

Autocrine/Paracrine Platelet-Derived Growth Factor Regulates Proliferation of Neural Progenitor Cells

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Abstract

Growth factors play an important role in regulating neural stem cell proliferation and differentiation. This study shows that platelet-derived growth factor (PDGF) induces a partial differentiation of neural stem/progenitor cells (NSPCs) in the absence of other mitogens *in vitro*. NSPCs thus acquire an immature morphology and display markers for both neurons and glia. In addition, these cells do not readily mature in the absence of further stimuli. When NSPC cultures treated with PDGF were exposed to additional differentiation factors, however, the differentiation proceeded into neurons, astrocytes, and oligodendrocytes. We find that NSPC cultures are endowed with an endogenous PDGF-BB production. The PDGF-BB expression peaks during early differentiation and is present both in cell lysates and in conditioned medium, allowing for autocrine as well as paracrine signaling. When the NSPC-derived PDGF was inhibited, progenitor cell numbers decreased, showing that PDGF is involved in NSPC expansion. Addition of a PDGF receptor (PDGFR) inhibitor resulted in a more rapid differentiation. Neurons and oligodendrocytes appeared earlier and had more elaborate processes than in control cultures where endogenous PDGFR signaling was not blocked. Our observations point to PDGF as an inducer of partial differentiation of NSPC that also sustains progenitor cell division. Such an intermediate stage in stem cell differentiation is of relevance for the understanding of brain tumor development because autocrine PDGF stimulation is believed to drive malignant conversion of central nervous system progenitor cells. (Cancer Res 2006; 66(16): 8042-8)

Introduction

During neural development, stem cell number and the subsequent formation of neurons, astrocytes, and oligodendrocytes must be tightly controlled. Failure in this regulation may lead to disorganization of the central nervous system (CNS) or the development of tumors. External signals are thought to play an important role for the regulation of neural stem/progenitor cell (NSPC) fate (1, 2). *In vitro*, fibroblast growth factor-2 (FGF2) and epidermal growth factor are potent mitogens for NSPC (3), whereas ciliary neurotrophic factor (CNTF) directs NSPC to an

astrocytic cell fate (4, 5) and the thyroid hormone triiodothyronine (T₃; ref. 4) augments oligodendrocyte differentiation. To date, no one-step neuronal inducer has been described, although activation of proneural genes is clearly involved in lineage restriction and the acquisition of a neuronal fate (6). Platelet-derived growth factor (PDGF) has been suggested to support neuronal differentiation (4, 7), and we have previously reported that PDGF acts as a mitogen for immature neurons (8).

PDGF binds to and signal through the two cell surface receptor tyrosine kinases, PDGF receptor (PDGFR) α and PDGFR β (9). The PDGF ligands are present as homodimers (AA, BB, CC, and DD) and heterodimer (AB). Binding of the ligand causes dimerization of the PDGFR followed by an activation of intracellular signaling cascade (10, 11). The PDGFRs have different binding capacities for the ligands. The β -receptor binds PDGF-B and PDGF-D, whereas the α -receptor binds PDGF-A, PDGF-B, and PDGF-C.

Both the PDGFRs and their ligands are expressed during CNS development. PDGF-A has been detected in the subventricular zone at embryonic day 11.5 (E11.5; ref. 12) and in neurons in the spinal cord and dorsal root ganglia of E12 mice. From E15 and onwards, PDGF-A is found in most neurons (13). Similar to PDGF-A, PDGF-B is expressed by neurons in the adult brain, and PDGF-B-positive cells were also found in the subventricular zone in E14 mice (14). PDGFR α is predominantly expressed by oligodendrocyte precursors during neural development (15), and loss-of-function studies further enhance the role for PDGF in oligodendrocyte development (9).

PDGF is a potent growth and differentiation factor during normal development and has also been implied in tumor development. Many types of brain tumors express the PDGFRs and their ligands (16), and retroviruses encoding PDGF have been reported to induce brain tumors in mice (17–19). Several recent studies suggest that gliomas arise from transformed NSPCs and stem cells have also been isolated from brain tumors (20, 21). We therefore sought to define the effect of PDGF in NSPC differentiation. In this study, we find that PDGF has growth-promoting capacity for progenitors from the CNS *in vitro*. Furthermore, autocrine/paracrine PDGF from NSPC supports early neurogenesis and gliogenesis. Autocrine PDGF signaling may therefore reflect a normal trait of progenitor cells that is retained in their transformed derivatives.

Materials and Methods

Cell culture. NSPCs from embryonic cerebral cortex of timed pregnant Sprague-Dawley rats (B&K, Sollentuna, Sweden), E15, were prepared and maintained as described previously (8). NSPCs were used for experiments 1 to 4 days after the first passage.

For differentiation of NSPC, FGF2 was withdrawn and PDGF-AA (10 ng/mL) or PDGF-BB (10 ng/mL) was added daily. After 4 days, PDGF

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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was withdrawn and the cells were grown for an additional 4 days. Control cells were grown for 8 days in the absence of PDGF and FGF2. B27 supplement (1:50), CNTF (10 ng/mL), and T3 (3 ng/mL) were added daily during the 4 last days after the withdrawal of PDGF-AA.

