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Immunocytochemical Studies on the Cellular Origin of Ectopic Striated Muscle Fibers in Monolayer Cultures of Rat Anterior Pituitary Cells

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ABSTRACT—We have previously reported that glia-like folliculo-stellate (FS) cells in the rat anterior pituitary may be involved in heterotopic differentiation of striated muscles which appear during pituitary cell culture. In the present immunocytochemical study, cytological alterations of FS cells *in vitro* were investigated by using antibodies to marker proteins (i.e., S-100 and vimentin) for FS cells. Expression of skeletal muscle-specific MyoD1 detected by immunocytochemistry, enabled identification of myogenic cells at a stage when they were still unicellular. Elongated myoblasts containing MyoD1-positive nuclei were found as early as the fifth day of monolayer culture of pituitary cells. The double immunostaining technique showed that some of these myoblasts reacted with antiserum to S-100. Although all of the myoblasts were immunoreactive to vimentin, this marker protein was unable to identify FS cells *in vitro* because rapidly proliferating fibroblasts were also immunoreactive to vimentin. Since the antiserum to S-100 that we used did not react with already differentiated striated muscle fibers, the demonstration of both MyoD1 and S-100-immunoreactive myoblasts in our pituitary cultures suggests that these myogenic cells are derived from FS cells. The present study does not rule out the possibility that fibroblasts, whose origin is presently unknown, are also involved in myogenesis in pituitary cultures.

INTRODUCTION

Cells in a culture system reorganize themselves in response to their new microenvironmental conditions. Under *in vitro* circumstances, cells sometimes behave and express phenotypic characteristics which are not observed in intact tissues. For example, striated muscle fibers are known to differentiate in monolayer cultures of non-muscular tissues (Watanabe *et al*., 1981; Wekerle *et al*., 1975) and rat anterior pituitary (Brunner and Tschank, 1982; Spira *et al*., 1988; Watanabe, 1989). The cellular origin of pituitary-derived striated muscles remains obscure. Inoue *et al*. (1987) observed that muscle fibers appeared in close topological association with folliculo-stellate (FS) cells when the anterior pituitary was grafted under the kidney capsule. Recently, we have provided quantitative data indicating a good correlation between the incidence of muscle fibers and the proportion of FS cells in pituitary cultures (Hosoya and Watanabe, 1997). These findings indicate that FS cells may be likely candidates for myogenic precursors.

Recent studies on differentiating skeletal muscle have shown that MyoD1, one of the skeletal muscle-specific regulatory factors, appears to play a critical role in the commitment of mesodermal progenitors to the myogenic lineage and subsequent differentiation of skeletal myoblasts into terminally differentiated myotubes (Olson and Klein, 1994). Since MyoD1 is a nuclear phosphoprotein whose expression is restricted to proliferating myoblasts and differentiated myotubes (Buckingham, 1994; Tapscott *et al*., 1988), this protein may serve as a specific cell marker for skeletal myoblasts.

This study was conducted to determine whether pituitary FS cells are actually involved in the heterotopic muscle differentiation. For the purpose of identifying myoblasts in pituitary cultures, we employed a specific monoclonal antibody to MyoD1 protein (Dias *et al*., 1992). Moreover, MyoD1-labeled cells were doubly immunostained by using antisera to S-100 protein or vimentin, marker proteins for FS cells in the rat pituitary (Cocchia and Miani,1980; Marin *et al*., 1989; Nakajima *et al*., 1980; Shirasawa *et al*., 1983).

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were used in the present study. Animals were kept in an environment of constant temperature and humidity, and on a 12 hr light and dark cycle. They were killed by decapitation under ketamine hydrochloride anesthesia.

Tissues

For immunohistochemical studies on the localization of S-100

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protein and vimentin, adult female pituitary glands were fixed in Bouin's solution or 2% paraformaldehyde/Dulbecco's phosphate buffered saline (PFA/DPBS) at pH 7.4 for 6–8 hr at 4°C, dehydrated in ethanol, and embedded in an Epon-Araldite mixture. Serial semithin sections were cut in a transverse plane. Resin was removed according to the procedure of Shimada (1992) with a modification. Briefly, sections were treated with 14% sodium methoxide/ethanol for 1 min at room temperature (RT) and then washed in ethanol. In addition, two kinds of rat skeletal muscles, soleus muscle from an adult and vastus lateralis from a neonatal rat (postnatal day 5), were fixed in Bouin's solution at RT overnight to check the immunohistochemical specificity of the anti-S-100. The muscles were dehydrated in ethanol and embedded in Paraplast. Cross sections were cut at 3 μm and mounted on APS-coated slides (Matsunami Glass IND, Osaka, Japan). Both the pituitary and muscle sections were subjected to immunohistochemistry as described below.

