Assessment of antiangiogenic effect of imatinib mesylate on vestibular schwannoma tumors using in vivo corneal angiogenesis assay

Laboratory investigation

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Object. Angiogenesis and the platelet-derived growth factor (PDGF) pathway are active in the pathogenesis of vestibular schwannomas (VSs). The purpose of this study was to test whether imatinib mesylate (Gleevec), a PDGF receptor (PDGFR) blocker, reduces angiogenic capacity in sporadic VS and in VS associated with neurofibromatosis Type 2 (NF2) using a corneal angiogenesis assay.

Methods. From 121 VS tissue samples stored in the tumor bank at the Marmara University Institute of Neurological Sciences, 10 samples (6 from sporadic cases, 4 from NF2-associated cases) were selected at random for use in this study. Expression of PDGF-A and PDGF-B and their receptors was evaluated in sporadic and NF2-associated VS as well as in glioblastoma (GBM) and normal brain tissue by means of immunohistochemistry and Western blot analysis. Corneal angiogenesis assay was then used to evaluate the angiogenic capacity of tissue specimens from sporadic and NF2-associated VS with and without imatinib treatment as well as positive and negative controls (GBM and normal brain tissue).

Results. The angiogenic potential of the sporadic and NF2-associated VS tumor tissue differed significantly from that of the positive and negative control tissues (p < 0.05). Furthermore, NF2-associated VS showed significantly lower angiogenic potential than sporadic VS (p < 0.05). Imatinib treatment significantly reduced the angiogenic potential in both the sporadic VS and the NF2-associated VS groups. The level of PDGF-A and PDGFR-α as well as PDGF-B and PDGFR-β expression in sporadic VS and NF2-associated VS also differed significantly (p < 0.05) from the levels in controls. Additionally the level of PDGFR-β was significantly higher in sporadic VS than in NF2-associated VS (p < 0.05).

Conclusions. The findings of this study indicate that NF2-associated VS has significantly more angiogenic potential than sporadic VS and normal brain tissue. Additionally, imatinib reduces the angiogenic activity of both sporadic and NF2-associated VS. The authors conclude that imatinib may be a potential treatment for VS, especially for NF2-associated lesions that cannot be cured with resection or radiosurgery.

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Key Words • angiogenesis • corneal angiogenesis assay • oncology • vestibular schwannoma • neurofibromatosis Type 2

Vestibular schwannomas are histologically benign, encapsulated slow-growing tumors. They arise from Schwann cells in the vestibular branch of the eighth cranial nerve (vestibulocochlear nerve) located in or next to the internal acoustic meatus. Probably due to the increased use of MRI over the last 4 decades, the annual incidence of VS has increased to 19 cases per million persons.51 It is sporadic, solitary, and found bilaterally in patients with NF2. Neurofibromatosis Type 2 is a rare autosomal dominant genetic disorder with an incidence rate of 1 case in 25,000 and its spontaneous mutation rate is the highest among human genetic diseases.4,23,35 Patients with NF2 characteristically have

Abbreviations used in this paper: bFGF = basic FGF; CU = colorimetric unit; FGF = fibroblast growth factor; GBM = glioblastoma; NF2 = neurofibromatosis Type 2; PDGF = platelet-derived growth factor; PDGFR = PDGF receptor; VEGF = vascular endothelial growth factor; VEGFR = VEGF receptor; VS = vestibular schwannoma.

This article contains some figures that are displayed in color online but in black-and-white in the print edition.
bilateral VSs, making management of these cases more difficult. Manifestations of the disease are loss of hearing, tinnitus, or dizziness. Although VSs are benign in nature, they trigger various symptoms by compressing and displacing nearby vital structures. Resection and radiosurgery are the 2 main effective modalities in the management of VS. Applied separately or in combination. However, there are NF2 patients for whom these proven treatments are not satisfactorily effective in preserving serviceable hearing and quality of life. Angiogenesis is a component of tumorigenesis in VS, and several angiogenic factors have been identified. Among the most studied molecules are VEGF, PDGF-A, PDGF-B, and their receptors. Vestibular schwannomas have been shown to express VEGF, VEGFR, PDGF-A, and PDGF-B. Furthermore, the expression of VEGF and VEGFR correlates with the tumor growth rate. The PDGF pathway plays a key role, with signaling from PDGF receptors causing expression of genes associated with angiogenesis.

