

Roles of insulin-like growth factors and their binding proteins in the differentiation of mouse tongue myoblasts

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ABSTRACT To study the roles of insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) in the differentiation of tongue myoblasts, we established a mouse tongue organ culture system and examined the effects of exogenous IGF-I, exogenous IGFBP4, 5, 6, and des(1-3)IGF-I, an IGF-I analogue with reduced affinity for IGFBPs, on the differentiation of tongue myoblasts. The exogenous IGF-I stimulated differentiation of tongue myoblasts and induced the expressions of endogenous IGFBP4, 5, and 6, suggesting that these IGFBPs were involved in the regulation of tongue myoblast differentiation by the IGF-I. Exogenous IGFBP4 and 5 slightly stimulated early tongue myoblast differentiation in which myogenin was involved. The stimulation seems to be due to the protection of endogenous IGFs from proteolytic degradation by the binding of these IGFBPs to endogenous IGFs. A low concentration of des(1-3)IGF-I stimulated tongue myoblast differentiation, whereas high concentrations of des(1-3)IGF-I inhibited it. The abnormal shape of the tongue, low cell density and low staining intensity with hematoxylin and eosin in tongues treated with high concentrations of des(1-3)IGF-I, suggest that the inhibition is due to abnormal reactions of tongue tissues to the toxicity caused by high concentrations of des(1-3)IGF-I. From these results, we suggest that IGFBPs may function to regulate the differentiation of mouse tongue myoblasts by controlling the concentration of free IGFs within a range suitable for the progress of tongue myoblast differentiation.

KEY WORDS: *Insulin-like growth factor, binding proteins, tongue organ culture, myoblast, differentiation*

Introduction

Many reports have shown that several peptide growth factors play important roles in the development of skeletal muscles. Fibroblast growth factor (FGF) and transforming growth factor (TGF) β strongly stimulate the proliferation of cultured myoblasts including C2 and L6, whereas they inhibit the differentiation of them (Olson, 1992; Buckingham, 1994). TGF α inhibits proliferation of primary cultures of fetal bovine skeletal muscle cells and rat L6 myoblasts (Blachowski *et al.*, 1993), but it does not affect the differentiation of C2 myoblasts (Luetke *et al.*, 1993). Hepatocyte growth factor (HGF) is involved in the migration of hind limb muscle precursor cells (Bladt *et al.*, 1995).

Insulin-like growth factors (IGFs) are well known to play very important roles in both the proliferation and differentiation of cultured myoblasts (Florini *et al.*, 1996). The treatments with 10 ~ 100 ng/ml of exogenous IGF-I and II stimulate the proliferation of myoblasts cultured for 6 ~ 24 hours and, subsequently, the differentiation of them cultured for 48 ~ 72 hours (Florini *et al.*, 1996). The

mitogenic action of IGFs utilizes the mitogen-activated protein (MAP) kinase signaling pathway, while the phosphatidylinositol 3-kinase/p70^{S6k} signaling pathway is essential for IGF-stimulated differentiation (Coolican *et al.*, 1997; Weyman and Wolfman, 1998). It has recently been reported that IGF-I is involved in the regulation of skeletal muscle hypertrophy and a shift in myofiber phenotypes through the Ca²⁺-calcineurin signaling pathway (Semsarian *et al.*, 1999; Olson and Williams, 2000).

It is known that both IGF-I and II can bind to IGF receptor (IGFR)1 and 2, and to insulin receptor (Florini *et al.*, 1996). However, the IGF signalings during skeletal myogenesis are mediated only by IGFR1 (Ewton *et al.*, 1987; Quinn *et al.*, 1994; Navarro *et al.*, 1997). It seems that IGFR2 serves IGF-II turnover in skeletal muscle tissue (Kiess *et al.*, 1987; Ludwig *et al.*, 1996).

Abbreviations used in this paper: IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor; IGFBP, insulin-like growth factor binding protein; MCK, muscle creatine kinase.

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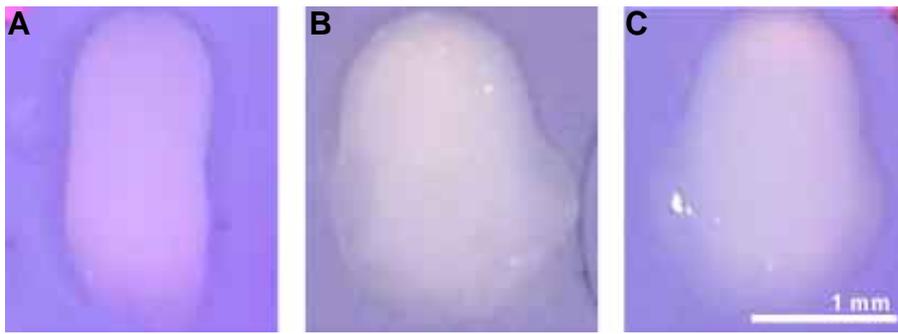


Fig. 1. A tongue dissected from E13 mouse embryos (A) and a tongue cultured for 4 (B) or 8 (C) days in serum-free and chemically-defined BGJb medium. The tongues appeared to become round after 4 or 8 days in culture.

The actions of the IGFs appear to be regulated and coordinated by a family of six high-affinity IGF binding proteins (IGFBP), designated as IGFBP1 to IGFBP6 (Jones and Clemmons, 1995). The IGFBPs are thought to have four major functions that are essential to the regulation and coordination of the biological activities of IGFs. These are 1) to act as transport proteins in plasma and to control the efflux of IGFs from the vascular space; 2) to prevent IGFs from being degraded and to prolong the half-lives of IGFs; 3) to provide a means of tissue and cell type-specific localization, and 4) to directly modulate the interaction of the IGFs with their receptors and thereby indirectly control biological actions. In addition, recent evidence suggests that the IGFBPs can have direct actions on cellular functions.

It has been reported that all six IGFBPs are expressed in skeletal muscles and that IGFBP4, 5, and 6 play important roles in the regulation of skeletal myogenesis (Ferguson *et al.*, 1992; Florini *et al.*, 1996). The treatment with 10 ~ 200 ng/ml of exogenous IGFBP4 inhibits the IGF-I induced proliferation and differentiation of L6 myoblasts (Ewton *et al.*, 1998) and that with 20 ~ 500 ng/ml of exogenous IGFBP6 inhibits the IGF-II induced differentiation of L6 myoblasts (Bach *et al.*, 1994). IGFBP5 has dual effects on skeletal myogenesis; the treatment of 10 ~ 200 ng/ml of IGFBP5

inhibits the IGF-I induced proliferation but stimulates the IGF-I induced differentiation of L6 myoblast (Ewton *et al.*, 1998).

