Int. J. Dev. Biol. 41: 597-605 (1997)

Original Article

Effect of platelet-derived growth factor isoforms on the migration of mouse embryo limb myogenic cells

SARAH E. WEBB and KENNETH K.H. LEE*

Department of Anatomy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong

ABSTRACT The effect of platelet-derived growth factor (PDGF) isoforms on limb myoblast migration was examined *in vitro*. Using Blindwell Chemotaxis chambers, the ability of PDGF-AA, -AB and -BB to stimulate the migration of myoblasts, obtained from the proximal region of 11.5 day mouse forelimb buds, was examined. Immunocytochemistry, with the anti-sarcomeric myosin antibody MF-20, was used to identify the myogenic cells in the heterogeneous cell population. Myoblasts, suspended in PDGF-free medium in the upper chamber, migrated across the polycarbonate filter of the Blindwell chamber to 1-10 ng/ml PDGF-AB and 1-100 ng/ml PDGF-BB situated in the lower well. At 1-10 ng/ml of either PDGF-AB or -BB migration increased in a dose-dependent manner. PDGF-AA, however, was unable to elicit a significant locomotory response in forelimb myoblasts. A Checkerboard assay, with various concentrations of PDGF-AA, -AB or -BB in the upper and lower wells of the chamber, indicated that -AB and -BB but not -AA stimulated the random migration of limb myoblasts. The differential effect of PDGF isoforms on myoblast migration was compared with other aspects of skeletal muscle development. At 0.1-10 ng/ml all three isoforms were able to stimulate an increase in the number of differentiated myoblasts, indicated by the expression of sarcomeric myosin, on examination after 48 h when cultured at low density. In high density cultures, however, these isoforms inhibited myoblast fusion when compared to the spontaneous fusion observed in untreated cultures. Immunohistochemical studies of both cultured limb cells and cryosections of 11.5 day whole limbs revealed that myoblasts expressed both PDGFα- and β-receptors which suggests that the action of PDGF isoforms on limb myoblasts is receptor-mediated. Finally, having demonstrated that the PDGF-B monomer stimulates migration in limb myoblasts, by immunohistochemistry, the presence of PDGF-B was confirmed and its distribution examined in the 11.5 day forelimb.

KEY WORDS: *mouse embryo, limb myogenic cells, PDGF isoforms, migration*

Introduction

It has been clearly established, from chick/quail somite transplantation experiments, that cells from the somites adjacent to the limb regions of avian embryos invade the somatopleure of the limb and differentiate exclusively into skeletal muscle cells (Chevallier *et al.*, 1977; Christ *et al.*, 1977; Newman *et al.*, 1981; Lance-Jones, 1988; Schramm and Solursh, 1990). In addition, we have recently demonstrated this morphological event in murine embryos by grafting DiI-labeled and desmin transgenic somites in unlabeled hosts (Lee and Sze, 1993; Sze *et al.*, 1995). In these embryos, the myogenic precursors migrate from the ventral lateral edge of the dermomyotome to the forelimb bud at the 22-somite stage (approximately 9.5 day p.c.). It has been demonstrated that this process involves active rather than passive movement (Sze *et al.*, 1995).

Within the limb, the myogenic cells retain their invasive property and actively colonize the limb in a proximo-distal direction to establish the pattern of skeletal musculature (Brand-Saberi *et al.*, 1989; Lee and Ede 1989a,b, 1990). In contrast, cells such as chondrocytes and soft connective tissue cells, derived from the somatopleure, do not migrate (Lee and Ede 1989a,b, 1990). Various mechanisms have already been proposed to explain the proximo-distal migration of myogenic cells in the limb. Interaction with the extracellular matrix, such as collagen (Stopak and Harris, 1982), fibronectin (Chiquet *et al.*, 1981) and hyaluronic acid (Krenn *et al.*, 1991) produced by the connective tissue have been implicated both *in vitro* and *in vivo*. Another mechanism by which myogenic cells may undergo a unidirectional migration in the limb is via chemotaxis. Signals produced by the apical ectodermal ridge (AER) are thought to be responsible for maintaining limb development, especially proximo-distal outgrowth; removal of the AER

***Address for reprints:** Department of Anatomy, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong. FAX: 852.26035031. e-mail: kaholee@cuhk.hk

0214-6282/97/$05.00

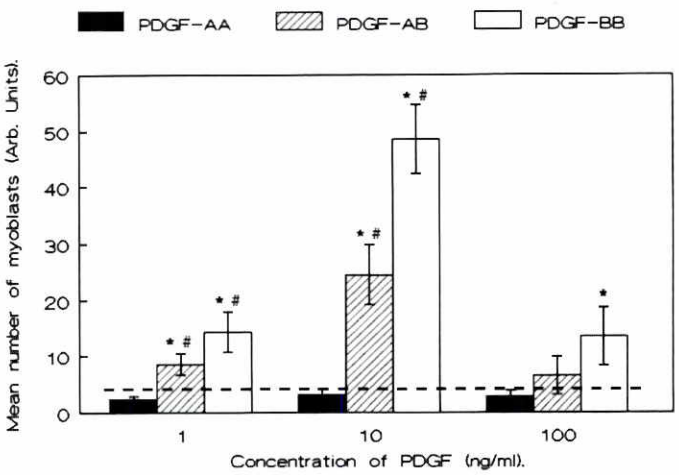

Fig. 1. The effect of PDGF-AA, PDGF-AB and PDGF-BB on limb myoblast migration. *Results are expressed as mean±SEM of 6 experiments. *indicates values which are significantly different to those obtained in the absence of PDGF, represented by the horizontal dotted line, and # indicates values which are significantly different from the values obtained at the adjacent lower concentration (Mann-Whitney U-test, P<0.05).*

results in the truncation of the limb along the proximo-distal axis as limb outgrowth is arrested (Gumpel-Pinot *et al.*, 1984). The identification of the AER signal is still unknown although various growth factors, including FGF-2, FGF-4, FGF-8, and the A-chain of platelet-derived growth factor (PDGF) are known to be expressed by this region of the limb (Niswander and Martin, 1992; Orr-Urtreger and Lonai, 1992; Savage *et al.*, 1993; Crossley and Martin, 1995). Long before the PDGF-A monomer was demonstrated to be produced by the AER, Venkatasubramanian and Solursh (1984) showed that PDGF was chemoattractive to chick limb myogenic cells. They subsequently suggested that muscle pattern is established via the chemotactic influence of PDGF synthesized by the endothelial cells lining blood vessels, as they and others noted that in the chick there is a close correlation between the distribution of skeletal myogenic cells and the vasculature (Caplan, 1981; Solursh and Meier, 1986).

In this study, Solursh's pioneering work has been re-evaluated because it is now known that PDGF is far more complex than was originally recognized; the cytokine can exist in 3 possible disulphide-linked dimeric forms, AA, AB and BB via the combination of two polypeptide chains, A and B (reviewed in Ross *et al.*, 1986). These isoforms interact with different affinities with two distinct receptors, α- and β-. PDGFα-receptors recognize both A and B chains whereas PDGFβ-receptors only recognize the B chain (Bowen-Pope *et al.*, 1989; Heldin and Westermark, 1989). The receptors are present in differing amounts on different cell types, many cells such as NIH 3T3 fibroblasts (Grotendorst *et al.*, 1991) express both α- and β-receptors but some only express one type of PDGF receptor; human skin fibroblasts and fetal bovine aortic smooth muscle cells, for example only express the PDGFβ-receptor (Grotendorst *et al.*, 1991).

