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IGF-I binding in primary culture of muscle cells of rainbow trout: changes during in vitro development

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Castillo, Juan, Pierre-Yves Le Bail, Gilles Paboeuf, Isabel Navarro, Claudine Weil, Benoit Fauconneau, and Joaquim Gutiérrez. IGF-I binding in primary culture of muscle cells of rainbow trout: changes during in vitro development. *Am J Physiol Regul Integr Comp Physiol* 283: R647–R652, 2002. First published June 6, 2002; 10.1152/ajpregu.00121.2002.—To characterize and study the variations of IGF-I binding during the development of trout muscle cells, in vitro experiments were conducted using myocyte cultures, and IGF-I binding assays were performed in three stages of cell development: mononuclear cells (*day 1*), small myotubes (*day 4*), and large myotubes (*day 10*). Binding experiments were done by incubating cells with IGF-I for 12 h at 4°C. Specific IGF-I binding increased with the concentration of labeled IGF-I and reached a plateau at 32 pM. The displacement of cold human and trout IGF-I showed a very similar curve (EC_{50} = 1.19 ± 0.05 and 0.95 ± 0.05 nM, respectively). IGF binding proteins did not interfere significantly because displacement of labeled IGF-I by either cold trout recombinant IGF-I or Des (1–3) IGF-I resulted in similar curves. Insulin did not displace labeled IGF-I even at very high concentrations (>1 μM), which indicates the specificity of IGF-I binding. The amount of receptor (R_0) increased from 253 ± 51 fmol/mg DNA on *day 1* to 766 ± 107 fmol/mg DNA on *day 10*. However, the affinity (K_d) of IGF-I receptors did not change significantly during this development (from 1.29 ± 0.19 to 0.79 ± 0.13 nM). On the basis of our results, we conclude that rainbow trout muscle cells in culture express specific IGF-I receptors, which increase their number with development from mononuclear cells to large myotubes.

insulin-like growth factor-I; receptor

IN VERTEBRATES, IGFs are involved in several muscle functions. They stimulate proliferation and differentiation in a number of cellular models of myogenesis (16, 18), and the overexpression of IGF-I (8) or the IGF-I receptor (42) in cultured mouse myoblasts accelerates cell differentiation. IGFs increase the expression of some crucial genes in muscle development, such as MyoD and myogenin in mammals (17) and fish (43). Moreover, IGF-I stimulates glucose uptake in avian muscle cells (12) and glycogen formation in rat skeletal muscle (19). Anabolic effects have been demonstrated in rat muscle satellite cells (1, 9), the RMO cell line (25), chicken muscle satellite cells (13, 23), turkey embryonic myoblasts and satellite cells (33, 35), and also in myotubes from porcine (24) and chick myoblasts (49).

In fish, IGF-I binding was first reported by our group in carp ovary (31). This binding was later characterized in most fish tissues (4, 6, 10, 28, 30, 36, 40, 41), in which the presence of this receptor was always remarkably abundant. In contrast to mammals (38), IGF-I binding in fish skeletal muscle is higher than insulin binding. Furthermore, this high ratio of IGF-I/insulin binding is established during development and maintained throughout adulthood (34). However, despite the literature on IGF-I binding, the role of the abundant IGF-I receptors in fish muscle still remains unclear.

The in vivo model has some limitations for the study of IGF-I in trout, because IGF-I interacts with the insulin receptor in target tissues and because IGF-I may stimulate insulin secretion (5), which could hinder the interpretation of results. Therefore, the in vitro model offers a useful alternative in studies on the regulation and function of IGF-I and its receptors in fish. Satellite cells have been isolated and cultured in many homeothermic species such as chicken or mouse (13, 45) and in two fish species: carp and zebrafish (26, 29). Culture of trout myocytes was first described by Rescan et al. (44), and a more precise protocol of isolation and optimization of conditions for the maintenance of rainbow trout satellite cells in culture was proposed by Fauconneau and Paboeuf (15). These cells in culture were functional, with simultaneous proliferation and differentiation, as shown by the expression of genes involved in developmental processes, such as MyoD and myogenin (43). On the basis of these observations, primary culture of trout satellite cells has become a useful tool with which to characterize the role of IGF-I receptors in fish skeletal muscle.

