J Neurooncol (2010) 98:11–19
DOI 10.1007/s11060-009-0049-4

LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Imatinib mesylate (Glivec) inhibits Schwann cell viability and reduces the size of human plexiform neurofibroma in a xenograft model

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Received: 28 April 2009 / Accepted: 26 October 2009 / Published online: 17 November 2009

Abstract Plexiform neurofibromas (PNF), one of the major features of neurofibromatosis type 1 (NF1), are characterized by complex cellular composition and mostly slow but variable growth patterns. In this study, we examined the effect of imatinib mesylate, a receptor tyrosine kinase inhibitor, on PNF-derived Schwann cells and PNF tumour growth in vitro and in vivo. In vitro, PNF-derived primary Schwann cells express platelet-derived growth factors receptors (PDGFR) α and β, both targets of imatinib, and cell viability was reduced by imatinib mesylate, with 50% inhibition concentration (IC_{50}) of 10 μM. For in vivo studies, PNF tumour fragments xenografted onto the sciatic nerve of athymic nude mice were first characterized. The tumours persisted for at least 63 days and maintained typical characteristics of PNFs such as complex cellular composition, low proliferation rate and angiogenesis. A transient enlargement of the graft size was due to inflammation by host cells. Treatment with imatinib mesylate at a daily dose of 75 mg/kg for 4 weeks reduced the graft size by an average of 80% ($n = 8$), significantly different from the original sizes within the group and from sizes of the grafts in 11 untreated mice in the control group ($P < 0.001$). We demonstrated that grafting human PNF tumour fragments into nude mice provides an adequate in vivo model for drug testing. Our results provide in vivo and in vitro evidence for efficacy of imatinib mesylate for PNF.

Keywords Neurofibromatosis · Plexiform neurofibromas · Imatinib mesylate · Xenografts · Glivec

Abbreviations

GST	Glutathione transferase
PNF	Plexiform neurofibromas
NF1	Neurofibromatosis type 1
MPNSTs	Malignant peripheral nerve sheath tumours
PDGFR	Platelet-derived growth factor receptor
DMEM	Dulbecco's modified essential medium
PAS	Periodic acid-Schiff
VEGF	Vascular endothelial growth factor
DAPI	4′,6-Diamidino-2-phenylindole
SCID	Severe combined immunodeficiency

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Background

Neurofibromatosis 1 (NF1) is an autosomal dominantly inherited disease characterized by multiple neurofibromas, melanogenic abnormalities, bone defects and cognitive deficits, among other characteristics. The genetic causes for NF1 are heterozygous inactivating mutations of the *NF1* tumour suppressor gene on chromosome 17.

Plexiform neurofibromas (PNF) are present in 30–50% of NF1 patients [1, 2]. Unlike cutaneous neurofibromas, the hallmark of NF1 in which tumours are mostly small and mainly of cosmetic relevance, PNF grow to various sizes, infiltrate the surrounding tissues and often have significant clinical consequences, including severe disfigurement, pain, organ compression and other functional impairments. PNF can become extremely large, and some of them affect the whole face, arm or leg. Occasionally, large lesions can erode adjacent bones and thus produce skeletal instability. Our recent study showed growth of PNF mostly in early childhood and adolescence [3]. PNF is the precursor of malignant peripheral nerve sheath tumours (MPNST), which develops in 6–13% of NF1 patients and is the leading cause of death due to this condition [4].

To date, treatment of PNF has been limited to surgical intervention. However, since the tumours often infiltrate adjacent tissues, complete resection is usually not possible without damaging nerves and healthy tissues. Currently, there is no established medical therapy available for PNF.

Imatinib mesylate (Glivec) is a receptor tyrosine kinase inhibitor that targets platelet-derived growth factor receptor (PDGFR)-α and -β, c-Kit, Bcr-Abl and Arg-kinase, and that exhibited efficacy for some cancers [5, 6]. We have previously shown that imatinib mesylate inhibits growth of MPNST cells in vitro [7], raising the hope that this drug may also be beneficial for benign PNF. A recent study showed that c-Kit inhibition by imatinib mesylate leads to reduced mast cell infiltration and neurofibroma growth inhibition in a transgenic mouse model of NF1 [8].

In the present study, we examined the effect of imatinib mesylate on the viability of primary PNF-derived Schwann cells in vitro and its impact on size and survival of PNF-derived tumour fragments in vivo.

Materials and methods

Patients and tumours

Plexiform neurofibromas were obtained from seven unrelated NF1 patients who underwent surgery at the University Hospital Hamburg Eppendorf (age range 12–57 years, mean 34 ± 7.208 years; five male, two female). Diagnosis of NF1 was conducted according to the modified National Institutes of Health (NIH) criteria [9]. All patients gave informed written consent, and the local ethics committee approved the study protocol. After saving sufficient material for histological examination, parts of each specimen were used to establish primary Schwann cell cultures and/or for xenografting into nude mice.