Monolayer cultures of anterior pituitary cells

The anterior pituitaries, obtained from 2- to 3-month-old female rats, were enzymatically dispersed into single cells and established in monolayer cultures, as previously described, with a modification (Hosoya and Watanabe, 1997). Briefly, 2×10^5 cells in 200 μl of serum-free α-minimum essential medium (αMEM) (Gibco, Grand Island, New York, USA) were plated onto the center of 35 mm tissue culture dishes (Nunc, Roskilde, Denmark) and incubated to allow the cells to attach to the dish at 37°C in a humidified 5% CO_2-air. After 6 hr of incubation, dishes were flooded with 1.6 ml of αMEM supplemented with 10% fetal bovine serum (FBS, Gibco). Thereafter, the culture medium was changed every 4th day. On various times between 1 and 21 days after plating, cultures were fixed with 2% PFA/DPBS for 10 min at RT. After washing in 90% ethanol at 4°C overnight, cells were immunostained as described below.

Monolayer cultures of striated muscle cells

Muscles were obtained from the hind limbs of 3 day-old rats. Tissues were minced with a pair of fine scissors and then treated with 1.25% collagenase (type V, Sigma, St Louis, Mo., USA) in αMEM for 1 hr at 37°C with agitation. The tissue fragments were suspended by gentle pipetting and floating cells were collected in a centrifuge tube. The tissue sediments were resuspended in 0.125% trypsin (type IX, Sigma) and 2 mM EDTA in DPBS containing 0.1% bovine serum albumin (fraction V, Sigma) and digested for 10 min at 37°C. The cell suspension was then filtered through gauze in order to remove tissue debris. The dissociated cells were collected by centrifugation at 200 × g for 3 min and resuspended in the culture medium. The subsequent procedures were performed in the manner described above except that cultures were fixed at 6, 12, 24, 48, and 96 hr after plating.

Antibodies

Rabbit polyclonal antibody (Pab) to bovine S-100 protein (Dakopatts, Denmark), mouse monoclonal antibody (Mab) to porcine vimentin (clone V9; Dakopatts, Carpinteria, CA), and Mab to mouse MyoD1 protein (clone 5.8A; medac, Hamburg, Germany) were used for immunostaining. The immunospecificity of these antibodies has been already described elsewhere (Dias *et al.*,1992; Kameda,1996; Watanabe, 1989).

Immunostaining

Immunostaining was carried out by the streptoavidin-biotin-peroxidase method. All staining procedures were performed at RT unless otherwise stated. Control reactions included replacing the primary antisera with normal serum (1 : 100) and omission of the first antibodies. All controls were negative. Semithin sections were pretreated with 3% H_2O_2 for 10 min to eliminate endogenous peroxidase activity and then incubated with vimentin Mab (prediluted product) for 1 to 2 hr. After washing with PBS (0.02 M, pH 7.4), they were treated with biotinylated sheep-anti-mouse Igs (a dilution of 1 : 500 in PBS) (Boehringer Mannheim, Indianapolis, USA) for 1 hr. Subsequently, peroxidase conjugated streptoavidin (prediluted) (Nichirei, Tokyo, Japan) was applied for 30 min. Finally, the reaction product was demonstrated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-buffer (pH 7.5) containing 0.001% H_2O_2. When S-100 Pab (1 : 200) was used as the primary antibody, the sections were preincubated with 10% normal goat serum (Nichirei) for 10 min, and incubated with biotinylated goat anti-rabbit IgG (prediluted) (Nichirei) instead of biotinylated sheep-anti-mouse Igs as the second antibody.