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Here we aimed to examine IGF-I binding in trout muscle cells in culture and characterize this binding to study the evolution of IGF-I receptors during myocyte differentiation, as a first step to understand the role of these receptors in fish muscle physiology.

MATERIALS AND METHODS

Chemicals. Trout recombinant IGF-I (rtIGF-I), human recombinant Des (1–3) IGF-I, and human recombinant IGF-I (rhIGF-I) were purchased from GroPep (Adelaide, Australia). Des (1–3) IGF-I is an analog of IGF-I that exhibits low affinity for most IGF binding proteins (IGFBP). Unlabeled porcine insulin was obtained from Lilly (Indianapolis, IN).

Recombinant human ^{125}I-IGF-I with a specific activity of 260 μCi/μg was purchased from Amersham Pharmacia Biotech Europe GmBH. Trout IGF-I was labeled following the Cloramine T method, as modified by Martal (32) (specific activity 80 to 100 μCi/μg). Other cell culture reagents were purchased from Sigma Aldrich Quimica, S. A.

Animals. We used rainbow trout (*Oncorhynchus mykiss*) with weights ranging from 1.8 to 2.5 g. These fish were maintained in the Rennes and Barcelona facilities in closed-circuit flow systems at 12°C, fed ad libitum with a commercial diet, and fasted for 24 h before the experiments. The fish (30 to 40 for each culture) were killed by a sharp blow to the head and immersed in 70% ethanol for 30 s to sterilize external surfaces.

Isolation of myosatellite cells. The protocol used was described by Fauconneau and Paboeuf (15). White myotomal muscle was excised under sterile conditions and collected in cold (0°C) DMEM, 9 mM $NaHCO_3$, 20 mM HEPES (pH 7.4, Posm 300 mosmol/kgH_2O), containing 15% horse serum and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, fungizone 0.25 μg/ml, gentamycin 75 μg/ml) at a concentration of 5 g/ml.

The tissue was minced, and fragments were centrifuged (300 *g*, 5 min) and washed twice in DMEM without horse serum to eliminate the serum components.

Enzymatic digestion was performed with collagenase (Type Ia Sigma) in DMEM (final concentration 0.2%) for 1 h at 18°C with gentle agitation. The suspension was centrifuged (300 *g*, 5 min), and the pellet was washed with DMEM, resuspended in DMEM (5 ml/g of muscle), triturated through a pipette five times, and centrifuged again (300 *g*, 5 min).

Fragments were resuspended in a trypsin solution 1:250 (Sigma) (0.1% final concentration in DMEM) for 20 min at 18°C with gentle agitation and centrifuged for 5 min at 300 *g*.

The supernatant was diluted in 2 vol of cold complete medium to block trypsin activity. The tissue fragments (pellet) were given a second similar trypsin digestion, and the suspension was then diluted. The two supernatants of digestion were pooled, diluted (1:1 vol/vol) in cold DMEM supplemented with 15% horse serum, and centrifuged (300 *g*, 20 min, 4°C). The resulting pellets were resuspended in 20 ml of basal medium and submitted to mechanical dissociation by trituration five times with a 10-ml pipette and five times using a 5-ml pipette. The suspension was then filtered successively on a 100- and 40-μm nylon cell strainer and centrifuged (300 *g*, 2 min, 4°C). The cells were resuspended in basal medium supplemented with 10% fetal calf serum (FCS) and diluted to reach a final concentration of 10^6 cells/ml of normal medium.

