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Uveal melanoma and macular degeneration: Molecular biology and potential therapeutic applications

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Stockholm 2007

Published by Karolinska Institute and printed by University Service-AB press
SE-171 77 Stockholm, Sweden

ISBN 978-91-7357-254-5

To my father *Solon*

ABSTRACT

Uveal melanoma is the most common primary intraocular malignant tumor in adults with 30% to 50% of patients that ultimately succumb to metastatic disease, mainly to the liver. Although new diagnostic and therapeutic tools have been developed during the most recent years, only the eye conservation rate has been achieved, while the survival rate remains poor. Neovascular age-related macular degeneration (AMD) is a disease where abnormal blood vessels grow in the macula and cause blindness and is the leading cause for legal blindness in many industrialised countries. Angiogenesis and more specifically growth factors are thought to be responsible for the development of both tumors and neovascular AMD. The aim of the study was to identify prognostic markers for uveal melanoma and potential new therapeutic applications for both uveal melanoma and exudative AMD.

Paraffin-embedded tumor specimens from 132 patients with primary uveal melanoma were analyzed with immunohistochemistry by using well-established specific antibodies against c-Met and IGF-1R. We found that the expression of both IGF-1R and c-Met was significantly associated with melanoma-specific mortality while IGF-1R showed to be a stronger prognostic marker than other currently used such as tumor cell type and tumor diameter. That could suggest a possible role of these two receptors in the spread of uveal melanoma while IGF-1R could be considered as prognostic tool and a therapeutic target.

The elevated mortality rate of uveal melanoma is due to a high incidence of metastases, which occur preferentially in the liver. We showed that intraperitoneal injections of picropodophyllin (PPP), an inhibitor of IGF-1R, efficiently blocks uveal melanoma growth *in vitro* using different uveal melanoma cell lines and *in vivo* in uveal melanoma xenografts. Furthermore PPP inhibited several mechanisms involved in metastasis, including tumor cell adhesion to extracellular matrix proteins, activity and expression of matrix metalloproteinase 2, and also significantly reduced the number of the micrometastases to the liver.

Oral chemotherapy generally represents a fundamental change and challenge at the same time in contemporary oncology practice. We demonstrated that oral administration of PPP completely blocks the growth of uveal melanoma xenografts and was well tolerated by the mice. PPP also reduced expression of IGF-1 dependent VEGF in uveal melanoma tumors. This effect and the anti-angiogenic response probably contribute to the high anti-tumor efficacy of PPP. PPP was also found to be superior to the other anti-tumor agents such as imatinib mesylate, cisplatin, 5-FU and doxorubicin in killing uveal melanoma cells. We could only detect a limited synergistic effect with some of them in combination with PPP.

There is now compelling evidence that targeting angiogenesis in general and VEGF signaling in particular is a meaningful approach for the therapy of both cancer and exudative AMD. We could show that PPP efficiently reduces experimental CNV and inhibits the VEGF secretion from RPE cells.

This thesis highlights the possibility of using IGF-1R as a prognostic marker for uveal melanoma as well as that targeting the receptor with inhibitors like PPP could offer a useful option in therapy of uveal melanoma and exudative AMD.

KEY WORDS: Uveal melanoma, exudative AMD, IGF-1R, c-Met, prognostic markers, metastasis, oral treatment, VEGF.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Economou MA**, All-Ericsson C, Bykov V, Girnita L, Bartolazzi A, Larsson O, and Seregard S.

Receptors for the liver synthesized growth factors IGF-1 and HGF/SF in uveal melanoma: intercorrelation and prognostic implications.
Invest Ophthalmol Vis Sci. 2005 Dec; 46(12):4372-5.

II. Girnita A*, All-Ericsson C*, **Economou MA**, Åström K, Axelson M, Seregard S, Larsson O, Girnita L.

The Insulin-Like Growth Factor-I Receptor Inhibitor Picropodophyllin Causes Tumor Regression and Attenuates Mechanisms Involved in Invasion of Uveal Melanoma Cells.
Clin Cancer Res. 2006 Feb 15; 12(4):1383-91.

III. **Economou MA**, Andersson S, Vasilcanu D, All-Ericsson C, Menu E, Girnita A, Girnita L, Axelson M, Seregard S, Larsson O.

Peroral administered picropodophyllin (PPP) is well tolerated in vivo and inhibits IGF-1R expression and growth of uveal melanoma.
(submitted)

IV. **Economou MA**, Wu J, Vasilcanu D, Rosengren L, All-Ericsson C, van der Ploeg I, Menu E, Girnita L, Axelson M, Larsson O, Seregard S, Kvanta A.