To study the effect of endogenous PDGF on NSPC fate determination, NSPCs were initially placed on polyornithine/fibronectin glass coverslips (3,700 cells/cm²) in the presence of FGF2. After 2 days, FGF2 was withdrawn and cells were treated with either PDGF-BB (10 ng/mL), STI 571 (1 μ mol/L), or vehicle and fixed in 4% paraformaldehyde in PBS (pH 7.4) after 1, 2, 4, and 6 days of differentiation and processed for immunocytochemistry. For staining with antibodies to PDGF-BB, NSPCs grown in the presence of FGF2 were fixed and processed for immunocytochemistry.

Immunocytochemistry. NSPCs were grown on polyornithine/fibronectin-coated glass coverslips and fixed in ice-cold acid ethanol (90% ethanol, 5% acetic acid) or in 4% paraformaldehyde (O4 staining and STI 571 treatment of NSPC during differentiation). Cells were permeabilized with 0.2% Triton X-100 in PBS (except for O4 staining), washed in PBS, and incubated in blocking solution [20% normal goat serum (DAKO A/S, Glostrup, Denmark), 0.1% saponin (Sigma, St. Louis, MO), and 4 mg/mL bovine serum albumin (BSA; Sigma)] in PBS for 30 minutes at room temperature. The cells were incubated with the following primary antibodies diluted in PBS with 0.1% saponin and 4 mg/mL BSA for 1 hour at 37°C: mouse β -III tubulin (BabCO, 1:500; Nordic Biosite AB, Täby, Sweden), rabbit anti-GEAP (1:200; DAKO A/S), mouse anti-CNPase (1:500; Sigma), mouse anti-O4 (1:30; Chemicon, Temecula, CA), mouse anti-PDGF-BB (007; 1:50; Biosciences Research Laboratories, Mochida Pharmaceutical, Tokyo, Japan), rabbit anti-PDGFR α (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse/rabbit anti-*nestin* (1:200; Chemicon). NSPCs were washed 5 to 6 times in PBS and incubated with Cy3-, AMCA-, or FITC-conjugated goat anti-mouse/rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Pictures were taken with a 20 \times objective on a Zeiss Axioplan 2 (Carl Zeiss, Oberkochen, Germany).

Cell counting. Following the immunostainings, positive cells were counted using a fluorescence microscope (Carl Zeiss). For duplicate samples, positively stained cells and the total cell numbers were counted in eight different fields. Cell counting for the STI 571 inhibition studies was done using a Coulter Z1 cell counter (Coulter Electronics, Herten, United Kingdom). The cells were removed from the dishes with a cell scraper, after which they were carefully dissociated and counted. Each time point was analyzed in triplicate.

Proximity ligation. Samples were analyzed for PDGF-BB by homogeneous proximity ligation using a polyclonal anti-PDGF-B chain antibody (PeproTech, Rocky Hill, NJ) as described before (22). Before analysis, the samples were boiled for 10 minutes and diluted 10 times in PBS containing 0.1% BSA. Each sample (1 μ L) was analyzed in triplicates. Quantification of the total protein content was done using the NanoOrange kit (Molecular Probes, Eugene, OR). Results of the analysis are expressed as percentage of maximal values (pg of PDGF-BB/mg of total amount of protein in lysates and pmol/L in the medium).

Statistical analysis. For statistical analysis, the ANOVA method was used followed by multiple comparison by Fisher's method as well as Student's *t* test. Values presented are the mean \pm SEM.

Results

PDGF delays NSPC differentiation. NSPCs from the embryonic rat cortex were expanded in medium containing FGF2, which inhibits differentiation. After FGF2 withdrawal, NSPC cultures were treated with PDGF during the first 4 days of differentiation. Following washout of PDGF, cultures were kept for an additional 4 days in the absence of growth factor, and the ability of PDGF-pretreated cells to differentiate to mature neurons was studied. Control cultures contained β -III tubulin-positive cells with a mature neuronal phenotype (Fig. 1A). In contrast, in cultures that had been pretreated with PDGF-AA or PDGF-BB, β -III tubulin-positive cells were immature: had a rounded appearance, lacked