It is known that during development, PDGF and its receptors are often expressed by adjacent cell layers, with epithelial cells secreting the cytokine and their neighboring mesenchymal cells expressing the PDGF receptors (Orr-Urtreger and Lonai, 1992). Sometimes PDGF and its receptors are expressed by the same cells in which case PDGF has an autocrine effect (Koyama *et al.*, 1994). It has already been demonstrated that in the day 11.5 mouse limb bud, PDGF-A mRNA is expressed in the myogenic primordia, ectoderm and AER while its receptor is distributed profusely at all levels in the limb (Orr-Urtreger and Lonai, 1992). However, the distribution of PDGF-B and the PDGFβ-receptor in the developing mouse limb has still not been established. Since as yet little is known about the action of the different PDGF isoforms on limb myogenic cells, we examined: (1) the ability of PDGF-AA, -AB and -BB to elicit a locomotory response in myoblasts obtained from the forelimbs of day 11.5 mouse embryos and the results were compared with the effect of PDGF isoforms on differentiation, determined by analyzing sarcomeric myosin expression and the formation of myotubes, (2) the distribution of PDGFα- and β-receptors in the limb with respect to the developing skeletal musculature and (3) the distribution of the PDGF-B monomer in the limb.

Results

PDGF-AB and PDGF-BB stimulate myoblast migration

The effect of PDGF isoforms on myoblast motility in the mouse embryo forelimb was studied. Initially, the day 11.5 limb was divided into proximal and distal regions, the cells from each region were cultured for 24 h, the myoblasts identified with MF-20 and then quantified (Table 1). As a significantly greater number of myoblasts were found to be located in the proximal region as compared with the distal region, this area alone was used for experimental studies on myoblast motility.

Using a Blindwell chemotaxis chamber, various concentrations of PDGF-AA, -AB and -BB (ranging from 1-100 ng/ml) were examined for their ability to attract limb myoblasts. PDGF-AA did not appear to significantly stimulate the migration of proximal forelimb myoblasts isolated from 11.5 day ICR embryos (Fig. 1). However, these cells migrated in a dose-dependent manner in response to 1-10 ng/ml of PDGF-AB and -BB, with optimal migration noted at 10 ng/ml of each isoform (Figs. 1 and 2). Cell migration decreased, however, at higher concentrations (100 ng/ml) of PDGF-AB or -BB, so that the response to PDGF-AB was not significantly different from the background level of migration measured when this cytokine was absent (Fig. 1).

It was necessary to determine whether the observed effect of PDGF-AB and -BB on myoblast migration was real, or whether the cytokines either stimulated the growth of cells which subsequently migrated across the filter stochastically, or enhanced the expres-

TABLE 1

QUANTIFICATION OF THE PERCENTAGE OF MYOGENIC CELLS FOUND IN THE PROXIMAL AND DISTAL REGIONS OF THE 11.5 DAY MOUSE FORELIMB

Region of limb bud studied	Number of assays	% of myogenic cells±SEM
Distal	4	4.83±0.44
Proximal	4	17.32±2.69

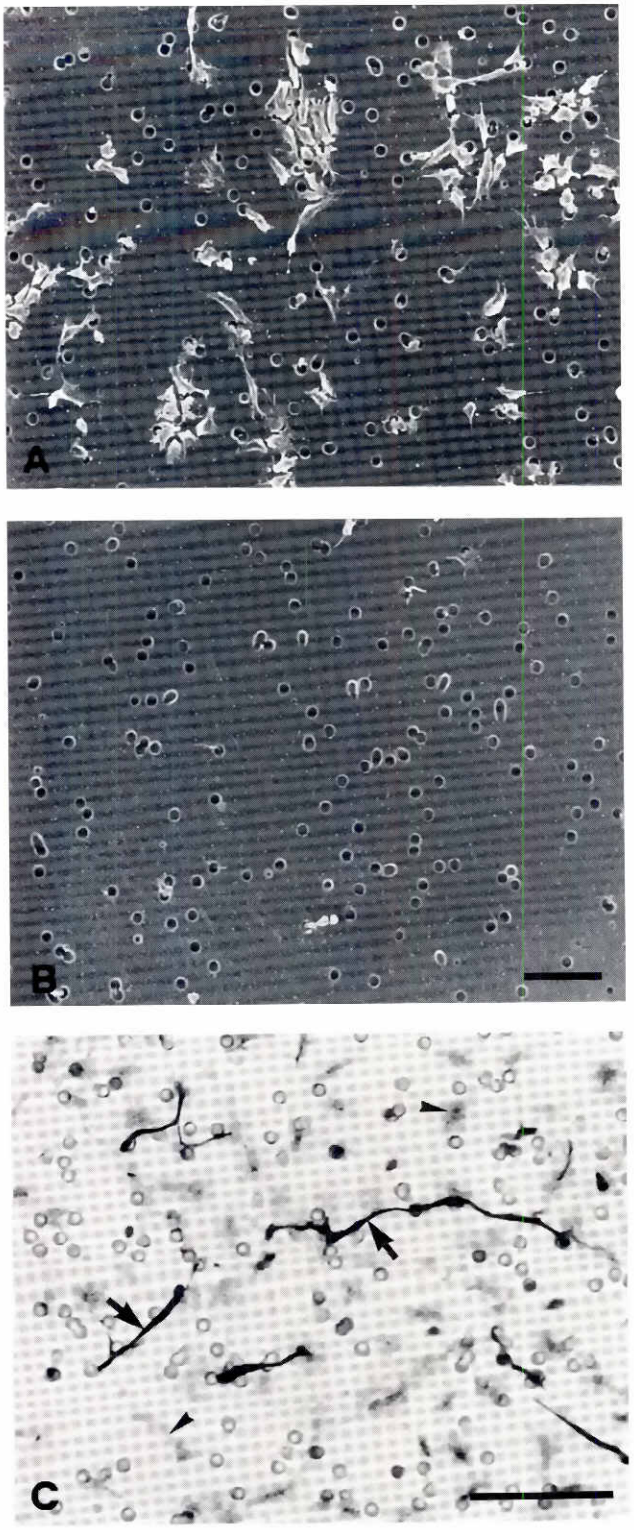

Fig. 2. Scanning electron micrographs (A and B) of the underside of Nuclepore filters on completion of the Blindwell assay. (A) *Forelimb cells have migrated through the filter after stimulation with 10 ng/ml PDGF-BB and* **(B)** *migration is negligible when PDGF-BB is absent from the lower well.* **(C)** *MF-20 staining of cells which have migrated to the lower surface of a polycarbonate filter after stimulation with 10 ng/ml PDGF-BB demonstrating the presence of myoblasts (arrows). MF-20-negative limb cells are stained with eosin (arrowheads). Bar, 50 μm.*

sion of sarcomeric myosins recognized by the MF-20 antibody. To account for these possible complications, limb cells, suspended in DMEM in the presence or absence of 10 ng/ml PDGF-AB or -BB, were plated onto coverslips which had been pre-coated with 100 μg/ml fibronectin. The cells were incubated at 37°C for 5 h after which the attached cells were rinsed with PBS and re-fed with DMEM containing 10% FBS for a further 20 h. It was possible to demonstrate that a 5 hour 'pulse' of either PDGF-AB or -BB was insufficient to stimulate an increase in the number of MF-20 myoblasts (22±2, 20±2, 20±1 myoblasts/mm^2, control, PDGF-AB, PDGF-BB, respectively).