Inhibition of VEGF secretion and choroid neovascularization by picropodophyllin (PPP), an inhibitor of the insulin-like growth factor-1 receptor.
(submitted)

***** Equal contributions

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LIST OF ABBREVIATIONS

Akt	Protein kinase B
AMD	Age related macular degeneration
CM	Cutaneous melanoma
CNV	Choroidal neovascularisation
ECM	Extracellular collagen matrix
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinase-1 and 2
5-FU	Fluorouracil
FDA	Food and drug administration
HGF/SF	Hepatocyte growth factor/scatter factor
IGF-1	Insulin-like growth factor
IGF-1R	Insulin-like growth factor receptor type 1
IHC	Immunohistochemistry
ip	intraperitoneal
IR	Insulin receptor
MAPK	Mitogen-activated protein kinase
MMP-2	Matrix metalloproteinase 2
po	peroral
PPP	Picropodophyllin
RTK	Receptor tyrosine kinase
RPE	Retinal pigment epithelial
SCID	Severe combined immunodeficiency
TGF-ß	Transforming growth factor-ß
UM	Uveal melanoma
VEGF	Vascular endothelial growth factor
WB	Western blotting

ACKNOWLEDGMENTS

I would like to express my gratitude to everyone who have contributed to this thesis. In particular I am grateful to:

Stefan Seregard, my supervisor, for giving me the opportunity to start research in Sweden, for sharing his big scientific experience in ocular oncology and medical statistics, for being generous with research leave, and for being always available for every possible matter that concerns research, clinical work and career planning.

Olle Larsson, my supervisor, for accepting me in his lab, for introducing me into basic research, for sharing his vast scientific knowledge, for his integrity and total devotion to his students, for being always and promptly helpful and for receiving with enthusiasm my new ideas.

Leonard Girnita, my co-supervisor, for having always an answer to my questions and for sharing his big skills on molecular biology techniques.

Magnus Axelson, for our fascinating discussions and for last moment assistance before experiments.

Anders Kvanta, my co-author, for all the stimulating discussions, constructive critisism and for sharing his knowledge on angiogenesis.

Jan Ygge, for help and support.

Armando Bartolazzi, my co-author, for interesting discussions about research and nice dinners talking about life in Sweden.

Daiana Vasilcanu, Sandra Andersson, Linda Rosengren, Ada Girnita my co-authors for being always helpful with experiments and with answering questions.

Bita Sehat, Radu Vasilcanu, Natalia Natalishvili, Pädraig Darcy for nice chats and for positive attitude towards life.

Ingeborg van der Ploeg for sharing her skills in PCR.

Jiangmei Wu for sharing her skills in flat mount technique and for company at late pizza dinner on Saturday evening after performing experiments together.

To the ophthalmology resident group (ST-gänget) for welcoming me warmly to the group from day one and being supportive and friendly.

To all the staff at the ward 42 (avdelning 42) and especially *Yvonne* for making me feel a bit more home here in Sweden by being so warm and friendly.

Sven Sahlin, my clinical tutor, for guidance and for being always available.

Talal Ali, for being such a warm person and such a skilled phaco teacher.

Per Montan, for our italian speaking dinners.

Johann Gudmunsson and the islandic basketball gang for their Viking version of basketball and for good friendship.

Jaakko Patrakka my footboll and lunch mate and to the guys from MBB.

Monica Aronsson and Helen Braxenholm at the animal facilities of St Eriks Eye Hospital and MTC respectively, for helping me with the animal experiments.

Margareta Rodensjö and Liss Garberg at the CCK for the help concerning immunohistochemical stainings.

Ann Britt Spåre, Ulla-Britt Peterson, Britt-Marie Karlheden, Carina Zahn, Catharina Kuylenstierna for secreterial assistance.

Colleagues and staff at St Erik`s Eye Hospital, for always being helpful.

David Mansfield, Consultant ophthalmologist in Scotland, for friendship and for having being more than a mentor for me, and his wife *Uma* for her positive spirit towards my move to Sweden.

Mark Hero, Consultant ophthalmologist, my italian speaking boss in Coventry for being so helpful and supportive.

Wendy Franks, Consultant ophthalmologist at Moorfields, for helping me so much at the beginning of my career in the UK and for continuing to be supportive and interested in my work even from distance.

Vincenzo Maurino, Consultant ophthalmologist at Moorfields, the italian link in England, for being helpful and educative on my first steps in ophthalmology.

David Jones and his wife *Vandana*, *Teresa Sandinha*, colleagues from Glasgow, for really nice dinners and friendship.

All my colleages at the eye department of the University of Ferrara, in Italy and especially Professor *Adolfo Sebastiani* and *Paolo Perri* for good advice.

Guido, Alessandro and Monia, Pasquale, Jean-Claude, Ifix and Frans, Giovanna Vallini, Haris, Manolis, Panos, Michalis for long friendship.

Anna and Erik, for nice dinners and talks.

