Abstract

Tongue is a complex muscular organ constituted of several intrinsic and extrinsic muscles, and involved in several important physiological tasks such as suckling, swallowing, mastication, breathing and vocalizing. Tongue striated muscles have several unique characteristics that are different from other skeletal muscles such as limb and trunk muscles. The roles of hepatocyte growth factor and transforming growth factor α in tongue myogenesis differ from those in other skeletal muscles. Insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) play essential roles in the development of trunk and limb skeletal muscles, but their roles in the development of tongue striated muscle have not been examined. Thus, the main purpose of the present study is to elucidate roles of IGFs and IGFBPs in the differentiation of mouse tongue myoblasts. The present study was designed to consist of four parts: 1) Temporal expressions of myogenic marker mRNAs including desmin, muscle creatine kinase and troponin C, and myoD family mRNAs during the development of mouse tongue were examined by competitive polymerase chain reaction in combination with reverse-transcription (competitive RT-PCR) to determine the stage for the myoblast differentiation and myofiber maturation. 2) Temporal and spatial expressions of nicotinic acetylcholine receptor (nAChR) α, δ, ε and γ subunit mRNAs and proteins were examined by competitive RT-PCR and immunohistochemistry to study the time course of nAChR subunit switch and elimination. 3) Temporal and spatial expressions of IGF-I, II, IGF receptor (IGFR) 1 and 2 mRNAs and immunolocalization of their proteins during the development of mouse tongue were analyzed to elucidate *in vivo* roles of IGFs and IGFRs. 4) Mouse tongue organ culture system with a serum-free and chemically-defined medium was established to study the differentiation of tongue

myoblasts. The effects of exogenous IGF-I, exogenous IGFBP4, 5, 6 and des(1-3)IGF-I, an IGF analogue with the reduced affinity of IGFBPs, on the differentiation of mouse tongue myoblasts were examined by this organ culture system to understand the roles of IGFs and IGFBPs in the differentiation of tongue myoblasts.

Part 1: Myotube formation and myofiber maturation began between embryonic day (E) 11 and E13 in both the tongue and hind limb muscles, but ended two days earlier in the tongue muscle than in the hind limb muscle. Expression of myoD and myogenin mRNAs began at E11, increased, and showed peak values earlier in the tongue muscle (E13) than in the hind limb muscle (E15). Expression of MRF4 mRNA appeared earlier in the tongue muscle (E13) than in the hind limb muscle (E15). These results suggest that myotube formation and myofiber maturation in the tongue muscle progress faster than in the hind limb muscle as a result of earlier expression of myoD, myogenin, and MRF4.

Part 2: The nAChR elimination and subunit switch began at E15 in the tongue and at E17 in the hind limb. They were nearly complete at birth in the tongue, but not in the hind limb. The early completion of synaptogenesis in the tongue at birth may be related to the early functional demands for the tongue, such as suckling and swallowing immediately after birth.

Part 3: IGF-I, II and IGFR 1 mRNAs were highly expressed between E13 and E15 during differentiation of myoblasts and formation of myotubes. IGF-I and II proteins were co-localized to differentiating myoblasts, myotubes and myofibers with IGFR 1 protein. High level expression of IGFR 2 mRNA was also observed between E13 and E15. However, the expression of IGFR 2 protein was sparsely

observed throughout the whole tongue tissues and not restricted to the striated muscle tissue. These data suggest that IGFR 1 is related to the IGF signal transduction and the differentiation of mouse tongue striated muscle, whereas IGFR 2 is not directly involved in them.

Part 4: Tongues obtained from E13 mouse embryos were cultured for 8 days in a serum-free and chemically-defined BGJb medium. Tongue myoblasts in this organ culture system were able to fuse and become myotubes. Endogenous IGF-I was mainly involved in this differentiation of the tongue myoblasts. E13 mouse tongues were cultured for 8 days in the BGJb medium containing exogenous IGF-I, exogenous IGFBP4, 5, 6 and des(1-3)IGF-I, an IGF-I analogue with the reduced affinity to IGFBPs. The exogenous IGF-I stimulated the 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 promoted the early tongue myoblast differentiation, to which myogenin was related, probably due to protection of endogenous IGFs from proteolytic degradation by binding to the endogenous IGFs. Low concentration of des(1-3)IGF-I stimulated the tongue myoblast differentiation, whereas high concentration of des(1-3)IGF-I inhibited it, probably due to toxic reactions such as the abnormal shape of tongue, low cell density and low staining intensity with hematoxylin and eosin. From these results, I hypothesized that IGFBPs function to control the concentration of free IGFs within a normal range in the differentiation of mouse tongue myoblasts. However, the concentration of des(1-3)IGF-I with the reduced affinity to IGFBPs was not able to be controlled by this mechanism. Since the present low concentration of des(1-

3)IGF-I was within the normal or optimal range for the differentiation, it stimulated the tongue myoblast differentiation. On the other hand, since the present high concentration of des(1-3)IGF-I exceeded the normal range, it inhibited the differentiation by inducing abnormal reactions.

The myogenesis and synaptogenesis in the mouse tongue striated muscle are almost competed at birth. They are controlled and progressed normally by the IGF-IGFBP regulatory mechanism and, thereby, are able to finish at birth to meet early functional demand such as suckling, swallowing and respiration immediately after birth.