DEVELOPMENTAL DYNAMICS 209:206–216 (1997)

Fibroblast Growth Factors 2 and 4 Stimulate Migration of Mouse Embryonic Limb Myogenic Cells

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ABSTRACT **Fibroblast growth factors (FGFs) are believed to be vital for limb outgrowth and patterning during embryonic development. Although the effect of FGFs on the formation of the skeletal elements has been studied in detail, their effect on the development of the limb musculature is still uncertain. In this study, we used Blindwell chemotactic chambers to examine the effect of FGF-2 and FGF-4 on the motility of myogenic cells obtained from the proximal region of day 11.5 mouse forelimbs. The limb myogenic cells were found to be chemotactically attracted to FGF-2 and FGF-4 at 1–50 ng/ml. Both FGFs increased myogenic cell migration in a dose-dependent manner, with maximal responses attained at 10–50 ng/ml for FGF-2 and at 10 ng/ml for FGF-4; however, FGF-2 was found to be a more potent chemoattractant than FGF-4. It was possible to inhibit the myogenic cells' response to FGF-2 and FGF-4 by the addition of the appropriate neutralizing antibody. The effects of FGF-2 on cell migration were further investigated by loading this cytokine into Affi-Gel blue beads and transplanting them into day 11.5 forelimb buds. The results showed that FGF-2 attracted DiI-labelled proximal cells to migrate toward the implanted beads and that the migration was more extensive than that observed in the absence of FGF-2. A checkerboard assay was performed in which various concentrations of FGF-2 and FGF-4 were introduced to both the upper and lower wells of the Blindwell chambers. The results indicated that both FGF isoforms can stimulate chemokinesis as well as chemotaxis in myogenic cells. In addition, the effect of FGF-2 and FGF-4 on other aspects of skeletal muscle development was investigated. FGF-2 at 0.1–10 ng/ml stimulated a significant increase in the number of myocytes expressing sarcomeric myosin on examination after 48 hr in culture, but the effect of FGF-4 was negligible at all concentrations analyzed; however, both FGF-2 and FGF-4 inhibited myocyte fusion compared with the spontaneous fusion observed in control cultures. Finally, we used in situ hybridization and immunohistochemical techniques to determine the distribution of myogenic cells and FGF-2 protein in the day 11.5 mouse forelimbs. *Dev. Dyn. 209:206–216, 1997.*** © 1997 Wiley-Liss, Inc.

Key words: fibroblast growth factor-2 and fibroblast growth factor-4; mouse limbs; myogenic cell migration

INTRODUCTION

During embryogenesis, myogenic precursor cells migrate from the ventrolateral edge of the dermomyotome to the lateral plate mesoderm of the prospective limb (Chevallier et al., 1977; Christ et al., 1977; Newman et al., 1981; Rutz et al., 1982; Lance-Jones, 1988; Schramm and Solursh, 1990; Lee and Sze, 1993; Sze et al., 1995; Daston et al., 1996). Within the limb bud, the myogenic cells retain their invasive property and actively migrate in a proximodistal direction to establish the skeletal muscle pattern of the limb (Brand-Saberi et al., 1989, 1996a; Lee and Ede, 1989a,b, 1990). It has been revealed that this migration is mediated by cell-cell contacts (Lee and Ede, 1989a; Brand-Saberi et al., 1996a), cell-matrix interactions (Krenn et al., 1991; Brand-Saberi et al., 1993), and cell growth-factor interactions (Venkatasubramanian and Solursh, 1984; Bladt et al., 1995; Brand-Saberi et al., 1996b). All of these three types of interactions are crucial to the migration process in the limb. For example, in mutant studies, we have demonstrated that altered cellular adhesion between myogenic cells and the connective tissues reduced the ability of myogenic cells to migrate (Lee and Ede, 1989a). Moreover, the myogenic cells require the presence of extracellular matrix components, such as fibronectin and hyaluronic acid, to maintain movement and confer directionality in the limb (Krenn et al., 1991; Brand-Saberi et al., 1993). Recent gene knockout studies also implicate the importance of growth factors; for example, target mutation of the c-met gene in mice revealed that myogenic precursors will not initiate migration from the somite in the absence of c-met receptors (Bladt et al., 1995). Hepatocyte growth factor

Grant sponsor: Croucher Foundation; Grant sponsor: Ho Sin-Hang Foundation.
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Received 25 July 1996; Accepted 25 February 1997

(HGF) is the ligand for the c-met receptor, and Brand-Saberi et al. (1996b) have demonstrated that ectopic application of exogenous HGF can induce Pax-3$^+$ myogenic cells to emigrate from chick somites in vivo.

Many types of growth factors are now known to be expressed in the developing limb. These include fibroblast growth factors (FGFs; Niswnder and Martin 1992; Dono and Zeller, 1994), HGF (Bladt et al., 1995), insulin-like growth factors (Dealy and Kosher, 1996), platelet-derived growth factors (PDGF; Orr-Urtreger and Lonai, 1992), transforming growth factors (Heine et al., 1987), and many more. In this study, we have concentrated on members of the FGF family (FGF-2 and FGF-4), because, during migration, limb myogenic cells express FREK, which is the FGF receptor (Marcelle et al., 1995). FGF-2, FGF-4, and FGF-8 genes are expressed very early on in limb development by the apical ectodermal ridge (AER), and it is now believed that these isoforms are likely candidates for the signaling molecules responsible for many of the inductive properties of the AER (Niswander and Martin, 1992; Crossley and Martin, 1995; Savage and Fallon, 1995). These cytokines can functionally replace the AER by providing the signals necessary to stimulate both the outgrowth and the patterning of the limb. For example, application of beads preadsorbed with either FGF-2, FGF-4, or FGF-8 to the mesenchyme of the progress zone following AER removal prevents truncation of the limb and results in the formation of a virtually normal limb skeleton in developing chicks (Niswander et al., 1993; Fallon et al., 1994; Crossley et al., 1996). In addition, it has recently been demonstrated that these three isoforms may be involved in initiating limb development; for example, beads soaked in FGF-2, FGF-4, or FGF-8 and implanted into nonlimb forming flank regions of chick embryos induced the production of ectopic limbs, either wing buds or leg buds, depending on the location of the implanted bead (Cohn et al., 1995; Crossley et al., 1996). FGF-2 and FGF-4 are also involved in maintaining ZPA signaling and sonic hedgehog expression (Niswander et al., 1993; Li et al., 1996). Aside from the AER, FGF-2 is also present in the early limb bud associate within the ectoderm, the peripheral mesenchyme, and migrating myogenic cells (Dono and Zeller, 1994; Savage and Fallon, 1995).

Although the effect of FGFs on the development of the limb skeletal elements has been studied in detail (Vogel and Tickle, 1993; Taylor et al., 1994; Cohn et al., 1995), their effect on the formation of limb musculature is still largely undefined. Here, by using Blindwell chemotactic chambers, we examined the ability of FGF-2 and FGF-4 to elicit a migratory response in myogenic cells obtained from the forelimbs of day 11.5 mouse embryos. We have also investigated the stimulatory effect of FGF-2 in situ by exogenously applying the cytokine to limbs in combination with cell labelling techniques. The effect of FGF-2 and FGF-4 on myogenic cell differentiation was also determined by analyzing the extent of sarcomeric myosin expression and myocyte fusion. Moreover, by immunohistochemistry and in situ hybridization, we determined the codistribution pattern of FGF-2 protein and Pax-3$^+$ myogenic cells in the day 11.5 mouse forelimb.