Cell culture. Culture plates were pretreated with a solution of 100 μg/ml of poly-L-lysine (Sigma MW300000) at a concentration of 16 μg/cm^2 for 2 h 30 min at 15°C. After this precoating, the poly-L-lysine solution was aspirated, substratum was washed twice with distilled water, air dried, covered with a solution of 20 μg/ml of laminin (L2020 Sigma) in DMEM at 2 μg/cm^2, and incubated for 24 h at 18°C. The laminin solution was aspirated immediately before the plating of the cell suspension, which contained a crude extract of muscle cells. Culture was performed with this crude extract, without selection of satellite cells.

Cells were cultured in complete medium at 18°C in air, in 6-well plastic plates (9.6 cm^2/well, NUNC), and medium was changed every 2 days. Observations of morphology were regularly made to control the state of the cells.

IGF-I binding assays. Binding studies were conducted with cells seeded at a density of 1.5 to 2 × 10^6 per well. In each well, monolayers were washed three times with 2.5 ml of cold DMEM without FCS and incubated for 12 h at 4°C in 1 ml DMEM containing 0.5% BSA (free of insulin, A-7888 Sigma) with ^{125}I-labeled rtIGF-I or ^{125}I-labeled rhIGF-I in the presence or absence of a range of concentrations of cold peptides (from 1 to 400 ng/ml for IGF-I and 1,000 times for cold insulin). Nonspecific binding (NSB) was obtained with a concentration of 400 ng/ml of cold rtIGF-I or cold rhIGF-I.

Incubation was stopped by aspiration of medium, the monolayer was washed twice with 2.5 ml of cold DMEM, and cells were triturated by incubation with 1 ml 1 N NaOH for 30 min at 40°C. Radioactive solution was counted using a gamma counter (Packard Bioscience, Meriden, CT). Each binding experiment was performed in duplicate at least three times for each developmental stage.

The DNA content of wells was determined using a RNA/DNA calculator Genequant II (Pharmacia Biotech, Barcelona, Spain). The protein content of each well was determined following the method described by Bradford (7).

Statistical analysis. The treatment was performed in duplicate in each experiment. All data are presented as means ± SE of at least three experiments (Scatchard assays). Statistical differences between conditions were tested by ANOVA (two-way ANOVA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

The phenotype of cells in different days of culture is shown in Fig. 1: in *day 1*, cells are mononucleated (Fig. 1*A*), and throughout their development, they fuse to form small myotubes (*day 4*; Fig. 1*B*) and large myotubes later on (*day 10*; Fig. 1*C*). Time course experiments involving the incubation of cells in *day 4* at 4°C with a fixed amount of labeled rtIGF-I (10^5 cpm/well) (Fig. 2) showed that the highest specific binding for IGF-I was obtained after 12 h and remained at this level up to 24 h. Maximum binding was ~2–2.5% of the labeled trout IGF-I incubated and ~0.4% of the labeled insulin. This temperature (4°C) and incubation time (12 h) were subsequently used to perform the binding experiments and receptor quantification.

Cells were incubated with increasing concentrations of labeled rtIGF-I. The NSB and total binding (TB) increased and did not reach a plateau, whereas specific binding (SB) (SB = TB − NSB) increased from ~4,000 to 20,500 cpm, then reached a plateau and remained at high amounts of labeled IGF-I (Fig. 3). The Scatchard transformation of the SB curve gave the affinity of the receptors (K_d = 0.91 ± 0.03 nM). This K_d was similar to that obtained when labeled rtIGF-I was displaced with cold rtIGF-I (K_d = 0.87 ± 0.08 nM).

