Delayed Myogenesis Associated with Muscle Fiber Hyperplasia in High-Growth Mice (44107)

PHILIP J. SUMMERS AND JUAN F. MEDRANO[1]
Department of Animal Science, University of California at Davis, Davis, California 95616-8521

Abstract. **The *high-growth* (*hg*) locus in the mouse produces a 40% increase in adult body weight and proportional hypertrophy of skeletal muscles, due to fiber hyperplasia. Using biochemical and histological criteria we tested the hypothesis that myogenesis is delayed in fetal high-growth mice, compared with normal mice, allowing an increase in the muscle stem cell population. Significant biochemical and weight differences between lines were first apparent at embryonic day 17 (E17). At this stage, high-growth hind limbs were smaller, contained fewer nuclei, less RNA, and showed less creatine kinase (CK) activity, than controls. Histologically, high-growth muscles contained fewer, but larger, myotubes and increased extracellular matrix (at E17) compared with controls. By embryonic day 19, high-growth limbs showed increases in wet weight, CK activity, protein, RNA, and DNA compared with controls. Our results are consistent with delayed production and/or fusion of high-growth myoblasts to form secondary myotubes from embryonic day 15 to 17 and accelerated production of secondary myotubes from day 17 to 19. Delayed fusion of high-growth myoblasts may allow an increase in the muscle stem cell population, resulting in fiber hyperplasia at birth.**
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Little is known about the molecular control of cell number and ultimate tissue size in animals. The high-growth mouse is a promising model for these studies since the *high-growth* (*hg*) locus is important in controlling muscle fiber number and adult body size. The high-growth phenotype results from a single autosomal, partial, recessive locus (*hg*) mapped to chromosome 10, which produces a 40% increase in adult body weight and greater plasma concentrations of insulin-like growth factor–I (IGF-I) than in control mice (1–3). Increased body size is accompanied by proportional growth of skeletal muscles due to fiber hyperplasia and moderate fiber hypertrophy (4).

Several studies of animals selected for large body size have demonstrated muscle fiber hyperplasia (5–8). In some species this is associated with delayed muscle differentiation and commitment to the myogenic lineage. Japanese quail selected for large body size show a 60% increase in muscle fiber number and delayed early somite formation (9, 10). Fiber hyperplasia is a characteristic of double-muscling (DM) in cattle (11), which is also associated with delayed myogenic differentiation. Quinn *et al.* (12) found that fetal DM myoblasts showed prolonged proliferation and delayed fusion in culture compared with control cells. DM fibroblasts also produced increased amounts of an unknown paracrine growth factor, and the authors suggested that myoblast proliferation in DM cattle may be controlled by growth factors originating from connective tissue cells. Muscle differentiation is also delayed in satellite cell cultures from heavily muscled turkeys (13). In a study of mice selected for large body size, Penney *et al.* (8) found no delay in myoblast fusion and suggested that muscle fiber hyperplasia, in growth-selected mice, was due to changes in myoblast pre-fusion proliferation rates.

The above studies in other species raise the possibility that the *hg* locus may act during myogenesis to alter the proliferation rate and/or the fusion kinetics of high-growth muscle cells compared with control cells. Fiber hyperplasia may result from delayed myoblast fusion

[1] To whom requests for reprints should be addressed at Department of Animal Science, University of California at Davis, Davis, CA 95616-8521.
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and differentiation allowing an increase in the muscle stem cell population (10). Using biochemical indices, we tested the hypotheses that high-growth muscle cells, at an early stage in muscle development, show increased proliferation rates and/or delayed differentiation.

Materials and Methods

Lines of Mice. Mice homozygous for the *hg* locus were obtained from our breeding colony at the University of California at Davis. The *hg* locus, described in Bradford and Famula (14), has been introgressed into the C57BL/6J background, creating congenic line C57BL/6J-*hghg* (HG) (2). In this study, a total of 84 HG mice and 58 control mice (C57BL/6J) were used.

