The mitochondrial-apoptotic pathway is triggered in *Xenopus* mesoderm cells deprived of PDGF receptor signaling during gastrulation

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Abstract

Platelet-derived growth factor receptor (PDGFR) signaling is required for normal gastrulation in *Xenopus laevis*. Embryos deprived of PDGFR signaling develop with a range of gastrulation-specific defects including *spina bifida*, shortened anteroposterior axis, and reduced anterior structures. These defects arise because the involuting mesoderm fails to move appropriately. In this study, we determine that inhibition of PDGFR signaling causes prospective head mesoderm cells to appear in the blastocoel cavity at the onset of gastrulation, stage 10. These aberrant cells undergo apoptosis via the caspase 3 pathway at an embryonic checkpoint called the early gastrula transition (EGT). They are TUNEL-positive and have increased levels of caspase 3 activity compared to control embryos. Apoptotic death of these mesoderm cells can be prevented by coinjection of mRNA encoding Bcl-2 or by injection of either a general caspase inhibitor or a caspase 3-specific inhibitor. Prevention of cell death, however, is not sufficient to rescue gastrulation defects in these embryos. Based on these data, we propose that PDGFR signaling is necessary for survival of prospective head mesoderm cells, and also plays an essential role in the control of their cell movement during gastrulation.

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Keywords: *Xenopus*; Platelet-derived growth factor receptor; Apoptosis; Caspase 3; Gastrulation

Introduction

Platelet-derived growth factor receptor (PDGFR) signaling is essential for normal embryonic development (reviewed in Betsholtz et al., 2001; Hoch and Soriano, 2003). When PDGFR signaling is disrupted during embryogenesis, the embryos develop with severe abnormalities, often resulting in death. For example, PDGFRα null mouse embryos die at specific stages of development that are associated with periods of cell movement and differentiation, such as neural crest cell migration and organogenesis (Soriano, 1997). Amphibian, sea urchin, zebrafish, and cow embryos are similarly affected when they are deprived of PDGFR signaling (Ataliotis and Mercola, 1997; Hoch and Soriano, 2003; Liu et al., 2002). At the time of death, all of these embryos display a range of developmental defects resulting from the failure of mesenchyme cells to migrate or differentiate.

Of the two PDGFRs (α and β) and four PDGF ligands (A, B, C, and D), PDGFRα and PDGF-A are the predominant forms present in the early embryo (Jones et al., 1993). The receptors and ligands are generally expressed in separate tissues that are brought into contact for the first time through cell rearrangements. This pattern of expression is reiterated throughout development in which motile cells expressing PDGF move across stationary cells expressing PDGF ligands. For example, during gastrulation in frog and mouse embryos, mesoderm cells expressing PDGFRα move across ectoderm cells that express PDGF-A (Ataliotis et al., 1995; Orr-Urtreger and Lonai, 1992). Later, during neural crest cell migration in zebrafish, mice, and frogs, neural crest cells expressing PDGFRα move through the branchial arches that express PDGF-A and PDGF-C (Aase et al., 2002; Ding et al., 2000; Ho et al., 1994; Liu et al., 2002; Orr-Urtreger and Lonai, 1992; Soriano, 1997). Moreover, during organogenesis, PDGFRs and their ligands tend to be expressed in the mesenchymal and epithelial tissues, respec-
tively, of organs such as the heart, kidney, eye, and lung (Aase et al., 2002; Betsholtz et al., 2001; Ding et al., 2000; Orr-Urtreger and Lonai, 1992; Sun et al., 2000). In cells in culture, PDGFR signaling also plays a role in cell motility and is known to affect cellular processes important for it, including rearrangement of the actin cytoskeleton, adhesion to fibronectin, and chemotaxis (reviewed in Heldin et al., 1998; Heldin and Westermark, 1999). Taken together, these studies suggest that PDGFR signaling controls cell motility and differentiation both in vivo and in vitro.

Inhibition of PDGFR signaling in *Xenopus* embryos using a dominant inhibitory PDGFRα (PDGFR-37) results in embryos with severe developmental defects including an open blastopore, spina bifida, and reduced anterior structures (Ataliotis et al., 1995). These defects arise because the cell movements of gastrulation are disrupted. During gastrulation, the first mesoderm cells to move into the embryo are the presumptive head mesoderm cells (Keller and Winklbauer, 1992). These cells migrate across a fibronectin matrix that lines the inner surface of the blastocoel roof. When PDGFR signaling is inhibited in *Xenopus* embryos, the presumptive head mesoderm cells fail to adhere to the blastocoel roof and accumulate in the blastocoel cavity. The fate of these detached cells is unknown; however, it has been suggested that they may simply die (Ataliotis et al., 1995).