Checkerboard analyses incorporating various concentrations of PDGF-AA, -AB or -BB in the upper and lower wells of the Blindwell chemotaxis chamber, were performed to determine whether these isoforms could stimulate an increase in non-directional migration (chemokinesis) of limb myoblasts (Table 2A and 2B). Again PDGF-AA had no significant effect on cell migration (data not shown), however, it was possible to demonstrate that in a uniform concentration of 10 ng/ml PDGF-AB, for example, the number of myoblasts which had accumulated on the lower surface of the Nuclepore filter (13±3.69 myoblasts/5 mm^2) was approximately 3-fold greater than the number which accumulated in a uniform concentration of DMEM alone (4.33±1.13 myoblasts/5 mm^2, Table 2A). In addition, small numbers of myoblasts (7.83±3.19/5 mm^2) were also observed on the lower surface of the filter even when subjected to a negative gradient of PDGF-AB (10 ng/ml) although these were significantly lower than cells subjected to a positive gradient of either cytokines. PDGF-BB also stimulated chemokinesis so that in a uniform concentration of 10 ng/ml PDGF-BB there was greater than an 8-fold increase in the number of myogenic cells which had accumulated on the lower surface of the filter when compared to the number which accumulated in a uniform concentration of DMEM alone (Table 2B). Although the random migration observed in response to PDGF-BB was considerable, it was still possible to distinguish directed migration (chemotaxis) — as PDGF-BB at 10 ng/ml in the lower well alone produced the maximal chemotactic response (48.50±6.22 myoblasts/5 mm^2), but at 10 ng/ml both above and below the filter no more than 35.30±4.62 myoblasts/5 mm^2 migrated across the filter. Similar results were also obtained for PDGF-AB (Table 2A).

PDGF isoforms influence the expression of sarcomeric myosin and fusion of myoblasts

PDGF isoforms at concentrations of 0.1-10 ng/ml, were tested for their ability to regulate other aspects of limb myoblast behavior (Fig. 3A and 3B). When cells were cultured at low density for 48 h, all PDGF isoforms stimulated an increase in the number of MF-20-positive cells when compared to control cultures (incubated for 48 h in the absence of PDGF). PDGF-AA had the most profound effect, stimulating almost a 100% increase in the number of myogenic cells which expressed MF-20 at 10 ng/ml. PDGF-AB and -BB also enhanced the number of MF-20-positive cells but were less potent than PDGF-AA, for example, 10 ng/ml of either -AB or -BB stimulated less than a 50% increase in the number of myogenic cells which expressed MF-20 (Fig. 3A).

When limb cells were cultured at a higher density it was possible to determine the effect of the PDGF isoforms on the formation of myotubes (Fig. 3B). In the control cultures (cultured in the absence of PDGF) approximately 40.9±1.01% of the MF-20-positive nuclei were present in myotubes. PDGF-AB and -BB both appeared to be

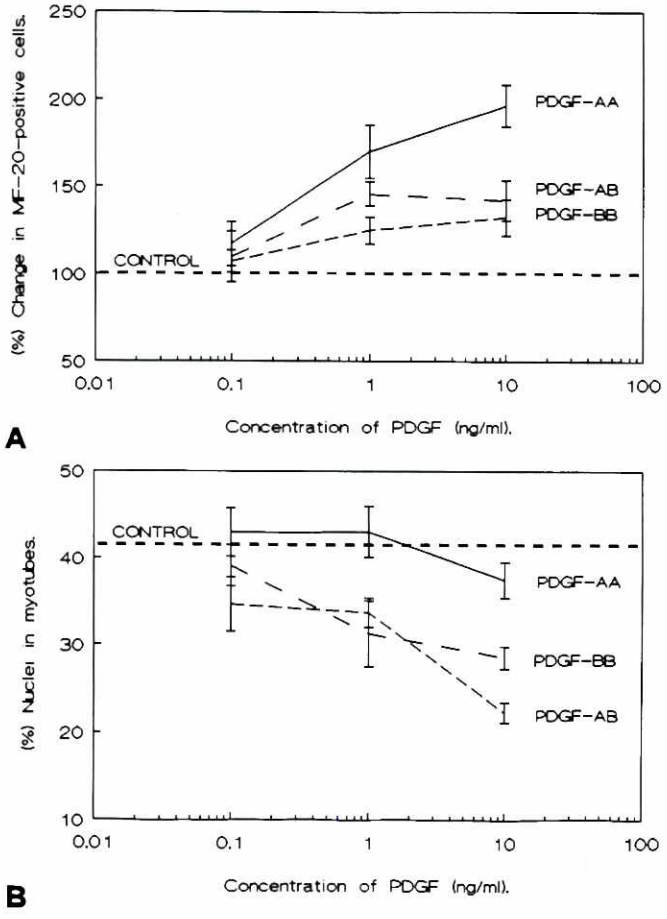

Fig. 3. PDGF isoforms stimulate expression of sarcomeric myosins, recognized by the MF-20 antibody, in myoblasts but inhibit myoblast fusion. *Limb cultures were incubated either at low or high density with 0.1-10 ng/ml PDGF-AA, PDGF-AB or PDGF-BB for 48 h after which the myogenic population was identified with MF-20. At low density* **(A)** *the plotted values represent the percentage difference in the number of MF-20-positive cells/mm² when cultured in the presence of each PDGF isoform with respect to the number of cells cultured in the absence of cytokine. At higher cell culture density* **(B)**, *each value represents the percentage of nuclei in myotubes. The mean and SEM of 6 experiments are shown.*

potent inhibitors of myogenic cell fusion such that at 10 ng/ml of PDGF-AB and -BB the percentage of nuclei in myotubes was only 22.38±1.16% and 28.62±1.27%, respectively. PDGF-AA, however, had negligible effect on cell fusion at 0.1 and 1 ng/ml, when compared to the spontaneous fusion observed in the absence of cytokines, although at 10 ng/ml fusion was inhibited by approximately 8.5% such that there were approximately 37.5±2.08% nuclei in myotubes (Fig. 3B).

Expression of PDGFα- and PDGFβ-receptors in the limb

Immunohistochemistry was used to study the expression pattern of the PDGFα- and β-receptors in the limb in an attempt to determine the likelihood of the observed effects of the various PDGF isoforms being receptor-mediated (Fig. 4). Using MF-20 to identify myoblasts in a heterogeneous limb culture (Fig. 4B,D) it was possible to demonstrate that the myogenic population, as well as the other limb mesenchyme which were cultured for 24 h, expressed both PDGFα- and β-receptors (Fig. 4A,C). These results were further confirmed on cryosections of 11.5 day limbs, which demonstrated that both α- and β-receptors were expressed, non-specifically, throughout the whole 11.5 day forelimb (Fig. 5).

Distribution of PDGF-B in the limb

As both PDGF-AB and -BB had a significant effect on myogenic cell migration, it was important to establish whether the PDGF-B chain was present in the 11.5 day limb. Using immunohistochemistry, the presence of the -B monomer in the limb bud was determined (Fig. 6). PDGF-B was strongly expressed in the AER, ectoderm and the mesenchyme of the progress zone (Fig. 6B). PDGF-B was also expressed in the myogenic regions (Fig. 6C). The chondrogenic regions were weakly stained for PDGF-B.