RESULTS

Effect of FGFs on Myogenic Cell Migration

Various concentrations of FGF-2 and FGF-4 were examined for their ability to stimulate myogenic cell migration by using the Blindwell chemotaxis chambers. Although the proximal region of the day 11.5 murine limb is enriched with myogenic cells, many other cell types are also present, so myogenic cells were identified by immunohistochemistry with the antisarcomere myosin antibody, MF-20. The presence of MF-20$^+$ cells could not be detected directly at the end of the chemotaxis incubation period despite the presence of numerous cells on the lower surface of the filters. Therefore, the filters containing the migrant cells were cultured for an additional 20 hr to allow the myogenic cells to differentiate and to express sarcomeric myosin. When FGF-2 (1–50 ng/ml) was placed in the lower well of the Blindwell chamber, myogenic cells, which were suspended in FGF-free medium in the upper well, migrated across the Nucleopore polycarbonate filter. At all concentrations of FGF-2 analyzed (1–50 ng/ml), myogenic cell migration was significantly greater than the random migration observed in the absence of this cytokine (Figs. 1A–C, 2). Migration increased in a dose-dependent manner from 1 ng/ml to 5 ng/ml, with an approximate threefold increase in the number of myogenic cells that migrated across the filter. At 10–50 ng/ml, however, there was no further significant increase in the number of migrating myogenic cells (Fig. 2). FGF-4 also stimulated an increase in limb myogenic cell migration but to a lesser extent than FGF-2 (Figs. 2, 3A–C). Indeed, at concentrations of 1–5 ng/ml FGF-4 in the lower well, the number of myogenic cells that migrated was not significantly different from the basal level of cell migration observed when FGF-4 was absent (Figs. 2, 3A,B). Significant myogenic cell migration was observed at 10 ng/ml and 50 ng/ml of FGF-4 in the lower well, with optimal migration being observed at 10 ng/ml (Fig. 2, 3C).

The Blindwell assays were repeated using FGFs that had been neutralized with the appropriate FGF antibody (Table 1, Figs. 1D–F, 3D–F). It was possible to demonstrate that using FGF-2 or FGF-4 at a concentration of 10 ng/ml, anti-FGF-2 at 0.1–10 μg/ml (Table 1, Fig. 1D–F), and anti-FGF-4 at 1–50 μg/ml (Table 1, Fig. 3D–F) indeed inhibited myogenic cell migration. In fact, anti-FGF-2 and anti-FGF-4 at 10 μg/ml neutralized their respective cytokine to levels equivalent to the basal level of migration exhibited by these cells when the FGFs were absent from the Blindwell chamber (compare Fig. 1A with Fig. 1F and compare Fig. 3A with Fig. 3F). Additional tests were performed to determine whether the ability of the FGF antibodies to reduce myogenic cell migration could be attributed to the

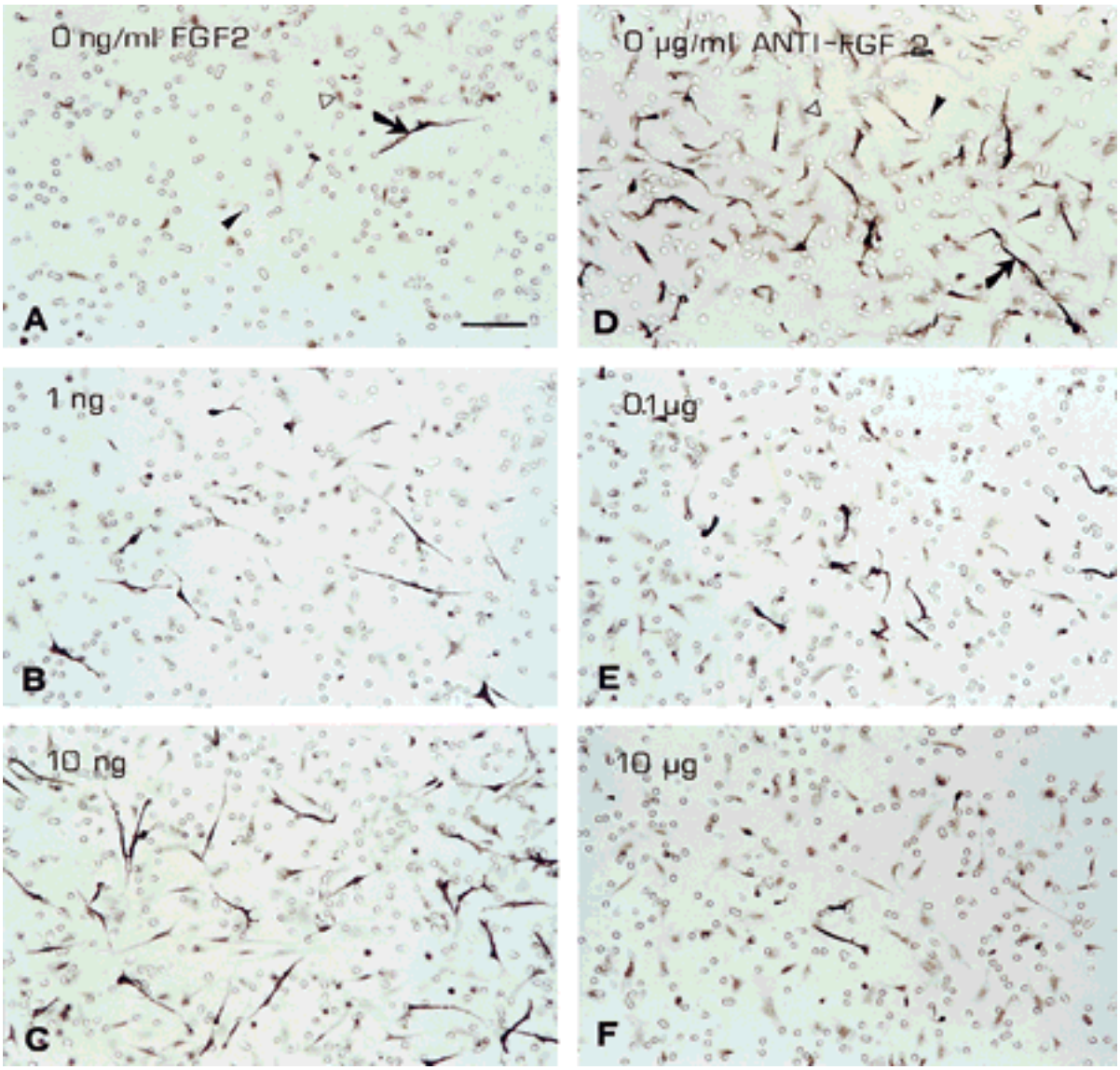

Fig. 1. Representative photomicrographs demonstrating the migration of myogenic cells (arrow) in response to fibroblast growth factor (FGF-2). Limb cells suspended in Dulbecco's modified essential medium (DMEM) minus FGF-2 were placed in the upper well of the Blindwell chamber after loading the lower well with DMEM alone (**A**), 1 ng/ml FGF-2 (**B**), 10 ng/ml FGF-2 (**C**), 10 ng/ml FGF-2 + 0 µg/ml anti-FGF-2 (**D**), 10 ng/ml FGF-2 + 0.1 µg/ml anti-FGF-2 (**E**), or 10 ng/ml FGF-2 + 10 µg/ml anti-FGF-2 (**F**). Some MF-20$^-$ cells (triangles) also migrated through the 8 µm pores (arrowhead) of the polycarbonate filter. Scale bar = 80 µm.