Tissues. All viable fetuses from litters of both lines of mice were sacrificed, weighed, and measured at embryonic days 13 (E13), 15 (E15), 17 (E17), and 19 (E19), and, as pups, at birth, day 6, and day 12. The day of coitus (embryonic day 0) was determined by the presence of a vaginal plug and developmental stage was confirmed by fetal length (15). Typical gestation period for both genotypes was around 20 days. For assay of creatine kinase, DNA, RNA, or protein, hind paws were removed and the remainder of the limb bud was weighed and quickly frozen in liquid nitrogen. At E13, limb buds were pooled from several fetuses to obtain enough tissue for analysis. The number of pooled limbs used was taken into account in calculations. Due to the fragility of early fetuses, skin was left intact at E13 and E15, but dissected from E17 and E19 fetuses. Hind-limb muscles (gastrocnemius, tibialis anterior, quadriceps, and hamstrings) were dissected from bone at birth and at 6 and 12 days of age, pooled, and frozen.

Biochemical Assays. Limbs and muscles were homogenized in water using a polytron homogenizer for 1 min at full speed, and an aliquot of the homogenate was assayed for protein using bovine serum albumin as a standard (16). Protein values were expressed per milligram of tissue (contribution of protein to limb weight) and per limb (a measure of limb size). The remainder of the homogenate was assayed for DNA and RNA. Briefly, acid-insoluble material was precipitated with 0.2 *N* perchloric acid (PCA), followed by solubilization in KOH and precipitation of DNA in 1.2 *N* PCA. Pelleted DNA was assayed by the fluorometric method of Labarca and Paigen (17) using Hoescht dye (33258). RNA in the supernatant was assayed by the dual-wavelength method of Fleck and Begg (18), reading RNA content from a nomograph. DNA, which is an index of cell number, and RNA, a measure of protein synthetic activity, were expressed per milligram of tissue and per limb.

Aliquots of limb or muscle homogenates were centrifuged at 5000*g* for 15 min and the supernatants assayed for creatine kinase activity using a 1-step ultraviolet (UV) absorbance kit from SIGMA (Procedure No. 47UV). One unit of activity (U) is defined as the amount of enzyme that produces one micromole of NADH per minute under the conditions of the assay procedure. Control and high-growth samples were treated identically and assayed simultaneously. CK activity was expressed per milligram tissue (a measure of the proportion of muscle in the limb), per milligram protein (an index of myogenic fusion relative to non-muscle protein), and per milligram of DNA (an index of DNA unit volume or volume of cytoplasm controlled by one nucleus). Means for biochemical parameters from at least five mice from each line were compared by independent, two-tailed Student's *t* tests at each time point.

Light Microscopy. Limb buds at E13 and E17 were fixed in 4% paraformaldehyde, washed, dehydrated in a series of alcohols, and embedded in methacrylate. Sections were cut at 2 μm and stained with toluidine blue.

Results

High-growth fetal body weights were significantly greater than controls at E15 (11%; $P < 0.05$), E19 (17%; $P < 0.01$), at birth (22%; $P < 0.01$), and at 12 days of age (25%; $P < 0.01$). There was no difference in body weight between lines at E13, E17, and 6 days of age (Fig. 1A). High-growth mouse limb weights (Fig. 1B), and limb weights normalized to body weights (data not shown) were 42% less than control at E17. At E19 high-growth limb weights were 25% greater than control values (Fig. 1B).

Biochemical Data. Fetal weight, limb bud weight, CK activity, protein, DNA, and RNA content of limbs all increased during the developmental period in both lines. There were no significant differences in CK units of activity per milligram of tissue (Fig. 1C), units per limb (data not shown), or units per milligram of DNA (Fig. 1E) at E13 and E15 between lines. At E17 total CK activity per limb in high-growth fetuses was decreased by 39% (not significant, data not shown). However, CK activity expressed on a protein basis was decreased by 44% in high-growth limbs at E17 ($P < 0.01$; Fig. 1D). CK activity per milligram of tissue was significantly increased in high-growth limbs compared with control at E19 and at birth ($P < 0.05$; Fig. 1C). At 6 days of age, CK activity (expressed per milligram of tissue, protein, and DNA) in high-growth muscles was around 20% less than control (Fig. 1C–E). CK activity expressed on a DNA basis was significantly ($P < 0.05$) greater than control at 12 days of age, suggesting that DNA unit volume may be increased in high-growth muscle fibers at this stages (Fig. 1E). This is consistent with weight data above and is borne out by the histological appearance (see below).