During embryogenesis, a specific form of cell death, called apoptosis, is used to shape tissues and organs and to remove damaged cells in the developing embryo (Hensey and Gautier, 1997, 1998; reviewed in Jacobson et al., 1997). Apoptosis occurs through the activation of specific cysteine proteases, termed caspases that lead to the disassembly of the cell through the cleavage of proteins necessary for cell integrity and survival (reviewed in Evan and Littlewood, 1998; Thornberry and Lazebnik, 1998). In *Xenopus*, apoptosis is first detected at the beginning of gastrulation at a checkpoint termed the early gastrula transition (EGT; Howe et al., 1995). At the EGT, two classes of cells can die. First, as part of normal development, a small percentage of ectoderm cells undergoes apoptosis (Hensey and Gautier, 1998). Second, cells that received an apoptotic stimulus much earlier in development, before the mid blastula transition (MBT), wait until the EGT to undergo apoptosis (Hensey and Gautier, 1997; Howe et al., 1995; Stack and Newport, 1997). In both cases, cell death occurs through the mitochondrial apoptotic pathway. This pathway is regulated by anti-apoptotic factors, such as Bcl-2 and pro-apoptotic factors. Once the apoptotic pathway is triggered, a cascade of signaling events leads to the activation of caspases 3 and 7 and the subsequent disassembly of the cell (Thornberry and Lazebnik, 1998).

In this study, we determine that inhibition of PDGFR signaling causes mesoderm cells to appear in the blastocoel cavity at the onset of gastrulation, stage 10. These detached cells undergo apoptosis via the mitochondrial pathway at the EGT. They are TUNEL positive and have increased levels of caspase 3 activity compared to control embryos. The death of these mesoderm cells is prevented by coinjection of mRNA encoding Bcl-2 or by injection of a general caspase inhibitor or a caspase 3-specific inhibitor. Although these cells are rescued from death, it is not sufficient to rescue gastrulation movements in these embryos. In contrast, inhibition of PDGFR signaling in Keller explants of the axial mesoderm neither prevents their convergent extension nor affects their cell survival. Based on these data, we propose that PDGFR signaling is needed for the survival of prospective head mesoderm cells and also plays a vital role in the control of their cell movement during gastrulation.

**Materials and methods**

**Embryos**

*Xenopus* embryos were fertilized in vitro, dejellied in 2% cysteine, pH 7.8, and cultured in 10% Marc’s Modified Ringer (0.1× MMR, Peng, 1991) at temperatures between 14°C and 23°C as previously described (Ataliotis et al., 1995). Embryos were staged according to Nieuwkoop and Faber (1967).

**mRNA synthesis and microinjection**

mRNA for microinjection was transcribed from template DNA with the mMessage mMACHINE kit (Ambion). Embryos were microinjected in a solution of 3% Ficoll in 1× MMR (Peng, 1991). At the 2- to 4-cell stage, embryos were injected into the dorsoanterior marginal zone of each blastomere with 100 pg mRNA encoding a dominant negative (PDGFR-37) or control, frameshift (PDGFR-FS) PDGFRα. In whole embryo experiments, as a lineage tracer and to equalize the amount of total mRNA introduced into embryos, 100 pg to 1 ng mRNA encoding β-galactosidase (β-gal) with a nuclear localization signal (β-gal-NLS) was co-injected. In the explant experiments, 1 ng of Gap43-GFP mRNA was injected as a lineage tracer (the generous gift of E.M. DeRobertis). For the rescue experiments, embryos were injected at the 2- to 4-cell stage as described above, but with the addition of either 1 ng mRNA encoding Bcl-2 or XPDGFRα. Caspase inhibitors, 100–400 μM z-DEVD-fmk or z-VAD-fmk (Enzyme Systems Products), were microinjected directly into the blastocoel cavity at stages 8 to 9. The embryos were stained for β-gal activity as described previously (Ataliotis et al., 1995).

