

Materials and Methods

Tissue

Pregnant ICR mice were purchased (Nippon Clea, Tokyo, Japan). Tongues were dissected from embryos at E11, 13, 15, and 17, and from newborn mice. The tissues for PCR analysis were immediately frozen and stored at -80°C until use. Five or six samples were collected at each developmental stage. Four preparations at E11 and two preparations at E13 were pooled due to their small amounts of RNA. The tissues obtained from E13, E15 and newborn mice for immunohistochemistry were immediately fixed in Bouin's solution. Experimental protocols concerning animal handling were reviewed and approved by the Institutional Animal Care Committee of Tsurumi University School of Dental Medicine.

Tongue organ culture

Pregnant ICR mice were killed by cervical dislocation at E13. Embryos were isolated from uterine deciduas and eliminated of their membranes under a dissection microscope. The tongues of embryos were carefully microdissected and explanted. The explants were supported by Millipore type AABP filters with 0.8 µm pore size on steel rafts and cultured in BGJb medium (Life Technologies, Gaithersburg, MD, USA) freshly supplemented with 100 µg/ml ascorbic acid and 100 unit/ml penicillin-streptomycin (Life Technologies, Gaithersburg, MD, USA). Cultures were maintained for 4 or 8 days at 37° in an atmosphere of 5% carbon dioxide and 95% air with medium changes every two days. IGF-I (Life Technologies, Gaithersburg, MD, USA), IGFBP4, 5, 6 and des(1-3)IGF-I (GroPep Limited,

Adelaide, SA, Australia) were added to the cultured medium. After the culture, the explants for the analysis of competitive PCR were frozen and for histological analysis were fixed in Bouin's fixative.

RNA extraction

Total RNA extraction was performed according to the specification of the manufacturer (Rapid total RNA isolation kit, 5 Prime→3 Prime Inc., Boulder, CO, USA). To remove contaminants of genomic DNA, the RNA was treated with 200 U ribonuclease-free deoxyribonuclease I (Life Technologies, Gaithersburg, MD, USA) at 37°C for 15 min, then heated at 65°C for 10 min to stop the reaction. The quantity of total RNA was determined by absorbance using a spectrophotometer (GeneQuant II, Amersham Pharmacia Biotech., Tokyo, Japan) at a wavelength of 260 nm.

Reverse transcription

The RNA was denatured at 65° for 5 minutes and then 6 µl of 5X reverse transcription buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 3 µl of 100 mM dithiothreitol, 1.5 µl of each 10 mM 2'-deoxynucleoside 5'-triphosphate (dNTP), 1.5 µl of random primer and 200 units of reverse transcriptase (SuperScript II, Life Technologies, Gainthersburg, USA) were added to the reaction mixture, resulting in a final reaction volume of 30 µl. Reverse transcription was performed at 45° for 60 min.

Competitive polymerase chain reaction (Competitive PCR)

Internal standards (competitors) for the competitive RT-PCR amplification were constructed according to the manufacturer's instructions (PCR MIMIC Construction Kit, Clontech Laboratory Inc., Palo Alto, CA, USA or Takara Biomedicals, Shiga, Japan). Total cDNA (50 ng) was amplified along with the internal standard in the presence of primers specific to the target genes in a thermal cycler (TP3000, Takara Biomedicals, Shiga, Japan). The cycling parameters were as follows: denaturation at 94°C for 30 sec, annealing at 46 ~ 55°C for 60 sec, and extension at 72°C for 60 sec. Sequences of primers specific to the target genes, annealing temperatures, numbers of cycles and concentrations of competitors are shown in Table 1. The specificity of primers was determined by analyzing the sequences of the resultant PCR products with an automated sequencer (Model 377, Perkin-Elmer Corp., Applied Biosystems Div., Foster City, CA, USA). Amplification products were isolated by electrophoresis of 5 µl of each sample on a 3% agarose gel containing ethidium bromide. Examples of electrophoretic gel patterns of myoD and its internal standards are shown in Figure 1. Fluorescent intensities in bands of the target genes (lower bands) and their respective internal standards (upper bands) were measured by image analyzers (Argus-100, Hamamatsu Photonics K.K., Hamamatsu, Japan or Molecular Imager FX, Bio-Rad, Hercules, CA, USA) and ratios of fluorescent intensities in the target gene bands to those in their respective internal standard bands were calculated.

To calculate the cDNA quantity of each target gene from the calculated ratio

of fluorescent intensity, I generated a standard curve for each target gene by plotting the logarithmic values of the ratios of fluorescent intensities in the bands of known quantities of cDNA standards to those in the bands of their respective internal standards against the logarithmic values of the known quantities of the cDNA standards. To obtain cDNA for the standard curve, PCR amplification was carried out in the presence of primers specific to the target genes. When a single band appeared on the electrophoretic gel, the PCR products were purified by passing them through a spin column (CHROMA SPIN+TE 100, Clontech Laboratory Inc., Palo Alto, CA, USA). The quantity of the amplified cDNA was measured by a spectrophotometer at a wavelength of 260 nm and 1:2 serial dilutions of the cDNA were made. The diluted cDNA standards and their competitors were amplified by PCR and analyzed in the same manner and time as the experimental samples. The same concentrations of internal standards (competitors) for the experimental samples were used for the diluted cDNA standards. The quantity of each target gene was extrapolated from its standard curve, divided by the quantity of glyceraldehyde-phosphate dehydrogenase (GAPDH) and the resulting ratio value was expressed as a percent value relative to the mean ratio value of each target gene in the tongue muscle at the newborn stage or to the mean ratio value of each target gene at 0 ng/ml of IGF-I, IGFBP4, 5, 6 and des(1-3)IGF-I (untreated control).

Immunohistochemistry

Specimens for immunohistochemistry were fixed in Bouin's fixative for two

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 frozen. Sagittal sections of tongues were prepared at a 10 µm thickness in a cryostat and air-dried for 1 hour at room temperature. The frozen sections were stained with by hematoxylin and eosin, and observed under light microscope. For immunostainig, they were post-fixed in acetone at -20°C, rehydrated in PBS, and incubated with 5% of normal goat serum for 30 min to block non-specific immunostaining. The sections were incubated with primary antibodies. The following primary antibodies were used in the present study; rabbit polyclonal antibodies against IGF-I, II (AUSTRAL Biologicals, San Ramon, CA, USA), IGFR 1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), IGFR 2 (a kind gift from Dr. S.D. Scott, Kolling Institute of Medical Research, Royal North Shore Hospital, St Leonards, NSW, Australia; Scott and Baxter, 1987), IGFBP4, 5, 6 (GroPep Limited, Adelaide, SA, Australia) and the mouse monoclonal antibodies against fast skeletal muscle myosin heavy chain (Sigma-Aldrich Japan Inc., Tokyo, Japan) and nAChR δ subunit (Affinity Bioreagents, Inc. Golden, CO, USA) . After washing 3 times in PBS, the sections were incubated with FITC-conjugated goat antibody against rabbit IgG and/or the rhodamine-conjugated goat antibody against mouse IgG (Sigma-Aldrich Inc.). After washing in PBS and subsequent incubating with Equilibration buffer of SlowFade-Light Antifade Kit (Molecular Probes Inc., Eugene, OR, USA), the stained sections were mounted in SlowFade-Light Antifade reagent and visualized with a confocal laser scanning microscope (PCM2000, Nikon, Tokyo, Japan). For control staining, the primary antibodies were replaced with normal rabbit, mouse IgG or PBS; all controls

showed no staining.

Statistical Analyses

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