Schwann cell cultivation and treatment with imatinib mesylate

PNF from five patients (age range 16–57 years, mean 42 ± 7.2595 years; four male, one female) were cut into small pieces and placed overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine and 2 mM sodium pyruvate (Invitrogen, Karlsruhe, Germany). Cultivation and enrichment of Schwann cells was carried out as previously described [10]; heregulin was kindly provided by S. Carroll (Department of Neurobiology, University of Alabama at Birmingham). Proportion of Schwann cells was defined as the number of S100-positive Schwann cells divided by the number of nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI, Vysis Inc., Downers Grove, USA).

For imatinib mesylate treatment, 10,000 cells were seeded in 100 µl medium into each well of a 96-well plate for viability assays or in chambers for immunocytochemistry. Imatinib mesylate (kindly provided by Novartis Pharma AG, Basel, Switzerland) was dissolved in water, and added at final concentrations of 2, 5 and 10 µM. Medium was changed every 5 days. Cell viability was evaluated on days 7, 14 and 28 of imatinib treatment using the XTT proliferation assay (Promega, Mannheim, Germany) by measurement of absorbance at 490 nm. Each drug concentration was tested in 12 replicates. Vitality and final number of cells was determined at the end of the experiment using Trypan Blue exclusion.

Xenograft implantation and imatinib mesylate treatment

Tumour tissue from freshly resected PNFs (12-year-old female and 16-year-old male) was placed in sterile DMEM and cut into 4–9 mm^3 pieces. These tumour pieces were soaked in Matrigel (R&D Systems, Wiesbaden, Germany) at 4°C and their sizes were determined using a calliper. Female athymic nu/nu Balb/c mice (Charles River, Sulzfeld, Germany) were anaesthetised with a mixture of xylasin and ketamin. A small incision was made into the skin to expose the sciatic nerve, and then an incision was made into the sciatic nerve, onto which one tumour piece was implanted. Muscle and skin layers were closed and sutured. To determine graft volume changes after transplantation,

mice were sacrificed at days 7 ($n = 4$), 21 ($n = 4$), 35 ($n = 3$) and 63 ($n = 3$) to determine size and for histological analysis.

For treatment with imatinib mesylate, Alzet mini-pumps containing either phosphate-buffered saline (control) or imatinib mesylate (treatment) were subcutaneously implanted into the back of mice [11]. Drug release was set to 75 mg/kg per day with pumping rate of 0.2 µl/h [12], giving an in vivo concentration in excess of the IC_{50} (10 µM) determined for inhibition of Schwann cell proliferation.

Imatinib mesylate treatment was started at day 7 and continued until day 35 post grafting. Mice were kept alive for further 28 days without treatment and sacrificed at day 63. Because of inflammation at the Alzet pump site, four mice of the treatment group had to be discontinued and were not included in the evaluation. Graft size was measured with a calliper and the volume was calculated as $(\pi/48)(\text{length} + \text{width})^3$. All animal experiments were approved by the local authority.

Histology

Tumours were fixed in 7% formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin (H&E) or with Periodic acid-Schiff (PAS) stain to specifically detect mast cells. In addition, a series of immunohistochemical stainings were carried out using anti-human S100 (rabbit polyclonal, 1:800, Dako, Hamburg, Germany), anti-human Ki67 (rabbit monoclonal 1:50, Neomarkers, Asbach, Germany), anti-mouse macrophage surface marker CD68 (mouse monoclonal, 1:100, R&D Systems) and anti-human glutathione transferase (GST) (mouse monoclonal, 1:100, ABCAM, Cambridge, USA). Antibodies against human vascular endothelial growth factor (VEGF) (rabbit polyclonal 1:500) and FLK-1 (rabbit polyclonal, 1:100, Santa Cruz Biotechnology, Heidelberg, Germany) were used to detect blood vessels, while isolectin B4 (biotinylated, 20 µg/ml, Vector Laboratories, Peterborough, UK) was used to detect specifically mouse and non-human endothelial cells. For antibody detection, Envision Kit dual system peroxidase (Dako, Hamburg, Germany) was used. For isolectin detection, the ABC reagent (Vector Laboratories) was used. Colour reactions were developed with VectorRed (Vector Laboratories), and the nuclei were counterstained with haematoxylin.

Expression of receptor tyrosine kinases was examined by immunofluorescence staining on paraffin-embedded sections. Antigen retrieval was enhanced by heating in a microwave. Rabbit polyclonal antibodies against PDGFR-α (1:50) and PDGFR-β (1:50) were from Santa Cruz Biotechnology, while the antibody against c-Kit (1:100) was obtained from Dako. As secondary antibodies we utilized 1:100 dilutions of Cy3- (Dianova) and Alexa Fluor 488-conjugated (Invitrogen) anti-rabbit Ig. Nuclei were counterstained with DAPI. As positive controls we used skin and tonsil tissue. Negative control stainings without primary antibodies did not produce signals.