The tongue is a complex muscular organ comprised of several intrinsic and extrinsic muscles and is involved in several important physiological tasks such as suckling, swallowing, mastication, respiration, and vocality. Tongue striated muscles have several unique characteristics different from other skeletal muscles such as limb and trunk muscles. For example, tongue muscles are capable of moving in three dimensions. The embryonic origin of connective tissue cells in tongue striated muscle is the neural crest, whereas that in trunk

and limb skeletal muscles is the mesoderm (Noden, 1983; Jacob *et al.*, 1986). Fast myosin heavy chain is expressed not only in the myotubes and myofibers of tongue muscles but also the myoblasts of tongue muscles, but is not expressed in the myoblasts of trunk and limb muscles (Dalrymple *et al.*, 1999). Tongue myogenesis and synaptogenesis are almost complete at birth, which is earlier than that in other skeletal muscles (Yamane *et al.*, 2000a, 2001). In addition to several unique developmental characteristics of tongue striated muscle, we have reported a difference in the roles of TGF α in myogenesis between tongue and other skeletal muscles: TGF α promotes early differentiation of mouse tongue myoblasts (Yamane *et al.*, 1997, 1998a, 1998b), while it does not affect the differentiation of the C2 myoblast (Luetteke *et al.*, 1993).

There are a few reports on the expression of IGFs and IGFBPs during embryonic development of tongue striated muscle (Ferguson *et al.*, 1992; Kleffens *et al.*, 1999), but the roles of IGFs and IGFBPs remain unclear. In the present study, to elucidate the roles of IGFs and IGFBPs in the differentiation of tongue myoblasts, we established a mouse tongue organ culture system and used it to examine the effects on the differentiation of tongue myoblasts of exogenous IGF-I, exogenous IGFBP4, 5, 6, and des(1-3)IGF-I, an IGF-I analogue with reduced affinity for IGFBPs.

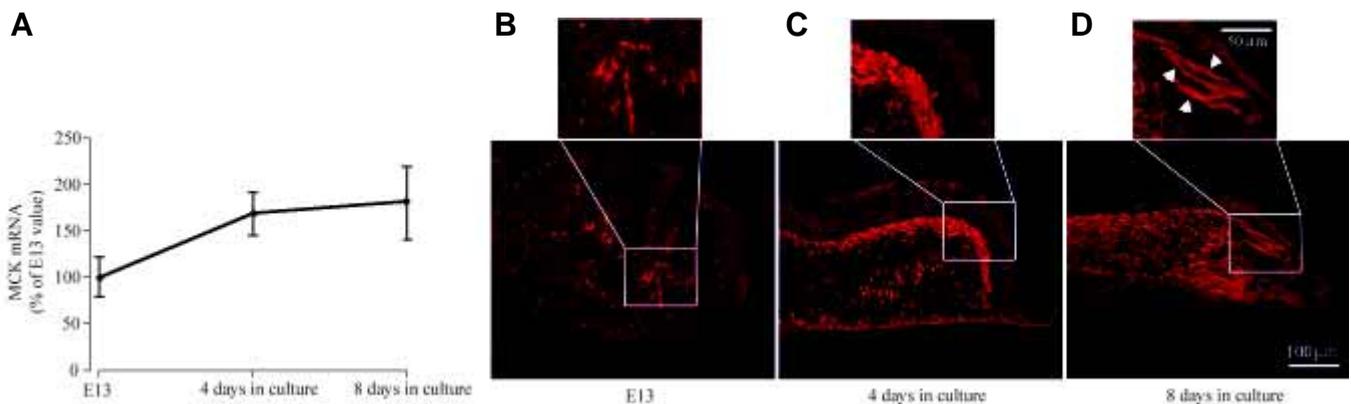


Fig. 2. The expression of muscle creatine kinase RNA and immunolocalization of fast myosin heavy chain. Relative changes in the mRNA level of MCK (A) in the E13 tongues cultured for 0, 4, or 8 days assessed by competitive RT-PCR. Each point and its vertical bar represent the mean \pm 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value for the E13 tongue. The level of MCK increased by 70% ($p < 0.01$) between 0 and 4 days in culture. Immunolocalization for fast myosin heavy chain in the proximal portions of E13 tongues cultured for 0 (B), 4 (C), or 8 (D) days. In the proximal portion of the E13 tongue, we observed a few fast myosin heavy chain positive-myoblasts. After 4 days in culture, the number of fast myosin heavy chain positive-myoblasts appeared to increase. After 8 days in culture, several elongated myoblasts were observed. Arrowheads in the upper panel of D indicate elongated myoblasts.

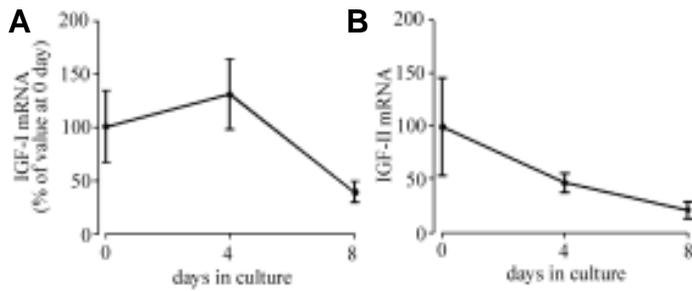


Fig. 3. Relative changes in the mRNA levels of IGF-I (A) and IGF-II (B) in the E13 tongues cultured for 0, 4, or 8 days assessed by competitive RT-PCR. Each point and its vertical bar represent the mean \pm 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value of the E13 tongue. IGF-I mRNA was highly expressed for the first 4 days of the culture period during which myoblast differentiation actively progresses. The level of IGF-II mRNA decreased throughout the whole culture period.

Results

Establishing of Tongue Organ Culture System

To establish a tongue organ culture system to study the differentiation of tongue myoblasts, the E13 mouse tongues, in which myoblasts just had begun to differentiate (Yamane *et al.*, 2000a), were cultured in serum-free and chemically-defined BGJb medium for 4 or 8 days. Figure 1 shows a tongue dissected from E13 mouse embryos (A) and a tongue cultured for 4 (B) or 8 (C) days. The tongues appeared to become round after both 4 and 8 days in culture.

The level of muscle creatine kinase mRNA was measured as a marker for the myoblast differentiation (Fig. 2A). The level increased by 70% ($p < 0.01$) for the first 4 days of the culture, then slightly increased. In the proximal portion of the E13 tongue, a few fast myosin heavy chain positive-myoblasts were observed (Fig. 2B). After 4 days in culture, the number of fast myosin heavy chain positive-myoblasts appeared to increase (Fig. 2C). After 8 days in culture, several elongated myoblasts were observed in the proximal region of the tongue (arrowheads in the upper panel of Fig. 2D). These results for the muscle creatine kinase and fast myosin heavy chain suggest that the differentiation of myoblasts was able to progress in E13 tongues cultured in serum-free and chemically-defined medium.