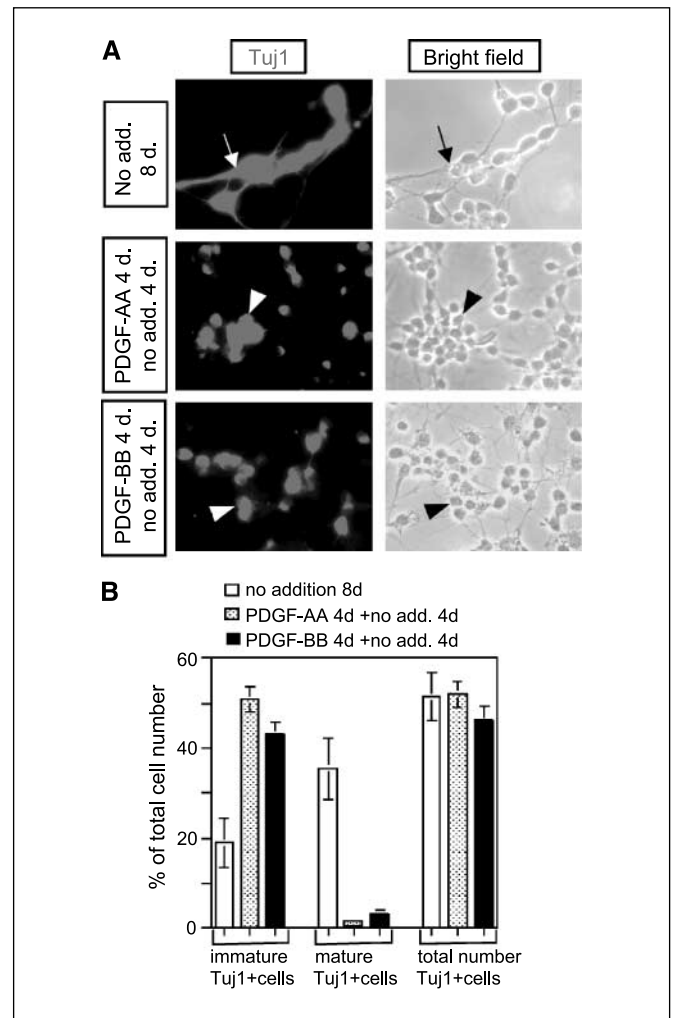


Figure 1. PDGF alters neuronal maturation without increasing the proportion of neurons. *A*, after FGF2 withdrawal, NSPCs were grown either for 8 days in medium without mitogens (*No add.*) or for 4 days in the presence of PDGF-AA or PDGF-BB followed by 4 days in medium without PDGF. Cells were fixed and stained for the neuronal marker β -III tubulin (*TuJ1*). Depending on their morphology, the *TuJ1*-positive cells were divided into two subgroups. Cells extending long processes were judged to be more mature neurons (*arrows*), whereas round cells bearing no processes were defined as immature neurons (*arrowheads*). *B*, quantification of cells with neuronal morphology. The proportion of *TuJ1*-positive cells that lacked processes (immature) was increased by a factor of 2 in PDGF-treated cell cultures (control, 19.0 \pm 5.5%; PDGF-AA, 50.8 \pm 2.6%; PDGF-BB, 42.9 \pm 2.8%). Mature neurons (bearing processes) were almost exclusively found in cultures that had not been exposed to PDGF. (control, 35.4 \pm 6.7%; PDGF-AA, 1.3 \pm 0.6%; PDGF-BB, 3.0 \pm 1.1%). The total number of *TuJ1*-positive cells remains unchanged by PDGF.

processes, and tended to grow in clusters. A slightly more potent effect of PDGF-AA than PDGF-BB was noticed; particularly, the cell density was higher with PDGF-AA and the cell clusters contained more cells. Thus, NSPC pretreated with PDGF did not readily mature further when kept without mitogen. Instead, they maintained an immature phenotype, suggesting that exposure to PDGF delayed terminal differentiation.

PDGF alters neuronal maturation without increasing the proportion of neurons. The number of immature (no processes) and mature (process bearing) β -III tubulin-positive cells was counted and displayed as the percentage of total cells (Fig. 1B). In control cultures, most neurons are mature, 35.4 \pm 6.7% of the

