Inhibition of vascular smooth muscle cell proliferation by a novel fibroblast growth factor receptor antagonist

Amit Segev\textsuperscript{a,b}, David Aviezer\textsuperscript{c}, Michal Safran\textsuperscript{a}, Zeev Gross\textsuperscript{d}, Avner Yayon\textsuperscript{a,c,*}

\textsuperscript{a}Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel
\textsuperscript{b}Department of Cardiology, Meir General Hospital, Kfar-Saba and Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel
\textsuperscript{c}ProChon Biotech Ltd, Weizmann Science Park, Rehovot, Israel
\textsuperscript{d}Department of Chemistry, Technion – Israel Institute of Technology, Haifa, Israel

Abstract

Objective: One of the key events in post-angioplasty restenosis is the migration and proliferation of medial smooth muscle cells leading to neo-intima formation. This phase is mediated by several growth factors, mainly platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF2/bFGF) and heparin-binding epidermal growth factor (HB-EGF). In this study, we have focused on the role of FGF2, which requires heparan sulfate proteoglycans (HSPG) as cofactors for binding and activation of its cell surface tyrosine kinase receptor. The aim of this study was to identify and explore the effect of novel FGF antagonists on vascular smooth muscle cell (VSMC) proliferation.

Methods: We have recently identified a novel class of small, positively charged molecules sharing a porphyrin core as inhibitors of FGF2 and vascular endothelial growth factor (VEGF) activity. Here we investigated the inhibitory effect of these compounds on VSMC proliferation and their effect on heparin-induced FGF receptor activity.

Results: We found that these molecules exert a marked inhibitory effect on FGF2-mediated smooth muscle cell (SMC) proliferation, manifested by reduced cell growth and DNA synthesis, which occurred in a dose-dependent manner with an IC\textsubscript{50} of \textasciitilde1 \textmu M of inhibitor. We demonstrate that the molecule, 5, 10, 15, 20-tetrakis (methyl-4-pyridyl)-21\textsubscript{H},2 3\textsubscript{H}-porphine tetra-p-tosylate salt (TMPP), inhibits binding of radiolabeled FGF2 to SMCs and to soluble FGF receptor 1 (FGFR1) in a manner that interferes with both ligand and receptor interactions with heparin, thereby blocking growth factor mediated SMC proliferation.

Conclusion: We have identified an FGF antagonist, which may serve in clinical practice as a preventive measure of restenosis. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Growth factors; Smooth muscle; Restenosis

1. Introduction

Restenosis after successful percutaneous transluminal coronary angioplasty (PTCA) remains a major complication leading to recurrent acute coronary events and even death. Acute closure occurs in \textasciitilde4/\% of patients and may be partially resolved by using anti-thrombotic agents such as glycoprotein IIb/IIIa inhibitors [1]. The rate of restenosis is \textasciitilde30/\% after 6 months [2] and remains at least 20/\% following intra-coronary stent deployment [3].

The pathogenesis of restenosis consists of three cellular phases: (i) disruption of the endothelial layer, platelet adhesion and aggregation, and the initiation of inflammatory response; (ii) the granulation phase, involving smooth muscle cell (SMC) migration and proliferation, and secretion of extra-cellular matrix; and (iii) the final remodeling phase involving re-endothelization and matrix organization [4]. Great experimental efforts have been focused on the crucial role of the granulation phase in the pathogenesis of restenosis. This phase is mediated by several growth factors, mainly platelet derived growth factor (PDGF) [5], heparin-binding epidermal growth factor (HB-EGF) [6] and basic fibroblast growth factor (FGF2) [7].

Fibroblast growth factors constitute a family of poly-
peptides involved in many vital processes including cell growth and differentiation, embryogenesis, angiogenesis and wound healing [8]. Most types of cells found in the restenotic area, e.g. endothelial cells, macrophages, SMCs and even T-cells, synthesize FGF2, which was shown to be involved in the atherosclerotic process [9]. FGF2 is highly mitogenic for vascular SMCs [10].