neutralization of FGFs or whether the antibodies were toxic to the myogenic cells. This was realized by adding FGF antibody in the presence of PDGF-BB, a cytokine that we have found to stimulate chemotaxis in limb myogenic cells (unpublished data). It was possible to demonstrate that the FGF antibodies did not interfere with myogenic cell migration induced by PDGF-BB (Table 2). Therefore, it can be concluded indirectly that, at the concentrations used, the FGF antibodies were not toxic, and the observed reduction in myogenic cell migration could be attributed to neutralization of the FGFs.

Checkerboard analyses incorporating various concentrations of FGF-2 or FGF-4 in the upper and lower wells of the Blindwell chemotaxis chamber were performed to determine whether these isoforms could stimulate chemokinesis as well as chemotaxis of limb myoblasts (Table 3). It was possible to demonstrate that, in a uniform concentration of 1 ng/ml of FGF-2 (i.e, 1 ng/ml of FGF-2 in both the upper and the lower wells of the chamber), the number of myogenic cells that had accumulated on the lower surface of the Nucleopore filter was approximately threefold greater than the number that had accumulated in a uniform concentra-

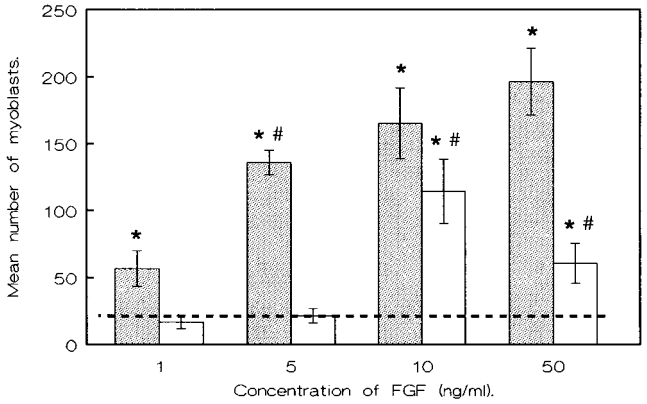

Fig. 2. Effect of FGF-2 and FGF-4 on limb myogenic cell migration. Limb cells suspended in DMEM alone were placed in the upper well of the Blindwell chamber after loading the lower well with either FGF-2 (shaded bars) at 1–50 ng/ml or FGF-4 (open bars) at 10–50 ng/ml. The means and standard errors of the mean (s.e.m.) experiments are shown for eight experiments. Asterisks indicate results that were significantly different from those obtained in the absence of cytokines (represented by the horizontal dashed line), and the pound symbol indicates values that were significantly different from the values obtained at the adjacent lower concentration (Mann-Whitney U-test; $P < 0.05$).

tion of Dulbecco's modified essential medium (DMEM) alone (Table 3). Similarly, in a uniform concentration of 1 ng/ml FGF-4, myogenic cell migration was approximately twofold greater than in the absence of cytokines (Table 3).

Effect of FGF Isoforms on Myogenic Cell Differentiation

The effect of FGF isoforms on sarcomeric myosin expression, which was recognized by immunohistochemistry with the MF-20 antibody, and myocyte fusion was studied. At concentrations of 0.1–10 ng/ml, FGF-2 significantly enhanced the number of MF-20$^+$ myogenic cells when examined after 48 hr in culture, such that, at 10 ng/ml, the total number of nuclei in myogenic cells was approximately 4.5-fold greater than cells cultured in the absence of this cytokine. In contrast, FGF-4 was unable to stimulate a significant increase in these cells (Fig. 4A); however, both isoforms did stimulate an increase in the number of connective tissue cells (Fig. 4B). The extent of myocyte fusion was estimated by calculating the percentage of nuclei found in multinucleated myotubes (Fig. 4C). In the control cultures, which were maintained in the absence of FGFs, approximately 54.7% ± 5.24% of the myogenic nuclei were present in myotubes. FGF-2 (at 0.1 ng/ml) and FGF-4 (at 0.1–1 ng/ml) had a negligible effect on muscle cell fusion compared with the spontaneous fusion observed in the absence of these cytokines. At 1–10 ng/ml of FGF-2 and 10 ng/ml of FGF-4, however, myogenic cell fusion was significantly inhibited, such that, at 10 ng/ml, the percentages of nuclei present in myotubes were 36.14% ± 5.02% and 34.06% ± 4.53% for FGF-2 and FGF-4, respectively (Fig. 4C).

Because FGF-2 stimulated a significant increase in the number of MF-20$^+$ myogenic cells, and because we did not examine the myogenic cells directly after the chemotactic assay but had to culture the cells for another 20 hr, it was necessary to determine whether the observed effect of FGF-2 on myogenic cell migration was genuine. To answer this question, cells were plated out onto fibronectin-coated coverslips in the presence or absence of 10 ng/ml FGF-2 for 5 hr, the duration that myogenic cells were exposed to FGF-2 in the Blindwell chambers. The attached cells were subsequently rinsed and refed with DMEM/F10 containing 10% fetal bovine serum (FBS) for another 20 hr. The number of differentiated myogenic cells was identified by staining with the MF-20 antibody, after which the total number of MF-20$^+$ cells/mm^2 was quantified. Averages of 52.7 ± 5.70 cells/mm^2 and 59.2 ± 6.63 cells/mm^2 were demonstrated for cells cultured in the presence of 10 ng/ml FGF-2 and in the absence of FGF-2, respectively. It was therefore obvious that a 5 hr "pulse" of FGF-2 was not sufficient to stimulate either proliferation or the expression of the myogenic marker recognized by MF-20. Thus, despite the fact that FGF-2 stimulated an increase in the number of myogenic cells expressing sarcomeric myosin when cultured for 48 hr, the apparent increase in the number of migrant cells observed after 5 hr stimulation in the Blindwell chemotaxis assay could be attributed to a stimulation of migration alone.

Effect of FGF-2 on Proximal Cell Migration in the Day 11.5 Limb

To investigate the effects of FGF-2 on cell migration in the limb, DiI was injected into the proximal regions of day 11.5 forelimbs. Beads loaded with and without FGF-2 were transplanted into the distal regions approximately 500–700 μm from the DiI-labelled proximal cells. The experimental limbs were then cultured for 36 hr. In 6 of 7 specimens examined, the presence of FGF-2 induced DiI-labelled cells to migrate extensively toward the beads. Some of the labelled cells were even found in the vicinity of the implanted beads (Fig. 5A). No attempts were made to identify the cells that were responding to the FGF; however, these cells originated from the periphery rather than from the central core of the limb, which suggests that they could be either fibroblasts or myoblasts or a mixture of both. In all of the control specimens examined, some labelled cells dispersed in a distal direction from the original labelled site, but the migration was not as extensive as that observed in the presence of FGF-2 (Fig. 5B).