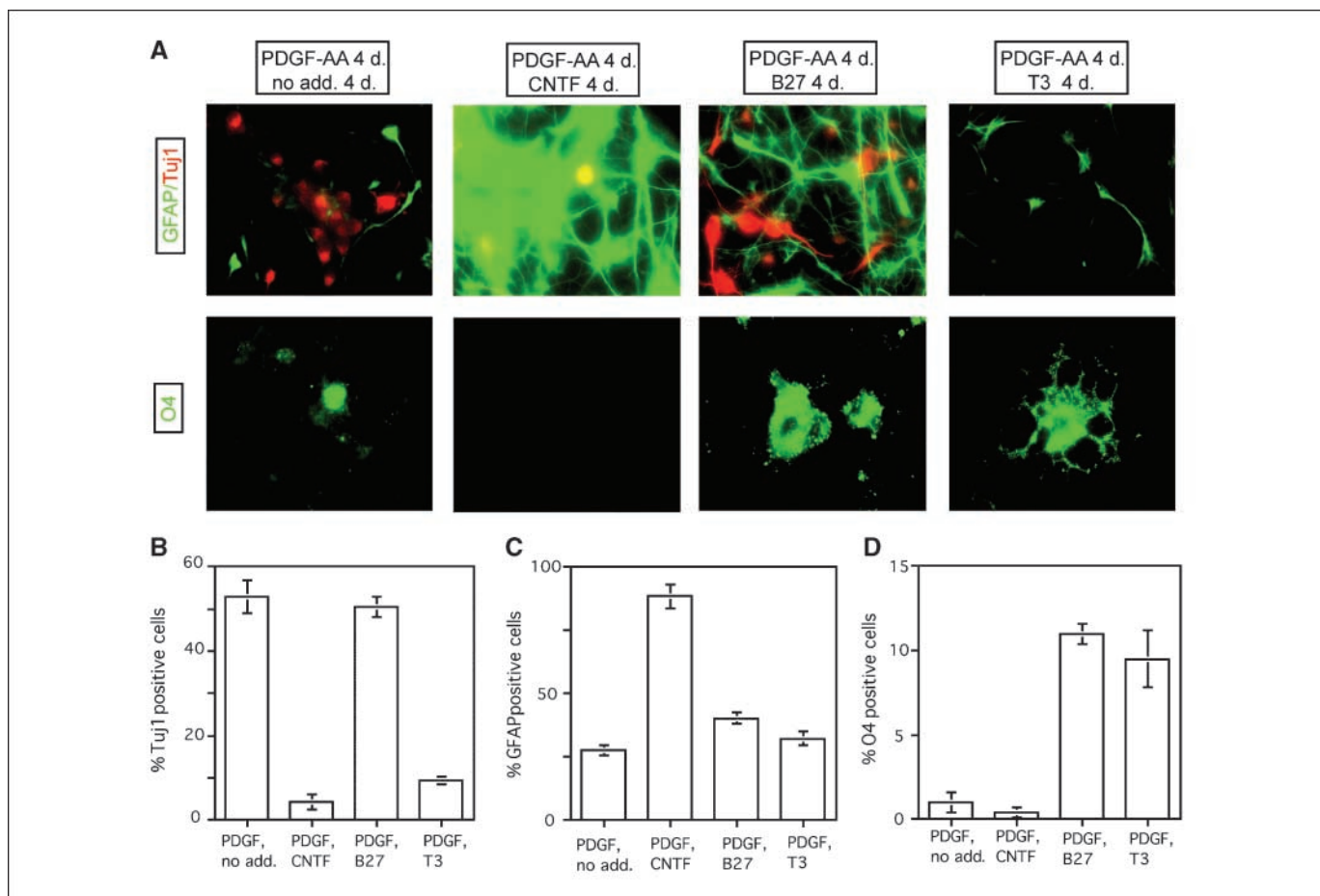


Figure 2. The PDGF-induced delay in differentiation can be overcome by additional stimulation. *A*, NSPCs were first treated with PDGF-AA for 4 days and then with CNTF, B27, or T3 for 4 days. Cells were fixed and stained for GFAP (green), Tuj1 (red), or O4 (green). Control cultures where PDGF was discontinued resulted in an immature morphology after 4 days, whereas B27 increased the maturity of all three lineages. CNTF converted PDGF-pretreated cells into almost exclusively astrocytes. T3 treatment decreased the number of neurons formed and increased the number of O4-positive cells. *B* to *D*, quantification of cell lineages formed after PDGF exposure that was followed by addition of B27, CNTF, or T3.

total cell number, compared with $19.0 \pm 5.5\%$ immature neurons. In contrast, the PDGF-treated cultures contained very few mature neurons but instead a large population of immature neurons. For the PDGF-AA-treated cultures, $1.3 \pm 0.6\%$ of the neurons were mature and $50.8 \pm 2.6\%$ were immature, whereas the corresponding numbers for PDGF-BB-treated cultures were $3.0 \pm 1.1\%$ and $42.9 \pm 2.8\%$, respectively. We did not notice any difference in the total number of β -III tubulin-positive cells between control and PDGF-treated cultures, indicating that PDGF does not increase the amount of neurons.

Astrocyte cell number is not affected by PDGF. Next, we investigated the effect of PDGF-AA and PDGF-BB on formation of astrocytes and oligodendrocytes. FGF2 was withdrawn, and NSPCs were treated with PDGF-AA or PDGF-BB or untreated for 4 days. This was followed by an additional 4 days without mitogens, after which the number of astrocytes and oligodendrocytes was counted. In control cultures, $34.6 \pm 3.3\%$ of the cells were positive for GFAP, and this remained unchanged by PDGF treatment ($36.4 \pm 1.4\%$ for PDGF-AA and $33.6 \pm 3.1\%$ for PDGF-BB). Although the amount of GFAP-positive cells was not affected by PDGF exposure, we noted a less developed morphology of astrocytes after PDGF treatment (data not shown). Oligodendrocytes derived from NSPC in culture appear later than neurons and astrocytes and are few in

number (2-10%). We noticed a slight reduction in O4-positive cells in cultures treated with PDGF-AA ($1.0 \pm 0.6\%$) or PDGF-BB ($2.1 \pm 0.5\%$) compared with untreated cultures ($6.4 \pm 0.9\%$).

The PDGF-induced maturation delay can be overcome by additional treatment. We next investigated if PDGF-treated cells that were unable to differentiate into mature neurons simply by withdrawing PDGF could be influenced to differentiate by additional stimuli. The medium supplement B27 used to enhance neuronal maturation (23, 24), CNTF, or the thyroid hormone T3 shown to facilitate differentiation into oligodendrocytes were used.

After PDGF-AA exposure in the absence of other mitogens, NSPCs were grown for another 4 days in medium alone or in medium containing B27, CNTF, or T3. The cells were fixed and stained with antibodies against β -III tubulin, GFAP, or O4 (Fig. 2A). In cultures that were kept in medium alone for 4 days after PDGF-AA washout, both neurons and astrocytes showed an immature morphology as mentioned above and only a few oligodendrocytes were detected. B27 treatment resulted in more mature cells of all three lineages, although the effect was most pronounced for neurons. In contrast to the round immature β -III tubulin-positive cells found in cultures treated with PDGF-AA, neurons in B27-treated cultures extended long processes. Cells that were treated with CNTF following PDGF-AA treatment had almost

exclusively differentiated into astrocytes with a mature phenotype. Thus, when exchanging PDGF for CNTF, hardly any β -III tubulin-positive cells or O4-positive cells could be detected, indicating that CNTF could exert a similar effect on PDGF-AA-treated stem cell cultures as on naive NSPC. This further suggests that PDGF-AA-treated cells were not committed to a neuronal cell fate. Treatment of the cells with T3 had a weakly enhancing effect on the number of O4-positive cells and caused a reduction in β -III tubulin-positive cells.

