone E1915) were cultured at 3×10^4 cells/16-mm well in MEM-based medium containing 10% horse serum and 5% chicken embryo extract for a period of 24 hours. Medium was then changed to MEM containing 2.5% FCS for 48 hours in the absence or presence of additives. MEM was fortified with iron in the form of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at 0.834 mg/l to provide iron for transferrin. Iron formulation and concentration was based on F-10 medium from Gibco. Highly purified chicken transferrin was from Organon Teknika Corporation (Cappel Research Products, Durham, NO); we used chicken transferrin for this study because species specificity was previously established for the effect of transferrin on chicken myogenesis. Transferrin (Tf) was added to MEM at 20 $\mu\text{g/ml}$ and PDGF-BB was added at 20 ng/ml. The 2.5% FCS-medium and additives were replenished every 24 hours. Prior to cell harvesting, the cultures were exposed for 4 hours to ^3H -thymidine at 1 $\mu\text{Ci/ml}$. and the extent of ^3H -thymidine incorporation into TCA-precipitable material was determined as for Figure 5. Plotted values represent the average of triplicate wells, (Yablonka-Reuveni, unpublished results.)

Fig. 8.

Effect of PDGF-BB and basic FGF on the proliferation of C2 cultures. Cultures were initiated at low density (1×10^3 cells/16-mm well) or high density (7.5×10^3 cells/16-mm well) in proliferation medium (MEM with 20% FCS and 0.5% chicken embryo extract). After 24 hours medium was changed to MEM containing 2% FCS and cultures were maintained in this serum-low medium for various length of time in the absence or presence of growth factors. PDGF-BB was added at 20 ng/ml and bFGF was added at 4 ng/ml. These concentrations of growth factors are maximally effective, as determined in separate experiments. The 2% FCS medium with or without growth factors was replaced every 24 hours. Two hours prior to cell harvesting the cultures were exposed to ^3H -thymidine at 1 $\mu\text{C}/\text{ml}$ and the extent of ^3H -thymidine incorporation into TCA-precipitable material was determined as for Figure 5. Plotted values represent the average of triplicate wells. Highly purified bFGF (human recombinant; produced in yeast) was kindly provided by Dr. S.D. Hauschka (University of Washington, Seattle, WA). (Yablonka-Reuveni and Balestreri unpublished data; study was briefly mentioned in an abstract by Yablonka-Reuveni et al., 1990b).

Fig. 9.

Effect of PDGF-BB and medium conditioned by C2 cells on the proliferation of C2 myoblasts. High-density C2 cultures were prepared as in Figure 5 and maintained in 2% FCS medium for 3 days with daily changes of medium. Medium from the third day at which time cells are responsive to PDGF, as was shown on parallel cultural was collected, cleared from cell debris by centrifugation (2,000g, 5 minutes), and added to fresh C2 cultures at the time of transition from the 20% FCS medium to 2%. Conditioned medium, with or without PDGF-BB (20 ng/ml) was replaced every 24 hours. Incorporation of ^3H -thymidine was determined as in Figure 5. Low- and high-density cultures were initiated at 2.5×10^3 cells/16-mm well and 1×10^4 cells/16-mm well, respectively. Note that proliferation of low-density cultures in conditioned medium is lower than in fresh medium but PDGF effects is similar. Also, the decline in proliferation in high-density cultures after 3 days is due to terminal differentiation of the cells, but more cells are still proliferating in the presence of PDGF even during the third day. As in Figure 5, FCS used in the 2% FCS medium was heat inactivated, as described in the legend to Figure 5. (Yablonka-Reuveni and Rivera, unpublished data.)