A common feature of FGF family members is their high affinity towards heparin and heparan sulfate proteoglycans (HSPGs). The interaction between FGF2 and HSPG enhances high affinity binding to the FGF cell surface, tyrosine kinase receptor (FGFR), and is essential for mediating receptor phosphorylation and receptor-mediated signal transduction [11,12]. Several models have been suggested for the mechanism by which heparin or heparan sulfates enhance FGF–FGFR interaction and activation. Recent studies have demonstrated that the interaction of FGF2 with heparin-derived oligosaccharides can lead to both FGF2 [13] and FGFR [14] dimerization and that either cis- [15] or trans-oriented [16] dimers of FGF2 can be formed. These can subsequently lead to dimerization and activation of the tyrosine kinase receptor.

In a search for compounds that can block these interactions, we have recently identified a novel group of small molecules capable of blocking FGF–FGFR interaction and tumor progression [17]. We found that these molecules directly interfere with the interaction of both FGF2 and FGFR with heparan sulfates on SMC. The porphyrin derivative, 5, 10, 15, 20-tetrakis (methyl-4-pyridyl)-21H, 23H-porphine tetra-p-tosylate salt (TMPP), can inhibit the interaction of FGF2 with its receptor on the surface of SMC, thereby blocking FGF2-induced SMC proliferation.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Materials and chemicals

Disuccinimidyl suberate, bis (sulfosuccinimidyl) suberate and Bolton-Hunter reagent (N-succinimidyl-3-(4-hydroxyphenyl) propionate) were from Pierce (Rockford, IL). Na-$^{125}$I was from Amersham Pharmacia Biotech (UK). Heparin Sepharose and CNBr-activated Sepharose were from Pharmacia Biotech (Uppsala, Sweden). Human recombinant FGF2 and biotinylated FGF2 were a generous gift from Andrew Seddon. Heparin was from Hepar Industries (Franklin, OH).

2.2. Identification of FGF receptor binding antagonists

A high throughput screening system composed of a heparin matrix, FGF2 and an alkaline phosphatase tagged FGFR (FRAP), was previously employed [17]. Briefly, the screen was conducted using 96-well plates to which heparin had been covalently attached. FGF2 was then bound to the plates through heparin–FGF2 interactions followed by the addition of FRAP and the compounds to be screened for their modulation of heparin–FGF, receptor–heparin and receptor–FGF interactions. The endpoint of the assay measures the alkaline phosphatase (AP) catalyzed formation of a chromogenic product [18], which is proportional to the amount of FRAP present on the plates as a heparin–FGF–FRAP ternary complex. Thus, a lowered or elevated AP value, relative to a control, would indicate modulation of binding at one or more of the three levels of interaction that describe the FGF–FGFR–heparin ternary complex. This screen has identified several compounds for their capability to inhibit soluble FGF receptor binding to immobilized ligand. One potent inhibitor was 5, 10, 15, 20-tetakis (methyl-4-pyridyl)-21H, 23H-porphine tetra-p-tosylate salt (TMPP).

2.3. Cells

SMCs isolated from bovine aortic media were kindly provided by Professor Israel Vlodavsky. Cells were cultured in DMEM supplemented with 10% bovine calf serum (BCS), at 37°C in 10% CO$_2$ humidiﬁed incubators. Experiments were performed on cells after six to 12 passages.

2.4. Cell growth assays

Bovine aortic smooth muscle cells were plated into 48-well plates (5×10$^3$ cells/well) in DMEM containing 10% BCS. Cells were allowed to attach to plates for 24 h at 37°C in 5% CO$_2$, 95% air. Then, 2 ng/ml FGF2 and increasing doses of TMPP were added. FGF2 was added again every 48 h for 4 days. Cell growth was determined by trypsinization and a camera counter counting the number of cells.