Cultured cells were permeabilized with 0.1% Triton X-100/DPBS for 20 min, washed with DPBS for 15 min, and pretreated with 3% H_2O_2 for 10 min. Immunocytochemical staining with vimentin Mab and S-100 Pab was performed in the manner described above. For immunostaining of MyoD1, cultures were first treated with 5% FBS/1% BSA/DPBS for at least 2 hr. Cells were then incubated with MyoD1 Mab (1 : 25) for at least 6 hr or overnight at 4°C and washed in PBS for 1 hr. The subsequent steps were the same as those described for staining of vimentin.

For double immunocytochemical staining for MyoD1 and vimentin, cultures were first stained with MyoD1 Mab and colored brown by DAB. After washing with 0.1 M glycine-HCl buffer (pH 2.2) for 2 hr, cells were incubated with vimentin Mab. Demonstration of reaction product at this point was done with the use of 4-chloro-1-naphthol. In the case of double staining for S-100 protein and MyoD1 or vimentin, monolayers were first stained with S-100-Pab and then with MyoD1 or vimentin Mab.

RESULTS

Immunostaining for S-100 protein and vimentin in the intact anterior pituitary gland

To compare the cytological characteristics of FS cells *in vivo* and *in vitro*, the intact anterior pituitary was first investigated by using antibodies to S-100 protein and vimentin. S-100-immunoreactive FS cells were diffusely distributed throughout the anterior pituitary gland. They were irregular, stellate in shape with long cytoplasmic processes extending between neighboring glandular cells (Fig. 1A). The distribution pattern and shapes of vimentin-immunopositive cells (Fig. 1B) were very similar to those of S-100-positive cells. Staining of consecutive sections with antisera to S-100 and vimentin revealed that some cells were immunoreactive to both antibodies (Fig. 1C and D). Vimentin was also detected in pituitary pia mater (Fig. 1E) and around some pituitary portal vessels (Fig. 1F).

Immunostaining for S-100 protein and vimentin in pituitary monolayer cultures

In monolayer cultures of anterior pituitary cells, S-100 protein was detected in many stellate-shaped cells bearing long cell processes and in polygonal cells. The cytological feature of the former cells were almost identical to that of FS cells *in situ*. They were found within glandular cell aggregates and showed strong immunoreactivity for S-100 throughout the course of the culture period (Fig. 1G). The latter polygonal cells (Fig. 1H), on the other hand, were observed only in areas where cells were allowed to spread freely. They were fewer in number than stellate-shaped S-100-positive cells. Morphologically, they were similar to fibroblastic cells which grew rap-