Imatinib mesylate (Gleevec, STI 571, Novartis AG) is a tyrosine kinase inhibitor that was first developed as a specific inhibitor for Bcr-Abl tyrosine kinase in chronic myelogenous leukemia. With increased understanding of the molecular aspects of imatinib-mediated tyrosine kinases, it was shown that imatinib inhibits both the α and β receptors. These findings led to further in vivo investigations, which demonstrated that imatinib inhibits VS growth by inhibition of the PDGF pathway. In addition, quantitative real-time polymerase chain reaction analysis of sporadic VS and NF2-associated VS showed that mRNA expression of imatinib-targeted receptors and ligands increased.

The present study was undertaken to test whether imatinib reduces angiogenic activity in sporadic and NF2-associated VS tumors by using a corneal angiogenesis assay. It is our hope that this in vivo study of therapeutic efficacy might provide a foundation for new clinical trials in the area of VS treatment, which might add a medical treatment option especially for patients with NF2-associated VS.

Methods

The study was performed at the Marmara University Institute of Neurological Sciences.

Tumor Tissue

A total of 121 microsurgically resected VS tissue samples were collected and stored in the liquid nitrogen tumor tank at the Institute of Neurological Sciences of Marmara University, Istanbul, Turkey, between 2000 and 2009. Ten randomly selected fresh-frozen VS tissue samples (6 from sporadic VS, 4 from NF2-associated VS) were used in this study. None of the patients from whom the VS tissue samples were obtained had received any other treatment for their tumors before surgery. Informed consent was obtained from the patients. Positive and negative control tissues were also randomly selected for experimental procedures. Six GBM specimens obtained from 6 different patients were used for positive controls and 4 specimens of normal human brain tissue obtained from 4 adult patients during epilepsy surgery were used as negative controls, as previously described. All diagnoses were confirmed by histopathological examination.

Immunostaining

Specimens from each of the tumors were evaluated by means of immunostaining. Multiple serial sections were cut from the fresh-frozen tissue using a cryostat and prepared for immunohistochemical analysis for PDGF-A, PDGF-B, PDGFR-α, and PDGFR-β. The details of the immunostaining procedures were described previously by Seker et al. The sections were rehydrated in alcohol gradient before analysis with the standard streptavidin-biotin technique. Endogenous peroxidase activity was blocked by incubation in 3% H2O2 (0.6 ml of H2O2 in 2.7 ml of distilled H2O). Slides were blocked with 5% normal goat serum and then incubated overnight with human monoclonal rabbit PDGF-A (sc-128), PDGF-B (sc-7878), PDGF-α (sc-338), PDGF-β (sc-339) (all from Santa Cruz Biotechnology, Inc.) at 4°C. Each slide was then incubated for 30 minutes at 25°C with secondary biotinylated goat anti–rabbit immunoglobulin G (Vector Laboratories, Inc.) and then with streptavidin-peroxidase complex (Vectorstain Elite ABC kit, Vector Laboratories, Inc.). Chromogenic reactions were completed with 3,3’-diaminobenzidine, yielding a positive brown stain (Vector Laboratories, Inc.). Each slide was counterstained with hematoxylin and then mounted and examined under the light microscope. Levels of expression were graded using a 4-point scoring scale, as follows: Grade 0, no expression; Grade 1, moderate expression; Grade 2, focal distribution; Grade 3, diffuse distribution. All slides were evaluated by 2 study-independent pathologists who were blind to the study conditions.

Western Blot Analysis

Tissue samples were homogenized using a glass pestle homogenizer in a buffer including 50 mM Tris (Sigma), 1 mM EDTA (Sigma), 0.1% sodium deoxycholate, and 1% Triton X-100 (pH 7.4). Insoluble materials were excluded by centrifugation at 10,000 rpm for 15 minutes. The protein level was measured using the Bradford method (Bio-Rad). Equal amounts of protein were loaded and electrophoresed on 8%–10% sodium dodecyl sulfate polyacrylamide gels. Subsequently, proteins were transferred on polyvinylidene difluoride membranes. The polyvinylidene difluoride membranes were blocked at room temperature for 1 hour, then probed with primary antibodies for PDGF-A, PDGFR-α, and PDGFR-β (Santa Cruz Biotechnology), and PDGF-B (Oncogene Research Products) for 1 hour, followed by an appropriate species-specific horseradish peroxidase–conjugated secondary antibody (TM–125-BN, Lab Vision). By using an enhanced chemiluminescence detection system (ECL, Santa Cruz Biotechnology), protein expression was detected and bands were visualized on film.