Protein in high-growth limbs, expressed on a tissue weight basis, was greater than control at E17 (77%;

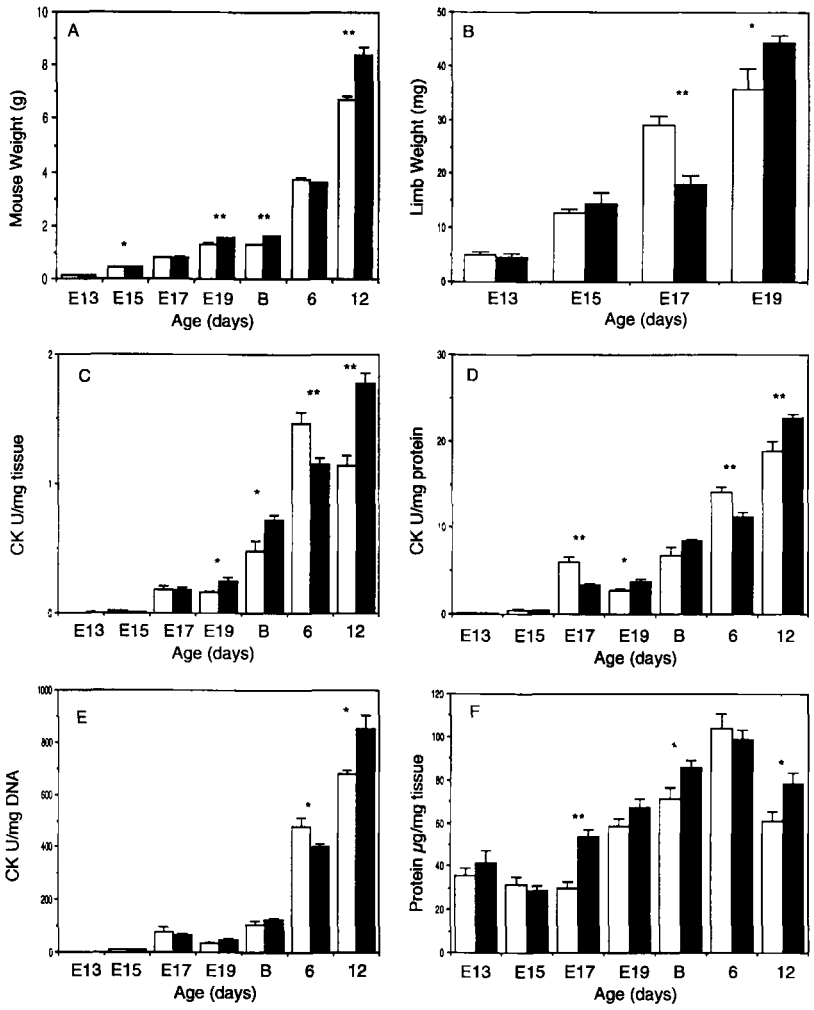

Figure 1. Weight and biochemical changes as a function of developmental stage in fetal mouse limb buds: (A) body weight (mg), (B) limb bud weight (mg), (C) creatine kinase (CK) U/mg tissue, (D) CK U/mg protein, (E) CK U/mg DNA, (F) total protein (μg/mg tissue). B, day of birth; □, C57BL/6J (control); ■, C57BL/6J-*hg/hg* (HG). Bars, at each time point the mean + SEM of 5–24 mice for Panel A, and of 5–11 mice for all other panels. Significant difference from control: $^{*}P < 0.05$; $^{**}P < 0.01$, Student';s *t* test.

$P < 0.01$; Fig. 1F). Presumably, high-growth values are high at E17 because tissue weight is lower than control (see above). There was no significant differences in protein expressed on a limb basis between lines from E13 to E17, but high-growth values were 50% greater than control at E19, which is consistent with fiber hyperplasia and increased limb weight at this stage ($P < 0.05$; Fig. 2A and 1B). Protein expressed on a DNA basis was increased by 63% in high-growth limbs at E17 (data not shown).