**Microdissection**

Open-faced Keller explants were dissected, cultured, and analyzed as described previously (Tahinci and Symes, 2003). Briefly, the explants were dissected at stage 10.25 from embryos that had been microinjected at the 2-cell stage in the dorsoanterior region (see above). Only explants with...
visible GFP were scored. The explants were flattened under a coverslip bridge and cultured in 1× Danilchik’s for Amy (DFA) at 14°C until stage 20. The explants were then photographed in bright field and GFP for analysis. Finally, the explants were fixed in MEMFA for 1 h at room temperature and stored in methanol before TUNEL staining.

TUNEL staining

Whole-mount TUNEL staining of Xenopus embryos was carried out using the In Situ Cell Death Detection Kit, POD (Roche Molecular Biochemicals) with the protocol modified as described by Hensey and Gautier (1997). Briefly, embryos were fixed for 1 h in 4% paraformaldehyde in PBS at room temperature. They were then blocked for 1 h in 3% hydrogen peroxide in methanol, rinsed in PBS, and permeablized in 0.1% Triton-X 100 in 0.1% sodium citrate for 30 min on ice. The embryos were rinsed in PBS and placed in the TUNEL reaction mixture at 37°C overnight for nicked-end labeling. As a positive control, permeablized embryos were incubated with 2 units of DNase I (Promega) for 30 min at 37°C before the labeling reaction. The embryos were rinsed in PBS, blocked with 20% goat serum in PBT, and incubated overnight at 4°C with the Converter-POD solution (anti-fluorescein HRP conjugated antibody). Before staining, the embryos were rinsed overnight in PBS. For the chromogenic horseradish peroxidase reaction, the embryos were transferred to DAB substrate in DAB buffer as described by the manufacturer (Roche Molecular Biochemicals) for 30 min to 1 h. The reaction was stopped by the addition of PBS.

Whole-mount TUNEL staining of Keller explants was performed as described above for embryos, except for the following changes. Before TUNEL staining, the explants were rehydrated by rinsing with 90% methanol in PBS (vol/ vol), 75% methanol, 50%, methanol, 25% methanol, and finally 1× PBS. In addition, TUNEL-positive, fluorescein-labeled cells were viewed directly using a SV6-GFP Microscope (Zeiss).

β-gal- and TUNEL-stained embryos were embedded for sectioning in JB-4 (Polysciences) and sectioned at 5–8 μm. The sections were mounted and viewed on an Axiovert LM35 Microscope (Zeiss).

Caspase 3 activity assay

At stages 10 and 11, uninjected embryos and embryos that had been injected with 200 pg PDGFR-37 mRNA or PDGFR-FS mRNA were frozen on dry ice. They were then thawed on ice and homogenized in 15 mM Tris, pH 6.8. The yolk was freon (1,1,2-trichlorotrifluoroethane, ICN) extracted from the lysates as described (Evans and Kay, 1991). As a positive control for apoptosis, embryos were cultured in 0.1 mg/ml cycloheximide in 0.1× MMR (Hensey and Gautier, 1997), and lysates were prepared as described above. The protein concentration of each sample was determined (Bio-Rad DC Protein Assay) and equal amounts of total protein were assayed in a 96-well plate. Each well contained 50 μg total protein, 200 nM of the caspase 3 substrate Ac-DEV-D-pNA (Calbiochem) with caspase 3 Incubation Buffer from the Caspase 3 Activity Assay (Roche Molecular Biochemicals). The plate was incubated overnight at room temperature, and the absorbance at 405 nm was determined.

Results

Rounded mesoderm cells appear in the blastocoel cavity at the beginning of gastrulation when PDGFR signaling is blocked

The role of PDGFR signaling during gastrulation in Xenopus embryos was previously examined by over-expression of PDGFR-37, a dominant-negative Xenopus PDGFRα (Ataliotis et al., 1995). When 50–200 pg PDGFR-37 mRNA is microinjected into the lateral marginal zone at the 2- to 4-cell stage, the embryos develop with gastrulation-specific defects including spina bifida and reduced anterior structures (Ataliotis et al., 1995 and see Fig. 1C). When examined at stage 11, it was found that the anterior mesoderm failed to migrate across the blastocoel roof and large cells collected on the floor of the blastocoel cavity. These defects are rescued by over-expression of wild type PDGFRα (Fig. 1D and Tables 1 and 2).

