

## **Part 4**

### **Effects of IGF-I, IGFBP4, 5, 6 and des(1-3)IGF-I on Differentiation of Cultured Tongue Myoblasts**

## Results

### *Establishing of tongue organ culture system*

To establish tongue organ culture system for studying the differentiation of tongue myoblasts, the E13 mouse tongues, in which myoblasts just had began to differentiate, were cultured in serum-free and chemically-defined BGJb medium for 4 and 8 days. Figure 19 shows a tongue dissected from E13 mouse embryos (A) and a tongue cultured for 4 (B) or 8 (C) days. The tongues appear to become round after 4 and 8 days in culture.

The level of muscle creatine kinase mRNA was measured as a marker for the myoblast differentiation (Figure 20A). The content increased by 70% for the first 4 days of the culture, then slightly increased later. In the proximal portion of E13 tongue, a few fast myosin heavy chain positive-myoblasts and myotubes were observed (Figure 20B). After 4 days in culture, the number of fast myosin heavy chain positive-myoblasts and myotubes appears to increase. Between 4 and 8 days of the culture period, marked change was not observed, but several elongated myotubes were observed after 8 days in culture. These results of muscle creatine kinase and fast myosin heavy chain suggest that myoblasts were able to fuse and become myotubes in E13 tongues cultured in serum-free and chemically-defined medium.

Since the autocrine secretions of IGF-I and II stimulate differentiation of cultured myoblasts such as C2C12 and L6 (Florini *et al.*, 1991b; Ewton *et al.*, 1994; Rosenthal and Cheng, 1995; Engert *et al.*, 1996; Yoshiko *et al.*, 1996), the levels of

endogenous IGF-I and II mRNAs were measured in the cultured tongues (Figure 21). IGF-I mRNA was highly expressed for the first 4 days of the culture period, then decreased later (Figure 21A). The tongue myoblast differentiation is suggested to occur actively suggested by the results of muscle creatine kinase and fast myosin heavy chain. The level of IGF-II mRNA decreased throughout the whole culture period (Figure 21B). These results suggest that IGF-I may be mainly involved in the differentiation of tongue myoblasts.

### *Effects of exogenous IGF-I on differentiation of mouse tongue striated muscle*

In order to study the function of IGF-I in the differentiation of tongue myoblasts, the effects of exogenous IGF-I on the tongue myoblasts were analyzed. E13 mouse tongues were cultured in BGJb medium containing 25, 50 or 100 ng/ml of IGF-I (Figure 22). A marked change in the shape and size was not observed between the control tongue cultured without IGF-I and the tongues cultured with IGF-I

The treatment with 50 ng/ml of exogenous IGF-I induced 30% ( $p < 0.01$ ) and 35% ( $p < 0.05$ ) increases in the contents of muscle creatine kinase (Figure 23) and myogenin (Figure 24) mRNAs, respectively. That with 100 ng/ml of exogenous IGF-I induced a 41% ( $p < 0.05$ ) increase in the content of myoD mRNA (Figure 23). To detect the differentiating myoblasts and myotubes in the cultured tongues, immunohistochemistry for fast myosin heavy chain was used (Figure 25).

In the middle portion of tongue treated with 50 ng/ml of exogenous IGF-I, the number of fast myosin heavy chain-positive myoblasts and myotubes appears to be

much greater than that in the control tongue cultured without IGF-I. In combination with the PCR results of muscle creatine kinase and myoD family, this immunohistochemical result suggests that exogenous IGF-I promotes the differentiation of the cultured tongue myoblasts.

To study the roles of endogenous IGFBPs in the differentiation of cultured tongue myoblasts, the expressions of endogenous IGFBP mRNAs were analyzed (Figure 26). No significant difference was found in the contents of exogenous IGFBP2 and 3 mRNAs (Figures 26A and 26B), 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 contents of endogenous IGFBP4 and 5 mRNAs (Figures 26C and 26D). Only the treatment with 100 ng/ml of exogenous IGF-I induced a 76% ( $p < 0.05$ ) increase in the mRNA content of endogenous IGFBP6 (Figure 26E). IGFBP1 mRNA was not able to be detected by this PCR technique (data not shown).