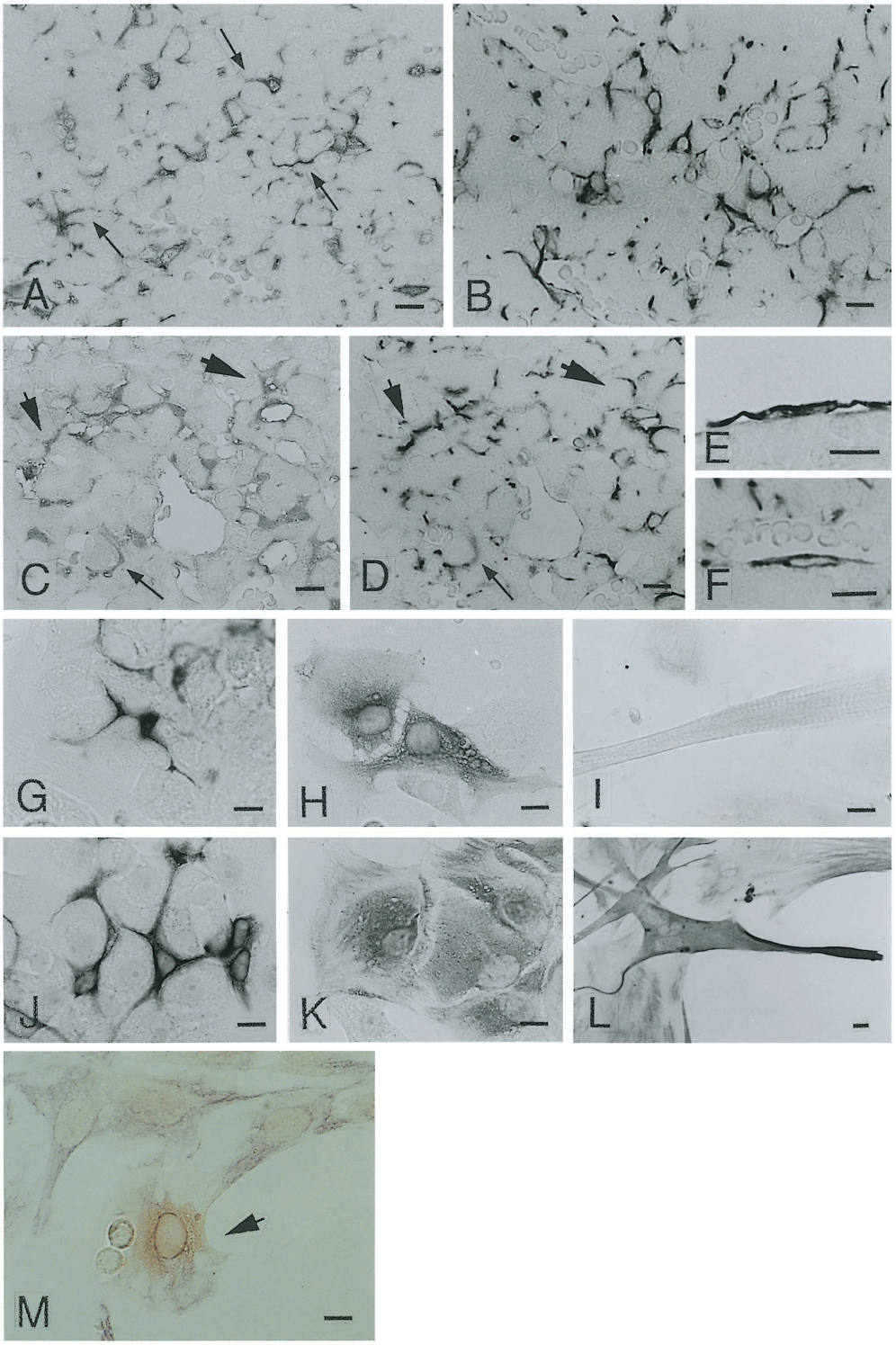

Fig. 1. Portions of rat anterior pituitary *in situ* (**A**–**F**) and in monolayer culture (**G**–**M**) immunostained with antisera to S-100 protein (**A**, **C**, **G**–**I**) and vimentin (**B**, **D**–**F**, **J**–**L**). (**A**) S-100 immunoreactive FS cells of stellate shape extending long cell processes (arrows) between neighboring cells. (**B**) Vimentin immunostaining also detected in FS cells as in **A**. (**C** and **D**) Two consecutive sections stained with anti-S-100 (**C**) and anti-vimentin (**D**). Arrows indicate FS cells immunostained for both markers. (**E**) Vimentin immunoreactive cell in the pituitary pia mater. (**F**) Vimentin localized in an elongated cell of a portal vessel. (**G** and **H**) Immunoreactivity for S-100 protein after 8 days of culture. (**G**) Stellate-shaped cells extending their processes among adjacent cells. (**H**) Flattened fibroblast-like cells with polymorphic cell processes. (**I**) Striated muscle fiber after 10 days of culture immunostained for S-100. Although striations are immunonegative, their presence is enhanced by adjusting the diaphragm of the microscope. (**J** and **K**) Vimentin detection after 7 days of culture. Positive staining is seen in stellate-shaped FS cells (**J**) and fibroblastic cells (**K**). (**L**) Vimentin detection in a myotube after 14 days of culture. (**M**) A fibroblast-like cell (arrow) doubly immunostained with anti-S100 (brown) and anti-vimentin (purple) after 4 days of culture. Bars are 10 μm.

idly in the periphery of cell aggregates. Their immunoreactivity to S-100 decreased during culture.

The results of immunostaining with vimentin-Mab were somewhat different to those with S-100 Pab. The incidence of vimentin-positive stellate cells (Fig. 1J) was nearly identical to that of S-100-containing cells. There were, however, a far greater number of large polygonal cells that contained vimentin (Fig. 1K). Double staining with these two antisera revealed that S-100-positive cells simultaneously expressed vimentin throughout the culture period (Fig. 1M). Multinucleated myotubes were observed after days 7–10 of culture. These myotubes grew both in size and number and finally differentiated into striated muscle fibers. Neither myotubes nor muscle fibers were stained with S-100 Pab (Fig. 1I). However, myotubes (Fig. 1L), but not muscle fibers, were immunoreactive for vimentin.

