Part 3

Expressions of IGF-I, II and Their Receptors

During Development of Tongue

Results

Standard curves

Figure 13 shows the electrophoretic gel pattern (upper panel) of IGF-I cDNA standard and its competitor after competitive PCR to generate a standard curve (lower panel). The sizes of PCR products for IGF-I cDNA standard and its competitor were 221 and 338 bp, respectively. The fluorescent intensities of IGF-I cDNA standard and competitor bands on the gel appeared to be inversely related. The formula of the regression line is represented by $y = 0.46x - 0.15$.

The formulae for the regression lines and correlation coefficients for IGF-I, II, IGFR 1 and 2 are included in Table 2. The correlation coefficients were greater than 0.99 for all the genes and were statistically significant from zero ($p<0.001$). This result indicated that the quantities of the target gene cDNAs could be reliably determined from these standard curves.

Changes in the mRNA level of IGF-I, II, IGFR 1 and 2 during the development of mouse tongue

The mRNAs of IGF-I (Figure 14A), II (14B), IGFR 1 (14C) and IGFR 2 (14D) were actively expressed between E13 and E15 stages in the mouse tongues. This time period corresponds with differentiation of myoblasts and formation of myotubes in tongue striated muscle. IGF-I mRNA was highly expressed between E11 and E15, then decreased in the level of expression until birth. Its quantity at

the newborn stage was less than 40% ($p<0.0001$) of the E15 value. The quantity of IGF-II mRNA increased by 43% ($p<0.05$) between E11 and E15 and showed a peak value at E15. After E15, the quantity decreased and became 53% ($p<0.001$) of the E15 value at the newborn stage. IGFR 1 mRNA was expressed at E11 and the expression decreased throughout all subsequent developmental stages. However, the quantities at E13 and E15, during which the tongue muscle differentiation actively occurred, were over 2-fold greater than those at E17 and newborn stages ($p<.01 \sim 0.0001$). High level expression of IGFR2 mRNA was observed between E11 and E15. After E15, the expression decreased and ultimately became less than 50% ($p<0.0001$) of the E15 value at the newborn stage.

Localization of IGF-I, II, IGFR 1 and 2 during the development of mouse tongue

IGF-I (Figure 15) and II (Figure 16) displayed similar immunostaining patterns and the proteins were present in differentiating myoblasts, myotube and myofibers in the developing mouse tongue. At E13, several cells with strong immunostaining for IGF-I and II were found in the proximal region of the developing tongue (Figures 15A and 16A). These cells were spindle-shaped and displayed strong immunostaining for fast myosin heavy chain, indicating that these cells were differentiating myoblasts (Figures 15B, 15C, 16B and 16C). At E15, strong immunostaining for IGF-I and II was observed in the myotubes and myofibers which displayed strong immunostaining for fast myosin heavy chain (Figures 15D, 15E, 15F, 16D, 16E and 16F). At the newborn stage, immunostaining for both IGF-I and II was observed in well-developed muscle fibers (Figures 15G

and 16G), but was weak in comparison with that in the myotubes at E15 (Figures 15D and 16D). The epithelial tissue of the developing tongue had a strong immunopositive reaction for both IGF-I and II at E15 and newborn stages (Figures 15D, 15G, 16D and 16G).

The immunostaining pattern for IGFR 1 was very similar to those for IGF-I and II in the developing mouse tongue (Figure 17). At E13, immunopositive cells for both IGFR 1 and fast myosin heavy chain were observed in the proximal region (Figures 17A, 17B and 17C). At both the E15 and newborn stages, the immunostaining for IGFR 1 was observed in myotubes and myofibers, which also contained fast myosin heavy chain (Figures 17D ~ 17I). The immunoreaction for IGFR 1 at the newborn stage was very weak (Figure 17G) in comparison with that at the E13 and E15 stages (Figures 17A and 17D). The epithelial tissue in the developing tongue was immunopositive for IGFR 1 at both the E15 and newborn stages (Figures 17D and 17G).

The immunostaining pattern for IGFR 2 in the developing mouse tongue differed from the other three proteins studied (Figure 18). Dot-shaped immunostaining for IGFR 2 was distributed sparsely throughout the developing tongue except for the epithelial tissue at all stages studied (Figures 18A, 18D and 18G). The immunostainig was not restricted to differentiating myoblasts, myotube and myofibers (Figures 18B, 18E and 18H). Immunostaining at the newborn stage (Figure 18G) was less than at the E13 (Figure 18A) and E15 (Figure 18D) stages.