There were no differences in DNA content expressed on a tissue weight basis between lines from stages E13 to E19, but DNA per milligram of tissue was increased in high-growth muscles at birth (26%; $P < 0.05$) and at 12 days of age (25%; $P < 0.05$), compared with control (Fig. 2B). DNA expressed on a weight basis decreases postnatally (19), which occurred in both lines from birth to 12 days of age (Fig. 2B). There were no significant differences in DNA expressed on a limb basis between lines at E13 and E15 (Fig. 2C), but high-growth values were 32% less than control at E17 ($P < 0.01$). By E19, DNA per limb was increased by 41% in high-growth fetuses compared with controls ($P < 0.01$; Fig. 2C). This is a reflection of greater limb size at this age. Identical results were, of course, obtained when DNA content was converted to nuclei per limb, assuming 6.2 pg DNA/nucleus (20).

RNA content expressed on a tissue weight basis was greater in high-growth limbs compared with control at E17 ($p < 0.01$) and E19 ($p < 0.05$) and at 6 days of age ($P < 0.05$) (Fig. 2D). This suggests that high-growth limbs have greater overall protein synthetic rates from E17. When RNA was expressed on a limb basis no differences between lines were found at E13 and E15 (Fig. 2E). At E17, high-growth values were 15% less than control, although not statistically significant. By E19, high-growth RNA content expressed on a limb basis was 53% ($P < 0.01$) greater than control, consistent with larger limbs and other biochemical data above (Fig. 2E).

Light Microscopy. The histological appearance of E17 control extensor digitorum longus (EDL) muscles was typical of late fetal muscle (Fig. 3A). Multinucleated myotubes are organized into fascicles surrounded by minimal interstitial tissue. Myofibrils are well developed, and pale-staining myonuclei are located centrally and peripherally. Numerous darkly stained mononucleated

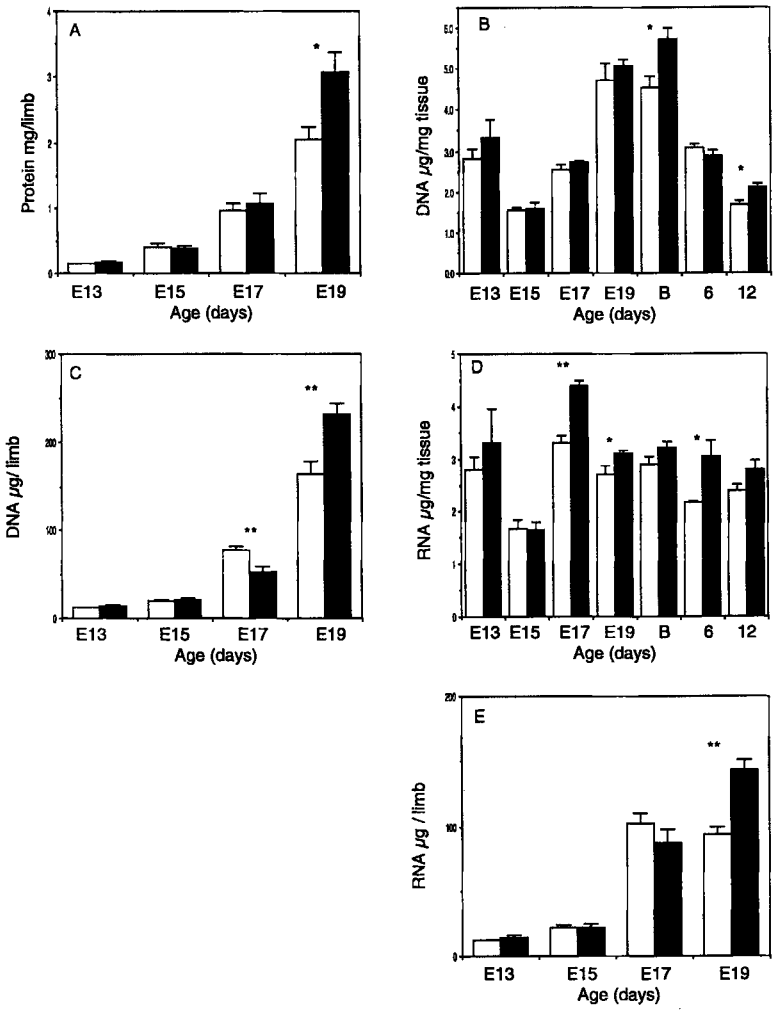

Figure 2. Biochemical changes as a function of developmental stage in fetal mouse limb buds: (A) total protein (mg/limb), (B) DNA (μg/mg tissue), (C) DNA (μg/limb), (D) RNA (μg/mg tissue), (E) RNA (μg/limb). B, day of birth; □, C57BL/6J (control); ■, C57BL/6J-*hg/hg* (HG). Bars, mean + SEM of 5–10 mice at each time point. Significant difference from control: $*P < 0.05$, $**P < 0.01$, Student's *t* test.