Immunostaining for MyoD1 protein in pituitary monolayer cultures

Figure 2 shows MyoD1-staining of monolayer cultures of pituitary cells. MyoD1 was found only in cell nuclei. The first MyoD1-immunoreactive cells were observed on days 5–6 of culture. The number of MyoD1-labeled cells increased gradually with time. Their shapes were either polygonal (Fig. 2A) like those of fibroblasts, or very elongated (Fig. 2B). The latter cells are identified as myoblasts in this study. These pituitary-derived myoblasts differed in appearance from those (Fig. 2C) seen in cultures of neonatal rat skeletal muscles. Moreover, MyoD1-positive cells were found far earlier, or 12 hr after the start of culture, in monolayers of skeletal muscles. In pituitary monolayer cultures, on the other hand, MyoD1 immunoreactive myotubes (Fig. 2D) were found to appear at about 10 days of culture. When non-immune mouse serum was substituted for MyoD1 Mab, no immunoreactive cells were observed (Fig. 2E).

Double-immunostaining for MyoD1 and S-100 protein or vimentin in pituitary monolayer cultures

The MyoD1-immunopositive cells were doubly immunostained for S-100 or vimentin to characterize the MyoD1-expressing cells in the pituitary monolayer cultures (Fig. 3). Weak staining for S-100 was shown in some polygonal MyoD1-positive (Fig. 3A, B) or negative (Fig. 3D) cells. Other myoblasts contained no immunoreactive S-100 (Fig. 3E). Vimentin was found in all MyoD1-labeled cells (Fig. 3C).

Immunostaining for S-100 protein in skeletal muscles

In order to check the immunoreactive specificity of the S-100 Pab used in this study, we examined the distribution of S-100 in the adult soleus muscle, which is known to be mainly composed of slow-twitch muscle fibers, and in the neonatal vastus lateralis. Adult slow-twitch muscle fibers and all developing skeletal muscles consist predominantly of S-100ao (Zimmer, 1991). In both cases, all muscle elements were negative to S-100 Pab, whereas immunoreactive materials were observed in peripheral nervous tissues (Fig. 4A, B).

DISCUSSION

Striated muscle fibers appear in the rat anterior pituitary under conditions of culture or grafting (Brunner and Tschank, 1982; Inoue *et al*., 1987; Spira *et al*., 1988; Watanabe, 1989). The significance of this phenomenon is presently not known. Different hypotheses have been proposed to explain the origin of these muscles: they originate from 1) pituitary folliculostellate (FS) cells, 2) contaminated myogenic cells, or 3) pre-