To examine when the mesoderm cells appear in the blastocoel cavity and their subsequent fate, embryos were co-injected with 200 pg mRNA encoding PDGFR-FS or PDGFR-37 and 200 pg mRNA encoding β-gal containing a nuclear localization signal (β-gal-NLS) and then fixed at stages before (stage 9; see Figs. 1E, I) and throughout early gastrulation (stages 10, 10 + and 10.5; see Figs. 1F–H, J–L). These embryos were stained for β-gal activity, embedded, and sectioned for analysis (see Materials and methods).

Before gastrulation, mesoderm cells that received either PDGFR-FS (Fig. 1E) or PDGFR-37 (Fig. 1I) mRNA are in the marginal zone and have distinct nuclear β-gal staining. As soon as gastrulation begins (stage 10), 33% of PDGFR-37-injected embryos have large cells with fragmented and non-nuclear β-gal staining present at the site of involution of the dorsoanterior mesoderm and in the blastocoel cavity (Figs. 1J, M). This number increases to 81% at stage 10+ and 68% at stage 10.5 (Fig. 1M). The number of large cells with non-nuclear β-gal staining in the blastocoel cavity of these embryos is approximately 6 at stages 10 and 10+ and 16 at stage 10.5 (Fig. 1M). At all these stages, sibling embryos injected with PDGFR-FS mRNA have normal involuting mesoderm cells with distinct nuclear β-gal staining (compare Figs. 1F–H with J–K). Not all PDGFR-37-injected cells appear in the blastocoel cavity. Many of these cells are expelled from the embryo and accumulate under the vitelline membrane (data not shown).
The breakdown of the nuclear envelope is a hallmark of apoptosis that has been identified previously by diffuse β-gal staining derived from a nuclear-localized β-gal (McCall and Steller, 1998). The appearance of diffuse β-gal staining in the detached mesoderm cells residing in the blastocoel cavity suggests that they are undergoing apoptosis.

**Inhibition of PDGFR signaling causes mesoderm cells to die by apoptosis at the EGT**

To determine whether inhibition of PDGFR signaling induces apoptosis in mesoderm cells, embryos injected with 200 pg PDGFR-37 (Figs. 2C, D) or PDGFR-FS (Fig. 2B) mRNA at the 2-cell stage were examined by whole-mount TUNEL staining throughout gastrulation (stages 9 to 11). As a positive control for the TUNEL assay, uninjected embryos were treated with DNase I to create nicked-ends in the DNA before incubation with the TUNEL enzyme (Fig. 2A, see Materials and methods). To view TUNEL staining, the embryos were embedded and sectioned. Mesoderm cells first stain TUNEL-positive at stage 10.5 in PDGFR-37-injected embryos. (I–L) When assessed earlier in development, mesoderm cells with diffuse β-gal staining are found to appear in the blastocoel cavity of PDGFR-37-injected embryos beginning at stage 10. (E–H) At all stages, PDGFR-FS-injected embryos develop normally, and their mesoderm cells have nuclear β-gal staining. (M) The number of detached cells in the blastocoel cavity at stages 10, 10+, and 10.5 was assessed. Error bars represent standard error.
mRNA have no TUNEL-positive mesoderm cells (Fig. 2B). In both PDGFR-37- and PDGFR-FS-injected embryos, a small number of TUNEL-positive cells are found in the ectoderm (Figs. 2B, C). This is consistent with previously reported data showing that a small percentage of ectoderm cells normally undergo apoptosis at these stages (Hensey and Gautier, 1998). These data suggest that inhibition of PDGFR signaling causes mesoderm but not ectoderm cells to die by apoptosis.

TUNEL staining detects nicked DNA and thus does not necessarily distinguish between apoptotic and necrotic cell death (Gold et al., 1994). To verify that inhibition of PDGFR signaling results in apoptosis of mesoderm cells, caspase 3 activity was assessed in PDGFR-37-injected embryos (Fig. 2E). A freon-extracted lysate derived from crushed embryos that had been injected with 200 pg PDGFR-37 or PDGFR-FS mRNA was incubated with the caspase 3 substrate Ac-DEVD-pNA (Miyanaga et al., 2002). As a positive control for caspase 3 activity, uninjected embryos were cultured in 0.1 mg/ml cycloheximide to induce apoptosis and treated in tandem with the injected embryos (Hensey and Gautier, 1997). PDGFR-37-injected and cycloheximide-treated embryos have approximately a 3-fold increase in caspase 3 activity compared to that of sibling uninjected or PDGFR-FS-injected embryos at stage 11 (Fig. 2E). These data suggest that disruption of PDGFR signaling results in apoptosis of mesoderm cells at the EGT and that it occurs through the mitochondrial-apoptotic pathway.