Carl-Friedrik and Christoffer for always showing interest in my work and especially the second one for assistance with power point slides.

My brother *Nick*, for being so encouraging and supportive on every “dangerous” step of my life, for having faith and trust on his big brother, for continuous challenge.

My mother *Mary*, for being a never-stop giving love person, for showing a lot of interest in my work and research and for supporting every choice of mine.

Lotta, for all her love and support.

This study was supported by grants from Swedish Cancer Society,
King Gustaf V Jubilee Foundation, St Erik's Eye Hospital Research Foundation, Carmen and Bertil Regner Foundation, Swedish Medical Council, Stockholm Cancer Society, Children Cancer Society, Lundberg's Research Foundation in Gothenburg, the Stockholm
County and the Karolinska Institute.

INTRODUCTION

Uveal Melanoma

Anatomy, histopathology, epidemiology

The iris, ciliary body and choroid constitute the uveal tract. The uveal tract is embryologically derived from the mesoderm and neural crest. It is a highly vascularised structure composed of melanocytes, supporting connective tissue with fibroblasts and blood vessels and peripheral nerves.
Histopathologically there are 4 different types of uveal melanoma identified: spindle cell, epithelioid, mixed and necrotic.
Approximately 5% of all uveal melanoma (UM) arise in ocular and adnexal structures. (Chang 1998) Most ocular melanomas (85%) are uveal in origin, whereas primary conjuctival and orbital melanomas are very rare. (Chang 1998) (Singh 2003) Approximately 90% of all UM develop in the choroid, 7% in the ciliary body and 3% in the iris. (Damato 2001) UM is the most common primary intraocular malignant tumor (Strickland 1981) with an annual incidence, which has remained stable for the last 50 years, of 8.4 to 11.7 cases per million in whites. (Bergman 2002)

Uveal and cutaneous melanoma, similarities and differences

The incidence of UM in whites is eight times that of Blacks and three times that of Asians. (Egan 1988) That fact could point to the casual role of ultraviolet (UV) light as a risk factor for UM. (Holly 1990) On the other hand, the incidence of UM in contrast to cutaneous melanoma (CM), has not increased during the last 40 years (Bergman 2002) possibly reflecting a greater role for UV light exposure in the development of CM than in UM. (Dolin 1994) Patients with a diagnosis of CM have a 10-fold increased risk of developing a second cutaneous lesion, but they have no additional risk for the development of a UM. (Shors 2002) The controversial role of UV exposure in causing UM is only one of a series of differences between the UM and CM. Both melanoma subtypes originate from a common precursor cell, the melanocyte, which migrates from the neural crest to the respective site during the embryonic development period. (Soufir 2000) UM metastasizes hematogenously, with the liver frequently being affected, (Didolkar 1980) whereas CM tends to spread through the lymphatic system, usually first affecting the regional lymph nodes. (Chang 1998) In addition, UM sometimes exclusively spreads to the liver, whereas single organ metastasis is extremely uncommon in CM. (Eskelin 2003) Despite the abundant evidence that UM and CM are distinctly separate tumors, few genotypic differences between these melanoma subtypes have been identified. Functional loss of the CDKN2A (p16INK4) tumor suppressor, whether through promoter methylation, mutation, and/or deletion, is observed in a significant fraction of both UMs and CMs. (Cannon-Albright 1992) (Edmunds 2002) (Merbs 1999) (van der Velden 2001) Likewise, p53 alterations are observed in both melanoma types in association with tumor progression. (Tobal 1992) (Weiss 1995)

Prognostic factors of uveal melanoma

The prognostic factors can be divided in clinical, histopathological, cytogenetical, and molecular. One of the most important clinical prognostic parameters is tumor location where the ciliary body melanoma has a worse prognosis (Seddon 1983) while iris melanomas have the lowest mortality of about 3–5% in 10 years. (Shields 2001, Rones 1958) Tumor size is another important clinical prognostic factor since high mortality rate is associated with large tumors. (Diener-West 1988) (COMS 1997) Also tumor configuration is another clinical predictive parameter, where diffuse choroidal melanomas have a poor prognosis. (Shields 1996) Cell type, mitotic activity, microcirculation architecture and tumor-infiltrating lymphocytes are the most significant histopathological prognostic factors. Specifically, epithelioid cell type (McLean 1983), high mitotic activity (McLean 1977), presence of microcirculation architecture (vascular patterns) (Rummelt 1995) (Folberg 1993) and presence of tumour-infiltrating lymphocytes (TILs) (de la Cruz 1990) are correlated with poor prognosis. The findings of monosomy 3 and duplication of 8q are associated with a survival rate of 40% at 3 years compared with 95% survival in the absence of these cytogenetic changes. These chromosomal aberrations constitute the most important cytogenetical prognostic factors. (White 1998) The overexpression of c-Myc, a potent inducer of malignant transformation, is known to promote cell growth and proliferation has been unexpectedly associated with improved prognosis (Chana 1998) while the nearby genes DDEF1 and NBS1 were more highly overexpressed and more strongly associated with poor prognostic features. (Ehlers 2005) (Ehlers 2005) Lastly, alterations in tumor-suppressor pathways, such as p53 pathway and the Rb pathway (Janssen 1996) (Brantley 2000) can play a role in uveal melanoma.