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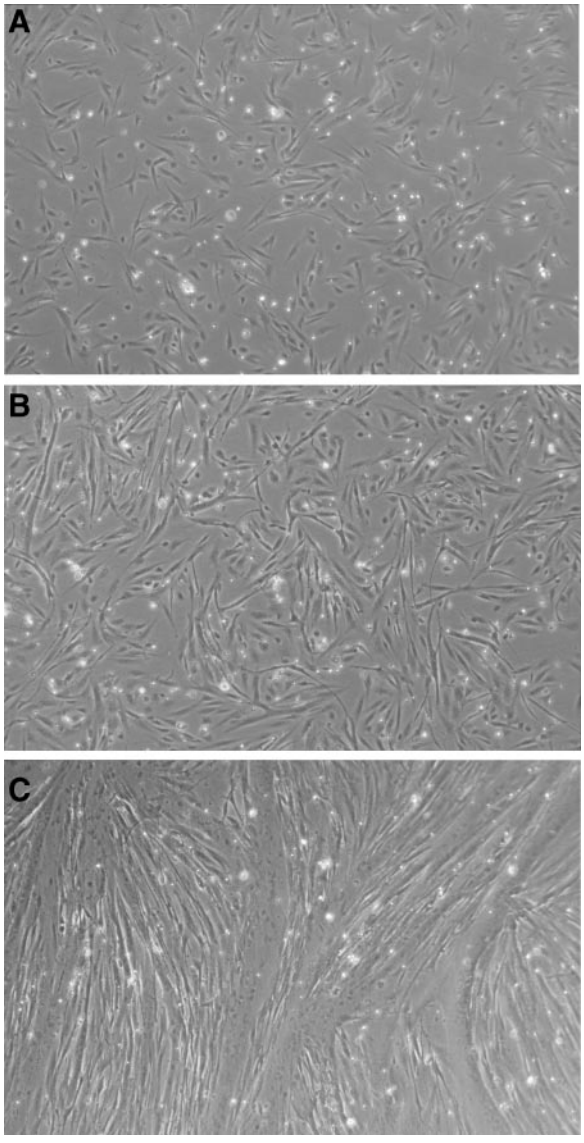


Fig. 1. Differentiation of satellite cells isolated from skeletal muscle of juvenile rainbow trout and cultured on a laminin substrate with DMEM 90%/fetal calf serum 10% medium at 18°C for 1 day (*A*), 4 days (*B*), and 10 days (*C*).

We then checked whether heterologous peptides could be used in our binding system. Cells were incubated with labeled rtIGF-I and increasing concentrations of cold rtIGF-I or cold rhIGF-I for comparison. Both the homologous (trout) and the heterologous (human) nonlabeled IGF-I displaced labeled rtIGF-I in a similar way (EC_{50} = 0.95 ± 0.05 and 1.19 ± 0.05 nM, respectively) (Fig. 4).

To assess the possible interference of IGFBP in the binding assay, we compared the displacement of labeled trout IGF-I with either cold trout IGF-I or Des (1–3) IGF-I (Fig. 5). Labeled rtIGF-I was similarly displaced by cold rtIGF-I and cold human Des (1–3) IGF-I (EC_{50} = 0.75 ± 0.06 and 1.04 ± 0.02 nM, respectively), thus showing that IGFBP did not interfere significantly in the binding of IGF-I to its receptor.

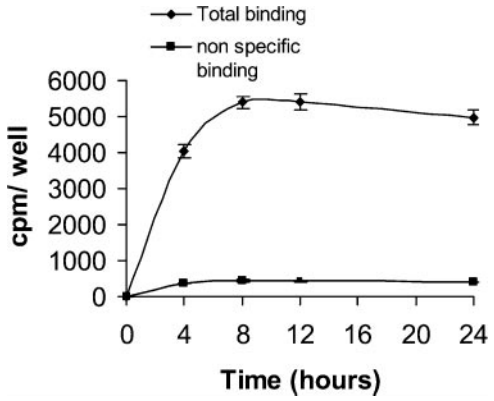


Fig. 2. Time course experiment of labeled trout IGF-I binding to primary culture of trout myosatellite cells. Cells were isolated and seeded as described in MATERIALS AND METHODS, and after 4 days, they were incubated at 4°C with a fixed concentration of labeled hormone at a range of incubation times (from 4 to 24 h). Data are means ± SE of 3 experiments.