2.5. DNA synthesis

Thymidine incorporation was measured in order to determine the effect of FGF2 and TMPP on DNA synthesis. Bovine aortic SMCs were plated on 96-well plates in DMEM and 10% BCS and were grown to subconfluency. After serum starvation for 24 h in DMEM, cells were stimulated with FGF2 in the presence or absence of increasing concentrations of TMPP for the following 18 h. Subsequently, [³H]thymidine (1 μCi/well) was added, and the incubation was continued for a further 6 h. DNA synthesis was assayed using a cell harvester to measure the radioactivity incorporated into trichloroacetic (TCA)-insoluble material.
2.6. Radiolabeling of recombinant growth factors

Recombinant growth factor proteins were labeled with Na-125I (1 mCi), using the chloramine-T method [11]. Briefly, iodinated FGF2 was separated from free iodine on a heparin-Sepharose column; the eluted factors were divided into aliquots and kept at −20°C until use.

2.7. Iodination of TMPP derivative P10016 by the Bolton-Hunter technique

The compound 5-(2,3,4,5,6-pentafluorophenyl)-10,15,20-tris(N-methyl-4-pyridylium) porphyrin triiodide was synthesized as previously described [17]. Bolton-Hunter reagent (5 mg) was dissolved in 1 ml of DMSO, and iodinated by the chloramine-T method according to the manufacturer’s recommendations. Coupling of the iodinated Bolton-Hunter reagent to P10016 was performed by adding 21.4 mg of P10016 dissolved in 1 ml methanol to 4.75 mg of the labeled Bolton-Hunter reagent dissolved in 1.5 ml of methanol. After 1 h of stirring, ether was added until the product formed a precipitate. The solvent was subsequently removed and the product was dried under a stream of nitrogen.

2.8. Binding of radiolabeled FGF2 to cell surface receptors

Subconfluent cultures of SMCs in 24-well plates (Falcon) were precooled and washed twice with cold DMEM supplemented with 20 mM HEPES (pH 7.5) and 0.1% bovine serum albumin (DMEM/BSA). Subsequently they were incubated for 1.5 h at 4°C with 2 ng/ml 125I-FGF2 in DMEM/BSA, increasing concentration of TMPP or Suramin (250 μg/ml). In order to remove low affinity bound FGF2, cells were incubated twice for 5 min with cold PBS (pH 7.5) containing 1.6 M NaCl, and 25 mM HEPES. High affinity binding of FGF2 was determined by incubating the cells for 5 min with cold PBS (pH 4) containing 1.6 M NaCl and 25 mM HEPES. The extract was counted in a γ counter.

2.9. Cross-linking of radiolabeled growth factors to soluble growth factor receptors

Conditioned medium from cells secreting an FGF receptor-1-AP or FGFR3-AP fusion proteins [18] was incubated with rabbit anti-human placental AP antibodies prebound to agarose-protein-A beads (Pierce) for 45 min, while gently shaking at room temperature. The binding reaction included: 20 μl of FGFR1 or FGFR3-AP prebound to protein-A beads, 125I-FGF2 (5 ng/ml), 125I-FGF9 (5 μg/ml) and 50 μg/ml heparin. TMPP was added when indicated. After 1.5 h, the beads were precipitated and washed three times with 1 ml of 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100 and 10% glycerol (HNTG). In parallel, soluble EGF receptor Fc-IgG fusion protein [20] was bound directly to protein-A beads and incubated with radiolabeled EGF (5 ng/ml), heparin and TMPP.

Growth factors were crossed-linked to their receptors by adding disuccinimidyldi sulfide (DSS) (15 mM) in PBS for 30 min at room temperature. Growth factor–receptor complexes were boiled for 5 min and separated under reducing conditions by 7% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The gel was dried and exposed to Kodak X-Omat AR film.

2.10. Competitive inhibition of FGF2 binding to heparin by TMPP

For competition assays, 50 ng of 125I-FGF2 was added to a heparin coated 96-well plate (Carmida, Sweden) with increasing doses of TMPP. After a 2-h incubation the wells were washed twice with cold PBS (pH 7.5) containing 1.6 M NaCl and 25 mM HEPES, in order to remove low affinity bound FGF. High affinity binding of FGF2 to heparin was determined by extracting the FGF with cold PBS (pH 4) containing 1.6 M NaCl and 25 mM HEPES. The extracts were counted in a γ counter.