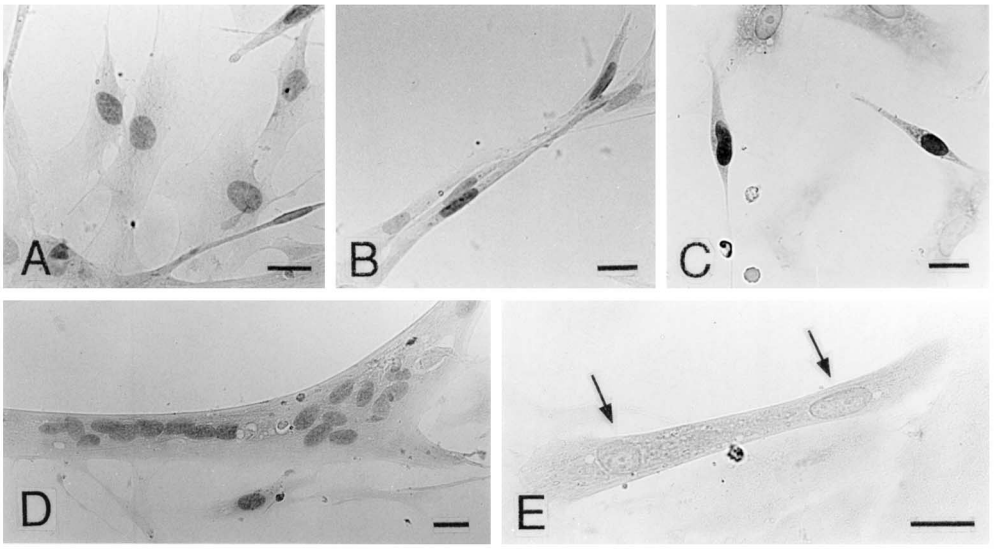

Fig. 2. Immunocytochemical detection of MyoD1 protein in monolayer cultures of pituitary (**A**, **B**, and **D**) and muscles (**C**). (**A**) MyoD1-immunoreactivity in fibroblast-like cells after 10 days of culture. (**B**) Elongated myoblast after 7 days of culture. (**C**) Spindle-shaped myoblasts appearing after 24 hr of culture. Note the bulging nucleus. (**D**) Multinucleated myotube after 10 days of culture. (**E**) Negative control shows a lack of nuclei staining (arrows). Bars are 20 μm.

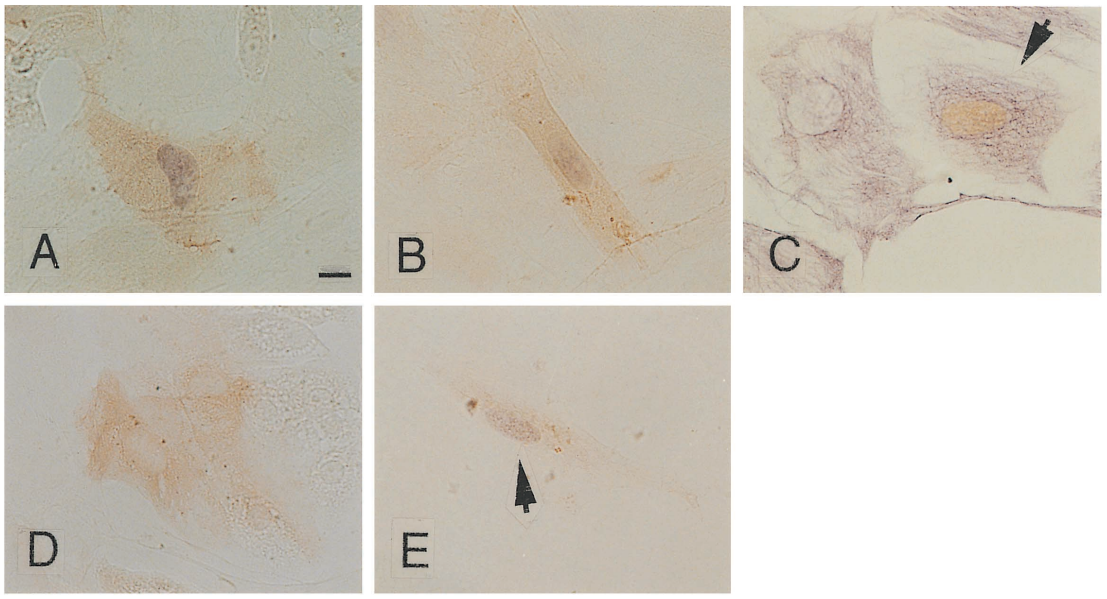

Fig. 3. Double immunostaining with anti-MyoD1 and anti-S-100 (**A**, **B**, **D**, and **E**) or anti-vimentin (**C**) after 8 days of pituitary culture. (**A** and **B**) Cells doubly stained with anti-MyoD1 (purple) and anti-S-100 (brown). (**C**) Arrow marks a cell doubly stained for MyoD1 (brown) and vimentin (purple), whereas an adjacent cell lacks nuclear staining. (**D**) S-100-positive cells (brown) without MyoD1-labeling. Reaction products are localized in their cytoplasm. (**E**) A fibroblast-like cell singly immunostained for MyoD1 (purple). Labeling is seen in the nucleus (arrow). Bar in **A** represents 10 μm.