For immunocytochemistry, Schwann cells were grown on chamber slides, fixed with 4% paraformaldehyde and stained with antibodies against S100, PDGFR-α, and PDGFR-β. Alexa Fluor 488-conjugated antibody (1:1,000) from Invitrogen was used as the secondary antibody. Nuclei were counterstained with DAPI.

Mast cells stained with PAS were counted within the graft and in the adjacent inflammatory area at 40× magnification. The total area of the section was measured using morphometry software (Zeiss, Axiovision, V4.6), and the mast cell density was calculated as mast cells/mm^2 for each section. Five sections from the control and three from the imatinib mesylate-treated group were counted.

Data analysis

The effect of imatinib mesylate on viability of cultured PNF Schwann cells was tested using non-parametric analysis of variance (ANOVA) (Kruskal–Wallis test), and if significant, post hoc comparisons using Dunn's test was performed.

We used non-parametric one-way ANOVA to compare graft sizes at days 7, 21, 35 and 63 (untreated), which were normalized to the original size at grafting. If changes were significant, Dunn's post hoc test was applied for further analysis. A non-parametric test (Mann–Whitney test) was used to examine difference in graft sizes between the control and the treated groups before transplantation. The same test was used to assess possible differences in mast cell density between grafts from the control group and those from the treatment group. To analyse the effects of imatinib mesylate on graft sizes, non-parametric one-way ANOVA was applied for pairwise comparison of each graft before and after treatment, and when significant, post–hoc analysis (Dunn's test) was used to compare changes within the groups. Statistical significance levels were set to $P = 0.05$. Graphpad Prism software was used for all statistical analyses. Averaged values are expressed as mean ± standard deviation (SD).

Results

Expression of c-Kit and PDGFR in PNF and in PNF-derived Schwann cells

Immunostaining revealed expression of the tyrosine kinase receptors PDGFR-α and PDGFR-β in the PNFs used to

graft nude mice (Fig. 1a–d). Double staining for S100 showed that PDFGR-α was mainly expressed in S100-positive Schwann cells (Fig. 1c), and also co-expressed with PDGFR-β (Fig. 1d insert). Co-expression of the PDGFRs was also seen in blood vessels (Fig. 1e). c-Kit-positive cells within the PNF morphologically resembled mast cells (Fig. 1f).

Expression of PDFGR-α and PDGFR-β was also detected in primary Schwann cell cultures derived from human PNF (Fig. 1h, i).

Imatinib mesylate reduces PNF Schwann cell viability in vitro

Primary Schwann cell cultures were derived from five human PNF. More than 85% of the cells were S100 positive and exhibited an elongated, spindle-shaped morphology, which is typical for Schwann cells (Fig. 1g). Treatment with 5 and 10 μM imatinib mesylate for 28 days reduced cell viability significantly, with $P < 0.05$ and $P < 0.001$, respectively (Fig. 2). The total number and the proportion of vital cells were counted at the end of the experiment for non-treated and 10 μM treated groups. While the number of cells increased to 608% (±101) in the control group, cells treated with imatinib only increased to 308% (±137). No differences were observed in the proportion of vital cells (non-treated: 86.74 ± 4.083%; treated 85.053 ± 6.96%). In addition, the cells changed to a more compact morphology after treatment with 10 μM imatinib mesylate for 28 days (Fig. 1j, k).

No effect on cell viability was detected at days 7 or 14 of treatment using the XTT assay (data not shown).

Volume changes of PNF tumour grafts

To obtain a baseline tumour growth pattern for grafted PNFs, changes in the volume of the grafts over time were determined in untreated animals (Fig. 3a). Animals