The distributions of IGFBP4, 5 and 6, which were increased by the IGF-I treatment, in the cultured tongues were examined by immunohistochemistry (Figure 27). In the striated muscle tissues in the middle portion of the cultured tongue, intense immunostaining for IGFBP4 (Figures 27A and 27B) and 5 (Figure 27C and 27D) was observed. The staining intensities for IGFBP4 and 5 in the striated muscle tissues appear to be slightly stronger in the tongues treated with 50 ng/ml of IGF-I (Figures 27B and 27D) than those in the control tongue (Figures 27A and 27C). In the epithelium and the tissues underneath the epithelium, very intense staining for IGFBP6 was observed. The area, which showed intense staining for IGFBP6, in the IGF-treated tongue (Figure 27F) appears to become

wide in comparison with that in the control tongue (Figure 27E). 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 differentiation of mouse tongue striated muscle*

To elucidate the role of IGFBPs in the differentiation of tongue myoblasts, effects of exogenous IGFBP4, 5 or 6 on the differentiation of tongue myoblasts were examined, because the expressions 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 content of myogenin mRNA but no significant difference was found in the content of muscle creatine kinase mRNA (Figure 28). 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 (Figure 29B). The mean values of muscle creatine kinase mRNAs at 200 and 400 ng/ml of IGFBP5 were 39% and 55% greater than that at 0 ng/ml, but these increase was not statistically significant due to large variation of the data (Figure 29A). IGFBP6 did not induce any significant changes in the contents of muscle creatine kinase and myogenin mRNAs (Figure 30). These results suggest that exogenous IGFBP4 and 5 promote the early differentiation of tongue myoblasts which myogenin is related to.

### *Effects of des(1-3)IGF-I on differentiation of mouse tongue striated muscle*

To further understand the role of IGFBPs in the differentiation of tongue myoblasts, effects of des(1-3)IGF-I, an IGF-I analogue with a reduced affinity of IGFBPs, on the differentiation of tongue myoblasts were analyzed. E13 mouse tongues were cultured in the BGJb medium containing 0, 10, 25, 50 and 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 content of MCK mRNA (Figure 31). 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% ( $p < 0.05 \sim 0.01$ ) decreases in the contents of myf5 (Figure 32A), myoD (Figure 32B) and myogenin (Figure 32C) mRNAs. The mean value of myogenin mRNA at 10 ng/ml was greater than that at 0 ng/ml (Figure 32C) and those of MRF4 at 25 ~ 100 ng/ml was less than that at 0 ng/ml (Figure 32D), 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, but those of 25 ~ 100 ng/ml inhibit it.

Figure 33 shows the tongues cultured with (A) or without 50 ng/ml des(1-3)IGF-I (B). The shape of tongue treated with des(1-3)IGF-I appears to be quite different from that of control tongue. Abnormal tissues were observed in the peripheral region of the tongue. Figures 33C and 33D show the middle portions in sagittal sections of tongues stained with by hematoxylin and eosin. Staining

intensity with hematoxylin and eosin, and cell density in the des(1-3)IGF-I treated tongue (Figure 33D) appear to be less in comparison with the control tongue (Figure 33C). Several multinucleated myotubes were observed in the control tongue (arrows in Figure 33C), but they were not observed in the des(1-3)IGF-I treated tongue (Figure 33D). These morphological results suggest that abnormal reactions to des(1-3)IGF-I happen in the tongues cultured with 50 ng/ml of des(1-3)IGF-I.

## Discussion

In the part 4, in order to study the roles of IGFs and IGFBPs in the differentiation of tongue myoblasts, I established the organ culture system of mouse tongue with serum-free and chemically-defined medium and, using this organ culture system, I examined 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.

I observed the increases in the mRNA content of muscle creatine kinase mRNA and the number of fast myosin heavy chain positive myoblasts and myotubes in the E13 mouse tongues (Figure 20), suggesting that tongue myoblasts are able to fuse and become myotubes in this organ culture system. The culture period during which the differentiation of tongue myoblast actively occurred (the first 4 days in culture) was correspondent to that for the high level expression of endogenous IGF-I mRNA in the cultured E13 mouse tongues. This result suggests that the IGF-I, which is secreted from the tongues tissues including 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.*, 1991b; Ewton *et al.*, 1994; Rosenthal and Cheng, 1995; Engert *et al.*, 1996; Yoshiko *et al.*, 1996). This supports the present view.