To check the specificity of the binding, cells were incubated with labeled rtIGF-I and increasing concentrations of salmon insulin for 12 h at 4°C (Fig. 6). No displacement of labeled trout IGF-I was observed (even for concentrations 1,000 times higher than the IGF-I concentration that completely displaced labeled rtIGF-I binding). This observation indicates that IGF-I binding is specific and is caused by the binding of IGF-I to its specific receptors. Furthermore, binding of insulin was very low (from 0.1 to 0.4% of the total radioactivity added).

Binding characteristics (affinity and number of IGF-I receptors) were studied using human labeled IGF-I in three cell stages: *day 1* (mononuclear cells), *day 4* (small myotubes), and *day 10* (large myotubes) (Table 1). The receptor affinity did not show significant differences among the three stages (K_d from 1.29 to

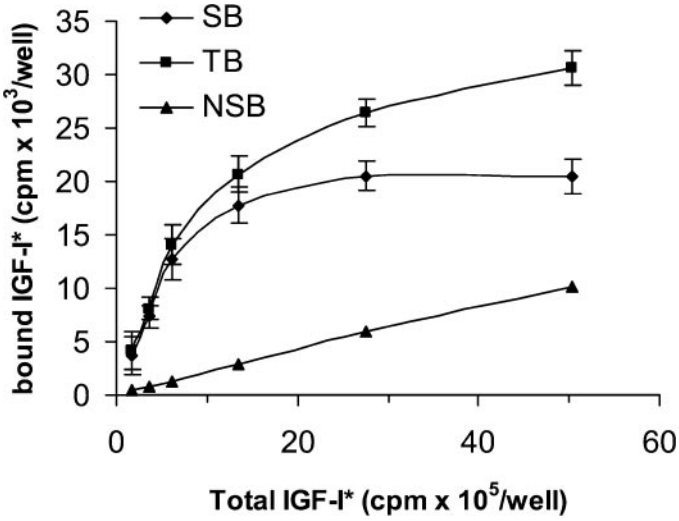


Fig. 3. Saturation with labeled trout IGF-I. Myosatellite cells were incubated with increasing concentrations of labeled trout IGF-I (from 1.77 × 10^5 to 50.42 × 10^5 cpm) for 12 h at 4°C. SB, specific binding; TB, total binding; NSB, nonspecific binding. Data are means ± SE of 3 experiments.

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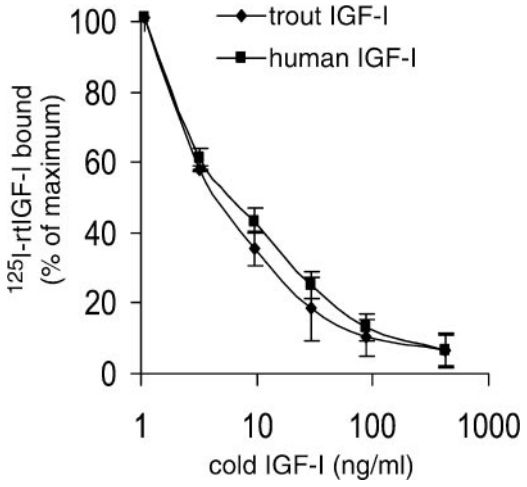


Fig. 4. Human vs. trout IGF-I binding to muscle cells. Cells were incubated for 12 h at 4°C with a fixed amount of labeled trout IGF-I and increasing concentrations of cold human IGF-I. Data are means ± SE of 3 experiments. rtIGF-I, trout recombinant IGF-I.