Discussion

In avian and mammalian embryos, myoblasts normally translocate over considerable distances from the somites to the limb bud and in the limb bud itself during morphogenesis (Lee and Ede, 1990; Lee and Sze, 1993; Hayashi and Ozawa, 1995; Sze *et al.*, 1995). These cells migrate exclusively in a proximo-distal direction in the chick limb bud during normal limb development and become localized into dorsal and ventral muscle masses (Brand-Saberi *et al.*, 1989; Lee and Ede, 1989b). In contrast, chondrocytes and soft connective tissue cells in the limb do not migrate (Solursh, 1984; Lee and Ede 1989a,b, 1990). Whilst the capacity of skeletal myogenic cells to migrate both *in vitro* and *in vivo* is well established, little is known as yet about the factors responsible for regulating the direction and pattern of muscle cell migration. A number of growth factors are known to be localized in the distal region in and adjacent to the AER (Niswander and Martin, 1992; Orr-Utreger and Lonai, 1992; Savage *et al.*, 1993; Crossley and

TABLE 2

CHECKERBOARD ANALYSIS OF PDGF-AB AND PDGF-BB

		Upper well		
A Lower well	[PDGF-AB] (ng/ml)	0	1	10
	0	4.33±1.13	3.00±1.92	7.83±3.19
	1	**8.50±1.89**	7.33±2.39	6.83±3.32
	10	**24.50±5.30**	**11.50±3.22**	13.00±3.69
		Upper well		
B Lower well	[PDGF-BB] (ng/ml)	0	1	10
	0	4.33±1.13	14.00±4.72	14.33±2.63
	1	**14.33±3.65**	23.83±6.57	23.00±3.42
	10	**48.50±6.22**	**49.25±6.09**	35.50±4.62

Myoblast migration in response to (A) PDGF-AB and (B) PDGF-BB which were placed at various concentrations above and below the filter of the Blindwell chamber. The results are expressed as mean number of myoblasts/5 mm²±SEM of 6 experiments.

Martin, 1995), and currently it is believed that these growth factors interact with the zone of polarizing activity to induce the release of a morphogen or group of morphogens, which form a diffusible signal, responsible for regulating the chondrogenic and muscle pattern in the limb. Moreover, the removal of the AER from the avian limb inhibits myogenic cell migration (Gumpel-Pinot *et al.*, 1984).

The PDGF-A chain is known to be expressed in the AER and so PDGF isoforms may play an important role in muscle patterning. Our present study, using the Blindwell chemotaxis assay system, demonstrates that of the 3 PDGF isoforms, only PDGF-AB and -BB (at 1-100 ng/ml) can elicit a migratory response in murine forelimb myogenic cells *in vitro*. These, and other unidentified cells derived from the proximal forelimb, reacted to both isoforms by migrating from low to high concentrations of the isoform across a polycarbonate filter coated with fibronectin, the fibronectin being vital for the initial cell attachment prior to migration (Venkatasubramanian and Solursh, 1984). The absolute concentration of PDGF in the developing mouse forelimb has not as yet been determined, however, it is known that the concentration of total PDGF in adult BALB/c mouse serum is 11-21 ng/ml and the concentration of PDGF released from different endothelial cell types ranges from 1-7 ng/ml (Bowen-Pope *et al.*, 1989). Therefore, the concentration range of PDGF which was used in this study is likely to represent, at least approximately, the physiological concentration available to responsive cells in the developing limb bud.

At 1-10 ng/ml of PDGF-BB in the lower well of the Blindwell chamber, myoblast migration was dose-dependent with maximal migration occurring at 10 ng/ml. At 100 ng/ml PDGF-BB the migratory response was still significantly greater than the background level of migration but it was much lower than that observed at 10 ng/ml. The migration in response to a gradient of PDGF-AB was not as dramatic but followed a similar pattern to that observed for PDGF-BB with a dose-dependent increase in migration from 1-10 ng/ml and optimal migration occurring at 10 ng/ml. Such bell-shaped response curves appear to be common in other cell types, having also been demonstrated for the migration of rat vascular smooth muscle cells and rabbit retinal endothelial cells in response to PDGF and to FGF-2 (Koyama *et al.*, 1992, 1994).

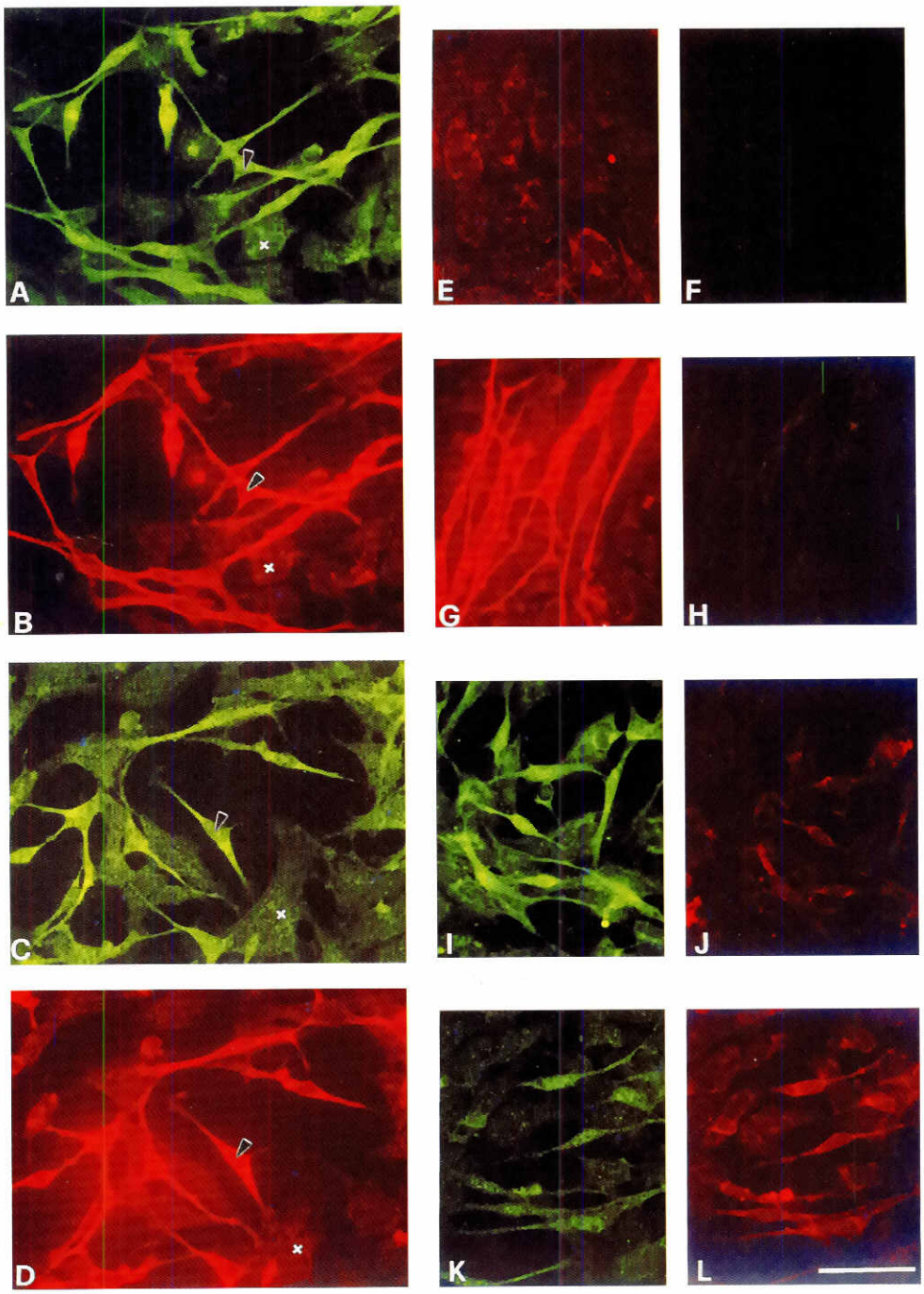

Fig. 4. Expression of PDGFα (A) and PDGFβ (C) receptors in 11.5 day forelimb cells, cultured for 24 h on gelatin-coated coverslips. *Myoblasts (arrowheads) are identified by MF-20, anti-sarcomere myosin antibody* **(B,D)**. *Controls* **(E-L)** *viewed with a blue filter* **(F,H,I,K)** *and a green filter* **(E,G,J,L).** *No antibodies* **(E,F),** *MF20 alone* **(G,H)**, *PDGFα alone* **(I,J)** *and PDGFβ alone* **(K,L).** *MF-20-negative cells - X. Bar, 50 μm.*