2.11. Specific binding of FGF2 to immobilized TMPP

TMPP was immobilized on CNBr-activated Sepharose beads (Pharmacia, Sweden) according to the manufacturer’s instructions. The TMPP-coupled beads (20 μl) were incubated with equal amounts of I25I-FGF2, I25I-FGF-9 or I25I-EGF (250,000 cpm per reaction) for 2 h. Beads to which TMPP was not bound served as a control. The beads were then washed three times with HNTG and counted in a γ counter.

2.12. Competitive inhibition of 125I-TMPP binding to FGFR-1 by heparin

Conditioned medium (200 μl) from cells secreting an FGF receptor-1-AP fusion protein was incubated with 20 μl of rabbit anti-human placental AP antibodies prebound to agarose-protein-A beads (Pierce) for 45 min, with gentle shaking at room temperature. The beads were centrifuged for 10 s at 6000 rpm, and washed twice with 1 ml 2 M NaCl and twice with PBS. Subsequently, 1 μg of 125I-TMPP was added in addition to increasing doses of heparin. All binding reaction tubes were gently shaken for 90 min at room temperature, washed three times with 0.5 ml NaCl 0.5 M, and counted by a γ counter.
Fig. 1. Inhibition of FGF2 binding to SMCs. Subconfluent cultures of SMCs were incubated at 4°C with \(^{125}\text{I}-\text{FGF2}\) and increasing concentrations of TMPP. Low affinity bound \(^{125}\text{I}-\text{FGF2}\) was excluded by incubation with a 1.5 M NaCl buffer (pH 7.5). High affinity receptor binding of FGF2 was determined by incubating the cells with a 1.5 M NaCl buffer (pH 4.0). The extracted solution was counted in a \(\gamma\) counter. Binding of FGF2 to SMCs was markedly inhibited at doses as low as 1 \(\mu\text{g/ml}\). Suramin was added as a positive control. Results are given as mean±S.D.

3. Results

3.1. TMPP inhibits binding of FGF2 to vascular smooth muscle cells

TMPP is a porphyrin analogue which we have recently identified for its capacity to inhibit FGF–FGFR binding in vitro and tumor progression in vivo [17]. In order to evaluate the capacity of TMPP to inhibit FGF2-receptor binding on SMC, we measured binding of radiolabeled FGF2 to SMCs, which are known to express FGF receptors. A significant dose-dependent inhibition of radiolabeled FGF2 binding occurred in the presence of TMPP at a concentration as low as 1 \(\mu\text{g/ml}\) (Fig. 1).

3.2. TMPP specifically inhibits FGF binding to soluble FGF receptors 1 and 3

In order to determine the specificity of this effect, chemical cross-linking of \(^{125}\text{I}-\text{FGF2}\) to soluble FGFR1 was carried out in the absence or presence of TMPP. As shown in Fig. 2, there is complete inhibition of the formation of a typical FGF2–receptor complex at TMPP concentrations as low as 5 \(\mu\text{g/ml}\), in agreement with the direct binding

![Graph showing inhibition of FGF2 binding to SMCs with increasing concentrations of TMPP.](image)

Fig. 2. Inhibition of FGF2 binding and cross linking to soluble growth factor receptors by TMPP. Soluble FGF receptor 1-AP, FGF receptor 3-AP or EGF receptor IgG-Fc fusion proteins were incubated with rabbit anti-AP antibodies bound to agarose-protein-A beads (FGF receptors) or directly to agarose-protein-A beads (EGFR-Fc). The radiolabeled ligands, \(^{125}\text{I}-\text{FGF2}\), \(^{125}\text{I}-\text{FGF9}\) or \(^{125}\text{I}-\text{EGF}\), respectively, were added to the binding reaction in the presence of heparin, with and without TMPP 5 \(\mu\text{g/ml}\). After 90 min, disuccinimidyl suberate (0.15 mM in PBS) was added. The protein complexes were separated by electrophoresis on a 7.5% SDS–polyacrylamide gel and analyzed on X-ray film.