The proportion of neurons (Fig. 2B) was markedly reduced by CNTF and T3, $4.3 \pm 1.8\%$ and $9.5 \pm 0.9\%$, respectively, compared with $52.8 \pm 3.9\%$ in the control culture. In B27-treated cultures, neurons were more mature but the total neuronal number was not affected. The proportion of astrocytes (Fig. 2C) was increased from $27 \pm 2.0\%$ to $88.5 \pm 4.7\%$ when the PDGF-AA-exposed cells were treated with CNTF, whereas T3 and B27 had no obvious effect on the astrocyte number. A low proportion ($6.4 \pm 0.9\%$) of the PDGF-treated cells that received no further stimulus were positive for O4 (Fig. 2C), and this proportion was even lower in CNTF-treated cultures ($0.4 \pm 0.3\%$). Both B27 and T3 increased the number of oligodendrocytes, resulting in $11 \pm 0.6\%$ and $9. \pm 1.7\%$ O4-positive cells, respectively.

Progenitors for neurons and oligodendrocytes express PDGF α receptors. We have previously shown that PDGFR α is expressed by proliferating NSPC in culture (8), whereas PDGFR β is not detectable until after several days of differentiation. It is not known which progenitor cell population continues to express PDGFR α on FGF2 withdrawal, and we therefore investigated if the

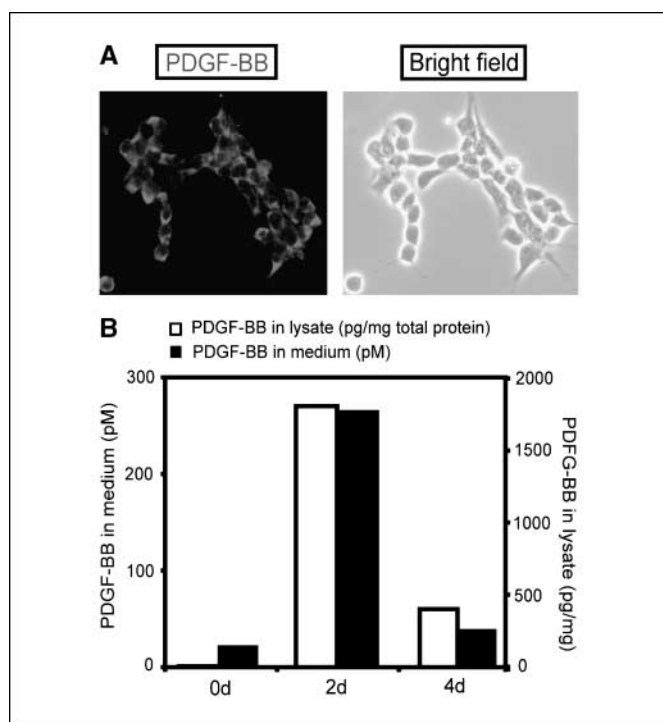


Figure 3. Endogenous PDGF-BB produced by NSPC. *A*, the vast majority ($95.7 \pm 1.5\%$) of NSPCs in culture are immunoreactive for PDGF-BB. *B*, levels of PDGF-BB were measured with the proximity ligation assay in cell lysates and in conditioned medium from NSPC during conditions of proliferation and differentiation. In the presence of FGF2, PDGF-BB can be detected at low levels. After FGF2 withdrawal, PDGF-BB levels in both lysate and conditioned medium increased by 10-fold at day 2 of differentiation. By 4 days, the levels of PDGF-BB have returned to 20% of its maximal value.

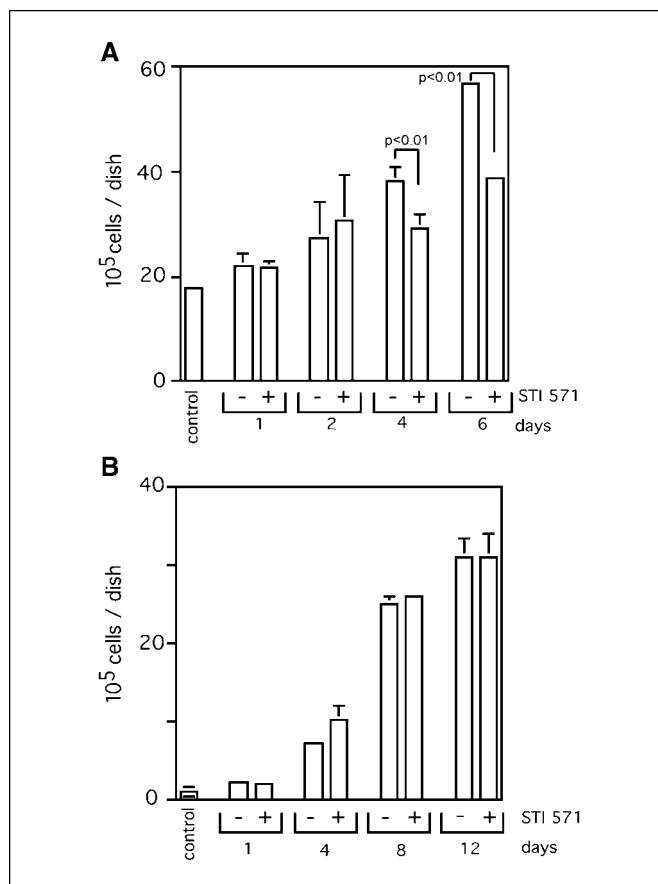


Figure 4. Endogenous PDGF from NSPC stimulates progenitor proliferation. *A*, after withdrawal of FGF2, NSPCs were treated daily with the tyrosine kinase inhibitor STI 571 or vehicle and counted at 1, 2, 4, and 6 days. STI 571 addition abolished the increase in cell numbers beyond 2 days of differentiation. *Bars*, SEM. *B*, endogenous PDGF had no effect on cell number in the presence of FGF2. NSPCs were treated daily with STI 571 or vehicle in the presence of FGF2. After 12 days in culture, the total cell number had increased 25 times, showing lack of toxic effect by STI 571. *Bars*, SEM.

distribution of PDGFR α differs between progenitor cell types. Immunostainings done after 3 days of differentiation show that PDGFR α -expressing cells mainly displayed markers for neurons and oligodendrocytes and, to a lesser extent, for astrocytes (Supplementary Fig. S1).