Normally, cells that are receptive to chemoattractants can respond by chemotaxis (directed migration along a concentration gradient), by chemokinesis (random migration observed in the

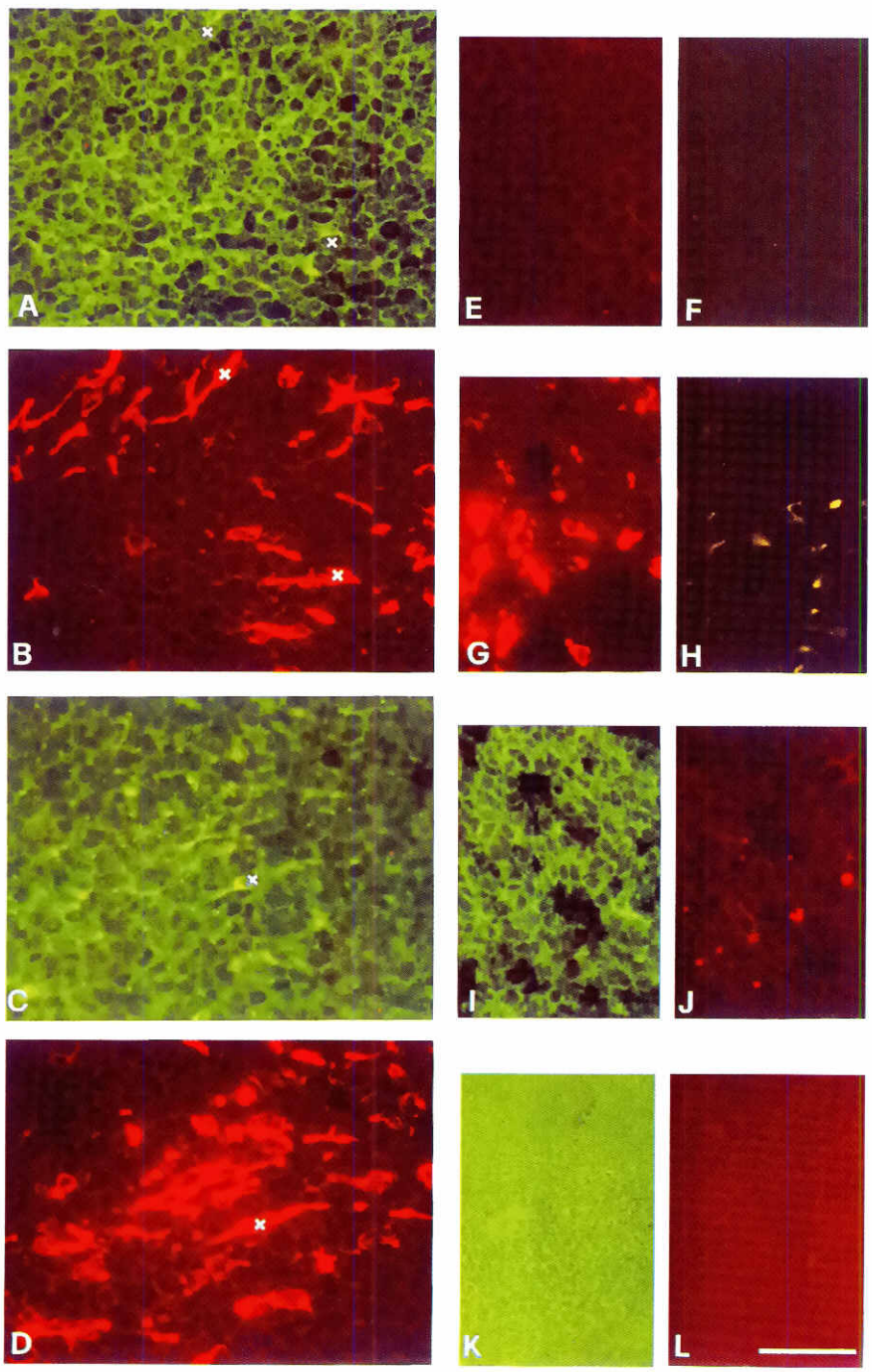

Fig. 5. Immunolocalization of PDGF receptors in the 11.5 day mouse forelimb bud. *Representative longitudinal sections of the proximal region of 11.5 day forelimbs to demonstrate the expression of PDGFα* **(A)** *and PDGFβ* **(C)** *receptors by myogenic cells (X), which have been identified by the anti-sarcomere myosin antibody, MF-20* **(B and D)**. *Controls* **(E-L)**, *viewed with a blue filter* **(F,H,I,K)** *and a green filter* **(E,G,J,L)**. *No antibodies* **(E,F)**, *MF20 alone* **(G,H)**, *PDGFα alone* **(I,J)** *and PDGFβ alone* **(K,L)**. *Bar, 50 μm.*

absence of a gradient), or a combination of both these processes. It is possible to distinguish chemokinesis from chemotaxis by performing a checkerboard analysis (Venkatasubramanian and Solursh, 1984). Our analysis shows PDGF-BB, when present at the same concentration in the upper and lower compartments of the Blindwell chamber, can induce a significant migratory response. However, this response was not as great as if PDGF-BB was present alone in the lower compartment. In the presence of a negative gradient (i.e. PDGF-BB present only in the upper compartment) a significant migratory response was also induced. Similar observations were also attained with PDGF-AB. These results suggest that PDGF-AB and -BB can simultaneously induce both chemokinesis and chemotaxis in limb myogenic cells.

We have found that, apart from myoblasts, other limb mesenchymal cells are able to migrate in response to PDGF-BB *in vitro*. We have not attempted to identify the non-myogenic cells in this study but it has been reported from quail/chick transplantation experiments that angioblasts, melanoblasts and Schwann cells normally migrate in the developing limb (Krenn *et al.*, 1991; Brand-Saberi *et al.*, 1995). Therefore, some of the non-myogenic cells which migrated in our assay system may represent cell types originating from these populations. In addition, some of these cells may be loose connective tissue cells, which normally do not migrate in the limb (Lee and Ede, 1989b), but which may be able to do so *in vitro* when constraints such as cell contact inhibition of movement are relaxed.

Whilst PDGF-AA had a negligible effect on myoblast migration, it was possible to demonstrate that this isoform, along with PDGF-AB and -BB stimulated an increase in the number of differentiated (MF-20-positive) myoblasts when cultured at low density. This effect could be attributed to PDGF either upregulating sarcomeric myosin expression or stimulating proliferation of undifferentiated myoblasts which subsequently expressed the markers for MF-20, or possibly a combination of both these processes. In addition, all three isoforms inhibited the formation of myotubes. Ideally, myoblast fusion should be studied with confluent myogenic cell cultures which are then maintained over a period of several weeks in differentiation medium (Jin *et al.*, 1991). However, in our experiments using high density short-term cultures, it was still possible to demonstrate that all three PDGF isoforms inhibited the spontaneous fusion of myoblasts during the early stages of myotube formation.