Since the autocrine secretions of IGF-I and II are reported to stimulate differentiation of cultured myoblasts such as C2C12 and L6 (Florini *et al.*, 1991; Ewton *et al.*, 1994; Yoshiko *et al.*, 1996), the levels of endogenous IGF-I and II mRNAs were measured in the cultured tongues (Fig. 3). IGF-I mRNA was highly expressed for the first 4 days of the culture period, then decreased (Fig. 3A). The level of IGF-II mRNA decreased throughout the whole culture period (Fig. 3B). The consist-

tency of the culture period for active myoblast differentiation and high level expression of IGF-I suggests that IGF-I may be mainly involved in the differentiation of tongue myoblasts.

Effects of Exogenous IGF-I on Differentiation of Mouse Tongue Myoblasts

In order to study the function of IGF-I in the differentiation of tongue myoblasts, we analyzed the effects of exogenous IGF-I on tongue myoblasts. E13 mouse tongues were cultured in BGJb medium containing 0, 25, 50, or 100 ng/ml of IGF-I. The treatment with 50 ng/ml of exogenous IGF-I induced a 30% ($p < 0.01$) increase in the levels of muscle creatine kinase mRNA, a marker for myoblast differentiation (Fig. 4A). To detect the differentiating myoblasts and myotubes in the cultured tongues, we performed immunolocalization for the fast myosin heavy chain (Fig. 4 B,C). In the middle portion of tongue treated with 50 ng/ml of exogenous IGF-I, the number of fast myosin heavy chain-positive myogenic cells seemed to be much greater than that in the control tongue cultured without IGF-I. In combination with the PCR result of muscle creatine kinase, this immunohistochemical result suggests that exogenous IGF-I promotes the differentiation of cultured tongue myoblasts.

Since IGF-I is reported to stimulate the differentiation of the L6 myoblast by changing the expression of the myoD family (Florini and Ewton, 1990; Mangiacapra *et al.*, 1992; Florini *et al.*, 1996), we analyzed the expression of myoD family mRNA in tongues treated with exogenous IGF-I (Fig. 5). Treatments with 50 and 100 ng/ml of exogenous IGF-I induced 35% ($p < 0.05$) and 41% ($p < 0.05$) increases in the levels of myogenin and myoD mRNAs, respectively.

To study the roles of endogenous IGFs in the differentiation of cultured tongue myoblasts, we analyzed the expressions of endogenous IGFBP mRNAs in tongues treated with exogenous IGF-I (Fig. 6). No significant difference in the levels of exogenous IGFBP2 and 3 mRNAs was found between the control and IGF-I treated tongues (Fig. 6 A,B), suggesting that their expressions were not affected by the IGF-I treatment. The treatments with 50 and 100 ng/ml of exogenous IGF-I induced approximately 50 ~ 60% ($p < 0.05 \sim 0.01$) increases in the levels of endogenous IGFBP4 and 5 mRNAs (Fig. 6 C,D). Only the treatment with 100

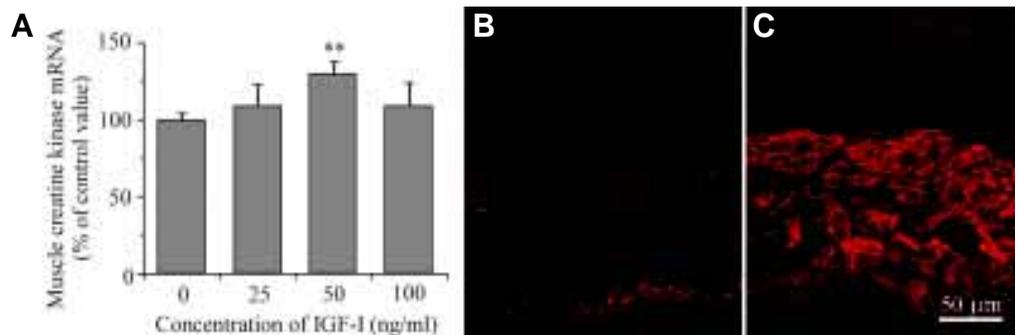


Fig. 4. The expression of muscle creatine kinase RNA and immunolocalization of fast myosin heavy chain. Relative changes in the mRNA level of muscle creatine kinase (A) in the E13 tongues cultured in BGJb medium containing 0, 25, 50, or 100 ng/ml of IGF-I assessed by using competitive RT-PCR. Each column and its vertical bar represent the mean \pm 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml of IGF-I. The treatment with 50 ng/ml IGF-I induced a 30% ($p < 0.01$) increase in the mRNA level of muscle creatine kinase. Significant difference from values at 0 ng/ml, ** $p < 0.01$. Immunolocalization of fast myosin heavy chain in the middle portions of the sagittal sections of tongues cultured without IGF-I (B) and with 50 ng/ml of IGF-I (C). The treatment with 50 ng/ml of exogenous IGF-I appeared to induce an increase in the number of fast myosin heavy chain-positive myoblasts.