Protein bands on the membranes were scanned using a densitometer (Bio-Rad GSH 800 and MagicScan32 V4.5 software). Scanned protein bands on the membranes were scanned using a densitometer (Bio-Rad GSH 800 and MagicScan32 V4.5 software).
Imatinib and inhibition of VS-induced angiogenesis

were analyzed using ImageJ software (NIH) to obtain quantitative measurements. Quantities of proteins were assessed in colorimetric units (CUs). A “CU” is a numerical value showing the sensitivity of the protein band concentration, ranging between 0 (least) and 255 (most). Values were linearized and normalized by loaded total protein concentration. All samples were scanned and analyzed using the same standard procedure.

Corneal Angiogenesis Assay

Thirty male Sprague-Dawley rats weighing 300–400 g were used for this study. Procedures for the corneal angiogenesis assay were as described by Konya et al.29 Four to five hours before the experimental procedure, tissue samples, which had been stored in liquid nitrogen (−187°C) were brought to room temperature, washed with dimethyl sulfoxide, and cut into pieces, approximately 2–3 mm in diameter, under a microscope.

General anesthesia was induced by means of intraperitoneal injection, and propacaine 0.5% was applied topically to one cornea of each rat using a surgical microscope under sterile conditions. The eye was displaced slightly anteriorly with the aid of jeweler’s forceps. A paracentral intrastromal linear keratotomy (~ 4 mm, at a right angle to the limbus) was performed with an arachnoid blade, and then a micropocket was formed within the corneal tissue using a microhook. A uniform amount of tissue was implanted into the micropocket in one cornea of each animal, and the other cornea was left intact. The day on which the procedure was performed was considered Day 0. Progression of angiogenesis was observed by microscope and photographed on Days 5, 10, 15, and 20 after tumor tissue implantation.

Two tissue sections (1 imatinib-treated and 1 untreated) were used from each of 10 VS specimens (6 sporadic VS, 4 NF2-associated VS). Thus 20 corneas were implanted with VS tissue—12 with tissue from sporadic VS, 8 with tissue from NF2-associated VS tissue. In each of these 10 animals, the implanted eye was treated with imatinib (200 μl of 10 μM imatinib applied topically daily for 30 days). Tissue from GBM tumors was implanted into one cornea in each of 6 rats (positive controls), and normal brain tissue from specimens obtained during epilepsy surgery was implanted into one cornea in each of 4 additional rats (negative controls). An antibiotic agent (200 ml of 0.1 mg/ml gentamicin) was applied topically once daily for 30 days after implantation. In animals treated with imatinib, the antibiotic was applied at least 6 hours after imatinib application. If signs of ocular infection (discharge, redness around the eye) appeared in either eye of any rat, the animal was excluded from the study, treated, and returned to its cage to be used later in another study. Each cornea was photographed daily, using a digital video system attached to a biomicroscope (Carl Zeiss Co.), and the degree of angiogenesis was assessed by counting the number of vessels by 2 people blinded to study condition.

All experiments were approved by the animal care and use ethics committee of Marmara University Faculty of Medicine.

Statistical Analysis

SPSS Version 15.0 (SPSS, Inc.) was used for data analysis and to draw graphs. Values of p < 0.05 were regarded as significant. The Mann-Whitney U-test was used to compare the expression levels of PDGF-A, PDGF-B, PDGFR-α, and PDGFR-β in the different groups. The results for corneal angiogenesis grading and vessel count were interpreted and compared as a function of time by means of “cell line charts” with error bar used to indicate SEM.

Results

Corneal Angiogenesis Assay

The cell line chart in Fig. 1 shows the extent of angiogenesis in the implants. The positive controls showed
significantly higher angiogenic potential and the negative controls showed significantly lower angiogenic potential when compared with both sporadic and NF2-associated VS tumor groups (p < 0.05 for both treated and untreated implant groups). The rats in the GBM-implanted cornea group (positive controls) were killed on Day 10 because of significant neovascularization and tumor formation (Fig. 1A). Vessel counts for sporadic VS were significantly higher in these animals than in the NF2-associated VS group (p < 0.05). Significant differences between the angiogenic potential of sporadic VS and that of NF2-associated VS were first observed on Day 10. Furthermore, imatinib-treated corneas showed less neovascularization than untreated corneas. The mean vessel counts on Day 20 for the imatinib-treated sporadic VS–implanted corneas, the untreated sporadic VS–implanted corneas, imatinib-treated NF2-associated VS–implanted corneas, and untreated NF2-associated VS–implanted corneas were 15.33, 19.17, 10.25, and 15.13, respectively. Thus, we can assume that imatinib activity significantly reduced angiogenic potential in both the sporadic VS and NF2-associated VS groups (p < 0.05) (Fig. 1).