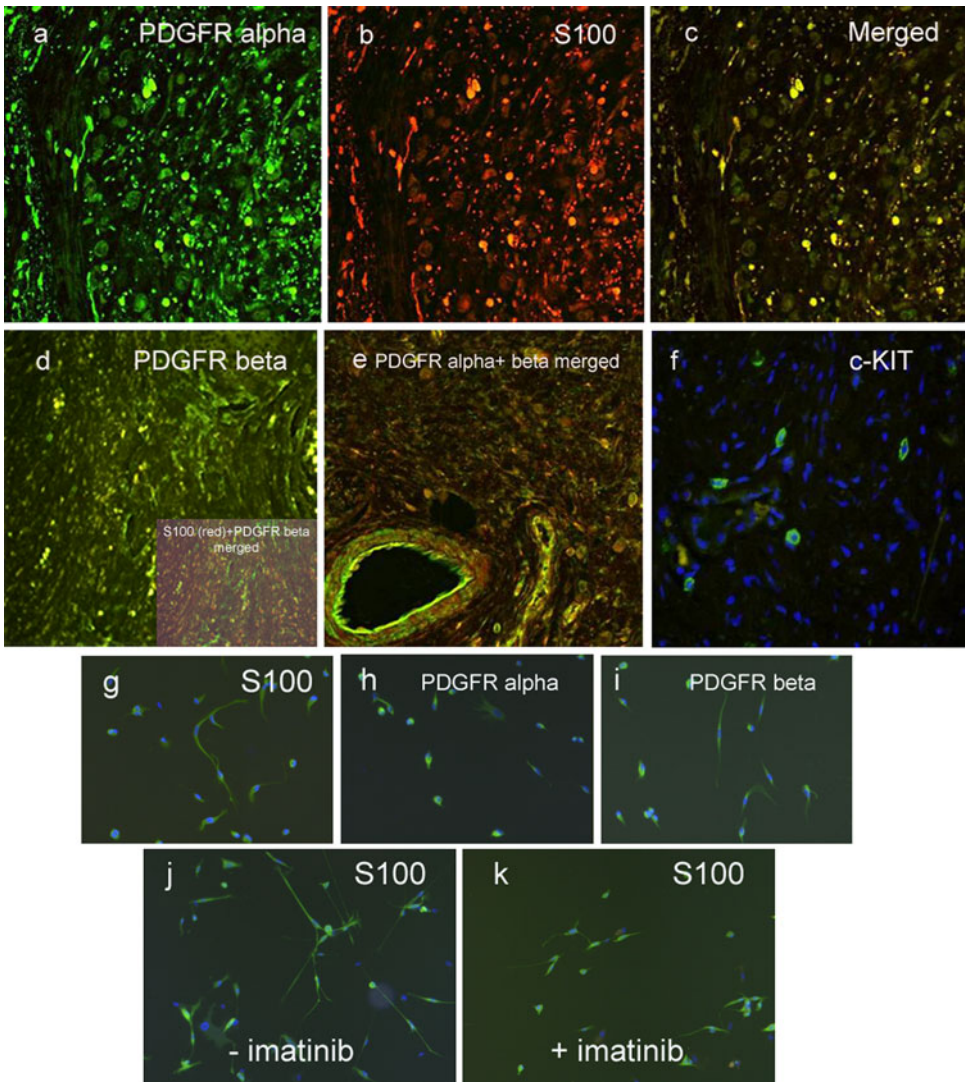

Fig. 1 Immunostaining of PNF (**a**–**f**) and cultured Schwann cells derived from PNF (**g**–**k**). **a** PDGFR-α expression (*green*) in PNF, **b** S100-positive Schwann cells (*red*) in PNF, **c** merged images of (**a**) and (**b**) showing expression of PDGFR-α in S100-positive Schwann cells. **d** PDGFR-β expression (*green*) in PNF. PDFGR-β was also co-localised with S100-positive cells (*insert*), **e** merged images of PDGFR-α (*green*) and PDGFR-β (*red*) indicating their co-expression in tumour cells and blood vessels, **f** c-Kit expression (*green*) in PNF, nuclei were counterstained with DAPI (*blue*), **g** S100-positive Schwann cells cultured from a PNF, **h**, **i** PDGFR-α and PDGFR-β expression in cultured PNF Schwann cells. **j**, **k** 28-day imatinib mesylate treatment (10 M) altered the morphology of S100-positive cells and the Schwann cell density. Original magnifications 20×, except for (**d**) 40×

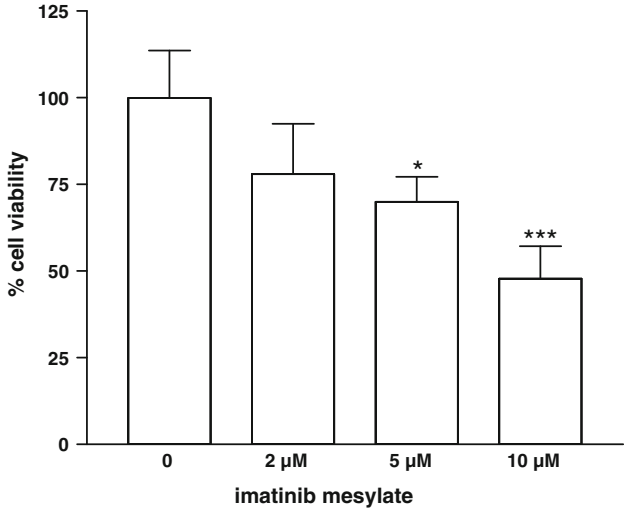

Fig. 2 Imatinib mesylate treatment reduced viability of PNF Schwann cells. Primary Schwann cell cultures derived from PNF were treated with imatinib mesylate at various concentrations for 28 days. Data was normalized from the absorbance values of untreated cells and data are expressed as percentage cell viability. Significant reduction in cell viability was detected for 5 and 10 μM imatinib mesylate treatment (*$P < 0.05$ and ***$P < 0.001$, respectively) when compared with untreated cells