Distribution of Myogenic Cells and FGF-2 in the Day 11.5 Limb

Desmin transgenic embryos were used to determine the distribution of myogenic cells in the day 11.5 forelimb. LacZ$^+$ myogenic cells were found in the proximal regions approximately 350 μm from the limb's distal tip (Fig. 6A). In situ hybridization was also

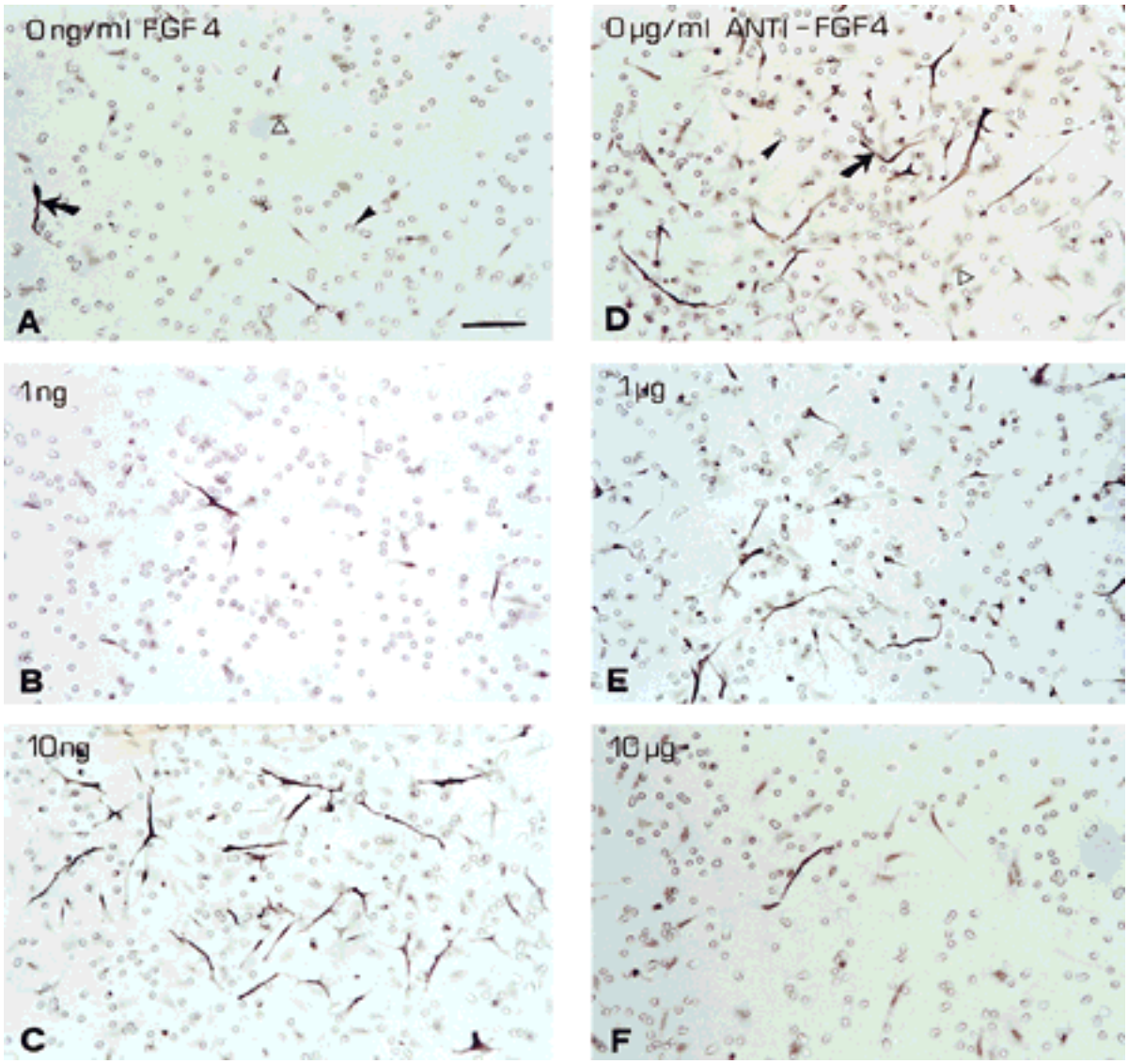

Fig. 3. Representative photomicrographs demonstrating the migration of myogenic cells (arrows) in response to FGF-4. Limb cells suspended in DMEM minus FGF-4 were placed in the upper well of the Blindwell chamber after loading the lower well with DMEM alone (**A**), 1 ng/ml FGF-4 (**B**), 10 ng/ml FGF-4 (**C**), 10 ng/ml FGF-4 + 0 µg/ml anti-FGF-4 (**D**), 10 ng/ml FGF-4 + 1 µg/ml anti-FGF-4 (**E**), or 10 ng/ml FGF-4 + 10 µg/ml anti-FGF-4 (**F**). MF-20$^-$ cells (triangles) also migrated through the 8 µm pores (arrowhead) of the polycarbonate filter. Scale bar = 80 µm.

performed with DIG-labelled Pax-3 riboprobes to determine the location of myogenic cells in the day 11.5 mouse forelimb buds (Fig. 6B,C). Pax-3 staining was detected in the dorsal and ventral regions of the subectodermal mesenchyme. The distribution of Pax-3-expressing myogenic cells was limited to the proximal regions of the limb; the distal region was totally devoid of Pax-3$^+$ cells (Fig. 6B).

Immunohistochemistry was performed to determine the distribution FGF-2 protein in the day 11.5 forelimb. FGF-2 was detected distally in the mesenchyme of the progressive zone and in the mesenchymal core of the limb bud (Fig. 6D). The ectoderm was also stained for FGF-2, but only a very few cells of the AER were stained. Moreover, clusters of FGF-2$^+$ cells were found in regions that expressed Pax-3, suggesting that migrating myogenic cells were also capable of expressing FGF-2 protein.

DISCUSSION

FGFs play an important role in inducing limb outgrowth and establishing the limb's pattern during embryonic development. Although the importance of FGF-2, FGF-4, and FGF-8 in the formation of the chondrogenic elements is already well established for the developing limb (Riley et al., 1993; Vogel and Tickle,

TABLE 1. Chemotactic Effect of FGF-2 and FGF-4 on Limb Myogenic Cells Can Be Inhibited by Neutralization With Anti-FGF-2 and Anti-FGF-4, Respectively

Growth factor	Mean number of migrated myogenic cells	Number of assays
Anti-FGF-2 (μg/ml)[a]		
0	123.75 ± 20.54	n = 4
0.1	60.50 ± 15.60	n = 4
1	33.25 ± 9.12	n = 4
10	20.67 ± 1.08	n = 4
Anti-FGF-4 (μg/ml)[b]		
0	98.38 ± 4.70	n = 4
1	71.50 ± 12.70	n = 4
10	19.50 ± 5.45	n = 4
50	22.00 ± 6.82	n = 4

[a]FGF-2 at 10 ng/ml was incubated with 0.1–10 μg/ml anti-FGF-2 neutralizing antibody.
[b]FGF-4, also at 10 ng/ml, was incubated with 1–50 μg/ml anti-FGF-4 neutralizing antibody. Results are expressed as mean ± s.e.m. of four experiments. FGF, fibroblast growth factor.