Bcl-2 and caspase inhibitors protect mesoderm cells from apoptosis but do not rescue cell movements when PDGFR signaling is disrupted

It has been shown previously that the normal death of ectoderm cells at the EGT can be rescued by over-expression of Bcl-2 (Yeo and Gautier, 2003) suggesting that apoptosis occurs through the mitochondrial-apoptotic pathway at this time. To determine if the gastrulation defects seen in PDGFR-37-injected embryos result from mesoderm cell death via this apoptotic pathway, we over-expressed Bcl-2 in PDGFR-37-injected embryos. Embryos were co-injected into both cells at the 2-cell stage with 2 ng Bcl-2 mRNA, 200 pg PDGFR-37 mRNA, and β-gal-NLS mRNA. The presence of displaced cells in the blastocoel cavity with diffuse or punctate β-gal staining at stages 11 to 11.5 was

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**Table 1**

Disruption of PDGFR signaling induces apoptosis of mesoderm cells through the mitochondrial apoptotic pathway

<table>
<thead>
<tr>
<th>Gastrula</th>
<th>No dead cells</th>
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<tbody>
<tr>
<td>PDGFR-FS</td>
<td>98</td>
<td>0</td>
<td>129</td>
</tr>
<tr>
<td>XPDGFR</td>
<td>100</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>100</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>PDGFR-37</td>
<td>30</td>
<td>68</td>
<td>199</td>
</tr>
<tr>
<td>XPDGFR/PDGFR-37</td>
<td>61</td>
<td>39</td>
<td>44</td>
</tr>
<tr>
<td>Bcl-2/PDGFR-37</td>
<td>71</td>
<td>27</td>
<td>77</td>
</tr>
<tr>
<td>PDGFR-37 + VAD</td>
<td>63</td>
<td>32</td>
<td>87</td>
</tr>
<tr>
<td>PDGFR-37 + DEVD</td>
<td>67</td>
<td>32</td>
<td>95</td>
</tr>
<tr>
<td>PDGFR-FS + VAD</td>
<td>100</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>PDGFR-FS + DEVD</td>
<td>100</td>
<td>0</td>
<td>66</td>
</tr>
</tbody>
</table>

Cell death is assessed by the presence of mesoderm cells with diffuse β-galactosidase staining in the blastocoel cavity at stage 11 to 11.5. Note that cell death in embryos deprived of PDGFR signaling is rescued by injection of caspase inhibitors or mRNA encoding wild type XPDGFR or Bcl-2. The remaining percent of embryos died.

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used as an indication of cell death (Table 1). We found that a 10-fold excess of Bcl-2 to PDGFR-37 mRNA was able to prevent mesoderm cell death (Table 1 and Fig. 3C). Sixty-eight percent of PDGFR-37-injected embryos contained cells with diffuse β-gal staining in the blastocoel cavity (Table 1; Fig. 3B). This number was reduced to 27% when Bcl-2 mRNA was co-injected with PDGFR-37 mRNA (Table 1). In no instance did injection of Bcl-2 mRNA alone cause mesoderm cells to die or appear in the blastocoel (Table 1). This rescue of cell death is similar to that obtained when mRNA encoding wild type XPDGFRα is co-injected with PDGFR-37 mRNA (Table 1). Cells rescued by co-injection of Bcl-2 and PDGFR-37 mRNAs have distinct nuclear β-gal staining (Fig. 3C). Taken together, these data further support the hypothesis that inhibition of PDGFR signaling leads to mesoderm cell death through the mitochondrial-apoptotic pathway.