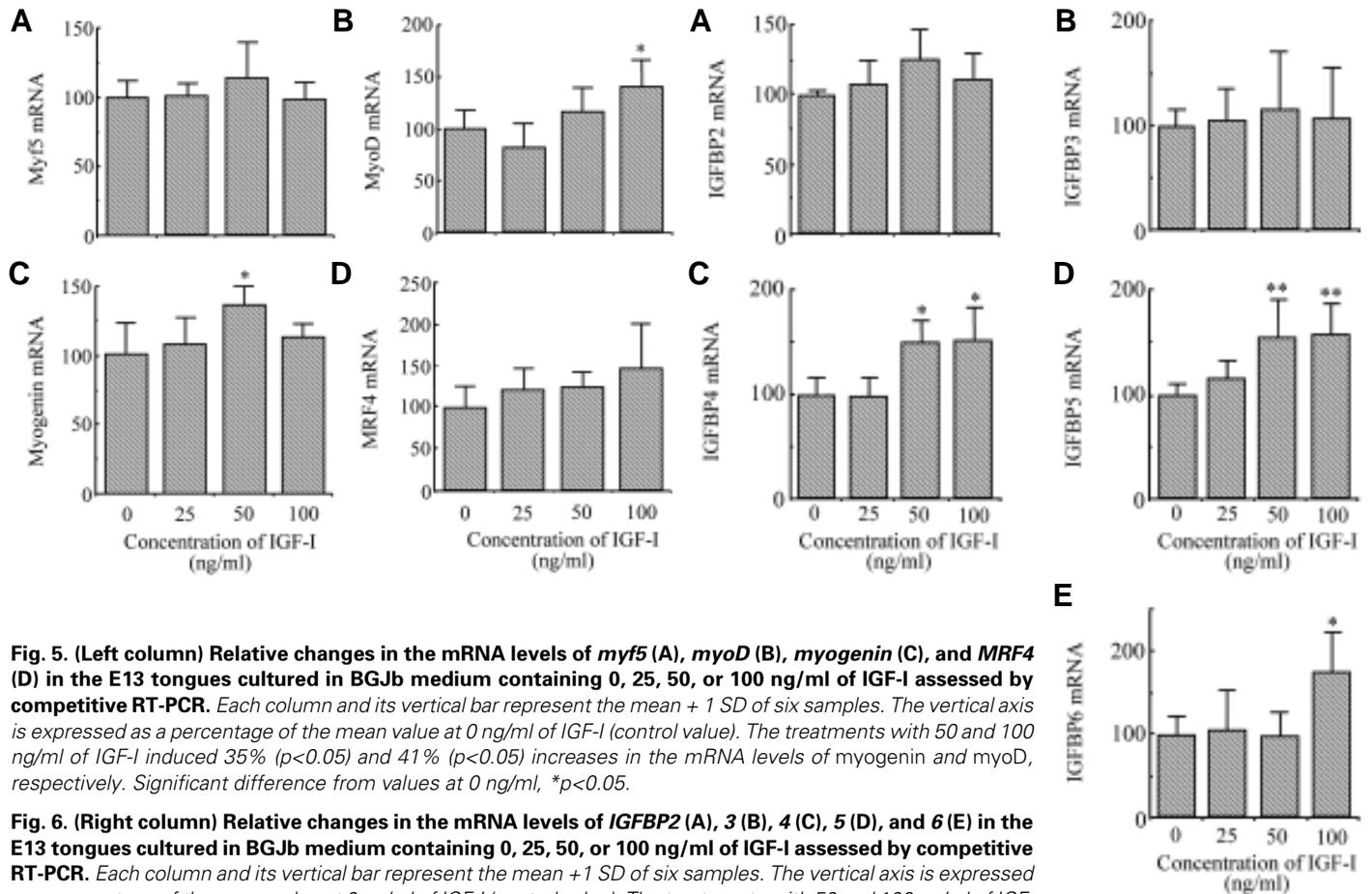


Fig. 5. (Left column) Relative changes in the mRNA levels of *myf5* (A), *myoD* (B), *myogenin* (C), and *MRF4* (D) in the E13 tongues cultured in BGJb medium containing 0, 25, 50, or 100 ng/ml of IGF-I assessed by competitive RT-PCR. Each column and its vertical bar represent the mean + 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml of IGF-I (control value). The treatments with 50 and 100 ng/ml of IGF-I induced 35% ($p < 0.05$) and 41% ($p < 0.05$) increases in the mRNA levels of myogenin and myoD, respectively. Significant difference from values at 0 ng/ml, * $p < 0.05$.

Fig. 6. (Right column) Relative changes in the mRNA levels of IGFBP2 (A), 3 (B), 4 (C), 5 (D), and 6 (E) in the E13 tongues cultured in BGJb medium containing 0, 25, 50, or 100 ng/ml of IGF-I assessed by competitive RT-PCR. Each column and its vertical bar represent the mean + 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml of IGF-I (control value). The treatments with 50 and 100 ng/ml of IGF-I induced approximately 50 ~ 60% ($p < 0.05$ ~ 0.01) increases in the levels of endogenous IGFBP4 and 5 mRNAs. Only the treatment with 100 ng/ml of IGF-I induced a 76% ($p < 0.05$) increase in the mRNA level of endogenous IGFBP6. Significant differences from values at 0 ng/ml, * $p < 0.05$, ** $p < 0.01$.

ng/ml of exogenous IGF-I induced a 76% ($p < 0.05$) increase in the mRNA level of endogenous IGFBP6 (Fig. 6E). We were not able to detect IGFBP1 mRNA by this PCR technique (data not shown).

The distributions of IGFBP4, 5, and 6, which were increased by the IGF-I treatment, were examined in the cultured tongues using immunohistochemistry (Fig. 7). In the striated muscle tissues in the middle portion of the cultured tongue, intense immunostaining for IGFBP4 (Fig. 7 A,B) and 5 (Fig. 7 C,D) was observed. The staining intensities for IGFBP4 and 5 in the striated muscle tissues appeared to be slightly stronger in the tongues treated with 50 ng/ml of IGF-I (Fig. 7 B,D) than those in the control tongue (Fig. 7 A,C). In the epithelium and the tissues underneath the epithelium including lamina propria, we observed very intense staining for IGFBP6. The area, which showed intense staining for IGFBP6 in the IGF-treated tongue (Fig. 7F), appeared to become wide compared with that in the control tongue (Fig. 7E). The results of PCR and immunohistochemistry for IGFBPs suggest that IGFBP4, 5, and 6 may be directly related to the regulation of the tongue myoblast differentiation by exogenous IGF-I.

Effects of Exogenous IGFBP4, 5 and 6 on the Differentiation of Mouse Tongue Myoblasts

To elucidate the role of IGFBPs in the differentiation of tongue myoblasts, we examined the effects of exogenous IGFBP4, 5, or 6 on the differentiation of tongue myoblasts, because the expres-

sions of these IGFBPs were increased by the IGF-I treatment. E13 mouse tongues were cultured in BGJb medium containing 100, 200, or 400 ng/ml of IGFBP4, 5, or 6.

The treatment with 200 ng/ml of exogenous IGFBP4 induced a 70% ($p < 0.01$) increase in the level of myogenin mRNA (Fig. 8B) but no significant difference in the level of muscle creatine kinase mRNA was found between the control and the IGFBP4-treated tongues (Fig. 8A). The treatments with 200 and 400 ng/ml of exogenous IGFBP5 induced 49% ($p < 0.01$) and 55% ($p < 0.01$) increases in myogenin mRNA expression, respectively (Fig. 9B). The mean values of muscle creatine kinase mRNA at 200 and 400 ng/ml of IGFBP5 were 39% and 55% greater than that at 0 ng/ml, but these increases were not statistically significant due to a large variation in the data (Fig. 9A). IGFBP6 induced no significant changes in the levels of muscle creatine kinase and myogenin mRNAs (Fig. 10). These results suggest that exogenous IGFBP4 and 5 promote the early differentiation of tongue myoblasts related to myogenin.

Effects of des(1-3)IGF-I on Differentiation of Mouse Tongue Myoblasts

To further understand the role of IGFBPs in the differentiation of tongue myoblasts, we analyzed the effects of des(1-3)IGF-I, an IGF-I analogue with a reduced affinity for IGFBPs, on the differentiation of tongue myoblasts. E13 mouse tongues were cultured in

a BGJb medium containing 0, 10, 25, 50, or 100 ng/ml of des(1-3)IGF-I.

The treatment with 10 ng/ml des(1-3)IGF-I induced a 48% ($p < 0.05$) increase in the level of MCK mRNA (Fig. 11A). The mean values of muscle creatine kinase mRNA at 25 ~ 100 ng/ml of des(1-3)IGF-I were slightly less than that at 0 ng/ml, but these decreases were not statistically significant. The treatments with 25 ~ 100 ng/ml of des(1-3)IGF-I induced 20 ~ 50% ($p < 0.05$ ~ 0.01) decreases in the levels of myf5 (Fig. 11B), myoD (Fig. 11C), and myogenin (Fig. 11D) mRNAs. The mean value of myogenin mRNA at 10 ng/ml was greater than that at 0 ng/ml (Fig. 11D) and those of MRF4 at 25 ~ 100 ng/ml were less than that at 0 ng/ml (Fig. 11E), but these changes were not statistically significant. These results suggest that the treatment with 10 ng/ml des(1-3)IGF-I stimulates the differentiation of tongue myoblasts, while those with 25 ~ 100 ng/ml inhibit it.