It has been suggested that the PDGFα- and β-receptors may use different signal-transduction pathways and have different functions, the β-receptor being concerned primarily with cell migration, whilst the α-receptor is perhaps associated with other biological actions, such as proliferation and differentiation (Yablonka-Reuveni *et al.*, 1990). It is well

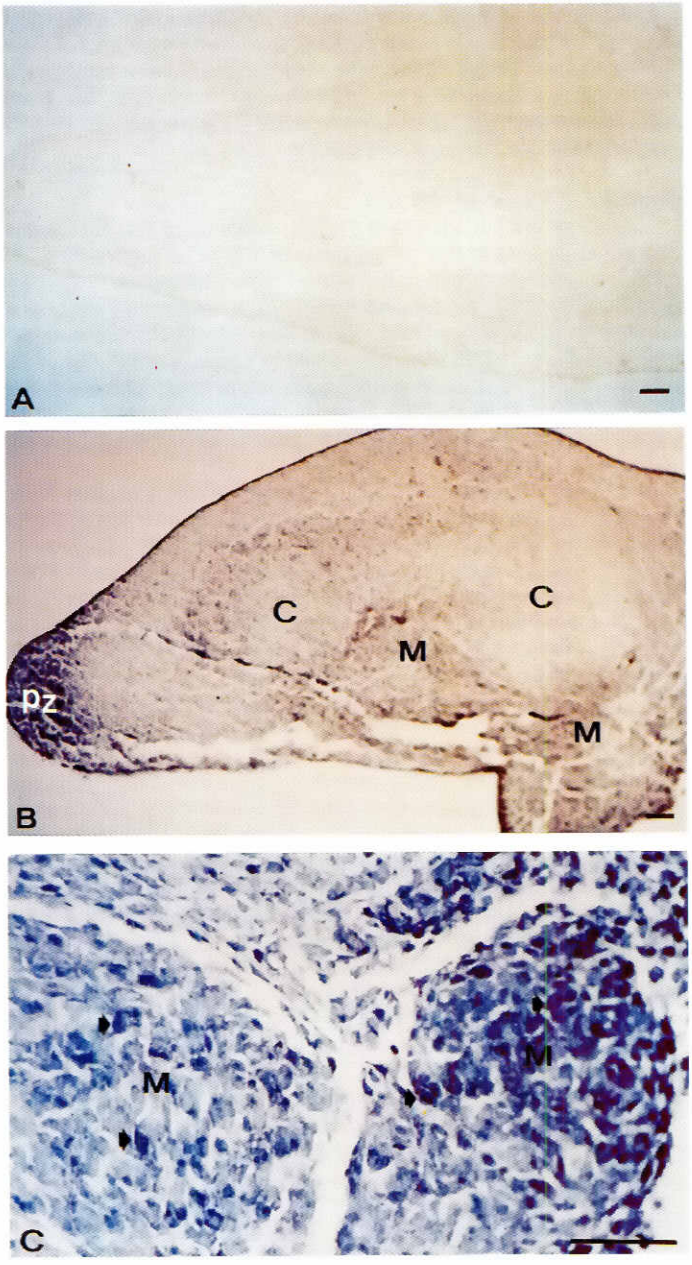

Fig. 6. Immunolocalization of PDGF-B in the 11.5 day mouse fore-limb bud. (A) *Negative control.* **(B)** *PDGF-B is expressed in the AER, ectoderm, mesenchyme of progressive zone (Pz) and myogenic regions (M). Chondrogenic regions* **(C)** *expressed very low levels of PDGF-B.* **(C)** *Enlargement of myogenic regions (M), showing myogenic cells expressing PDGF-B peptide (arrows). Bars, 50 μm.*

known that β-receptors bind PDGF-BB with high affinity and -AB with low affinity but do not bind the -AA isoform (Heldin *et al.*, 1988). Thus, even though limb myoblasts express both α- and β-receptors, the fact that PDGF-BB stimulated a greater migratory response in these cells than PDGF-AB, and PDGF-AA had a negligible effect on myoblast migration suggests that this process is mediated via the β-receptors alone. Furthermore, our results support the view that the α-receptors, which bind all three PDGF isoforms, are involved with non-locomotory activity in limb myoblasts as PDGF-AA, as well as PDGF-AB and -BB, stimulated an increase in the number of MF-20-positive myogenic cells and inhibited myotube formation.

Using *in situ* hybridization techniques, the expression pattern of the PDGF-A gene has already been reported in the 11.5 day mouse where it is expressed in the muscle anlagen, ectoderm and AER of the forelimb (Orr-Urtreger and Lonai, 1992). We demonstrated, by immunohistochemistry, that the PDGF-B monomer is also located in the 11.5 day forelimb. It appears to be particularly concentrated in the mesenchyme of the progress zone, the AER, ectoderm and the myogenic regions, with very low levels being found in the presumptive chondrogenic regions. This latter observation correlates with results obtained by Chen *et al.* (1992) who demonstrated that PDGF actually inhibits chondrogenesis *in vitro*. As all three PDGF isoforms have a significant effect on limb myoblast differentiation and migration *in vitro*, and both PDGF-A and PDGF-B can be detected in the 11.5 day limb, we believe that PDGF may play an important role in the development of the limb musculature *in vivo*. For example, the presence of PDGF-B peptide in the myogenic regions may act to stimulate chemokinesis in limb myoblasts – thereby sustaining myoblast migration in the limb. Moreover, PDGF-B's presence in the progress zone may act to chemotactically direct myoblasts to move in a proximo-distal fashion.

It is now well established that members of other growth factor families are also expressed in the limb, these include various members of the FGF family and hepatocyte growth factor (HGF) (Niswander and Martin, 1992; Savage *et al.*, 1993; Myokai *et al.*, 1995). It has been revealed that FGF-2 and -4 can chemotactically stimulate proximal limb cells to migrate in the chick (Li *et al.*, 1996; Li and Muneoka, personal communication) and mouse (Webb *et al.*, 1997) limbs. Similar effects have also been obtained for TGFβ on chick limb cells (Lucas and Caplan, 1988). Moreover, it has recently been demonstrated that knocking out the c-*met* receptor for HGF completely inhibits the migration of myogenic precursors from the somite to the limb bud (Bladt *et al.*, 1995; Maina *et al.*, 1996). Thus, we believe that rather than a single growth factor acting as a diffusible signal to stimulate the proximo-distal migration of myogenic precursor cells, the development of the limb musculature is probably regulated by a complex interaction of several cytokines which stimulate the proliferation and differentiation as well as migration of myogenic cells.

Materials and Methods

Embryos

Day 11.5 embryos were obtained from pregnant ICR mice, the presence of a vaginal plug being designated as embryonic day 0.5. The mice were killed by cervical dislocation and the embryos were isolated from the decidua in pre-warmed Dulbecco's phosphate-buffered saline (Sigma, St. Louis, MO, USA) containing 0.4% bovine serum albumin.

Preparation of a limb cell suspension

Using sharp forceps, the forelimbs were removed and by further dissection the distal and proximal halves of each limb bud were isolated. Initially the two groups of limb bud sections were rinsed with PBS minus BSA. The tissues were then 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 and 10 min at 37°C followed by

trituration with a Pasteur pipette to mechanically disrupt the tissues. The trypsin was inhibited by the addition of fetal bovine serum (FBS) 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 cells were pelleted by centrifugation at 250g for 3 min, then resuspended in DMEM medium containing 5% serum. Cell concentration was determined using an Improved Neubauer hemocytometer (American Optical, Buffalo, NY, USA) and cell viability was analyzed with Trypan blue. The cell suspension was then repelleted and resuspended to the required concentration in the medium appropriate for the assay under investigation.

Blindwell cell migration assay

Migration assays were performed using Nuclepore Blindwell chemotaxis chambers (Costar Scientific Corporation, Cambridge). Nuclepore polycarbonate filters of 8.0 μm pore size (Costar), which had been sterilized by UV-irradiation, were incubated with 100 μg/ml fibronectin solution (Life Technologies Inc., Grand Island) overnight at 4°C and then air-dried. In a typical experiment PDGF-AA, PDGF-AB or PDGF-BB (R and D Systems Inc., Minneapolis) was prepared in DMEM minus FBS at 1-100 ng/ml and added to the lower well of each Blindwell chamber. Each chamber was assembled with a fibronectin-coated filter and filter retainer, and 200 μl cell suspension, at $1x10^6$ cells/ml in DMEM medium minus FBS, but containing the appropriate concentration of PDGF isoform, was added to the upper well. The chambers were then incubated at 37°C and 5% CO_2 in air, in a humidified incubator for 5 h. Approximately $3.6x10^3$ cells will coat the upper surface of the Nuclepore filters after 1 h incubation.