Over-expression of Bcl-2 prevents the apoptosis of mesoderm cells when PDGFR signaling is inhibited but it does not rescue gastrulation defects in these embryos (Figs. 3F, I; Table 1). A low percentage (29%) of embryos co-injected with Bcl-2 and PDGFR-37 mRNAs, however, develop with abnormal lumps along the dorsal axis (Fig. 3F). These lumps are visible at the tailbud stage and contain β-gal staining, indicating that they are derived from cells that received the injected mRNA (Fig. 3F). This “lump” phenotype is not seen in embryos co-injected with wild type XPDGFRα and PDGFR-37 mRNAs (see Fig. 1D). It is

<table>
<thead>
<tr>
<th>Tailbud</th>
<th>Gastrulation defects</th>
<th>n</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Mild</td>
</tr>
<tr>
<td>PDGFR-FS</td>
<td>76</td>
<td>15</td>
</tr>
<tr>
<td>XPDGFR</td>
<td>70</td>
<td>12</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>61</td>
<td>24</td>
</tr>
<tr>
<td>PDGFR-37</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>XPDGFR/PDGFR-37</td>
<td>55</td>
<td>17</td>
</tr>
<tr>
<td>Bcl-2/PDGFR-37</td>
<td>14</td>
<td>32</td>
</tr>
<tr>
<td>PDGFR-37 + VAD</td>
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<td>40</td>
</tr>
<tr>
<td>PDGFR-FS + VAD</td>
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<td>18</td>
</tr>
<tr>
<td>PDGFR-FS + DEVD</td>
<td>82</td>
<td>16</td>
</tr>
</tbody>
</table>

Tailbud staged embryos (stage 26–38) are scored according to phenotype: normal, mild or severe. Mild phenotype includes embryos with a slightly shortened axis, bent axis, and “bubble belly”. Severe phenotype includes significantly shortened axis, spina bifida and reduced anterior structures. The remaining percent of embryos died.
likely that such lumps are formed from cells destined to die when PDGFR signaling is blocked, but rescued from death by Bcl-2. The rescued cells are then presumably swept along with involuting trunk mesoderm cells and subsequently end up in an inappropriate location. In support of this notion, this lump phenotype is not seen when embryos are injected with Bcl-2 mRNA alone (Fig. 3G). Injection of Bcl-2 mRNA alone, however, does cause severe gastrulation defects in a small percentage of embryos (10%; Table 2, and Fig. 3G). These defects are most likely due to the rescue of

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**Fig. 4.** Inhibition of caspases rescues cell death but not gastrulation movements in PDGFR-37-injected embryos. Embryos were co-injected dorsoanteriorly with mRNAs encoding β-gal NLS and (A–F) PDGFR-FS or (G–L) PDGFR-37 at the 2- to 4-cell stage. At the blastula stage (stages 8–9), the blastocoel cavity was injected with 400 μM of (B, E, H, K) a general caspase inhibitor (z-VAD-fmk) or (C, F, I, L) a caspase 3 inhibitor (z-DEVD-fmk). The embryos were cultured until stage 11.5 or 28 before being fixed and stained for β-gal. Note, no β-gal-positive cells in the blastocoel of PDGFR-FS-injected embryos (A–C) and in the PDGFR-37-injected embryos, the rescue of cells with diffuse β-gal staining by caspase inhibitors (compare G with H, I). Caspase inhibitor-injected embryos, however, still exhibit gastrulation defects at stage 28 (compare J with K, L). Cells with diffuse β-gal staining are indicated (arrows).
neural ectoderm cells that would normally undergo apoptosis (Hensey and Gautier, 1998; Yeo and Gautier, 2003).

A similar rescue of apoptosis, but not gastrulation, is seen when caspase inhibitors are injected into the blastocoel of PDGFR-37-injected embryos (Fig. 4). Embryos were injected in both cells at the 2-cell stage with 200 pg PDGFR-37 mRNA and cultured until the blastula stages (stages 8 to 9). At this time, 400 μM of either the general caspase inhibitor z-VAD-fmk or the caspase 3 inhibitor z-DEVD-fmk was injected directly into the blastocoel cavity. The embryos were then scored at stages 11 (Figs. 4A–C, G–I) and 28 (Figs. 4D–F, J–L). Both caspase inhibitors were able to rescue the death of mesoderm cells (Figs. 4 H and I and Table 1) but not their gastrulation movements (Figs. 4K, L and Table 2).

Taken together, these data show that apoptosis of mesoderm cells resulting from disruption of PDGFR signaling can be rescued by inhibition of the mitochondrial-apoptotic pathway, but the normal migratory behavior of these cells is not restored. This suggests that PDGFR signaling is not simply a survival mechanism for mesoderm cells during gastrulation but plays an essential role in the control of their movement during gastrulation.