($n = 14$) were sacrificed at different time points after transplantation [days 7 ($n = 4$), 21 ($n = 4$), 35 ($n = 3$) and 63 ($n = 3$)]. The average graft size increased until day 35. At day 63, graft size was comparable to the size of the original graft at day 0 (Fig. 3a). However, only grafts at day 21 post grafting were statistically significantly larger ($P < 0.05$) than those at grafting (day 0) and those at the end of experiment period of 63 days. At the other time points tumour volume variability was too large to reach statistical significance.

To investigate changes in tumour volume, PNF-derived tumour transplants were analyzed histologically at day 35 post grafting (Fig. 4). Tumour grafts, like the original tumours, consisted mostly of S100-positive cells (Fig. 4a). Staining with mouse-specific isolectin B4 antibody revealed newly formed blood vessels surrounding and invading the grafts (Fig. 4b). Angiogenesis at the graft periphery was confirmed by FLK and VEGF expression in the same area (not shown). Only a few cells in the S100-positive graft were Ki67 positive, suggesting that Schwann cell proliferation was slow. This is in concordance with the low proliferation rate of PNF tumours in humans. In contrast, proliferating Ki67-positive cells were mostly present in areas containing granulose tissue (Fig. 4 c, d) surrounding the grafts (Fig. 4 e, f), where S100-positive cells were only occasionally detected (Fig. 4g, h). In addition, CD68-positive mouse macrophages were mainly found in the granulose-containing periphery of the graft, but not within the tumour grafts themselves (not shown),

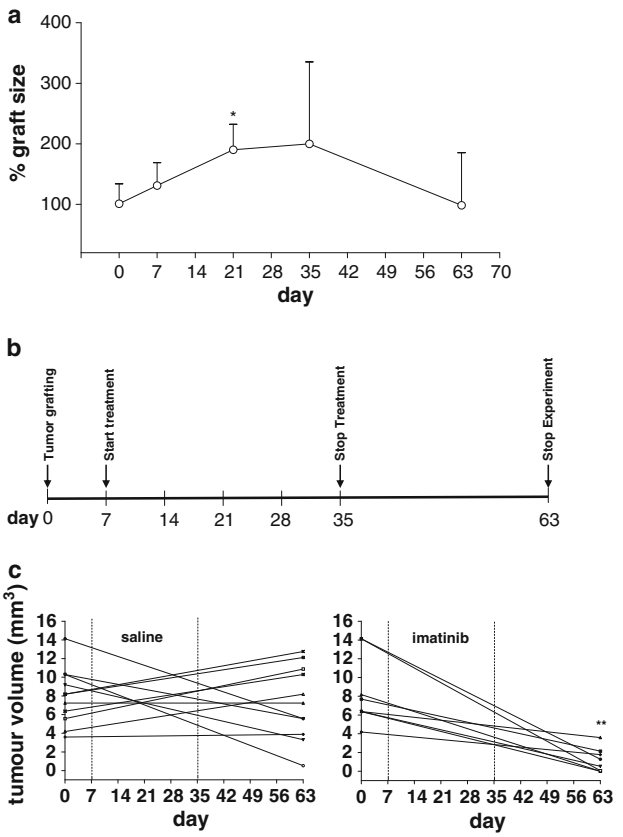

Fig. 3 Volume changes of PNF grafts in nude mice. **a** The growth pattern of non-treated grafted neurofibromas was assessed and the volume normalized at various time points post grafting. A significant increase in tumour volume compared with the time of grafting (day 0) was only seen on day 21 (*$P < 0.05$). **b** Time scale for imatinib mesylate treatment of grafted neurofibromas. **c** Changes in tumour volume are shown for grafts in untreated (saline, $n = 11$) and treated (imatinib mesylate, $n = 8$) mice, respectively. Only data at the beginning and at the end of the observation period are shown (group differences are significant at day 63, **$P < 0.01$). *Dotted lines* mark the 4-week treatment period

suggesting that the proliferating cells are not of human tumour origin but rather inflammatory cells. Human-specific GST antibody did not stain cells in the granulose tissue, supporting our interpretation that the inflammatory cells are of mouse origin (Fig. 4h, insert). These results suggest that the observed enlargement of the tumour grafts in the first 35 days post grafting is likely due to invasion of murine proliferating inflammatory cells rather than human tumour cell proliferation. The high variability of this increase in volume is most likely due to the variable dynamics of the physiological inflammatory process.