TABLE 2. Chemotactic Effect of PDGF-BB on Myogenic Cells Cannot Be Inhibited by Either Anti-FGF-2 or Anti-FGF-4

Growth factors	Anti-FGF-2 (10 μg/ml)	Anti-FGF-4 (10 μg/ml)
FGF-2 (10 ng/ml)	1.48 ± 2.15%	ND
FGF-4 (10 ng/ml)	ND	6.35 ± 6.65%
PDGF-BB (10 ng/ml)	84.17 ± 0.72%	79.84 ± 1.86%

FGF-2 and FGF-4, both at 10 ng/ml, were incubated with either anti-FGF-2 or anti-FGF-4 neutralizing antibody at 10 μg/ml for approximately 1 hr at 37°C before being added to the lower well of the Blindwell chemotaxis chamber. Platelet-derived growth factor (PDGF)-BB at 10 ng/ml was also incubated with anti-FGF-2 and anti-FGF-4 neutralizing antibodies at 10 μg/ml. Forelimb cells were suspended in Dulbecco's modified essential medium (DMEM) alone. Results are expressed as the mean percentage difference in number of myogenic cells that migrated with respect to the basal level of migration observed in the absence of cytokines. Mean ± s.e.m. of four experiments is shown. ND, not done.

TABLE 3. Checkerboard Analyses Using Various Concentrations of FGF-2 or FGF-4 in the Upper and Lower Well of the Blindwell Chemotaxis Chamber[a]

Lower well	Upper well		
FGF-2 (ng/ml)	**0**	**1**	**10**
0	21.25 ± 5.43	11.0 ± 1.42	42.5 ± 8.07
1	**56.63 ± 14.33**	69.0 ± 10.59	42.25 ± 8.07
5	**135.8 ± 9.76**	**108.0 ± 14.06**	45.25 ± 8.47
10	**165.3 ± 28.32**	**116.75 ± 33.9**	89.5 ± 4.36
FGF-4 (ng/ml)	**0**	**1**	**10**
0	17.0 ± 6.54	34.0 ± 8.02	36.0 ± 8.68
1	**16.62 ± 4.84**	33.25 ± 8.63	22.0 ± 4.06
5	**21.5 ± 5.47**	**40.0 ± 4.36**	30.5 ± 4.18
10	**114.4 ± 24.49**	**114.25 ± 9.33**	66.75 ± 5.30

[a]Numbers in bold indicate cells that migrated along a positive gradient; underlined numbers indicate random migration in the absence of a gradient. Results are expressed as mean ± s.e.m. of four experiments.

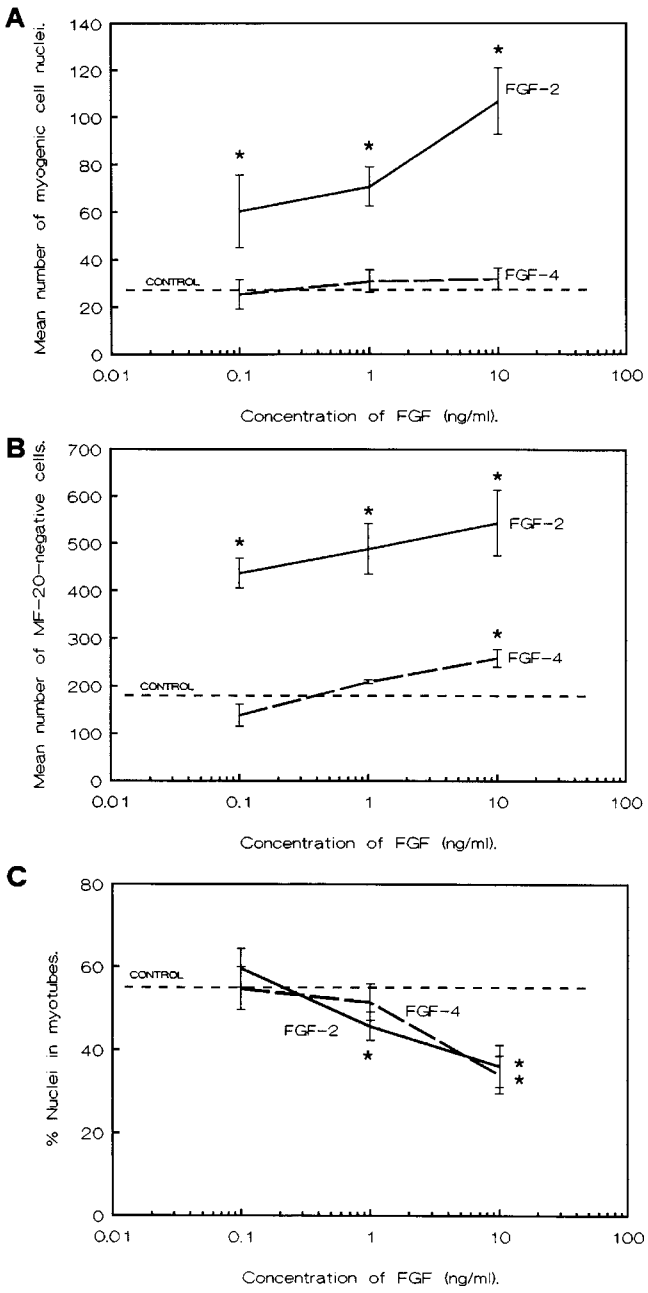

Fig. 4. Effect of FGF-2 and FGF-4 on the expression of MF-20 and formation of myotubes. Limb cultures were incubated with 0.1–10 ng/ml FGF-2 or FGF-4 for 48 hr, after which the myogenic population was identified with MF-20. **A:** FGF-2 (solid line) stimulated an increase in the number of differentiated myocytes expressing sarcomeric myosins, whereas FGF-4 (broken line) had no significant effect on the number of MF-20$^+$ cells. **B:** However, both FGF-2 and FGF-4 stimulated a significant increase in the number of MF-20$^-$ limb cells. The plotted values represent the mean number of MF-20$^+$ cells/mm^2 and MF-20$^-$ cells/mm^2, respectively. **C:** In addition, FGF-2 and FGF-4 both inhibited the fusion of myocytes to form myotubes; each value represents the percentage of nuclei in myotubes. The mean and s.e.m. are shown for six experiments. Values significantly different (Mann-Whitney U-test, $P < 0.05$) from the control (horizontal dashed line) are indicated by asterisks.