Figure 12 shows the tongues cultured without (A) or with 50 ng/ml des(1-3)IGF-I (B). The shape of the tongue treated with des(1-3)IGF-I appeared to be quite different from that of the control tongue. Abnormal tissues were observed in the peripheral region of the tongue. Figure 12C and 12D shows the middle portions of the sagittal sections of tongues stained with hematoxylin and eosin. The staining intensity with hematoxylin and eosin, and the cell density in the des(1-3)IGF-I treated tongue (Fig. 12D) appeared to be less compared with those of the control tongue (Fig. 12C). Several elongated myoblasts were observed in the control tongue (arrows in Fig. 12C), but they were not observed in the des(1-3)IGF-I treated tongue (Fig. 12D). These morphological results suggest that abnormal reactions to 50 ng/ml of des(1-3)IGF-I occur in the tongue.

Discussion

We observed increases in the mRNA level of muscle creatine kinase (Fig. 2A) and in the number of fast myosin heavy chain positive myoblasts (Fig. 2B ~ 2D) in the cultured E13 mouse tongues, suggesting that the differentiation of tongue myoblasts can progress in this organ culture system. The culture period during which the differentiation of tongue myoblasts actively progressed (the first 4 days in culture) corresponded to that for the high level expression of endogenous IGF-I mRNA (Fig. 3A) in the cultured E13 mouse tongues. This result suggests that IGF-I, which is secreted from tongue tissues such as muscle and epithelial tissues, is mainly involved in the differentiation of tongue myoblasts. It is already known that the autocrine secretion of IGF-I stimulates the differentiation of cultured myoblasts (Florini *et al.*, 1991; Ewton *et al.*, 1994; Yoshiko *et al.*, 1996). This supports our present view.

Exogenous IGF-I induced the increases in the level of muscle creatine kinase mRNA (Fig. 4A) and in the number of fast myosin heavy chain positive myoblasts (Fig. 4B) in the cultured E13 mouse tongue, suggesting that it stimulates the differentiation of tongue myoblasts. It was previously reported that exogenous IGFs pro-

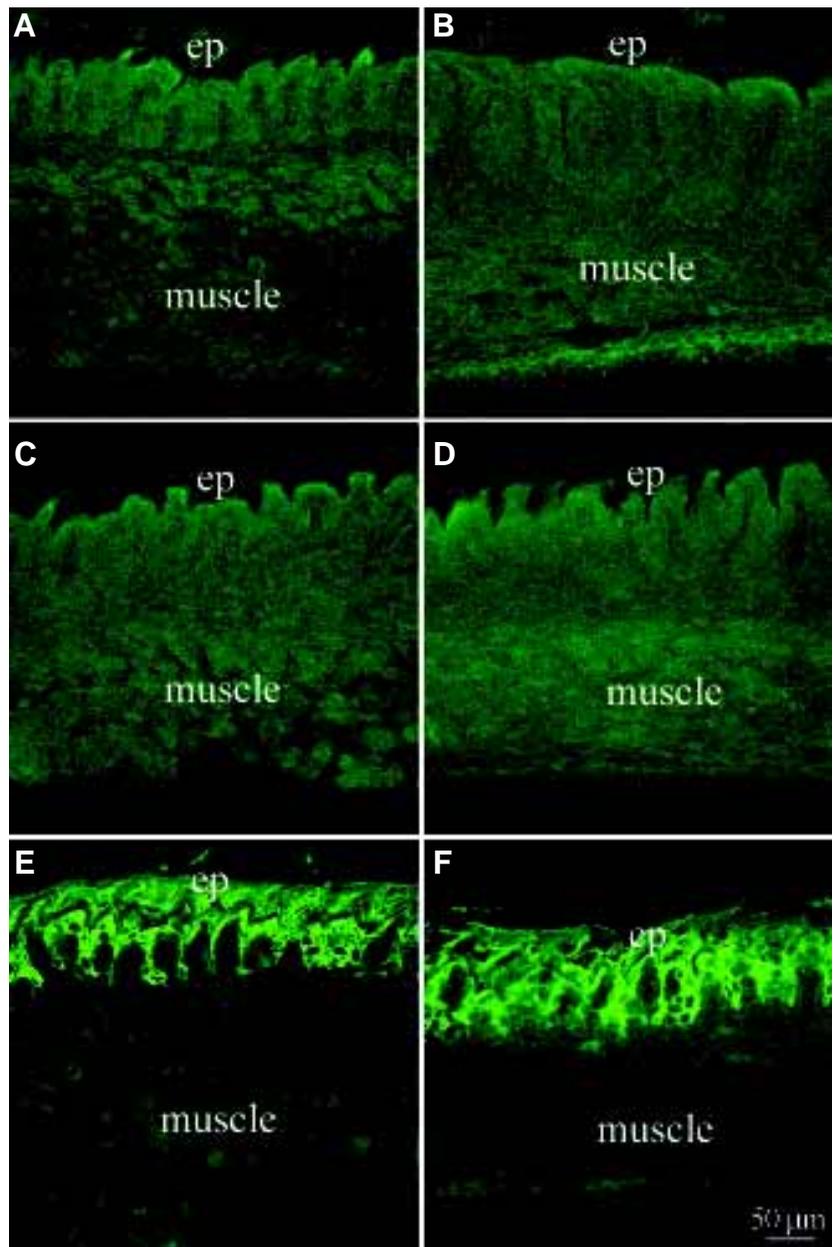


Fig. 7. Immunolocalization of IGFBP4 (A,B), 5 (C,D), and 6 (E,F) in the middle portions of the sagittal sections of tongues cultured without IGF-I (A,C,E) and with 50 ng/ml of IGF-I (B,D,F). In the striated muscle tissues, intense immunostaining for IGFBP4 (A,B) and 5 (C,D) was observed. In the epithelium and the tissues underneath the epithelium including lamina propria, very intense staining for IGFBP6 was observed (E,F). ep, epithelium.

mote the differentiation of several kinds of cultured muscle cells (Florini *et al.*, 1996), which is consistent with the present study. In association with the differentiation of tongue myoblasts, the increases in the levels of myoD and myogenin mRNAs were induced by the exogenous IGF-I (Fig. 5 B,C). It appears that myogenin is essential for the differentiation of myoblasts (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993) and myoD is involved in not only the determination of myogenic precursor cells, but also the initiation for the myoblast differentiation (Buckingham, 1994; Kitzman *et al.*, 1998). Thus, exogenous IGF-I seems to promote the differentiation of tongue myoblasts by inducing the expression of myoD and