At the end of the incubation period, the chambers were dismantled and the upper surface of each filter was wiped with sterile tissues to remove cells which had attached but which had not migrated through to the lower surface. Cells which had migrated were either: a) prepared directly for scanning electron microscopy by fixation with 2.5% glutaraldehyde; or b) cultured on the filters and maintained for 20 h in DMEM containing 10% FBS at 37°C and 5% CO_2, after which they were fixed for 30 min with 70% ethanol at room temperature. The presence of myoblasts was then determined by immunohistochemistry using a monoclonal anti-sarcomere myosin antibody, MF-20 (Developmental Studies Hybridoma Bank) diluted 1:300 in antibody buffer (PBS) which was viewed by the Vectastain ABC kit (Vector Laboratories, Burlingame) using metal-enhanced DAB (Amersham, Buckinghamshire). Eosin was used as a counterstain for MF-20-negative mesenchyme cells.

Scanning electron microscopy

Cells which had migrated to the lower surface of Nuclepore filters during the Blindwell Chemotaxis assay were initially fixed with 2.5% glutaraldehyde, made up in 0.1 M cacodylate buffer at 4°C for 2 h. They were then rinsed with 0.1 M cacodylate buffer, post-fixed with 1% osmium tetroxide for 15 min at room temperature and dehydrated through a series of increasing concentrations of ethanol. The filters were critical point dried with freon as the transitional fluid and sputter-coated with palladium gold. Specimens were then examined with a JEOL JSM-35CF scanning electron microscope at 10 KV.

Effect of PDGF isoforms on terminal differentiation and fusion of myoblasts

A single-cell suspension of the proximal regions of 11.5 day mouse embryo forelimbs was obtained as described above. Cells suspended in DMEM/F12 medium (Sigma) containing 1% FBS and 0, 0.1, 1 or 10 ng/ml PDGF-AA, -AB or -BB, were plated out at either $1.4x10^3$ cells/mm^2 onto 13 mm coverslips in 24-well tissue culture plates (Nunc, Roskilde), or $2.8x10^3$ cells/mm^2 into 16-well tissue culture chambers (Nunc), all of which had been pre-coated with 0.1% gelatin (Sigma) for 24 h at 37°C and then air-dried. The cells were incubated for 48 h at 37°C and 5% CO_2 in air, in humid conditions after which they were fixed with 95% ethanol/acetic acid (at a 95:5 ratio) for 20 min and the myoblasts and myotubes were identified by immunohistochemistry, using MF-20 (procedure described above). The low density cultures grown on the coverslips were viewed at a x40 magnification and the number of myogenic cells was quantified by calculating the total number of nuclei in MF-20-positive cells per mm^2. Subsequently, the percentage difference in myogenic cell number, compared to parallel cultures incubated in the absence of PDGF, was determined. The cultures plated at a higher density were viewed at 20x magnification and the percentage of nuclei in myotubes was calculated.

Detection of PDGF and PDGFR

A proximal limb bud cell suspension was prepared as described above, to give a concentration of $1x10^6$ cells/ml in DMEM containing 5% FBS. The cells were grown on gelatin-coated coverslips and maintained at 37°C and 5% CO_2 in air under humid conditions for 24 h, they were then fixed in acetone for 30 sec at room temperature. Whole forelimbs dissected from 11.5 day embryos were either embedded in TissueTek OCT compound (Miles Inc., Elkhart, IN, USA) and cryosectioned at 10 μm, after which they were fixed in acetone, again for 30 sec at room temperature, or else they were fixed with 4% paraformaldehyde prior to embedding in wax and sectioning at 7 μm.

A double-labeling technique was performed on both the cultured forelimb cells and the cryosectioned entire forelimbs in order to identify the location of PDGF-α and PDGFβ-receptor expression with respect to the myogenic cell population in the limb. MF-20 was used, in conjunction with Texas Red-conjugated anti-mouse IgG (Vector Laboratories) to identify the myogenic population. In addition, either anti-PDGF(R)-A(951) (at 1:25 dilution; Santa Cruz Biotechnology, Inc.) or anti-PDGF(R)-B(958) (1:25 dilution; Santa Cruz Biotechnology, Inc.) was used, along with a secondary antibody of FITC-conjugated anti-rabbit IgG (Sigma), to identify the location of the PDGFα- and β-receptors respectively. The specimens were then examined using a 40x objective under the Zeiss fluorescent microscope.

As well as having a simple conjugate control (no primary antibody), some of the specimens were treated with MF-20 alone and others with the appropriate anti-PDGF receptor antibody alone, along with both the FITC and Texas Red secondary antibodies in order to eliminate the possibility of obtaining false-positive results. All these control specimens were viewed with the excitation filters for both FITC and Texas Red in the same way that the experimental specimens were examined.

The presence of PDGF-B was also determined immunohistochemically using wax-sections of whole 11.5 day forelimbs. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide in methanol. After inhibiting non-specific protein interactions with goat serum, forelimb sections were probed with rabbit anti-PDGF-B (N-30) (Santa Cruz Biotechnology, Inc.) at a 1:10 dilution in PBS and then viewed with the rabbit Vectastain ABC kits using nickel-enhanced DAB as the peroxidase substrate. Antigen specificity was ensured by replacing the primary antibody either with PBS or with normal rabbit antiserum.

Acknowledgments

This work was supported by grants from the Ho Sin-Hang and Croucher Foundations. S.E.W. is a recipient of a post-doctoral fellowship awarded by CAPAD. The authors would like to thank Y.M. Cheung, L.Y. Sze, L.S. Kung, and D.Q. Cai 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, MD, and the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD.

References

BLADT, F., RIETHMACHER, D., ISENMANN, S., AGUZZI, A and BIRCHMEIER, C. (1995). Essential role for the c-*met* receptor in the migration of myogenic precursor cells into the limb bud. *Nature 376:* 768-771.

BOWEN-POPE, D.F., HART, C.E. and SEIFERT, R.A. (1989). Sera and conditioned media contain different isoforms of platelet-derived growth factor (PDGF) which bind to different classes of PDGF receptors. *J. Biol. Chem. 264:* 2502-2508.

BRAND-SABERI, B., KRENN, V. and CHRIST B. (1989). The control of directed myogenic cell migration in the avian limb bud. *Anat. Embryol. 180:* 555-566.

BRAND-SABERI, B., SEIFERT, R., GRIM, M., WILING, J., KUHLEWEI, M. and CHRIST, B. (1995). Blood vessel formation in the avian limb bud involves angioblastic and angiotrophic growth. *Dev. Dynamics 202:* 181-194.

CAPLAN, A.I. (1981). The molecular control of muscle and cartilage development. In *Levels of Genetic Control in Development* (Eds. S. Subtelny and U.K. Abbott). Alan R. Liss, New York, pp. 37-38.

CHEN, P., CARRINGTON, J.L., PARALKAR, V.M., PIERCE, G.F. and REDDI, H. (1992). Chick limb bud mesodermal cell chondrogenesis: inhibition by isoforms of platelet-derived growth factor and reversal by recombinant bone morphogenetic protein. *Exp. Cell Res. 200:* 110-117.

CHEVALLIER, A., KIENY, M. and MAUGER, A. (1977). Limb-somite relationship: origin of the limb musculature. *J. Embryol. Exp. Morphol. 41:* 245-258.