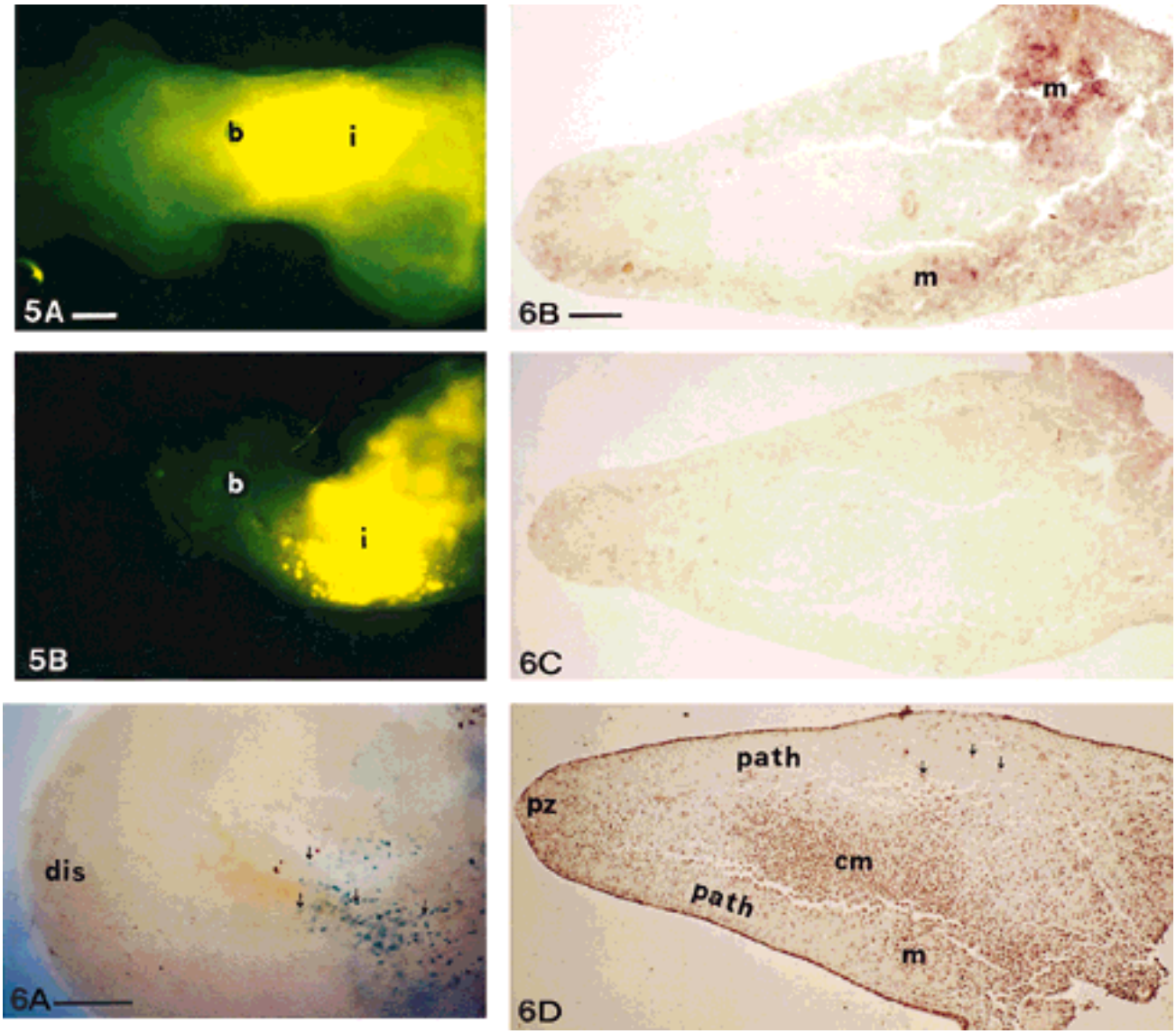

Fig. 5. Representative appearance of forelimbs that were labelled proximally with DiI, implanted with beads loaded with or without FGF-2 distally, and cultured for 36 hr. **A:** In the presence of FGF-2, DiI-labelled proximal cells migrated extensively towards the beads. **B:** Proximal cell migration was less extensive in the absence of FGF-2. i, Injection site; b, implanted bead. Scale bar = 300 μm.

Fig. 6. Day 11.5 forelimbs. **A:** Wholemount of a desmin transgenic limb showing that the distal region (dis) is devoid of myogenic cells (arrow). In situ hybridization was also performed on the limb section with Pax-3 riboprobe to demonstrate the distribution of myogenic cells. **B:** Pax-3$^+$ cells present in the proximal (m) but not the distal regions of the limb. **C:** Sense control. **D:** Immunohistological staining shows that FGF-2 protein is expressed in the limb ectoderm, the core mesenchyme (cm), the progress zone (pz), and the myogenic regions (m). Arrows indicate the possibility that some migrating myogenic cells might also express FGF-2. No staining was detected in the myogenic pathway (path). Scale bar = 100 μm.

1993; Taylor et al., 1994; Cohn et al., 1995; Crossley et al., 1996), little is known about the effect of these cytokines on the development of the skeletal musculature. In the present study, by using the Blindwell chemotaxis chamber, we have demonstrated that FGF-2 and FGF-4 can elicit a migratory response in mouse forelimb myogenic cells in vitro. These myogenic cells reacted to the FGF isoforms by migrating from low concentrations to high concentrations of the isoform across a polycarbonate filter, which had been precoated with fibronectin. We observed that myogenic cell migration was dependent on the presence of fibronectin, because migration was negligible when the filters were either left untreated or coated with gelatin. It has been suggested that fibronectin is required in these in vitro assays to stimulate the initial cell attachment to the polycarbonate filter (Venkatasubramanian and Solursh, 1984); however, it is also thought to be important

for myogenic cell migration in vivo, because the injection of fibronectin antibodies into the limb inhibits migration of these cells (Brand-Saberi et al., 1993). The limb myogenic cells responded to both FGF-2 and FGF-4; however, compared with FGF-2, FGF-4 was less effective at stimulating myogenic cell migration. By treating FGF-2 and FGF-4 with their respective neutralizing antibodies prior to the initiation of the Blindwell assay, it was possible to demonstrate that, rather than a possible contaminant, FGF-2 and FGF-4 alone were responsible for stimulating the migration observed.

We observed that, in addition to MF-20$^+$ myogenic cells, other limb mesenchymal cells were stimulated by the FGF isoforms to migrate in vitro. Although no overt attempt was made to identify these cells, it was obvious from the different cell morphologies observed that several cell types were present on the filter. It is possible that some of these cells are undifferentiated myogenic cells, whereas others may be endothelial and neural crest cells, which are known to normally invade and migrate in the developing limb bud (Beddington and Martin, 1989; Serbedzija et al., 1990). In addition, some of the cells may be loose connective tissue cells, which normally do not migrate in the limb (Brand-Saberi et al., 1989; Lee and Ede, 1989a) but which may be able to do so in vitro when constraints, such as cell contact inhibition of movement, are relaxed.