Platelet-Derived Growth Factor–A and its Receptor

In Western blot analyses, expression of both PDGF-A and its receptor PDGFR-α in sporadic VS and NF2-associated VS was significantly higher than in the negative control specimens (normal brain tissue) and less than in the positive controls (GBM tissue) (p < 0.05 for both comparisons). Additionally, NF2-associated VS showed significantly lower expression of PDGF-α than sporadic VS tissue (p = 0.032), but there was no significant difference in expression of PDGF-A (Fig. 2). In immunohistochemical analyses, sporadic VS and NF2-associated VS showed a significantly higher level of PDGF-A and PDGFR-α than the negative control specimens (normal brain tissue) and less angiogenic potential than GBM.

Platelet-Derived Growth Factor–B and its Receptor

Western blot and immunohistochemical analyses showed that PDGF-B expression was significantly higher in both sporadic and NF2-associated VS than in normal brain tissue (p < 0.05). Additionally, Western blot analyses revealed that the level of PDGFR-β was significantly higher in both forms of VS than in normal brain tissue (p = 0.034), while there was no significant difference in the level of PDGFR-β expression between sporadic VS and NF2-associated VS (Fig. 2). However, IHC analyses revealed that the PDGFR-β level is significantly higher in sporadic VS than in NF2-associated VS (Fig. 3).

Discussion

Human schwannomas are benign tumors that may grow slowly and silently for many years without causing any symptoms. Vestibular schwannoma may be treated by means of resection or radiosurgery, but total resection may result in increased morbidity, especially in larger lesions. In selected cases, a relatively small asymptomatic tumor may be managed with serial MRI monitoring. Although improvements in microsurgery and radiosurgery have been shown to reduce morbidity, patients may still experience a loss of function after treatment. The lack of management options is particularly problematic in NF2-associated VS, where, in the absence of a permanent cure, treatment is still controversial. The aim of treatment is preservation of hearing function and brainstem decompression.

Corneal Angiogenesis Assay and VS

Since it was first described by Gimbrone et al. in 1974, corneal angiogenesis assay has become one of the best methods for in vivo quantification of angiogenesis. The method is highly replicable and well described.

The angiogenic potential of vestibular schwannoma and NF2-associated vestibular schwannomas, focusing on different issues of angiogenic behavior with different angiogenic factors, was studied and resulted in the demonstration of the association between angiogenesis and tumor behavior. These studies are descriptive, however, and are based on immunohistochemistry data with no specific information on the level of inhibition of angiogenesis by antiangiogenic molecules. To our knowledge, the present study is the first to demonstrate the angiogenic potential of sporadic and NF2-associated VS and provide quantitative and semiquantitative data for future experimental trials.

As in any solid tumor, angiogenesis is an indispensable process for VS tumorigenesis. When the tumor volume exceeds more than 2 mm³, angiogenesis becomes vital for the growth of tumor tissues. The potential role of angiogenesis in VS and the correlation of several proangiogenic factors with tumor growth have been revealed in recent studies. In the light of these findings, angiogenic factor–directed anti-neovascularization therapies have become one of the most attractive potential treatment approaches.

Corneal angiogenesis assay assesses in vivo vessel formation in a dynamic manner. In contrast to Western blot and immunohistochemical analysis, which are static modes of investigation, corneal angiogenesis assay demonstrates angiogenic activity as a function of time. The results of the present study indicate that NF2-associated VS has significantly lower angiogenic potential than sporadic VS. Moreover, both sporadic and NF2-associated VS had significantly higher angiogenic potential than normal brain tissue and less angiogenic potential than GBM.

Several Angiogenic Factors are Expressed in VS

Angiogenesis is the process of formation of new blood vessels and is characterized by a cascade of processes, during which pathways involving several growth factors, such as VEGF, FGF, and PDGF, play an important role. It was previously demonstrated that several potent proangiogenic factors are expressed in VS tumors. These factors, including PDGF receptors and ligands, play important roles in tumor angiogenesis. PDGF influences angiogenesis by acting directly as a mitogen for
endothelial cells. In addition, endothelial expression of VEGF is induced by PDGF, thus making an autocrine loop through stimulation of VEGF receptors. The present study demonstrates high expression levels of PDGF ligands and receptors in VS tumors compared with normal brain tissue, indicating a potential relationship with the transformation of Schwann cells to schwannomas as previously stated by Mukherjee et al. Cayé-Thomasen et al. reported that VS tumor cells express VEGF and VEGFR and observed a correlation between the tumor growth rate and concentration of proteins. Murphy et al. showed elevated bFGF expression levels in schwannomas. Recent animal model and in vitro studies have demonstrated that inhibition of VEGF- and PDGF-induced angiogenesis by antiangiogenic antibodies reduces tumor growth. In light of these previously published findings, combined therapies—involving the use of growth factor inhibitors following surgery or radiosurgery—may be more effective in schwannomas than single therapies.