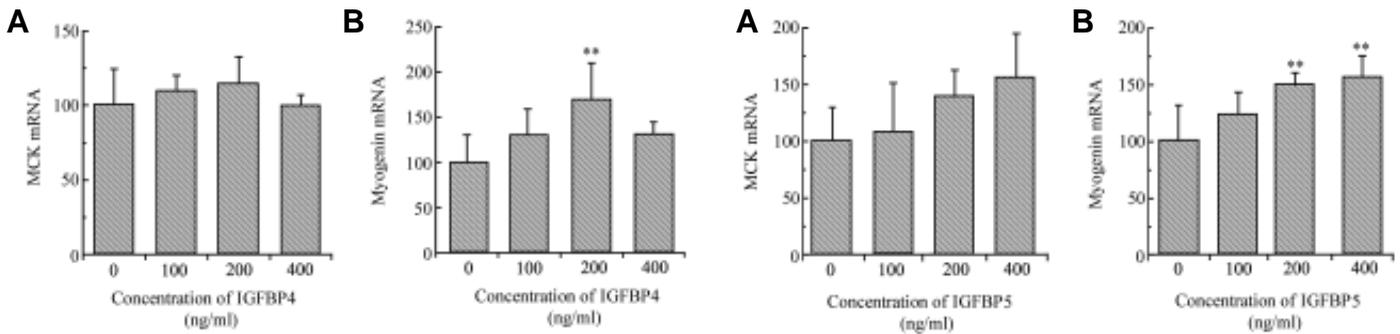


Fig. 8. (Left column) Relative changes in the mRNA levels of *muscle creatine kinase* (A) and *myogenin* (B) in the E13 tongues cultured in BGJb medium containing 0, 100, 200, or 400 ng/ml of IGFBP4 assessed by competitive RT-PCR. Each column and its vertical bar represent the mean + 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml of IGFBP4 (control value). The treatment with 200 ng/ml of exogenous IGFBP4 induced a 70% ($p < 0.01$) increase in the level of myogenin mRNA. MCK, muscle creatine kinase. Significant difference from values at 0 ng/ml, ** $p < 0.01$.

Fig. 9. (Right column) Relative changes in the mRNA levels of *muscle creatine kinase* (A) and *myogenin* (B) in the E13 tongues cultured in BGJb medium containing 0, 100, 200, or 400 ng/ml of IGFBP5 assessed by competitive RT-PCR. Each column and its vertical bar represent the mean + 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml of IGFBP5 (control value). The treatments with 200 and 400 ng/ml of exogenous IGFBP5 induced 49% ($p < 0.01$) and 55% ($p < 0.01$) increases in the myogenin mRNA expression, respectively. MCK, muscle creatine kinase. Significant difference from values at 0 ng/ml, ** $p < 0.01$.

myogenin. Myf5 is known to play a role in the determination and maintenance of myogenic precursor cells (Braun *et al.*, 1992; Rudnicki *et al.*, 1992, 1993; Braun and Arnold, 1994, 1995). MRF4 is reported to express at a high level after birth (Bober *et al.*, 1991; Hinterberger *et al.*, 1991), suggesting that it is involved in myofiber maturation and maintenance. Since no significant change in the mRNA level of myf5 and MRF4 was induced by exogenous IGF-I, myf5 and MRF4 do not seem to play an important role in the differentiation of tongue myoblasts.

The increases in the expression of IGFBP4, 5, and 6 induced by the exogenous IGF-I (Fig. 6 C,D,E) suggest that these IGFBPs are involved in the promotion of tongue myoblast differentiation by exogenous IGF-I. The induction of IGFBP4, 5, and 6 in the L6 myoblast cell line by exogenous IGFs has been reported (Ewton and Florini, 1995; Ewton *et al.*, 1998). The present results seem to accord with that reported for the L6 myoblast cell line. It has been reported that the expression of IGFBP4 and 5 in several myogenic cell lines is suppressed by transforming growth factor β 1, epidermal growth factor, and basic fibroblast growth factor, which inhibit the differentiation of myogenic cells (McCusker and Clemmons, 1994). Taken together, several growth factors including IGF-I seem to control the differentiation of myogenic cells by regulating the expression levels of IGFBPs.

In the present study, exogenous IGFBP4 and 5 induced the expression of myogenin in the E13 cultured mouse tongue (Figs. 8B and 9B). These results suggest that the IGFBPs stimulate the early differentiation of tongue myoblasts, since studies of knockout mice indicate that myogenin is necessary to begin the fusion of myoblasts (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). We assume how exogenous IGFBPs stimulated the differentiation of tongue myoblasts as follows. The exogenous IGFBPs bind to IGFs secreted from tongue tissues and protect the IGFs from proteolytic degradation in the culture medium. The IGFs are then released from the IGFBP-IGF complexes and stimulate the early differentiation of tongue myoblasts.

In the present study, the exogenous IGFBP6 did not affect the expression of myogenin and muscle creatine kinase (Fig. 10). In the L6 myogenic cell line, IGFBP6 is reported to inhibit the

differentiation of myoblasts (Bach *et al.*, 1994), which is inconsistent with the present result. We observed very intense immunostaining for IGFBP6 in the epithelial tissue of the cultured tongue (Fig. 7 E,F), suggesting that a high concentration of IGFBP6 was secreted into the culture medium from the epithelial tissue of the tongue. Thus, the endogenous IGFBP6 may abolish the effects of exogenous IGFBP6.

The induction of muscle creatine kinase by 10 ng/ml of des(1-3)IGF-I (Fig. 11A) suggests that a low concentration of des(1-3)IGF-I stimulates the differentiation of tongue myoblasts, whereas the suppression of myf5, myoD, and myogenin by 25 ~ 100 ng/ml of des(1-3)IGF-I (Fig. 11 B,C,D) suggests that high concentrations of des(1-3)IGF-I inhibit the differentiation of tongue myoblasts. Since the abnormal shape of tongues, the decrease in the cell density, and the weak staining intensity by hematoxylin and eosin were observed in tongues cultured in high concentrations of des(1-3)IGF-I (Fig. 12), the inhibitory effect on the differentiation

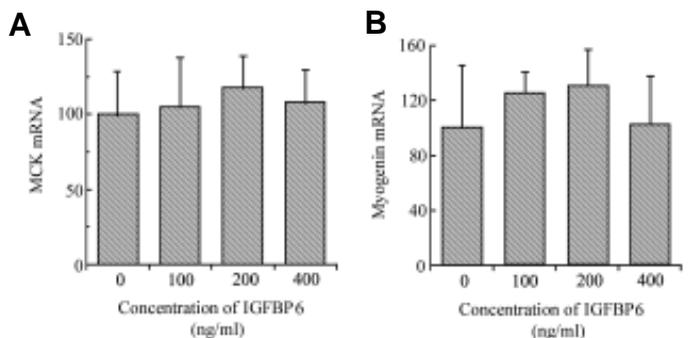


Fig. 10. Relative changes in the mRNA levels of *muscle creatine kinase* (A) and *myogenin* (B) in the E13 tongues cultured in BGJb medium containing 0, 100, 200, or 400 ng/ml of IGFBP6 assessed by using competitive RT-PCR. Each column and its vertical bar represent the mean + 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml of IGFBP6 (control value). IGFBP6 induced no significant changes in the levels of muscle creatine kinase and myogenin mRNAs. MCK, muscle creatine kinase.