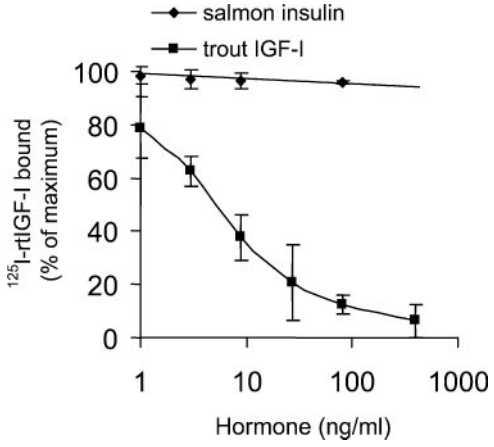


Fig. 6. Specificity of IGF-I binding to trout muscle cells. Cells were incubated with increasing concentrations of insulin for 12 h at 4°C. Binding values are expressed as percentage of maximum binding. Data are means ± SE of 3 experiments.

0.79). However, the number of IGF-I receptors per DNA content in the culture wells increased progressively with culture time and showed the highest values after 10 days (from 253 to 766 fmol/mg DNA). When results were expressed for the protein content, the number of IGF-I receptors ranged from 127 fmol/mg of protein on *day 1* to 268 fmol/mg on *day 10* of culture.

DISCUSSION

This study is the first report on the presence of specific IGF-I receptors in primary culture of fish muscle cells. Although several established muscle cell lines have been used in mammals, the lack of these lines in fish led us to examine the effects of IGFs in a primary muscle cell culture, which, in addition, resembles physiological conditions in vivo. In our study, cell culture contained fibroblasts (~15%) and satellite cells that presented similar characteristics to those observed by Rescan et al. (44) and Fauconneau and Paboeuf (15) in terms of growth, proliferation, and fusion of satellite cells to form myotubes. We used small fish because the number of satellite cells extracted from large ones is lower (20). The number of cells used for each binding experiment (~1.5–2 million of cells/well) was comparable to that used by other authors in other vertebrate species (13). Within the first 24 h of culture, cells attached to the culture surface and started to proliferate and differentiate; they then fused at the same time and were visible as small myotubes after 2–3 days. At 10 days, long multinucleated myotubes were clearly observed.

A temperature of 4°C and overnight incubation (12 h) were the optimal conditions selected from preliminary assays. These conditions are consistent with previous data on IGF-I binding in fish (6, 27, 30, 36, 39) and in higher vertebrates (3, 13). Our results in fish myocytes coincided with those observed in muscle cells from other species: Duclos et al. (13) obtained 3% of SB for IGF-I in chicken satellite cells, a percentage similar to that observed in our study (2–2.5%). When expressed per milligram of protein, SB in trout muscle cells (15%) present similar levels to those in mouse myoblasts (16.1%) (45). These results confirm that IGF-I binding in trout muscle cells is in the same range as that in cultured myocytes and in semipurified receptor preparations from other vertebrates.

Given that most of the previous IGF-I binding studies performed in fish used mammalian IGF, one of our

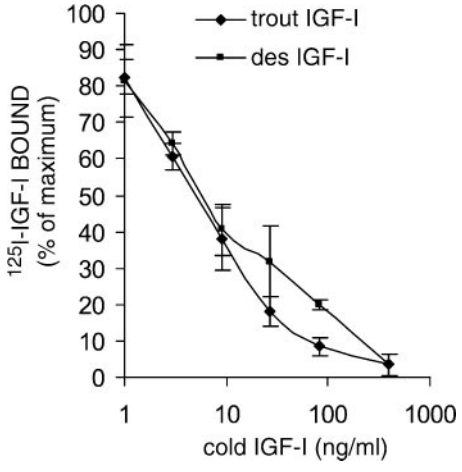


Fig. 5. Binding to IGF-I receptor. Cells were incubated with increasing concentrations of rtIGF-I and human Des (1–3) IGF-I for 12 h at 4°C. Data are means ± SE of 3 experiments.