![Image showing inhibition of FGF2 binding to soluble growth factor receptors.](image)
data (Fig. 1). In order to determine the specificity of this effect, we tested the capacity of TMPP to inhibit the binding and cross-linking of $^{125}$I-EFG to the EGF receptor and of $^{125}$I-FGF9 to FGFR3, a clone homologue of FGFR1. As shown in Fig. 2, TMPP at 5 μg/ml inhibits FGF2 and FGF9 receptor binding and the formation of an FGF–receptor complex, but has no effect on the binding of $^{125}$I-EGF, a non-heparin binding protein, to the EGF receptor.

3.3. TMPP binds FGF2

To evaluate whether TMPP can directly interact with the FGF protein, TMPP was chemically immobilized on cyanogen bromide activated Sepharose beads. The beads were then incubated with radiolabeled FGF2. Regardless of extensive washing, FGF2 remained specifically bound to TMPP (Fig. 3). In contrast, incubation with EGF showed no binding affinity to TMPP.

3.4. TMPP competitively inhibits FGF2 binding to immobilized heparin

In order to investigate the role of heparin in TMPP-mediated FGF inhibition, $^{125}$I-FGF2 was bound to heparin covalently immobilized to a 96-well plate. As can be seen in Fig. 4, the addition of increasing doses of TMPP results in competitive inhibition of FGF2–heparin interactions.

3.5. TMPP binding to soluble FGFR1 can also be competitively inhibited by heparin

In order to evaluate whether TMPP directly interacts with the FGFR protein, we radiolabeled its close analogue P10016 [17] with $^{125}$I using the Bolton-Hunter technique. TMPP can bind specifically to the extracellular domain of FGFR1 (Fig. 5, panel A), suggesting a direct interaction with the receptor protein. The addition of heparin or FGF-1 (acidic FGF) results in reduced binding. The addition of increasing doses of heparin to this experimental model results in a competitive inhibition of TMPP–FGFR binding (Fig. 5, panel B).

3.6. TMPP inhibits smooth muscle cell proliferation and DNA synthesis in a dose-dependent manner

Since SMCs are the key players in the development of a neo-intima after successful PTCA, and FGF2 is known to be a potent mitogen for SMCs, we investigated the influence of TMPP on FGF2-mediated vascular SMC growth by directly measuring proliferation and also by a thymidine incorporation assay. Fig. 6, panel A demonstrates a marked dose-dependent inhibitory effect on SMC growth over a period of time. At a dose of 2 μg/ml, TMPP exerts more than 50% inhibition on cell growth. TMPP also significantly inhibits DNA synthesis in SMCs in a dose-dependent manner (Fig. 6, panel B), with a similar response (Fig. 1).

4. Discussion

Smooth muscle cell proliferation and migration play a crucial role in the pathogenesis of restenosis after successful PTCA. There are multiple examples whereby inhibition of proliferation of SMCs can significantly reduce the
development of arterial thickening produced in response to balloon injury. These models have utilized non-specific, small molecular weight tyrosine kinase inhibitors [21], antisense oligodeoxynucleotides to several cell cycle regulatory proto-oncogenes [22], targeted gene delivery [23] and toxin-linked growth factors [24]. Other attempts to inhibit proliferation of SMCs included the use of troglitazone [25], cyclosporin A [26], beta-interferon [23], halofuginone [27], dipyridamole [28] and a prostacyclin analogue [29].

Porphyrins have been of interest to chemists and medical scientists for over a century. It has been known for many years that porphyrins interact with neoplastic tissues [37] and the fact that porphyrins demonstrate high affinity to tumorigenic cells in vitro and solid tumors in vivo, is well established [38,39]. Moreover, porphyrin derivatives have been used for the treatment of malignant tumors in combination with electromagnetic radiation or radioactive emissions. Since they strongly absorb light, many porphyrins are still being used as photosensitizers in photodynamic therapy (PDT) [39].