It is known that many chemoattractants that stimulate chemotaxis, or the directed migration of cells along a concentration gradient, also stimulate chemokinesis, the nondirectional migration of cells in the absence of a chemotactic gradient. To differentiate between these two mechanisms, we performed a standard checkerboard analysis with FGF-2 and FGF-4 (Zigmond and Hirsch, 1973; Adelmann-Grill and Cully, 1990). It was found that, when either of the FGFs were present at a uniform concentration, that is, the same concentration in both the upper and the lower wells of the Blindwell chamber, a substantial chemokinetic response was elicited. It was possible, however, to distinguish the random migration observed in response to FGF-2 from the directional migration. In this experiment, the concentration of FGF-2 that produced a maximal chemotactic response when it was present in the lower well alone (10 ng/ml) caused a significantly lower number of cells to migrate to the lower side of the filter when it was present as a uniform concentration above and below the filter. The random migration observed in response to FGF-4 was also considerable, but, once again, it could be distinguished from the directed migration. Again, a concentration of 10 ng/ml in the lower well elicited the maximal chemotactic response, but, when 10 ng/ml were present both above and below the filter, a significantly lower number of cells migrated across the filter.

Because FGF-2 elicited a greater effect on both directed and random myogenic cell migration than the same concentration range of FGF-4, it was of interest to determine the effect of these two isoforms on other aspects of myogenesis. It has already been demonstrated that FGF-2 and FGF-4 can stimulate the early mouse limb mesenchyme to proliferate (Niswander and Martin, 1993). We were able to confirm that both isoforms can stimulate the proliferation of limb cells not expressing the MF-20 (sarcomeric myosin) myogenic marker, and, in addition, we demonstrated that the effect of FGF-2 was greater than that of FGF-4. Furthermore, we demonstrated that FGF-2 but not FGF-4 could stimulate a significant increase in the number of MF-20$^+$ myocytes compared with cells cultured in the absence of cytokines. This increase could be attributed to FGF-2 either up-regulating sarcomeric myosin expression or stimulating the proliferation of the undifferentiated myogenic cells, which subsequently express the markers for MF-20, or possibly a combination of both these processes. In addition, the effect of FGF-2 and FGF-4 on the fusion of myocytes to form myotubes was analyzed. We found that both FGF-2 and FGF-4 inhibited myocyte fusion. Thus, the difference in potency of FGF-2 and FGF-4 is not unique to myogenic cell migration but can also be observed in other, but not all, aspects of skeletal myogenesis in vitro. These findings agree with previous reports that FGF-2 maintains myogenic cells in the cell cycle and prevents myogenic differentiation (Olwin and Hauschka, 1988; Seed et al., 1988). Those authors have also reported that it was possible to activate myogenic cell differentiation in vitro by withdrawing FGF-2 from the culture medium. Furthermore, a comparison of FGF-2 and FGF-4 indicated that FGF-4 was less potent at inhibiting myogenic cell differentiation than FGF-2 (Hannon et al., 1996). The effects of FGFs on myogenic cells are so distinct that Savage et al. (1993) used myogenic cell lines as an assay system for determining the biological activity of FGFs.

In the day 11.5 mouse forelimb, we have detected the presence of FGF-2 protein in the ectoderm, the core mesenchyme, the mesenchyme of the progressive zone, and the myogenic regions. This distribution pattern has also been observed in chick limbs of equivalent stages of development (Savage et al., 1993; Dono and Zeller, 1994). We compared the expression pattern of FGF-2 and Pax-3 and found that migrating myogenic cells were capable of expressing FGF-2. Moreover, these cells also expressed FREK, the FGF receptor (Marcelle et al., 1995; Szebenyi et al., 1995). Because our Blindwell chemotactic assays showed that FGF-2 can stimulate chemokinesis as well as chemotaxis, it is possible that myogenic cells maintain their own ability to migrate in the limb by producing FGF-2, which then acts through some form of autocrine mechanism.

Li et al. (1996) demonstrated that beads impregnated with FGF-2 and transplanted into chick limb buds can induce DiI-labelled proximal limb cells to migrate towards the beads. However, those authors did not identify the cell types involved, i.e., whether they were myogenic cells or fibroblasts. We have reproduced this result in the mouse and found that proximal cells can chemotactically respond to FGF-2 over a distance of

500–700 μm. In the limb, myogenic cells migrate specifically in a proximodistal direction (Brand-Saberi et al., 1989; Lee and Ede, 1989b). However, we do not know whether chemotaxis mediated by FGF-2 plays a role in directing this migration, because our immunohistological staining revealed that FGF-2 protein is expressed in the progressive zone but not along the myogenic pathway. Nevertheless, there are currently many models available that can explain this type of directed cell migration, such as hyaluronic acid (Krenn et al., 1991), juvenility factor (Brand-Saberi and Krenn, 1991), and N-CAM (Brand-Saberi et al., 1996a).

EXPERIMENTAL PROCEDURES

Embryos

Day 11.5 embryos were obtained from pregnant ICR mice. The presence of a vaginal plug was designated as embryonic day (E)0.5. The mice were killed by cervical dislocation, and the embryos were isolated from the decidua in prewarmed Dulbecco's phosphate-buffered saline (PBS; Sigma, St. Louis, MO) containing 0.4% bovine serum albumin (BSA). The forelimb buds were removed and subdivided into proximal and distal halves. Only the proximal portion was used in this study, because this region is enriched with myogenic cells.

Preparation of Forelimb Cell Suspension

After rinsing the limb fragments with PBS, they were dissociated by incubation with 0.5% trypsin and 0.25% pancreatin in MEM-HEPES medium (Sigma) containing 2.2% sodium bicarbonate for 30 min at 4°C, then for 10 min at room temperature, followed by trituration with a Pasteur pipette to mechanically disrupt the tissues. The enzymatic reaction was inhibited by the addition of FBS (Gibco BRL), and any remaining clumps of cells were removed by filtration through a Nylon filter (21 μm pore size) to produce a single-cell suspension. The dissociated cells were pelleted by centrifugation at 250*g* for 3 min and then resuspended in DMEM (Sigma) containing 10% FBS. Cell concentration was established with an Improved Neubauer haemocytometer, and viability was assessed with Trypan blue. The cell suspension was subsequently repelleted and resuspended to the required concentration in the medium appropriate for the experiment.

Effect of FGF-2 and FGF-4 on Myogenic Cell Migration

Migration assays were performed in Nucleopore Blindwell chemotaxis chambers (Costar Corporation, Cambridge, MA) with Nucleopore polycarbonate filters of 8 μm pore size (Costar) that had been coated with 100 μg/ml human plasma fibronectin (Gibco BRL) overnight and then air dried. FGF-2 or FGF-4 (R and D Systems, Inc., Minneapolis, MN) at 1–50 ng/ml in DMEM were added to the bottom well of each chamber, and each chamber was assembled with a filter and a filter retainer. A suspension of limb cells in DMEM containing 0–10 ng/ml FGF-2 or FGF-4 was then added to the upper well at 2×10^5 cells/chamber. After incubation at 37°C and 5% CO_2 in a humid incubator for 5 hr, the chambers were dissembled, and the upper surface of each filter was wiped with a sterile tissue to remove cells that had attached but not migrated. The filters were inverted (so that the cells that had migrated were uppermost), rinsed, and then flooded with a 1:1 ratio of DMEM/F10 Ham medium (Gibco BRL) containing 10% FBS. The cells on the filters were incubated at 37°C for another 20 hr, after which they were fixed for 30 min with 70% ethanol. The presence of skeletal myogenic cells was established by immunohistochemistry with the monoclonal antisarcomere myosin antibody, MF-20 (Developmental Studies Hybridoma Bank, Baltimore, MD, and Iowa, IA), which was viewed with the mouse Vectastain ABC kit (Vector Laboratories, Burlingame, CA) by using a substrate of nickel chloride-enhanced 3,3′-diaminobenzidine tetrahydrochloride (DAB; Amersham International, United Kingdom). The extent of myogenic cell migration was subsequently determined by quantifying the total number of nuclei in MF-20^+ cells in five fields chosen at random about the center of each filter.