**Imatinib and Inhibition of VS Angiogenic Activity**

Imatinib mesylate was first developed as a tyrosine kinase inhibitor to treat chronic myelogenous leukemia by blocking Bcr-Abl tyrosine kinase (expressed by the BCR-ABL fusion oncogene. Imatinib also inhibits c-Kit and PDGFRs. With expanding understanding of information on imatinib, a new spectrum of antitumor research and clinical assays was started. It has been shown that imatinib inhibits the growth of c-Kit–positive small cell lung cancers and gastrointestinal stromal tumor lines. Imatinib also inhibits PDGFR-expressing GBM cells and ovarian cells through the PDGF pathway. The expression of PDGFRs and other growth factors in VS is believed to be the target of imatinib, because schwannomas do not include BCR-ABL oncogenes. Mukherjee et al. and Altuna et al. showed in vitro antitumorigenic effects of imatinib through inhibition of PDGFR-α, PDGFR-β, and c-Kit signaling pathways in human schwannomas. In their in vitro study, Mukherjee et al. demonstrated that immortalized schwannoma and NF2-associated schwannoma cells express a significantly higher level of PDGFR-α and PDGFR-β than normal tissues. Additionally, the authors showed that different concentrations of imatinib inhibit the expression of those receptors, but as they indicated in their final comments, their results require in vivo confirmation.

Buchdunger et al. showed antiangiogenic effects of imatinib in animal models. It was reported that vascular endothelial cells express PDGFR-β and that imatinib inhibits both α and β receptors of PDGF. The authors hypothesized that VEGF and bFGF activate endothelial cells. Minimal neovascularization in normal brains indicates that the PDGF pathway is inactive in normal circumstances. But immunohistochemical studies proved...
that brain tissue expresses PDGF-A and PDGFR-β, without the expression of the corresponding receptors and ligands. The expression of molecules without their complements prevents the self-activation of the pathway by autocrine signaling and formation of a so-called “closed loop.” Expression of both ligands and receptors in the PDGF pathway is a clear indication of formation of a closed loop in sporadic and NF2-associated schwannomas, which supports the role of angiogenesis in these tumors. PDGF is locally produced as a result of endothelial cell stimulation and acts on smooth muscle cells, pericytes, and endothelial cells. Inhibition of PDGF may prevent stabilization of newly formed vessels.

The present study is in line with the studies of Mukherjee et al. and Buchdunger et al., showing the angiogenic potential of both sporadic and NF2-associated VS and the response of these tumors to imatinib in an in vivo corneal angiogenesis assay. Our model demonstrated that imatinib significantly inhibits the angiogenic potential of sporadic and NF2-associated VS. This result correlates with the findings of previous studies cited above. Additionally, the comparison of sporadic VS and NF2-associated VS in the present paper gives new insight into the molecular differences between these clinically closely related lesions.

**Clinical Significance**

Surgery and radiosurgery, used separately or in combination, are clinically proven, first-line therapy options for the treatment of VS. However, current neurosurgical treatment is occasionally insufficient in patients with NF2. Hence, an alternative strategy that inhibits the growth of VS or induces regression of the lesion could potentially stabilize the clinical symptoms and decrease the risk of morbidity. An orally active, tyrosine-kinase inhibitor, imatinib, targeting the specific growth factors and inhibiting both tumorigenesis and angiogenesis of schwannomas, might have a future role in the treatment of surgically and radiosurgically incurable VS, most often the NF2-associated lesions. So, like some other novel molecular therapeutic options, such as bevacizumab, trastuzumab, erlotinib, and lapatinib, the possible first-line treatment field for imatinib is NF2 patients in whom all treatment modalities had been used but failed.

**Conclusions**

The greater the understanding of underlying molecular mechanisms in VS tumorigenesis, the more novel therapeutic approaches have been yielded. With further research it may be possible to show significant increase of effectiveness of anti-tumorigenesis–targeted drugs. The current study provides further in vivo experimental data on the potential of imatinib for the treatment of sporadic VSs and, in particular, NF2-associated VS. Additional studies are needed to investigate the mechanisms of PDGFR and imatinib interactions at the gene and protein levels.

**Disclosure**

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Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Kılıç. Statistical analysis: Avsar.

Study supervision: Kılıç.

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