cells are present between fibers, some of which are probably unfused myoblasts. Ontell and Kozeka (21) have classified the larger myotubes as primary fibers and smaller myotubes as secondary fibers at this stage, but this distinction cannot be reliably made in these micrographs.

In contrast, the E17 high-growth EDL muscle appears to contain larger but fewer myotubes than control, with fewer, small (presumably secondary) fibers. The myotubes are not organized into discrete fascicles, and there is a marked increase in the darkly stained interstitial nuclear population and amount of extracellular matrix (Fig. 3B).

Discussion

We have previously shown that increased body size in high-growth mice is associated with muscle fiber hyperplasia (4). Here, we examined the possibility that fiber hyperplasia may be due to changes in early muscle development due to *hg*. We found no major differences in weight, CK activity, protein, or nucleic acids between control and high-growth mice at E13 and E15, which corresponds to the period when primary myotubes are formed by the fusion of embryonic type myoblasts (21, 22). By E15, normal mouse limbs contain a full complement of primary fibers (23). From the biochemical data, primary fiber production in high-growth mice therefore appears no different from control.

Major biochemical and weight differences between lines are first apparent at E17. At this stage, high-growth limbs are smaller, show less CK activity expressed on a protein basis, and contain fewer nuclei and less RNA per limb. Histologically, high-growth EDL muscle contains fewer, but larger, myotubes. Total protein is increased at this time in high-growth limbs, but this may not be myofibrillar protein since CK activity is reduced. This is supported by the histological finding of increased extracellular matrix in the high-growth EDL muscle. E15–E17 corresponds to the period of secondary myotube formation by fusion of fetal type myoblasts (21, 22). These results are consistent with delayed production and/or fusion of high-growth fetal type myoblasts to form secondary myotubes from E15–E17.

The largest differences between high-growth and control fetuses were apparent at E19. High-growth limbs

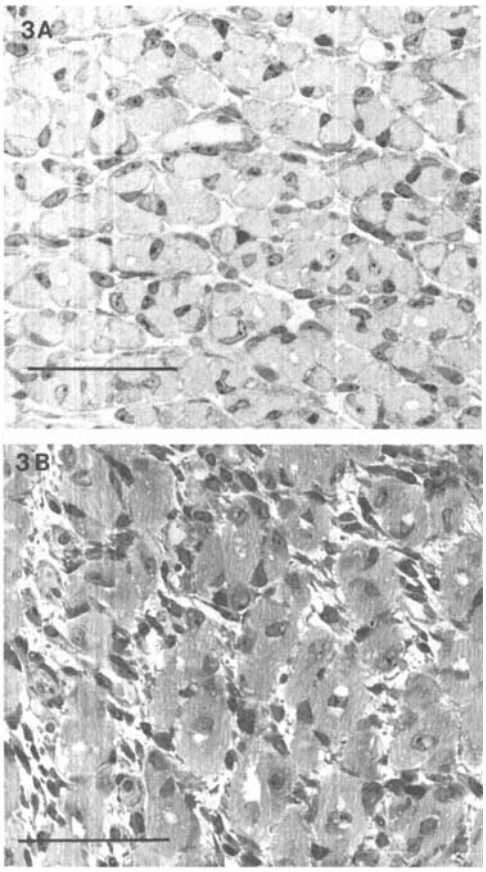

Figure 3. Methacrylate section (2 μm) of control mouse (A) and high-growth mouse (B) extensor digitorum longus (EDL) muscle at E17. Toluidine blue stain. Bar, 5 μm.

showed increases in almost all parameters: wet weight, CK activity, protein, RNA, DNA per limb (nuclear number) compared with controls. In normal mice, the adult number of muscle fibers is established by E19 (23). The data are consistent with accelerated production of secondary muscle fibers and/or fetal type myoblasts at E17–E19, resulting in fiber hyperplasia in adult high-growth mice (4). Since secondary fibers account for the majority of fast fibers found in the adult (22), this supports our previous finding of increased proportions of fast type 2 fiber sub-groups in high-growth muscles (4). Overall, therefore, high-growth mouse myogenesis is characterized by an initial delay in secondary fiber production (prolonged proliferation stage), allowing an increase in the muscle stem cell population, followed by accelerated production of secondary fibers at a later stage.