Autocrine/paracrine PDGF-BB from NSPC. The concept of autocrine/paracrine signaling has been discussed in the context of PDGF-induced brain tumors as it enables cells to respond to their own growth factor or PDGF secreted by close neighboring cells. We have noticed that NSPC density is a determining factor in growth arrest and that dense cultures continue to proliferate for a few days after withdrawal of FGF2 presumably because of supportive factors from cells in close contact, whereas sparse cultures do not.³ We reasoned that PDGF might be one factor that signals in the microenvironment of NSPC. To investigate if NSPCs produce PDGF, we used two independent methods: immunostaining and the more recently developed proximity ligation method. Figure 3A shows that NSPCs grown in the presence of FGF2 are immunopositive for PDGF-BB ($95.7 \pm 1.5\%$ of the nestin-positive cells also displayed

³ A. Erlandsson, unpublished observation.

PDGF-BB immunoreactivity). Small amounts of protein as well as changes in protein levels can accurately be detected by proximity ligation. PDGF-BB is produced (in the range of pg/mg protein) and is present in cell lysates of NSPC in FGF2-containing medium (Fig. 3B). On withdrawal of FGF2, the levels increase 10-fold at 2 days of differentiation, and by 6 days, the PDGF-BB concentration has returned to 15% of its maximal value. PDGF-BB is also secreted into the medium of NSPC cultures with the same peak at 2 days of differentiation (Fig. 3B). Thus, although some PDGF-BB remained cell associated or within the cell, some of the secreted protein was also made available to other cells pointing at possibilities for both short- and long-range effects.

NSPC-derived PDGF-BB drives progenitor cell proliferation during early differentiation to neurons and glia. To investigate if the PDGF-BB produced by NSPC during early differentiation plays a role in progenitor proliferation, we blocked signaling through PDGFRs. Cells were grown in the presence or absence of a receptor tyrosine kinase inhibitor, STI 571, and counted at the starting point of the experiment and at different times after initiation of differentiation. In the absence of FGF2, there was a 3-fold increase in the total cell number after 6 days (Fig. 4A). Clearly, one or several endogenous factor/s are able to expand progenitor cells at this stage. In cultures treated with STI 571, further cell division was stopped beyond 2 days of differentiation, which coincides with the peak of PDGF expression. In control

cultures, STI 571 was added to cells growing in the presence of FGF2 (Fig. 4B). After 12 days, the total cell number was 25 times higher than at the starting point of the experiment regardless of whether STI 571 was added or not. The inhibitor thus had no unspecific toxic or growth-arresting effects on NSPC.

The above result prompted us to find out how the inhibition of PDGF signaling affected different cell lineages deriving from NSPC. During differentiation induced by withdrawal of FGF2, the amount of nestin-positive cells declines as a result of cell fate choice and markers of neurons and glia gradually appear. At 2 days of differentiation, there were fewer nestin-positive cells in STI 571-treated cultures compared with control cells, indicating a more rapid differentiation when PDGFR signaling was blocked (Fig. 5A). In the presence of STI 571, neurons (Fig. 5B) and oligodendrocytes (Fig. 5C) were more abundant at an earlier stage than in control cultures. A similar trend, which could not be statistically confirmed, was also seen for astrocytes (Fig. 5D).

In addition to an altered cell number, the morphology of neurons and oligodendrocytes was altered (Fig. 6). In cultures treated with STI 571 for 4 days, elaborate neurite extensions with long processes were seen on β -III tubulin-positive cells. Oligodendrocytes were also morphologically more mature. Thus, the NSPC-derived PDGF expands the pool of immature progenitors during early differentiation and has most profound effects on neurons and oligodendrocytes.