Exogenous IGF-I induced the increases in the content of muscle creatine kinase mRNA and the number of fast myosin heavy chain positive myoblasts and myotubes in the cultured E13 mouse tongue (Figures 23 and 25), suggesting that it

stimulates the differentiation of tongue myoblasts. It is already reported that exogenous IGFs promote 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 contents of myoD and myogenin mRNAs were induced by the exogenous IGF-I. MyoD and myogenin are known to regulate the myogenesis by binding to E-boxes in the regulatory region of muscle specific genes such as muscle creatine kinase, myosin heavy chain, desmin etc and controlling the expressions of these genes (Weintraub, 1993; Buckingham, 1994, 1996). Thus, the exogenous IGF-I seems to promote the differentiation of tongue myoblasts by inducing the expression of myoD and myogenin. Other reports of L6 myogenic cell line provide supportive evidence for the present view (Florini *et al.*, 1991a, 1994).

The induction of the expression of IGFBP4, 5 and 6 by the exogenous IGF-I (Figure 26) suggests that these IGFBPs are involved in the promotion of tongue myoblast differentiation by the exogenous IGF-I. The expressions of IGFBP4 and 5 in several myogenic cell lines are reported to be suppressed by transforming growth factor  $\beta$ 1, epidermal growth factor and basic fibroblast growth factor, which inhibit the differentiation of the myogenic cells (McCusker and Clemmons, 1994). Taken together, several growth factors including IGF-I seem to regulate the differentiation of myogenic cells by inducing the expression of IGFBPs.

In the present study, exogenous IGFBP4 and 5 induced the expression of myogenin in the E13 cultured mouse tongue (Figures 28 and 29). These results suggest that the IGFBPs stimulate the early differentiation of tongue myoblasts, because studies of knockout mice indicate that myogenin is necessary to begin the

fusion of myoblasts (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). Probably, it seems that the exogenous IGFbps bind to IGFs secreted from tongue tissues, protect the IGFs from proteolytic degradation in the culture medium and then the IGFs are released from the IGFbp-IGF complexes and stimulate the early differentiation of tongue myoblasts. On the other hand, in C2 and L6 myogenic cell lines, IGFbp4 and 5 seem to function mainly as an inhibitor of the differentiation induced by IGFs, although IGFbp5 has the additional capability of stimulating myogenesis of these cell lines under the proper conditions (James *et al.*, 1993; Ewton and Florini, 1995; Rotwein *et al.*, 1995; Silverman *et al.*, 1995; Ewton *et al.*, 1998). This seems to be different from the present result of tongue striated muscles.

In the present study, the exogenous IGFbp6 did not affect the expression of myogenin and muscle creatine kinase (Figure 30). In L6 myogenic cell line, IGFbp6 is reported to inhibit the differentiation of myoblasts (Bach *et al.*, 1994), which is inconsistent with the present result. The effect of IGFbp6 in the tongue organ culture system seems to be weaker in comparison with the effects of IGFbp4 and 5 in the same organ culture system or the effect of IGFbp6 in L6 cell line. One possible reason for this weaker effect of IGFbp6 is that abundant IGFbp6 was secreted from the epithelial tissues of tongue into the cultured medium and attenuated the effects of exogenous IGFbp6.

The induction of muscle creatine kinase by 10 ng/ml of des(1-3)IGF-I (Figure 31) suggests that the low concentration of des(1-3)IGF-I stimulates the differentiation of tongue myoblasts, whereas the suppressions of myf5, myoD and myogenin by 25 ~ 100 ng/ml of des(1-3)IGF-I (Figure 32) suggest that the high concentration of des(1-3)IGF-I inhibits the differentiation of tongue myoblast. Since

the abnormal shape of tongues, the decrease in the cell density and weakness of staining intensity by hematoxylin and eosin were observed in the tongues cultured in the high concentration of des(1-3)IGF-I (Figure 33), the inhibitory effect on the differentiation of tongue myoblasts seems to be caused by toxic reaction to the high concentration of des(1-3)IGF-I.