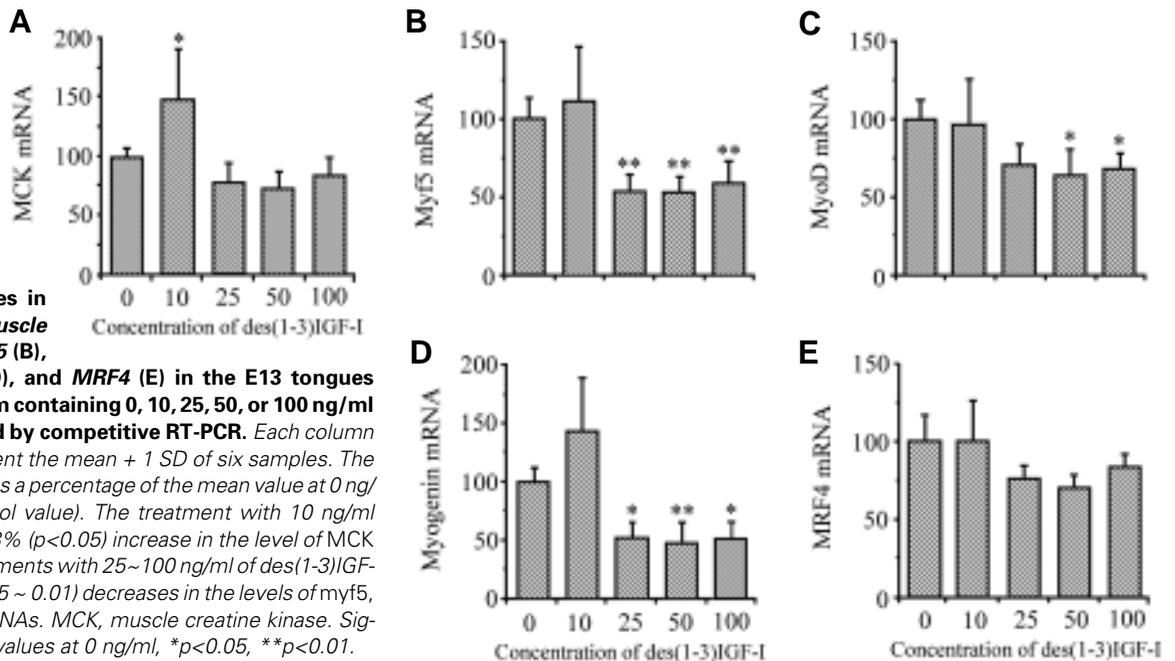


Fig. 11. Relative changes in the mRNA levels of muscle creatine kinase (A), myf5 (B), myoD (C), myogenin (D), and MRF4 (E) in the E13 tongues cultured in BGJb medium containing 0, 10, 25, 50, or 100 ng/ml of des(1-3)IGF-I assessed by competitive RT-PCR. Each column and its vertical bar represent the mean + 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml of des(1-3)IGF-I (control value). The treatment with 10 ng/ml des(1-3)IGF-I induced a 48% ($p < 0.05$) increase in the level of MCK mRNA, whereas the treatments with 25~100 ng/ml of des(1-3)IGF-I induced 20~50% ($p < 0.05 \sim 0.01$) decreases in the levels of myf5, myoD, and myogenin mRNAs. MCK, muscle creatine kinase. Significant differences from values at 0 ng/ml, * $p < 0.05$, ** $p < 0.01$.

of tongue myoblasts seems to reflect a toxic reaction of tongue tissues to high concentrations of des(1-3)IGF-I. IGF analogues with reduced affinity for IGFbps including des(1-3)IGF-I are reported to be much more potent than native IGF-I in stimulating L6 myoblast differentiation and no inhibitory effect of des(1-3)IGF-I at 25 ~ 100 ng/ml on the myoblast differentiation was reported (Ewton and Florini, 1995; Silverman *et al.*, 1995; James *et al.*, 1996).

In the present study, exogenous IGF-I stimulated myoblast differentiation (Fig. 4) and induced the expression of IGFbp4, 5, and 6 in the cultured tongue (Fig. 6). A low concentration of des(1-3)IGF-I promoted tongue myoblast differentiation (Fig. 11), whereas high concentrations of this analogue inhibited it due to toxic reactions such as the abnormal shape of the tongue, low cell density, and low staining intensity with hematoxylin and eosin (Figs. 11 and 12). Based on these results, we may hypothesize that IGFbps function to control the concentration of free IGFs

within a range suitable for progress of the differentiation of tongue myoblasts. When the concentration of free IGFs falls below this range, the IGFbp-IGF complex releases IGFs to raise the concentration of free IGFs. When the concentration of free IGFs exceeds the suitable range, IGFbps bind to free IGFs to decrease the concentration of free IGFs. Since, in the present study, this mechanism functioned to control the concentration of exogenous IGF-I within the suitable concentration range, we observed only a stimulatory effect of exogenous IGF-I. However, this mechanism was not able to control the concentration of des(1-3)IGF-I, because this analogue has a reduced affinity to IGFbps. Since the 10 ng/ml of des(1-3)IGF-I was within the concentration range suitable for the progress of the differentiation, it stimulated the differentiation of the tongue myoblasts. On the other hand, high concentrations of this analogue (25 ~ 100 ng/ml), which exceeded the suitable range to the point of toxicity, inhibited the differentiation by inducing abnormal reactions in the tongue tissues.

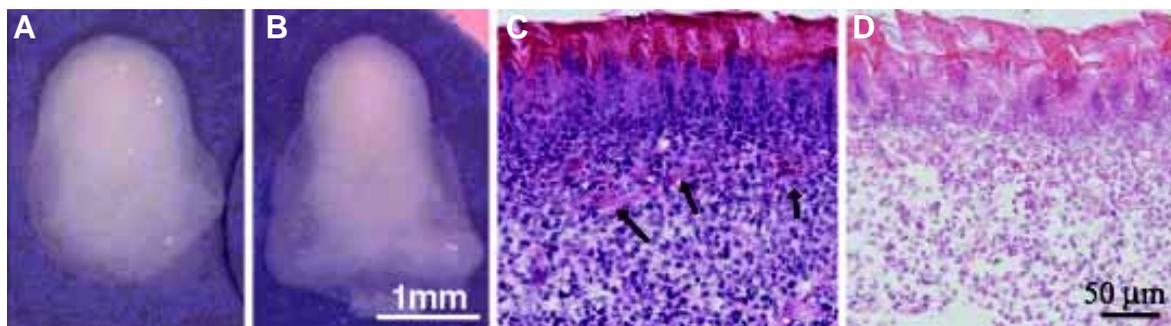


Fig. 12. Tongues cultured without (A) or with (B) 50 ng/ml des(1-3)IGF-I and the middle portions in sagittal sections of the control (C) and des(1-3)IGF-I treated (D) tongues stained with hematoxylin and eosin. The shape of tongue treated with des(1-3)IGF-I appeared to be quite different from that of control tongue. Abnormal tissues were observed in the peripheral region of the tongue. The staining intensity with hematoxylin and eosin, and the cell density in the des(1-3)IGF-I treated tongue appeared to be less in comparison with the control tongue. Arrows in C indicate in elongated myoblasts.