Imatinib mesylate reduces size of transplanted human PNFs

Treatment with imatinib mesylate started at day 7 post PNF grafting and continued for 28 days (Fig. 3b). This

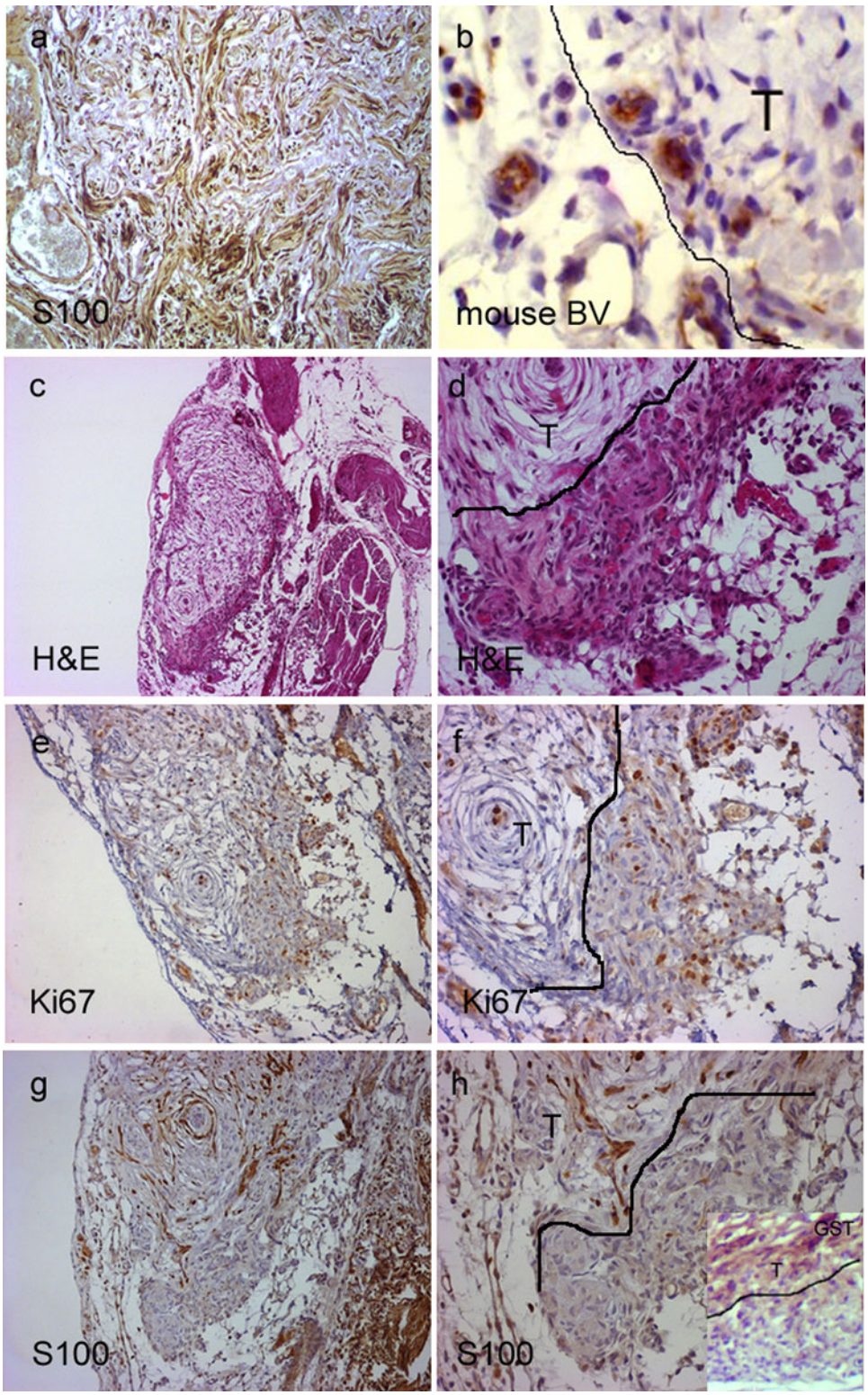

Fig. 4 Histology of PNF grafts at 35 days post grafting. Grafts areas (*lines*) are marked with **T** in **b**, **d**, **f** and **h**. **a** S100 staining indicates Schwann cells in the graft. **b** Mouse-specific isolectin B4 antibody stained mouse endothelial cells invading the tumour graft. **c**, **d** H&E staining revealed granulose tissue in area between the PNF graft and the mouse nerve. **e**, **f** Ki67-positive proliferative cells were mostly found in the inflammatory area surrounding the graft. **g**, **h** in contrast, S100-positive cells were mainly found within the graft but rarely in the inflammatory area. GST antibody labelled only human cells in the graft area but not the granulose tissue around (**h**, *insert*), suggesting non-human origin. *Original magnifications:* **c**, **e** >5×; **a**, **h** 10×; **d**, **f**, **g**, 20×; **b**, *insert in* **h**, 40×