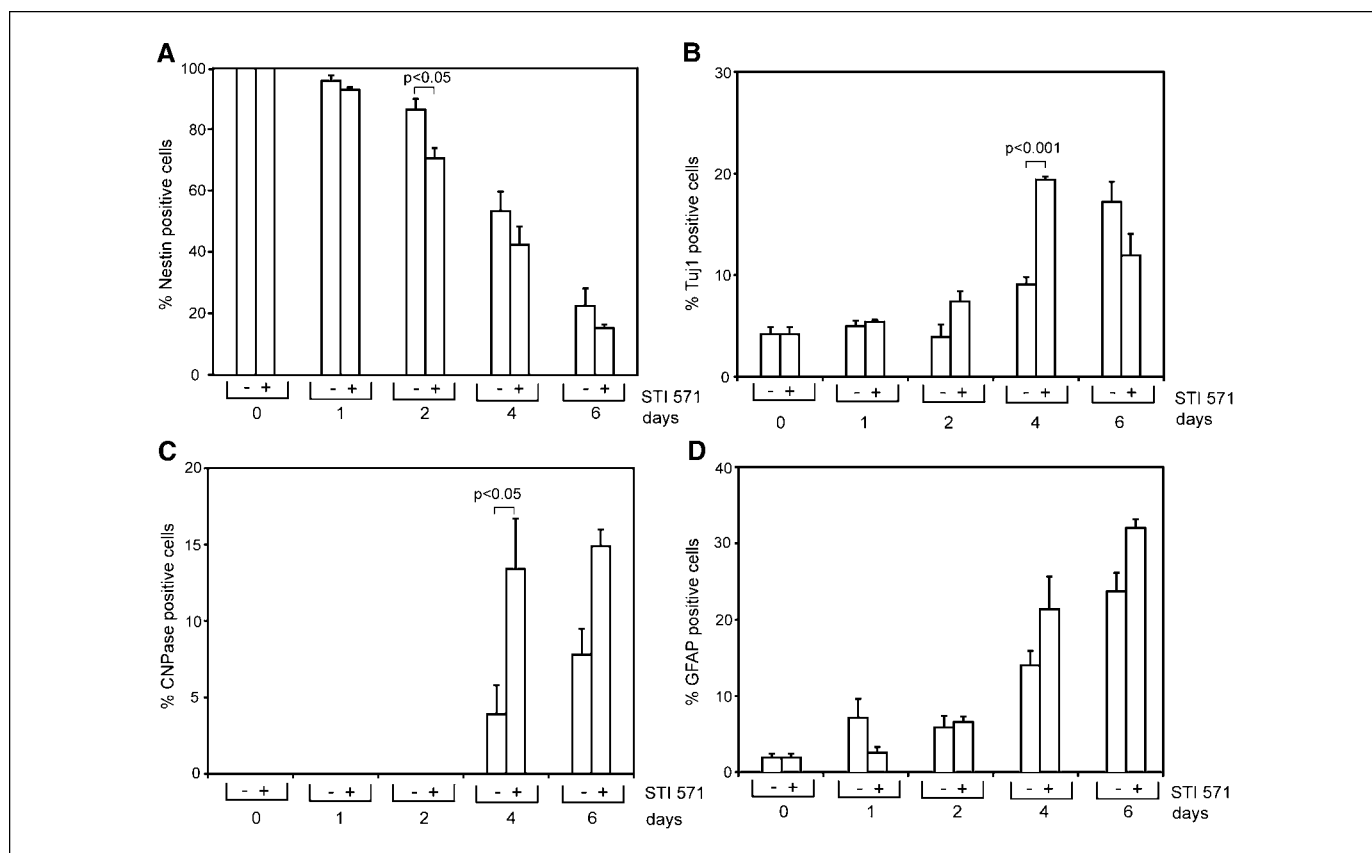


Figure 5. Inhibition of endogenous PDGF alters the differentiation kinetics of NSPC. NSPCs were differentiated by withdrawal of FGF2 in the presence or absence of STI 571 for 0, 1, 2, 4, and 6 days. Cells were fixed and processed for immunocytochemistry. *A*, in STI 571-treated cultures, there are fewer nestin-positive cells at day 2 of differentiation. *B*, STI 571 results in an increase in the number of neurons expressing β -III tubulin at day 4 of differentiation. *C*, the number of oligodendrocytes increases when STI 571 is added. *D*, STI 571 has no significant effect on the generation of astrocytes.

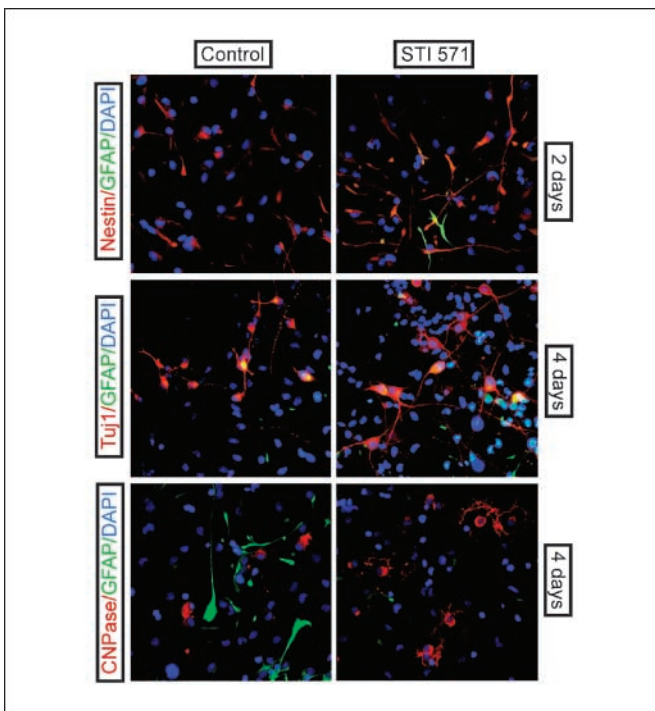


Figure 6. Blocking of PDGFR signaling increases the morphologic maturation of neurons and oligodendrocytes. STI 571 increases the maturity of β -III tubulin and CNPase-positive cells with regard to neurite outgrowth and process arborization, respectively. DAPI, 4',6-diamidino-2-phenylindole.

Discussion

We here describe that PDGF retains progenitors from NSPCs in an immature state *in vitro*, thus keeping them from terminal differentiation. This is particularly shown for the neuronal and oligodendrocyte lineages, although PDGF did not markedly affect differentiation to astrocytes. Furthermore, we show that a peak of autocrine/paracrine PDGF-BB produced by NSPC during initial differentiation acts to expand precursors of neurons and oligodendrocytes.