Surprisingly, we found a slight slowing of growth from birth to 6 days of age in high-growth mice. Although high-growth mice were larger at birth (22%), differences between lines were not apparent at 6 days of age. This suggests that high-growth muscles matured less rapidly than controls immediately after birth. Reduced milk supply, due to increased demand, may be a limiting factor in the growth of high-growth pups.

There have been few previous studies of muscle development in growth-selected animals. Penney *et al.* (8), studying mice selected for small (QS) and large size (QL), found no differences in fiber size (protein/DNA), timing of fusion (CK activity), and protein synthetic rate (RNA per nucleus) at 5 days before birth (approximately equivalent to E16 in our study), at birth, or at 20 days of age. However, QL mouse muscles contained more nuclei at all of these stages. They concluded that hyperplasia in QL mice was not due to a delay in fusion but to increased myoblast proliferation rates before fusion. In contrast, in high-growth mice at approximately the same stage (E17) we found delayed production of secondary myotubes (and presumably delayed fusion), increased protein per DNA ratio, and fewer nuclei. This, together with the histological appearance, might suggest that cell size is increased in high-growth mice *at* E17, but since in both studies an unknown amount of protein originates from extracellular matrix, no reliable conclusion can be made regarding cell size. In our study nuclear hyperplasia was not apparent until E19. Our findings may differ from those of Penney *et al.* (8) due to our use of a more sensitive CK assay, histological verification, and control mice with identical genetic backgrounds. We demonstrate here a delay in secondary fiber production due to a single locus.

Our findings agree with other studies which have reported delays in fetal myogenic differentiation associated with adult fiber hyperplasia. A Japanese quail line selected for high-growth rate (P) showed fiber hyperplasia and delayed early fetal development followed by accelerated growth (9, 24). Several aspects of muscle differentiation were also delayed compared with an unrelated control line. The P line showed delayed somite formation and expression of qmfl at 47 hr incubation. Expression of other muscle-specific genes qmf2, qmf3, and MHC was also delayed at later stages although somite number was the same in both lines (10). qmf1, 2, and 3 are homologous to mammalian myoD, myogenin, and myf5 respectively.

Muscle differentiation is also delayed in cell cultures established from animals showing fiber hyperplasia. Fusion is delayed in satellite cell cultures from heavily muscled turkeys (13). Myoblasts isolated from fetal DM cattle show prolonged proliferation and delayed, but increased, production of fused myotubes *in vitro* compared with control cells. DM fibroblasts produce increased amounts of a paracrine growth factor, which may be important in delaying myoblast fusion (12). The authors suggested that extracellular matrix growth factors may regulate the proliferative state of myoblasts *in vivo*. The increase in extracellular matrix and protein content we observed in the present study may play a role in delaying secondary fiber production in high growth mice.

Plasma concentrations of IGF-I in adult high growth mice are 10%–30% greater than control mice (3). IFG-I stimulates myoblast proliferation and myogenic differentiation *in vitro* (25). IGF-I is expressed in developing mouse muscle (26) and is elevated under conditions of increased muscle growth (27, 28). Increased exposure to IGF-I acting in an autocrine or paracrine manner could account for delayed differentiation and fiber hyperplasia in high-growth mice (12, 25). The relationship between *hg* and increased plasma IGF-I levels needs further study. However, from recent work we know that the high-growth phenotype is due to a deletion in mouse Chromosome 10 (29), which allows speculation that high-growth mice may lack a developmental factor directly or indirectly involved in the control of expression of IGF-I.

The *hg* locus clearly has a dramatic effect on cell proliferation and body size in mice. Characterization of the protein product of the *hg* locus in mice and its homolog in humans can potentially enhance our understanding of the molecular events controlling cell proliferation and ultimate tissue size.

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