schedule overlapped with the increase in tumour size due to inflammation. Mice were sacrificed at day 63 post transplantation, 28 days after treatment was discontinued, in order to observe whether tumours would recur post treatment (Fig. 3b). Initial sizes of grafts at grafting were similar in the control and the treatment group (7.93 ± 3.45 mm^3, $n = 11$ versus 8.428 ± 3.710 mm^3, $n = 8$) (non-parametric Mann–Whitney test, $P > 0.5$). At the end of the experiment at day 63, all eight PNF grafts in the treatment group decreased in size by an average of

82 ± 18.1% (Fig. 3c). This decrease is significant within the group (post hoc analysis using Dunn's test; $P < 0.001$). In contrast, only 4 out of 11 grafts in the control group decreased in size (by an average of 66%), while 5 increased in size and 2 remained unchanged (Fig. 3c). When all the tumours in the 11 control mice were averaged, there was no change in the graft size (0.4 ± 53%). At 63 days, graft sizes in the treatment and the control group were significantly different (non-parametric repeated-measures one-way ANOVA; $P < 0.001$).

Since insufficient nutritional supply may contribute to shrinkage of the grafts, especially in larger grafts (in both the control and treatment groups), we repeated the statistical analysis with only grafts that were smaller than 9 mm^3 at grafting (pieces of tumour larger than 9 mm^3 had all regressed in the control and treated groups), including six mice from the treatment and seven from the control group. The average reduction in graft size remained at 82 ± 8% in the treatment group, in comparison with a 45 ± 27% increase in the control group ($P < 0.001$, non-parametric repeated-measures one-way ANOVA). Post hoc analysis revealed that the size reduction within the treatment group itself was also significant ($P > 0.01$).

Histological analysis of grafts at day 63 showed healthy tumour tissue with mostly reduced inflammation in the control group (Fig. 5a). In the treatment group, a large inflammatory component was observed, which may be due to diminished viability of the tumour cells at this time point (Fig. 5b, c).

In order to assess whether imatinib mesylate influenced mast cell infiltration, mast cell density was assessed in grafts of both groups at the end of the experiment. No difference in mast cell density was observed between the control and the treatment groups: 34.64 ± 8.610/mm^2 versus 64.12 ± 0.2991/mm^2.