In the context of neural stem cell culture, PDGF was first shown to stimulate the formation of neurons from NSPC (4). Subsequently, the potential by which PDGF acts as a neurogenic inducer has been a subject of some debate. Although there are reports that a short exposure to PDGF suffices to induce neuronal differentiation (7), others, including our own laboratory, find the effect of PDGF mainly to expand neural progenitors (8). Data to support this conclusion come from the study by Mohapel et al. (25) who detected an overall increase in striatal cell number but not in newly generated neurons after intracerebroventricular infusion of PDGF. Others suggest that PDGF instead reduces the number of neurons in human neural stem cell cultures (26). We report that NSPC-derived neurons generated in the presence of PDGF were almost completely devoid of neurites, thus only partially differentiated. However, the total number of neurons did not differ from control cultures and this shows that the effect of PDGF relates to suppression of maturation as shown by the shift in neuronal morphology.

More mature cell types of all three lineages were induced by addition of B27 after washout of PDGF. In contrast, cultures that were not replenished with other supplements after removal of

PDGF were slower to establish a differentiated phenotype. This is indicative of a block of terminal differentiation that continued over several days. In accordance, addition of T3, which is known to augment the differentiation of oligodendrocytes, led to a higher proportion of oligodendrocytes compared with untreated cultures (4). The instructive effect of CNTF on NSPC is well documented (4, 5). We found that it was possible to direct PDGF-treated cells toward the astrocytic lineage using CNTF. This means that cultures, in which 50% of the cells express β -III tubulin, can be redirected to 90% GFAP-positive cells. The cytoskeletal protein β -III tubulin is commonly used as a neuronal marker (27, 28), but transient β -III tubulin expression has also been reported in telencephalic subventricular zones comprising putative neural and glial precursors (reviewed in ref. 29). We can therefore not conclude whether β -III tubulin-positive cells switching into GFAP expression reflect a transient marker expression of cells that can adopt a glial fate or whether this reflects lineage plasticity.

We have observed that NSPCs in culture continue to proliferate even after FGF2 withdrawal in a density-dependent manner.³ This would indicate that diffusible factors and/or cell-cell contacts inherent to the stem cell culture stimulate proliferation in the absence of exogenous FGF2. A previous study reported that conditioned medium from dense neural stem cells increased the number of viable cells and clone size when stem cells were seeded at clonal density (30), but the diffusible factor(s) secreted remained unidentified. Given our findings that PDGF can act as a mitogen for progenitors from stem cells and reduces apoptosis (8), PDGF could be one such inherent factor. Using two independent detection methods, we find that NSPC cultures produce PDGF-BB. The highly specific and sensitive proximity ligation assay (22, 31) revealed a peak of PDGF production during early NSPC differentiation. This method of measuring protein concentrations, which converts the actual protein detection to the analysis of a DNA sequence, enabled detection of PDGF-BB both in cell lysates and in conditioned medium. PDGF-BB in the cell lysate may be located either intracellularly or associated with proteoglycans of the extracellular matrix (32, 33), owing to a retention signal in the PDGF-BB protein. This could result in a localized high concentration of the growth factor. The proximity ligation assay also detected PDGF in the conditioned medium. Making PDGF available to other cells allows for paracrine as well as autocrine signaling and may account, at least in part, for proliferative effect described above.

Because addition of the small-molecule inhibitor STI 571 reduces cell number in progenitor cultures, we suggest that endogenous PDGF-BB stimulates progenitor proliferation. STI 571 blocks the activation of type III tyrosine kinase receptors, including the receptors for PDGF, stem cell factor (SCF), and the abl tyrosine kinase (34). We have earlier reported that SCF has no effect on neural stem cell proliferation (35) and its involvement is therefore less likely. The importance of the ableson nonreceptor kinase (abl) for proper axon outgrowth and path finding was shown for *Drosophila* (36), and cytoskeletal rearrangements in developing mammalian neurons involve abl (reviewed in ref. 37). A role for abl in neural stem cells has not been suggested but can at present not be ruled out.

When PDGFR signaling was blocked during differentiation of NSPC, neurons and oligodendrocytes differentiated more rapidly. For the astrocytic lineage, there was a similar trend, which could not be statistically confirmed. Neurons that form from NSPC in the presence of STI 571 have longer and more elaborate processes than those of control cultures. The same was true for oligodendrocytes

that exhibited an extensive arborization of their processes, indicative of a mature morphology. Thus, PDGF inherent to NSPC cultures from the embryonic rat cortex expands pools of immature precursors during early differentiation.

Progenitors of neurons and glia with an autocrine PDGF signaling have been suggested as an origin of malignant brain tumors (18). PDGF and PDGFRs are frequently coexpressed in human glioma cell lines, and some glioma cell lines can be growth arrested by PDGFR inhibitors (16). *In vivo*, during normal development, the ligand is generally confined to one cell type and the receptor is expressed by an adjacent cell layer (9). The concomitant expression of both PDGF and its receptor in cultures of normal cells from the CNS has to our knowledge not been

reported before. This study is based on *in vitro* cell culture; nonetheless, the finding that cultures of primary NSPC regulate their own proliferation by autocrine/paracrine PDGF provides a new link between regulation of normal cell growth and the perturbed growth pathways of malignant brain tumors. Autocrine PDGFR signaling in glioma cells may reflect a natural trait of their normal progenitor.

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