Here we demonstrate that TMPP, a positively charged porphyrin analog, significantly inhibits FGF2 induced SMC proliferation by specifically interfering with the essential interaction of both FGF2 and its receptor with heparan sulfates. Interaction of the non-heparin-binding dermal growth factor with its receptor is, however, not inhibited by TMPP. TMPP seems to directly bind with significant affinity to both FGF2 and FGFR1 and this binding could be competitively inhibited by heparin, suggesting that TMPP may block heparin-dependent interactions of FGF2 with its receptor. These compounds exert high anti-mitogenic properties towards vascular SMCs as shown by a reduction in FGF2 induced proliferation and thymidine incorporation.

All FGF members including FGF2 [11] and FGF9 [19] bind to their receptors. Both free and cell surface immobilized heparin-like molecules can interchangeably promote the formation of a stable, ternary complex composed of FGF, FGFR and a defined heparan sulfate molecule [31]. It has been proposed that the minimum active structural unit of an FGF2–heparin complex is a properly cis-oriented dimer bound to a sulfated decasaccharide [15]. Furthermore, heparin was also found to directly interact with the extra-cellular domain of FGFR and this interaction is essential for receptor activation [32,33]. Recently, the three-dimensional structures of FGFR and of FGF in complex with its receptor and heparin has been described [14,34,36]. An attractive way to inhibit FGF2-receptor binding and activation would therefore be to interfere with the essential interaction of heparin with both the ligand and the receptor.

It is not at all clear how a small, moderately charged molecule such as TMPP inhibits heparin binding of both FGF2 and FGF9, but not that of FGF to their corresponding high affinity receptors. One possible explanation is that this compound may structurally mimic a heparin-binding motif shared by both FGF and FGFR. This is not obvious, as TMPP by itself does not seem to bind heparin with high affinity. However, we and others have shown by experiments of site directed substitution of residues involved in heparin binding in FGF2, that heparin binding of
both FGF [35] and FGFR [32,33] is highly sensitive to restricted changes within their putative heparin-binding domains. A small, positively charged molecule such as TMPP may competitively inhibit one such interaction, which may be sufficient to cause a deleterious effect on the formation of functional ligand–receptor complexes.

A critical question still remains as to why an inhibitor of only one of several pathways participating in the process of intimal hyperplasia results in significant attenuation of the process. There is data to suggest that several of these strategies converge on a limited number of final common pathways responsible for the restenotic phenotype. Lindner and Reidy [30] showed that systemic injection of a neutralizing antibody against FGF2 prior to balloon catheterization significantly decreased the induced SMC proliferation by ~80%. This study and several others support the concept that FGF2 signaling is one of these main pathways. It is therefore possible that the identified compound,
Fig. 6. Inhibition of cell growth and DNA synthesis by TMPP. (A) Vascular SMCs were plated into 48-well plates and grown in the presence of FGF2 and different doses of TMPP. Cell growth was determined over 4 days by counting cells with a camera counter. (B) Vascular SMCs were plated into 96-well plates and were grown to subconfluency. After serum starvation for 24 h, cells were stimulated with FGF2 in the presence of increasing doses of TMPP. [³H]Thymidine was added to the growth medium and incubation was continued for a further 6 h. DNA synthesis was determined using a cell harvester. Results are given as mean±S.D.
which can act directly on cell surface FGF receptors, may serve as an adjuvant for the treatment and prevention of restenosis and related disorders.

Acknowledgements

This study was supported in part by a grant from Yael Research Fund (to Dr A. Segev).

References

[17] Aviezer D, Cotton S, David M et al. Porphyrin analogues as novel antagonists of fibroblast growth factor and vascular endothelial growth factor receptor binding that inhibit endothelial cell prolifer-