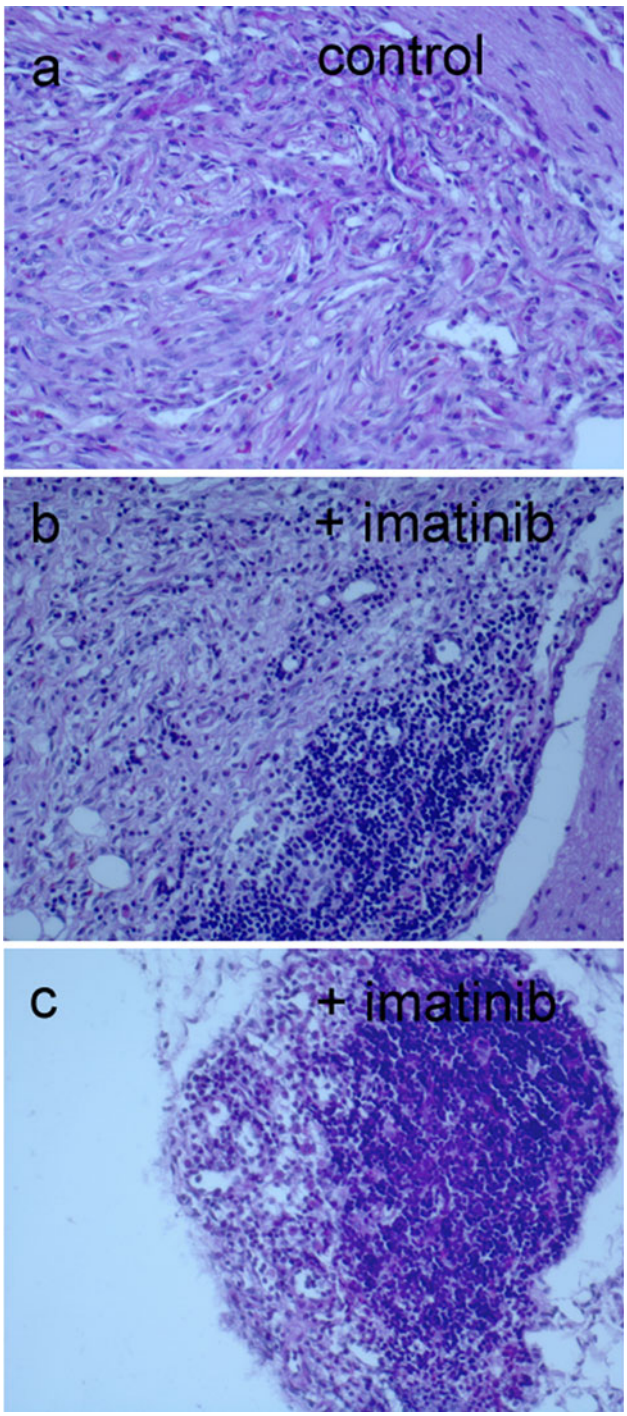

Fig. 5 Histology of PNF grafts at 63 days post grafting from control and treated mice. Sections were stained with Periodic acid-Schiff for mast cells, and counterstained with haematoxylin and eosin. **a** representative graft from a control mouse. **b**, **c** representative grafts from imatinib mesylate-treated mice. Inflammatory cells are abundant around but also within the remaining tumour tissue. Original magnifications 20×

Discussion

We grafted fragments of human PNF tumours semi-orthotopically into injured sciatic nerve of nude mice and found that the grafts persisted for at least 63 days without significant cell loss. The observed temporary increase in size at about 21 days post grafting appeared to be due to infiltration of Ki67-positive and S100-negative inflammatory cells of murine origin. After inflammation ceased, grafts reverted to their original size. According to our recent observation using magnetic resonance imaging, PNFs in patients do not grow, or grow only very slowly, in most cases [3]. Detectable growth of PNF grafts in mice within a period of 9 weeks clearly would not reflect the authentic growth pattern of this kind of tumour in patients. In contrast, lack of growth, as observed in this study, is the expected outcome in non-stressed, non-pregnant adult mice. For clinical trials for PNFs, it is becoming increasingly accepted that reduction in tumour size is the proper

measure of drug efficacy, but not growth inhibition. This is also applicable to in vivo models for PNF. Babovic-Vuksanovic et al. [13] used the persistence rate of PNF grafts as the measure of therapeutic outcome of anti-fibrotic pirfenidone treatment in a severe combined immunodeficiency (SCID) mouse xenograft model. PNF graft enlargement in nude mice within weeks has been interpreted as tumour growth in previous studies, which did not investigate inflammatory effects [14]. Another study reported growth of PNF-like tumours generated by xenografting cells of a tumour-derived Schwann cell line into nude mice [15]. This model using immortalized cells does not reflect the characteristic cellular heterogeneity and benign nature of PNFs.

The initial size of the grafts seems to affect their survival in vivo. In both, the control and treatment group, tumour grafts larger than 9 mm^3 decreased in size at the end of the 63-day experimental period. In contrast, smaller grafts remained unchanged or increased in size, if untreated. Size reduction of large grafts may be at least partially due to necrosis as a result of insufficient nutritional supply. The most drastic effect of imatinib mesylate treatment was observed for grafts >9 mm^3, possibly reflecting additive effect of the drug and intrinsic survival disadvantage due to large size.

Yang et al. [8] recently reported an effect of imatinib mesylate for neurofibromas in a transgenic mouse model and speculated that the observed tumour inhibition was via inhibition of *NF1*+/− mast cell infiltration. Here we show that PNF and PNF-derived Schwann cells express PDGFR-α and -β, both known targets of imatinib mesylate. The observation that imatinib mesylate indeed reduces viability of PNF-derived Schwann cells in vitro suggests a direct effect of the drug on the tumour cells. Furthermore, inhibition of neovascularization via the PDGF signalling pathway may have also played a role in the regression of these tumour grafts in mice. In recent studies, it has been shown that *NF1*+/− mice have increased neointima formation and vessel lumen occlusion in response to mechanical arterial injury; administration of imatinib mesylate to these mice prevented neointima formation, suggesting that neointima formation in NF1 is dependent on an imatinib mesylate-sensitive pathway [16]. It is unlikely that imatinib mesylate acts on mast cells via the c-Kit pathway in our model, as only very few infiltrating mast cells were found in both the control and the treated grafts. In addition, there was no difference in mast cell infiltration between imatinib mesylate treated and non-treated animals, and the inflammatory response follows similar patterns. Although we have not looked at systemic effects of the drug, it is unlikely that differences will be observed in immune-compromised nude mice.

Our findings support further exploitation of the established anti-cancer drug imatinib mesylate for treatment of PNF. Additional studies should be carried out to further clarify the mechanisms by which imatinib mesylate affects PNF survival.

Acknowledgments This study was sponsored by US Department of Defense (W81XWH-06-1-0280 and W81XWH-07-1-0359 to V. Mautner). We are very grateful to Novartis AG for the kind gift of imatinib mesylate.

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