CHIQUET, M., EPPENBERGER, H.M. and TURNER, D.C. (1981). Muscle morphogenesis: evidence for an organising function of exogenous fibronectin. *Dev. Biol. 88:* 220-235.

CHRIST, B., JACOB, H.J. and JACOB, M. (1977). Experimental analysis of the origin of the wing musculature in avian embryos. *Anat. Embryol. 150:* 171-186.

CROSSLEY, P.H. and MARTIN, G.R. (1995). The mouse FGF 8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development 121:* 439-451.

GROTENDORST, G.R., IGARASHI, A., LARSON, R., SOMA, Y. and CHARETTE, M. (1991). Differential binding, biological and biochemical actions of recombinant PDGF AA, AB, and BB molecules on connective tissue cells. *J. Cell. Physiol. 149:* 235-243.

GUMPEL-PINOT, M., EDE, D.A. and FLINT, O.P. (1984). Myogenic cell movement in the developing avian limb bud in the presence and absence of the apical ectodermal ridge (AER). *J. Embryol. Exp. Morphol. 80:* 105-125.

HAYASHI, K. and OZAWA, E. (1995). Myogenic cell migration from somites is induced by tissue contact with the medial region of the presumptive limb mesoderm in chick embryos. *Development 121:* 661-669.

HELDIN, C-H. and WESTERMARK, B. (1989). Platelet-derived growth factors: three isoforms and two receptor types. *Trends Genet. 5:* 108-111.

HELDIN, C-H., BACKSTROM, G., OSTMAN, A., HAMMACHER, A., RONNSTRAND, L., RUBIN, K., NISTER, M. and WESTERMARK, B. (1988). Binding of different dimeric forms of PDGF to human fibroblasts: evidence for two separate receptor types. *EMBO J. 7:* 1387-1393.

JIN, P., SEJERSEN, T. and RINGERTZ, N.R. (1991). Recombinant platelet-derived growth factor-BB stimulates growth and inhibits differentiation of rat L6 myoblasts. *J. Biol. Chem. 266:* 1245-1249.

KOYAMA, N., MORISAKI, N., SAITO, Y. and YOSHIDA, S. (1992). Regulatory effects of platelet derived growth factor-AA homodimer on migration of vascular smooth muscle cells. *J. Biol. Chem. 267:* 22806-22812.

KOYAMA, N., WATANABE, S., TEZUKA, M., MORISAKI, N., SAITO, Y. and YOSHIDA, S. (1994). Migratory and proliferative effect of platelet-derived growth factor in rabbit retinal endothelial cells: evidence of an autocrine pathway of platelet-derived growth factor. *J. Cell. Physiol. 158:* 1-6.

KRENN, V., BRAND-SABERI, B. and WACHTLER, F. (1991). Hyaluronic acid influences the migration of myoblasts within the avian embryonic wing bud. *Am. J. Anat. 192:* 400-406.

LANCE-JONES, C. (1988). The somitic level of origin of embryonic chick hindlimb muscles. *Dev. Biol. 126:* 394-407.

LEE, K.K.H. and EDE, D.A. (1989a). The capacity of normal and *talpid*^{3} mutant fowl myogenic cells to migrate in quail limb buds. *Anat. Embryol. 179:* 395-402.

LEE, K.K.H. and EDE, D.A. (1989b). A study on skeletal myogenic cell movement in the developing avian limb bud. *Anat. Embryol. 180:* 293-300.

LEE, K.K.H. and EDE, D.A. (1990). An autoradiography study of myogenic cell movement in avian limb buds following heterospecific and homospecific transplantation. *Anat. Embryol. 182:* 547-551.

LEE, K.K.H. and SZE, L.Y. (1993). Role of the brachial somites in the development of the appendicular musculature in rat embryos. *Dev. Dynamics 198:* 86-96.

LI, S.G., ANDERSON, R., REGINELLI, A.D. and MUNEOKA, K. (1996). FGF-2 influences cell movements and gene expression during limb development. *J. Exp. Zool. 274:* 234-247.

LUCAS, P.A and CAPLAN, A.I. (1988). Chemotactic response of embryonic limb bud mesenchymal cells and muscle-derived fibroblasts to transforming growth factor-beta. *Connect. Tissue Res. 18:* 1-7.

MAINA, F., CASAGRANDA, F., ANDERO, E., SIMEONE, A., COMOGLIO, P.M., KLEIN, R. and PONZETTO, C. (1996). Uncoupling of Grb2 from the met receptor *in vivo* reveals complex roles in muscle development. *Cell 87:* 531-542.

MYOKAI, F., WASHIO, N., ASAHARA, Y., YAMAAI, T., TANDA, N., ISHIKAWA, T., AOKI, S., KURIHARA, H., MURAYAMA, Y., SAITO, T., MATSUMOTO, K., NAKAMURA, T., NOJI, S. and NOHNO, T. (1995). Expression of the hepatocyte growth factor gene during chick limb development. *Dev. Dynamics 202:* 80-90.

NEWMAN, S.A., PAUTOU, M. and KIENY, M. (1981). The distal boundary of myogenic primordia in chimaeric avian limb buds and its relation to an accessible population of cartilage progenitor cells. *Dev. Biol. 84:* 440-448.

NISWANDER, L. and MARTIN, G.R. (1992). *Fgf-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development 114:* 755-768.

ORR-URTREGER, A. and LONAI, P. (1992). Platelet-derived growth factor-A and its receptor are expressed in separate, but adjacent cell layers of the mouse embryo. *Development 115:* 1045-1058.

ROSS, R., RAINES, E.W. and BOWEN-POPE, D.F. (1986). The biology of platelet-derived growth factor. *Cell 46:* 155-169.

SAVAGE, M.P., HART, C.E., RILEY, B.B., SASSE, J., OLWIN, B.B. and FALLON, J.F. (1993). Distribution of FGF-2 suggests it has a role in chick limb bud growth. *Dev. Dynamics 198:* 159-170.

SCHRAMM, C. and SOLURSH, M. (1990). The formation of premuscle masses during chick wing bud development. *Anat. Embryol. 182:* 235-247.

SOLURSH, M. (1984). The migratory capacity of myogenic cells *in vitro*. *Dev. Biol. 102:* 509-513.

SOLURSH, M. and MEIER, S. (1986). The distribution of somite-derived, myogenic cells during the early development of the wing-bud. In *Somites in Developing Embryos*, Series A: Life Sciences, Vol. 118 (Eds. R. Bellairs, D.A. Ede and U.W. Lash). Plenum, New York, pp. 261-276.

STOPAK, D. and HARRIS, A.K. (1982). Connective tissue morphogenesis by fibroblast traction I: culture observation. *Dev. Biol. 90:* 383-398.

SZE, L.Y., LEE, K.K.H., WEBB, S.E., LI, Z. and PAULIN, D. (1995). The migration of myogenic cells from the somites to the fore-limb buds of developing mouse embryos. *Dev. Dynamics 203:* 324-336.

VENKATASUBRAMANIAN, K. and SOLURSH, M. (1984). Chemotactic behavior of myoblasts. *Dev. Biol. 104:* 428-433.

WEBB, S.E., LEE, K.K.H., TANG, M.K. and EDE, D.A. (1997). FGF-2 and -4 stimulate migration of mouse embryonic limb myogenic cells. *Dev. Dynamics 209:* 206-216.

YABLONKA-REUVENI, Z., BALESTRERI, T.M. and BOWEN-POPE, D.F. (1990). Regulation of proliferation and differentiation of myoblasts derived from adult mouse skeletal muscle by specific forms of PDGF. *J. Cell Biol. 111:* 1623-1629.

Revised version received: February 1997
Accepted for publication: April 1997