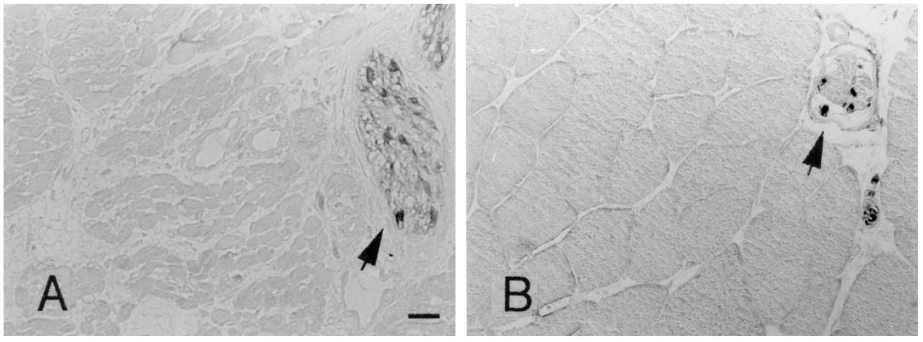

Fig. 4. Immunocytochemistry with anti-S-100 in the rat skeletal muscles. Transverse sections of developing rat vastus lateralis (**A**) and adult soleus muscles (**B**). Both types of muscle are devoid of reaction products for S-100 protein. Note positive staining in peripheral nerve tissues (arrows). Bar represents 20 μm.

existing primitive mesenchymal cells. Investigations of the cellular origin of ectopic myogenesis in the pituitary are hampered by cellular heterogeneity of the pituitary and the lack of a suitable marker which allows us to examine the myogenic cell lineage. Recent studies on the differentiation of skeletal muscle have demonstrated that the MyoD family of transcriptional regulatory factors appears to play important roles in commitment, differentiation and maintenance of the myogenic cell lineage. MyoD1 is a member of the MyoD family (Buckingham, 1994; Olson and Klein, 1994). Immunocytochemical studies revealed that MyoD1 protein is present in the nuclei of proliferating myoblasts and differentiated myotubes (Dias *et al*., 1992; Tapscott *et al*., 1988). Therefore, MyoD1 protein is thought to be a specific marker of skeletal myogenesis. In this study, a specific monoclonal antibody to mouse MyoD1 protein (Dias *et al*., 1992) was found to stain some cells of monolayer cultures of the rat anterior pituitary. This immunoreactivity was restricted to the nuclei of mononuclear cells and multinucleated myotubes. The first expression of MyoD1 protein in myoblasts of our cultures was in accord with other investigators (Brunner and Tschank, 1982; Spira *et al*., 1988; Watanabe, 1989) who observed that the appearance of elongated cells, resembling myoblasts, became detectable from days 5–6 of culture.

S-100 protein and vimentin are useful markers for FS cells in the adult rat anterior pituitary (Cocchia and Miani, 1980; Marin *et al*., 1989; Nakajima *et al*., 1980; Shirasawa *et al*., 1983; Tsuchida *et al*., 1991). S-100 protein is particularly known to be specific for the FS cells. In monolayer cultures of the anterior pituitary, FS cells maintain S-100-immunoreactivity (Vila-Porcile *et al*., 1992). To our knowledge, there is as yet little *in vitro* information concerning S-100-expression in

fibroblasts, glandular or endothelial cells. Therefore, employment of an antibody against S-100 protein is considered to be useful for the identification of cultured FS cells. Our results of double-immunostaining demonstrated that pituitary-derived myoblasts contained S-100 protein and/or vimentin. This fact is in harmony with the previous assumption that FS cells are involved in the heterotopic differentiation of striated muscles (Hosoya and Watanabe, 1997; Inoue *et al*., 1987).