PDGFR signaling is not required for convergent extension of Keller explants

In *Xenopus* gastrulation, the prospective head mesoderm cells actively migrate across the blastocoel roof whereas the prospective axial mesoderm cells carry out a different type of cell movement. These cells undergo mediolateral intercalation in a tissue rearrangement called convergent extension (Keller, 1991; Wallingford and Harland, 2002). In our previous study, we examined the role of PDGFR signaling in convergent extension in exogastrulae (Ataliotis et al., 1995). These experiments suggested that PDGFR signaling is not involved in convergent extension, since PDGFR-37-injected exogastrulae elongate to a similar extent as control embryos. To strengthen these data and to determine whether deprivation of PDGFR signaling affects cell survival of the axial mesoderm, convergent extension and apoptosis were assessed in Keller explants (Fig. 5). Keller explants were dissected at stage 10.25 from embryos that had been injected with either PDGFR-FS or PDGFR-37 mRNA and Gap43-GFP mRNA at the 2-cell stage in the dorsoanterior marginal zone. The explants were cultured to stage 20 and scored. No difference in elongation of PDGFR-37-injected
explants versus control PDGFR-FS-injected explants was observed (compare Figs. 5A, D). Furthermore, no difference between TUNEL staining in PDGFR-37- and PDGFR-FS-injected explants at stage 20 was detected (compare Figs. 5B, C with E, F). Note a similar and low number of TUNEL positive—fluorescein-labeled—cells in Figs. 5B, C and E, F). Taken together, these data suggest that PDGFR signaling is not required for cell intercalation or survival of the axial mesoderm but plays a significant role in motility and survival of prospective head mesoderm cells.

Discussion

In this study, we demonstrate that inhibition of PDGFR signaling in **Xenopus** embryos leads to apoptosis in mesoderm cells at the onset of gastrulation. The involuting mesoderm cells normally express PDGFRα as they migrate across the ectoderm that expresses PDGF-A (Ataliotis et al., 1995). When PDGFR signaling is blocked in these cells, they do not adhere to the blastocoel roof, fail to migrate, and appear in the blastocoel cavity. These mesoderm cells enter the mitochondrial-caspase 3 apoptotic pathway at the early gastrula transition (EGT; Howe et al., 1995) when embryos become competent to undergo programmed cell death. This cell death can be prevented by over-expression of the anti-apoptotic factor Bcl-2 or by caspase inhibitors. However, the prevention of cell death is not sufficient to prevent gastrulation defects, suggesting that PDGFR signaling is not only serving as a survival factor, but also is required for proper migration of these cells. In contrast, cells of the prospective axial mesoderm can undergo convergent extension and do not undergo apoptosis when PDGFR signaling is blocked. This suggests that PDGFR signaling is less important for cell motility and survival of the axial mesoderm than the prospective head mesoderm during gastrulation.

**Inhibition of PDGFR signaling results in death of prospective head mesoderm cells**

PDGFR signaling promotes cell survival both in vivo and in a variety of cultured cells (reviewed in Heldin et al., 1998). For example, apoptosis is triggered in vascular endothelial cells in neonatal mice (Sano et al., 2002) and fibroblasts (Brennand et al., 1997) when signaling via PDGFRβ and PDGFR-BB are inhibited, respectively. Apoptosis also occurs in the neural crest derived tissues of PDGFRα null mice (Soriano, 1997). Initially reported in PC12 cells (Yao and Cooper, 1995), PDGFR signaling has been shown to promote cell survival through phosphatidylinositol 3-kinase (PI3K) and the anti-apoptotic kinase, Akt (for reviews, see Datta and Greenberg, 1999; Bergmann, 2002). In response to PDGFR signaling, PI3K activates Akt, which in turn phosphorylates the pro-apoptotic Bcl-2 family member BAD. If phosphorylated, BAD protein becomes bound in an inactive complex and thus, cells are protected from undergoing apoptosis. This pathway was recently shown to also function in vivo. Using a knock-in strategy, a mutant mouse was created in which BAD cannot be phosphorylated (BAD3SA; Datta et al., 2002). Cells cultured from these transgenic mice have a decreased rate of survival even in the presence of PDGF.