Materials and Methods

Tongue Organ Culture

Pregnant ICR mice were killed by cervical dislocation at E13. Embryos were isolated from uterine deciduas and removed from their membranes under a dissection microscope. The tongues of the embryos were carefully microdissected and explanted. The explants were supported by Millipore type AABP filters, having a 0.8 μm pore size, on steel rafts and were then cultured in BGJb medium (Life Technologies, Gaithersburg, MD, USA) freshly supplemented with 100 $\mu\text{g/ml}$ ascorbic acid and 100 unit/ml penicillin-streptomycin (Life Technologies, Gaithersburg, MD, USA). Cultures were maintained for 4 or 8 days at 37°C in an atmosphere of 5% carbon dioxide and 95% air with medium changes every 2 days. IGF-I (Life Technologies, Gaithersburg, MD, USA) was used at the final concentrations of 25, 50 or 100 ng/ml, and IGFBP4, 5, and 6 (GroPep Limited, Adelaide, SA, Australia) at those of 100, 200 or 400 ng/ml, and des(1-3)IGF-I (GroPep Limited, Adelaide, SA, Australia) at those of 10, 25, 50 or 100 ng/ml. They were added to the cultured medium and replaced every 2 days. After the culture, the explants were stored at -80°C for the analysis of competitive PCR and fixed in Bouin's fixative for histological analysis.

RNA Extraction, Reverse Transcription and Competitive PCR Amplification

Total RNA extraction, reverse transcription, and competitive PCR amplification were performed as previously described (Yamane *et al.*, 2000a, b). Briefly, total RNA extraction was performed according to the manufacturer's specifications (Trizol, Life Technologies, Gaithersburg, MD, USA). The RNA was treated with 2 units of ribonuclease-free deoxyribonuclease I (Life Technologies, Gaithersburg, MD, USA), and was then reverse transcribed with 200 units of reverse transcriptase (SuperScript II, Life Technologies, Gaithersburg, MD, USA).

TABLE 1

SEQUENCES OF TARGET GENE-SPECIFIC PCR PRIMERS, TARGET AND COMPETITOR SIZES

Target genes	Sequences	References
IGFBP1	Forward	Schuller <i>et al.</i> , 1994
	Reverse	
	Target size	
	Competitor size	
IGFBP2	Forward	Schuller <i>et al.</i> , 1994
	Reverse	
	Target size	
	Competitor size	
IGFBP3	Forward	Schuller <i>et al.</i> , 1994
	Reverse	
	Target size	
	Competitor size	
IGFBP4	Forward	Schuller <i>et al.</i> , 1994
	Reverse	
	Target size	
	Competitor size	
IGFBP5	Forward	James <i>et al.</i> , 1993
	Reverse	
	Target size	
	Competitor size	
IGFBP6	Forward	Schuller <i>et al.</i> , 1994
	Reverse	
	Target size	
	Competitor size	

In the conventional PCR technique, a small difference in the starting amount of target DNA can result in a large change in the yield of the final product due to the exponential nature of the PCR reaction. A plateau effect after many cycles can lead to an inaccurate estimation of final product yield. Furthermore, since the PCR amplification depends on the reaction efficiency, small changes in the efficiency can lead to major differences in the final product yield. To overcome these problems, the competitor (internal standard), which has the same primer sequences as those of the target DNA at the 3' and 5' ends, was amplified simultaneously with the target (Yamane *et al.*, 1998a, b). The competitors constructed according to the manufacturer's instructions in the Competitive DNA Construction Kit (TaKaRa Biochemicals, Shiga, Japan) were amplified with 50 ng of the total cDNA in the presence of a primer pair specific to the target genes in a thermal cycler (TP3000, TaKaRa Biochemicals, Shiga, Japan). Table 1 shows the primer sequences and product size for IGFBPs; those for IGFs, muscle creatine kinase, and myoD family were previously reported (Yamane *et al.*, 1998b, 2000a, b). The amplification products were separated by electrophoresis on an agarose gel containing ethidium bromide. The fluorescent intensities of the bands of the target cDNAs and their respective competitors were measured by an image analyzer (Molecular Imager FX, Bio-Rad, Hercules, CA, USA). We then calculated the ratios of the fluorescent intensities of the target cDNA bands to those of their respective competitors. The logarithmic value of the fluorescent intensity ratio was used to calculate the amount of endogenous target mRNA based on the line formula derived from a standard curve for each target gene. The standard curve was generated as described previously (Yamane *et al.*, 2000a, b). The quantity of each target mRNA was normalized by the quantity of glyceraldehyde-phosphate dehydrogenase (GAPDH).

Immunohistochemistry

Specimens for immunohistochemistry were fixed in Bouin's fixative for 2 hours at 4°C, immersed in a graduated series of sucrose solutions (5-40 % w/v) in phosphate buffered saline (PBS) at 4°C, embedded in Tissue-Tek Oct Compound (Miles Laboratory, Elkhart, IN, USA), and then frozen. Sagittal sections of tongues were prepared at a 10 μm thickness in a cryostat and then air-dried for 1 hour at room temperature. The frozen sections were stained with hematoxylin and eosin, and observed under a light microscope. The immunofluorescent method was performed as described previously (Yamane *et al.*, 2000b). Briefly, the sections were post-fixed in acetone at -20°C, rehydrated in PBS, incubated with 5% normal goat serum for 30 min to block non-specific immunostaining, and then incubated with primary antibodies. The following primary antibodies were used in the present study: rabbit polyclonal antibodies against IGFBP4, 5, and 6 (GroPep Limited, Adelaide, SA, Australia); and a mouse monoclonal antibody against fast skeletal muscle myosin heavy chain (Sigma-Aldrich Japan Inc., Tokyo, Japan). The sections were then incubated with FITC-conjugated goat antibody against rabbit IgG or rhodamine-conjugated goat antibody against mouse IgG (Sigma-Aldrich Japan Inc., Tokyo, Japan). Fluorescence was monitored using a confocal laser scanning microscope (PCM2000, Nikon, Tokyo, Japan). For control staining, the primary antibodies were replaced with normal mouse or rabbit IgG, or PBS.

Statistical Analyses

For multiple comparisons, Tukey-Kramer's method was used to compare the mean values between two groups.

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