Discussion

I observed that mRNA and protein of IGF-I and II were highly expressed in differentiating myoblasts and myotubes of mouse tongue (Figures 14, 15 and 16). Previously, few studies examined *in vivo* expression of IGF-I and II in skeletal muscle tissues (Ishii, 1989; Ferguson *et al.*, 1992). Ferguson *et al.* (1992) detected IGF-I and II gene expression in developing mouse tongue between E12 and E15, which is consistent with the present observation. Ishii (1989) reported that IGF-II mRNA increased with accumulation of polyneuronal innervation and decreased with elimination of superfluous synapses in rat hind limb muscle. Polyneuronal innervation accumulated between E11 and E15, and elimination of superfluous synapses occurred after E15 in mouse tongue striated muscle (Yamane *et al.*, 2001). The present results seem to accord with the results in rat hind limb muscle (Ishii, 1989).

It has been already reported that autocrine secretion of IGF-I and II stimulates differentiation of cultured myoblasts such as C2C12 and L6 (Florini *et al.*, 1991b; Ewton *et al.*, 1994; Yoshiko *et al.*, 1996). Thus the expression of IGF-I and II in differentiating myoblasts and myotubes (Figures 15 and 16) suggests that the autocrine signals of IGF-I and II regulate differentiation of mouse tongue myoblast and formation of myotube, too. IGF-I and II gene expression persisted in the mouse tongue myofibers at the newborn stage (Figures 15 and 16). Recently, it has been reported that stable expression of IGF-I in C2C12 myogenic cells results in a switch to glycolytic metabolism, suggesting that IGF-I may be related to a change in myofiber phenotypes (Semsarian *et al.*, 1999). Since mouse tongue myofibers

mature to be fast-twitch glycolytic between E15 and newborn stages (Prigozy *et al.*, 1997; Yamane *et al.*, 2000a), IGF-I and II expression in tongue striated muscle at the newborn stage may be involved in maturation into fast-twitch glycolytic fibers.

Strong immunostaining for IGF-I and II was observed in the mouse tongue epithelial tissue adjacent to the striated muscle tissue at E15 and newborn stages (Figures 15 and 16). The development of craniofacial organs such as tooth and Meckel's cartilage is thought to depend on inductive interactions between epithelium and mesenchyme (reviewed by Slavkin, 1988). The present data suggest the potential that paracrine secretion of IGF-I and II from the tongue epithelial tissue may play a role in differentiation and maturation of mouse tongue striated muscle. Since there has been no report on the involvement of interactions between epithelial and muscle tissues in the development of mouse tongue striated muscle, further studies seem to be needed.

IGFR 1 was co-localized to differentiating myoblasts, myotubes and myofibers with IGF-I and II (Figures 15, 16 and 17). Since many studies have shown that IGF-I and II control differentiation of myoblasts *in vitro* (reviewed by Florini *et al.*, 1994, 1996), the co-localization suggests that IGFR1 is closely involved in the autocrine signal transduction of IGFs, differentiation of myoblasts and formation of myotubes in the developing mouse tongue. It has been reported that null mutants for the IGFR 1 gene exhibit a severe growth deficiency with poor skeletal muscle formation (45% of normal size) (Liu *et al.*, 1993); overexpression of IGFR 1 affects proliferation and differentiation (Quinn *et al.*, 1993, 1994; Quinn and Roh, 1993), and abolishes the exogenous IGF requirement for differentiation of cultured myoblasts (Navarro *et al.*, 1997). These results support the present

conclusion.

IGFR2 displayed a different localization pattern from IGFs and IGFR 1 in the developing mouse tongue (Figure 18). IGFR 2 protein was expressed sparsely throughout the developing tongue. This expression pattern suggests that IGFR 2 is not directly related to the signal transduction of IGFs, differentiation of myoblasts and formation of myotubes in the developing mouse tongue. There are several reports that provide supportive evidence for this conclusion. Blocking antiserum against IGFR 2 does not inhibit IGF-induced myogenesis in L6 cells (Kiess *et al.*, 1987) and IGF-I analogs with a low affinity for IGFR 2 exhibit the identical activity to native IGF-I for myogenesis in L6 cells (Ewton *et al.*, 1987).