S-100 protein, an acidic and calcium-binding protein with a molecular weight of about 20,000, is composed of three forms, S-100ao, S-100a, and S-100b, which are dimers with the subunit composition of αα, αβ, and ββ, respectively (Isobe and Okuyama, 1978; Isobe and Okuyama, 1981; Isobe *et al*., 1983). These S-100 proteins have different tissue distributions (Suzuki *et al*., 1982; Takahashi *et al*., 1984; Zimmer and Van Eldik, 1987; Zimmer *et al*., 1991). The αβ- and ββ-forms of S-100 protein predominate in the mammalian brain (Isobe *et al*., 1977; Isobe and Okuyama, 1978; Isobe and Okuyama, 1981). In contrast, adult slow-twitch muscle fibers and developing muscles contain predominantly the αα-form (Zimmer, 1991; Zimmer and Landar, 1995). In the rat pituitary gland, FS cells express immunoreactivity for the α- and β-subunits (Watanabe and Hashimoto, 1993). In this regard, we speculate that the antibody against S-100 protein used in this study recognizes S-100b and/or S-100a rather than that of S-100ao, since this antibody was raised with an S-100 protein mixture purified from the bovine brain, which is mostly composed of S-100b and S-100a (Isobe and Okuyama, 1978; Isobe and Okuyama, 1981). Our immunohistochemical results using the S-100 Pab showed that there was no detectable S-100-staining in any muscle elements, whereas the peripheral nervous tissues contained α- and β-subunits. For this reason, we consider that S-100-immunoreactivity in some pituitary-derived myoblasts was mainly due to S-100b and/or S-100a. If this is the case, the demonstration of these types of S-100 proteins uniquely observed in the pituitary-derived myoblasts indicates that they are in the course of transformation without losing the marker protein of FS cell.

In the present immunohistochemical study, pituitary-derived myoblasts were divided into two types, one expresses vimentin alone and the other both S-100 and vimentin. As for the former type of cell, there are two possible explanations. First, it is an FS cell which contains a form of S-100ao that was immunonegative to our anti-S-100. As stated before, the S-100 Pab used in this study seems to lack immunoreactivity to S-100ao. Second, it may be derived from a mesenchymal cell because vimentin is primarily known to be the major component of the cytoskeleton in mesenchymal cells such as skeletal muscles, endothelial cells and fibroblasts (Babai *et al*., 1990; Bornemann and Schmalbruch, 1992; Giometto *et al*., 1997; Kumar *et al*., 1991; Lazarides, 1980). It is possible that vimentin-positive myoblasts originated from contaminating myogenic satellite cells or myoblasts, because it has been reported that active satellite cells and myoblasts in regenerating muscles contain high levels of MyoD1 protein (Koishi *et al*., 1995). However, this latter hypothesis seems unlikely in view of the observation that myoblasts appeared far earlier in cultures of skeletal muscles than in pituitary cultures. The MyoD1-positive myoblasts appeared in cultures of neonatal rat muscles after only 6 hr of incubation, whereas in pituitary cultures they were not seen until 5 days of culture. Moreover, pituitary-derived myoblasts differed morphologically from the myoblasts that were found in cultures of muscles.

Our immunohistochemical study revealed the presence of vimentin in the pituitary pia mater and occasional portal vessels besides FS cells. This finding suggests that pituitary-derived fibroblastic cells may also originate from these tissues. As the pia mater is thought to be a fibrillar loose connective tissue, it may contain primitive mesenchymal cells. Striated muscle fibers have actually been reported in non-neoplastic leptomeninges (Angelov and Vasilev, 1989; Nakamura *et al*.,1984).

In addition to occurring in pituitary glands, heterotopic muscles have also been reported in the central nervous system, including the cerebellum, pineal body, and leptomeninges (Angelov and Vasilev, 1989; Nakamura *et al*., 1984; Ohanian, 1968; Watanabe *et al*., 1981). According to Nakamura *et al*. (1984) these muscles were associated with ectopic neuroglial tissues that were immunoreactive for S-100 protein and glial fibrillary acidic protein (GFAP). It has also been reported that a nitrosoethylurea-induced rat glial cell line, which contained S-100 protein and 14-3-2 protein, became converted into skeletal muscle fibers (Lennon and Peterson, 1979). In birds it is well established that the cephalic neuroectoderm, which is referred to as the neural crest, migrates throughout the head and neck to give rise to cranial mesenchyme, sensory ganglia, Schwann cells, leptomeninges, and skeletal muscle (Le Liévre and Le Douarin, 1975; Noden, 1978). These results led all of them to propose that the mammal neuroectoderm has a potential to differentiate into skeletal muscles also. Interestingly, it has been demonstrated in lower vertebrates that the anterior portion of the neural ridge is involved in organogenesis of the adenohypophysis (Couly and Le Douarin, 1987; Le Douarin *et al*., 1986). Although no evidence is at present available for mammals, neuroectoderm or its derivatives may contribute to the formation of the adenohypophysis. Thus study of the myogenic progenitor cells in pituitary cultures may provide clues to the developmental origin of the mammalian adenohypophysis.

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