Table 1. *Binding characteristics of IGF-I receptors in rainbow trout muscle cells*

	Day 1	*Day 4*	*Day 10*
K_d, nM	1.29 ± 0.19*	0.94 ± 0.12*	0.79 ± 0.13*
R_0, fmol/mg DNA	253.12 ± 50.97*	397.16 ± 24.57†	766.48 ± 107.14‡
R_0, fmol/mg protein	127.47 ± 12.42*	162.36 ± 1.34†	268.17 ± 9.80‡

Data are means ± SE of 4 separate experiments, each performed in duplicate. *, †, and ‡Significantly different values at $P < 0.05$.

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objectives was to verify that this heterologous system was similar to the homologous one and, therefore, appropriate for the measurement of IGF-I binding. A comparison of the displacement curves with cold trout IGF-I and cold human IGF-I showed an equivalent EC_{50}, a finding that is consistent with data obtained in other binding models (21, 22, 28). On the basis of these results, IGF-I receptors in trout myosatellite cells can be quantified using the heterologous peptide system. Moreover, the specific activity of commercially labeled rhIGF-I is higher (more than two times) than that obtained in our study with rtIGF-I and is therefore more suitable for this model in which binding is not very high.

In their studies on primary cultures of satellite cells of chicken, Duclos et al. (13) found that IGFBP interfered with the IGF-I binding. Other authors also reported the presence of autocrine IGFBP (especially IGFBP-4, -5, -6) in L6 rat myoblasts in culture, which inhibits the effects produced by IGF-I (2, 47). Similar binding proteins also occur in the blood of rainbow trout (50). Nevertheless, in our experiments, we did not detect interference of IGFBP in the IGF-I binding assays, because Des (1–3) IGF-I displaced bound labeled IGF-I in the same way as native IGF-I. This observation thus indicates that the IGF-I binding observed was mainly due to the binding of the peptide to its specific receptors.

Several authors working on higher vertebrate species, who found specific IGF-I binding in satellite cells in culture (3, 13, 45), observed that the IGF-I receptor bound insulin with much less affinity. A similar situation was observed in a range of fish preparations (34, 36). In this study, salmon insulin was unable to compete with rtIGF-I for binding to the IGF-I receptor, indicating the specificity of IGF-I receptors, and that this high specificity seems to be a characteristic of this culture.

We did not observe significant changes in receptor binding affinity during the differentiation of myocytes in culture. This result is in agreement with previous studies where the affinity of IGF-I receptors was maintained throughout the distinct stages of trout larvae development (34).

Specific binding of IGF-I (expressed per mg of protein content of the culture wells) increased from *day 1* (myosatellite cells) to *day 4* (small myotubes) and peaked on *day 10* of culture (large myotubes), suggesting an increase in the presence of IGF-IR during muscle cell differentiation in fish. These results contrast with the data obtained for mouse satellite cell lines by Rosenthal et al. (45) who reported a decrease of ~60% in IGF-I binding (expressed per mg of protein) when myoblast cells develop into myotube cells. These differences cannot be explained by a variation in protein synthesis in cell culture between species or between stages because the same tendency of an increase in IGF-I receptor binding is observed when expressed per milligram of DNA of the wells. We conclude that the crucial role of IGF-I binding in trout muscle, as seen in the abundance of IGF-I receptors in trout muscle through the whole life cycle (34, 38), explains this situation. In fact, we obtained higher IGF-I binding than for insulin in satellite cells (data not shown), which shows a similar tendency to trout cardiac cells (36) or wheat germ agglutinin-purified preparations of trout muscle tissue (37). This is the inverse pattern of the binding ratio IGF-I/insulin obtained in the muscle of endothermic vertebrates.

We conclude that trout muscle cells in culture show specific IGF-I binding sites and that the binding characteristics of this growth factor are similar to those previously observed in preparations of semipurified muscle receptor. The methodology used here allowed us to obtain the first results on the changes in IGF-I receptor number during multiplication and differentiation in fish muscle cells. Our results show that in vitro experiments are useful to study the role of IGF-I and its receptor in fish muscle metabolism and growth.

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