The rescue of the apoptosis of **Xenopus** mesoderm cells deprived of PDGFR signaling, however, is not sufficient to restore their motility. This suggests that during gastrulation, PDGFR signaling acts as more than a survival factor for these cells. In support of this notion, PDGFR signaling has been shown to affect a wide variety of behaviors in cells in culture, in tissues and during embryogenesis including cell motility, reorganization of the actin cytoskeleton, chemotaxis, and the control of cell division (for review, see Betsholtz et al., 2001; Heldin and Westermark, 1999; Hoch and Soriano, 2003). In **Xenopus** embryos, PDGFR signaling through PI3K has been shown to induce spreading of gastrula-staged mesoderm cells in culture (Symes and Mercola, 1996) and appears to provide guidance cues for prospective head mesoderm cells during gastrulation (T. Nagel, E. Tahinci, K. Symes and R. Winklbauer, unpublished observations). Likewise, PDGF signaling through PI3K in zebrafish mesendoderm has been shown to induce protrusions and polarization of the cells (Montero et al., 2003). Also, upon PDGF treatment, PKB/Akt becomes localized to the leading edge of the cells in a PI3K-dependent manner (Montero et al., 2003).

PDGFR activation can stimulate several different signaling pathways including those mediated by c-Src, Grb2, Grb7, PI3K, phospholipase Cγ (PLCγ), RasGAP, and the phosphotyrosine phosphatase Shp-2 (for review, see Heldin and Westermark, 1999). It is therefore likely that multiple signaling pathways are induced simultaneously and it is the balance of their activities that defines PDGFR function in a particular cell type. An example of this balance has been reported in fibroblast cells. In this case, PDGFR induces NFκB which in turn transmits two signals: one is required for the induction of the proto-oncogene c-myc and proliferation and the other an anti-apoptotic signal that is transmitted through the PI3K/Akt pathway and counterbalances c-myc (Romashkova and Makarov, 1999). During gastrulation in **Xenopus** embryos, PDGFR signaling likely induces an anti-apoptotic signaling pathway and one or more additional pathways important for cell motility.

**PDGFR signaling does not control convergent extension of Keller explants**

Inhibition of PDGFR signaling in Keller explants of the axial mesoderm neither prevents convergent extension nor affects cell survival. This is perhaps because the majority of axial mesoderm cells may not normally be subject to PDGFR signaling in vivo. During **Xenopus** gastrulation, while the prospective head mesoderm cells are in direct...
contact with ectoderm cells as they actively migrate across the blastocoel roof, the axial mesoderm cells undergo mediolateral intercalation within a tissue that is initially several cell layers deep (Keller, 1991). Thus, prospective axial mesoderm cells mostly do not appose the ectoderm, the site of expression of PDGFR-A (Ataliotis et al., 1995).

Additional signals may be necessary to induce apoptosis in prospective head mesoderm cells deprived of PDGFR signaling

Inhibition of PDGFR signaling alone may not be sufficient to induce apoptosis in future head mesoderm cells. Explants or dissociated single cells of *Xenopus* prospective head mesoderm can be cultured on a fibronectin matrix in a simple balanced salts solution that does not contain growth factors, (Symes et al., 1994; Winklbauer and Keller, 1996; Winklbauer and Nagel, 1991). These cells do not express PDGF (Ataliotis et al., 1995), yet they do not die, are actively motile, and can differentiate in vitro (Godsave and Slack, 1991; Symes et al., 1994; Winklbauer and Keller, 1996; Winklbauer et al., 1996). In addition, explants of *Xenopus* presumptive head mesoderm that have been dissected from embryos in which PDGFR signaling has been blocked can also migrate on fibronectin or a blastocoel roof matrix (T. Nagel, E. Tahinci, K. Symes and R. Winklbauer, unpublished observations). These data suggest that additional signals within the embryo may be necessary to initiate apoptosis in embryos deprived of PDGFR signaling.

It is clear that environment directly affects cell activity. For example, spatial organization is important for the growth and adhesion of human mammary epithelial cells. When organized in three-dimensional basement membrane cultures, the signaling pathways that control growth and adhesion of these cells are coupled and bi-directional. When such cells are cultured as a two-dimensional, monolayer, however, the same signaling pathways become uncoupled (Wang et al., 1998). It is likely that in the embryo, signaling pathways are similarly coupled and may account for the differences in the survival of *Xenopus* presumptive head mesoderm in situ and in vitro. The challenge now is to identify the role of different signaling pathways acting downstream of the PDGFR and to determine how these pathways are coupled to control cell survival and motility during gastrulation.

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References


Jones, S.D., Ho, L., Smith, J.C., Yordan, C., Stiles, C.D., Mercola, M.,...