Effect of FGF-2 and FGF-4 Neutralization on Myogenic Cell Migration

In an attempt to neutralize the effect of FGF-2 and FGF-4 on limb myogenic cell migration, FGF-2 or FGF-4 at 10 ng/ml were incubated at 37°C for 60 min in sterile Eppendorf tubes with 0–10 μg/ml neutralizing anti-FGF-2 or with 0–50 μg/ml neutralizing anti-FGF-4 (R and D Systems, Inc.). The Blindwell chemotaxis chambers were subsequently assembled as described above with the appropriate FGF/anti-FGF solution in the lower well and the limb cells, suspended in DMEM alone, in the upper well. At the end of the 5 hr incubation, the Blindwell chambers were dissembled, the upper surface of the filters were wiped clean, and the cells situated on the lower surface of the filter were returned to culture for another 20 hr, as described in detail above. Again, the myogenic cells were identified by immunohistochemistry with MF-20.

To determine whether the anti-FGF antibodies indeed neutralized the effect of FGFs on myogenic cell migration or whether the concentrations of antibodies used were cytotoxic to the myogenic cells, PDGF-BB (R and D Systems, Inc.) at 10 ng/ml was incubated with either neutralizing anti-FGF-2 or neutralizing anti-FGF-4, both at 10 μg/ml, for 60 min at 37°C prior to the assembly of the Blindwell chemotaxis chambers. PDGF-BB was selected for use, because it had previously been demonstrated to elicit a chemotactic response in limb myogenic cells. The chambers were subsequently assembled with the PDGF-BB/anti-FGF-2 or PDGF-BB/anti-FGF-4 mixtures in the lower well and the limb cells, suspended in DMEM alone, in the upper well. The remainder of the assay was conducted as described above.

Effect of FGF-2 on Cell Migration in the Limb

Forelimb buds were isolated intact from day 11.5 embryos in PB1 medium. By using a micropipette, approximately 2–4 µl of 5% DiI fluorescent dye (Molecular Probe, Eugene, OR) were microinjected into the proximal regions of the limb. An area 200–300 µm wide of proximal mesenchyme was labelled by using this method. Affi-Gel blue beads (150–200 µm size; BioRad) loaded with 500 µg/ml FGF-2 (R and D Systems, Inc.) for 3 hr were then implanted approximately 500–700 µm distal to the DiI-labelled cells. Beads loaded with PB1 medium were used as the control.

After the operation, the limbs were introduced into 25 ml bottles containing 3 ml DMEM/F12 medium supplemented with 10% heat-inactivated FBS. Four limbs were placed in each of the bottles. The cultures were then gassed with 5% CO_2, 40% O_2 balance in N_2, maintained on a roller incubator rotating at 30 rpm, and kept at 37°C for 36 hr. Following culture, the limbs were fixed with 4% paraformaldehyde, cleared in 30% sucrose, and examined under a fluorescent microscope.

Differentiation Assays

Differentiation assays were performed in 24-well tissue culture plates on 13 mm coverslips (Menzel-Gläser, Germany) that had been precoated with 0.1% gelatin (Sigma) at 37°C for 24 hr and then air dried. A single-cell suspension obtained from the proximal region of day 11.5 mouse embryo forelimbs in DMEM containing 5% FBS and 0–10 ng/ml FGF-2 or FGF-4 was plated out at 2×10^5 cells/well and incubated at 37°C for 48 hr. The attached cells were then fixed with acetone for 10 min at room temperature, the myogenic cells were identified by immunohistochemistry using the MF-20 antibody as described above, and the MF-20^- cells were detected with eosin. The total number of nuclei in MF-20^+ cells/mm^2, the number of MF-20^- cells/mm^2, and the percentage nuclei in myotubes were subsequently calculated in order to determine the effect of FGFs on various aspects of myogenic cell differentiation.

In Situ Hybridization

Pax-3 riboprobe (kindly provided by Prof. Peter Gruss) was used to determine the location of myogenic cells in paraffin sections of day 11.5 mouse forelimb buds. The cDNA was linearized, and digoxigenin-labelled antisense riboprobes were produced by using T7 RNA polymerase (Boehringer Mannheim Biochemica). In situ hybridization was performed according to protocol described by Goulding et al. (1994). Briefly, the limb sections were dewaxed, cleared in xylene, and hydrated. The sections were then treated with 10 µg/ml of proteinase K for 10 min, postfixed in 4% paraformaldehyde, and incubated in hybridization buffer containing 1 µg/ml of digoxigenin-labelled antisense riboprobes. The sense probe was used as the control. Localization of transcripts was visualized by using alkaline phosphatase-conjugated antidigoxigenin Fab fragments with Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoyl phosphate as chromagens.

Desmin Transgenic Limbs

Aside from using Pax-3 to determine the location of myogenic cells, transgenic embryos carrying the regulatory sequence of the desmin gene and LacZ marker were used (Lee et al., 1995). Day 11.5 transgenic embryos were fixed in 4% paraformaldehyde and incubated in X-gal solution (2 mM 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside, 10 mM potassium chloride, 10 mM potassium ferricyanide, and 1 mM magnesium chloride in PBS) at 37°C for 24 hr. The forelimbs were then excised, wet mounted onto slides, and photographed.

Immunocytochemistry

Whole forelimbs dissected from embryos at day 11.5 p.c. were fixed overnight in acetone, then embedded in paraffin wax, and sectioned at 7 µm. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in methanol. After inhibiting nonspecific protein interactions with horse serum, forelimb sections were probed with a 1:50 dilution of rabbit polyclonal antibody to human FGF-2 (SeroTec) and then viewed with a rabbit Vectastain ABC kit using nickel-enhanced DAB as the peroxidase substrate. Sections treated with PBS in place of the primary antibody were used as negative controls.

ACKNOWLEDGMENTS

We thank Professors Cheryl Tickle and Ken Muneoka for their advice on the preparation of this paper; Professor P. Gruss for the Pax-3 riboprobe; Professors D. Paulin and Z. Li for the Desmin transgenic mice; and Charles Cheung, Albert Wong, and Paul Sze for their technical assistance. The MF-20 antibody was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, under contract from the NICHD. S.E.W. is a recipient of a postdoctoral fellowship from the Chinese University of Hong Kong.

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