Treatment and prognosis

Treatment modalities of uveal melanoma include: plaque radiotherapy (brachytherapy) usually with either 106Ru or 125I, (Shields 2000), proton beam radiotherapy (Gragoudas 2005), transpupillary thermotherapy (TTT) (Journee-de Korver 2005), trans-scleral local resection (Damato 2006), and enucleation (Zimmerman 1978).
Systemic disease only rarely responds to treatment and is invariably fatal, usually within 6–9 months of the onset of symptoms. Survival can be much longer (i.e., >1 year) when asymptomatic metastases are detected by screening. Uveal melanoma is the cause of death in approximately 50% of all patients with this disease. (Kujala 2003)

Age-related macular degeneration

History, epidemiology, types

Since 1874, when it was first described in the medical literature as "symmetrical central choroido-retinal disease occurring in senile persons," (Hutchinson 1874) age-related macular degeneration has also been referred to as senile, or disciform, macular degeneration, among

many other terms. About 25 years ago, the term "age-related maculopathy" was coined and its end stage was acknowledged as age-related macular degeneration (AMD).
AMD is the leading cause of blindness in individuals aged 55 years and older in industrialized countries (Klein 1995) (Dimitrov 2003) (Buch 2004) Approximately 1.7% of individuals aged 55 years and older are affected with AMD in Europe (Vingerling 1995) while in the USA an estimated 8 million people are affected from age-related macular degeneration (AMD). (AREDS 2003) Two million people suffer from the advanced form of disease and with the expected increase of people over the age of 85 years, this number will increase to 3 million by the year 2020 (Eye Diseases Prevalence Research Group 2004) (Thylefors 1998)
AMD is clinically divided into two forms; the dry or nonneovascular (nonexudative) form comprising about 90% of cases. It is characterised by soft indistinct drusen (63 µm), hyperpigmentation, or depigmentation, and geographic atrophy. Only 10% of patients will develop the wet or neovascular (exudative) form of AMD, which is responsible for nearly 90% of those with severe vision loss. Features of neovascular AMD include subretinal fluid, retinal swelling, hemorrhage, exudation, detachment of the retinal pigment epithelium and the development of choroidal neovascularisation (CNV) in addition to those described for nonneovascular AMD. (Smith 2007)

Pathogenesis, treatment

The etiology of AMD is multifactorial and it is believed that four main processes are involved: the accumulation of lipofuscin granules composed mainly of lipids and proteins, as a result of an age-dependent phagocytic and metabolic insufficiency of retinal pigment epithelium (RPE) cells, the accumulation of drusen, amorphous deposits between retinal pigment epithelium (RPE) and the inner collagenous zone of the Bruch´s membrane, the complement factor H (CFH) in the complement cascade which is believed to play an important role in AMD development. (Klein 2005) (Donoso 2006) Last but certainly not least, choroidal neovascularization, the proliferation of blood vessels into the subretinal space from the underlying choriocapillaries, is a very complex phenomenon where molecules known as growth factors, among others, regulate angiogenesis (vessel formation). Vascular endothelial growth factor (VEGF) has been identified as a major angiogenic stimulus in the development of CNV (Witmer 2003) but a many other growth factors such as transforming growth factor-ß (TGF-ß) (Kliffen 1997) and insulin-like growth factor 1 (IGF-1) (Lambooij 2003) and angiogenic molecules such as angiopoietin (Hera 2005) are believed to play an important role as well.
Vitamin and mineral supplementation can reduce the risk of moderate visual loss among some patients with the dry form of AMD as shown from the Age-Related Eye Disease Study (AREDS). (AREDS 2001) For many years laser photocoagulation remained the only available treatment option, although a destructive one, for patients with well-defined extrafoveal CNVs. (Moisseiev 1995) Photodynamic therapy with verteporfin, was developed as an alternative to thermal laser photocoagulation could safely reduce the risk of vision loss in patients with subfoveal CNV caused by AMD. (TAP 1999) Pegaptanib sodium, an aptamer that binds to VEGF isoform 165 was the first drug to target VEGF and to get Food and Drug Administration (FDA) approval for use against wet AMD. Pegaptanib showed some efficacy in stopping the development of CNV by inhibiting angiogenesis and/or reducing vascular permeability. (Gragoudas 2004)

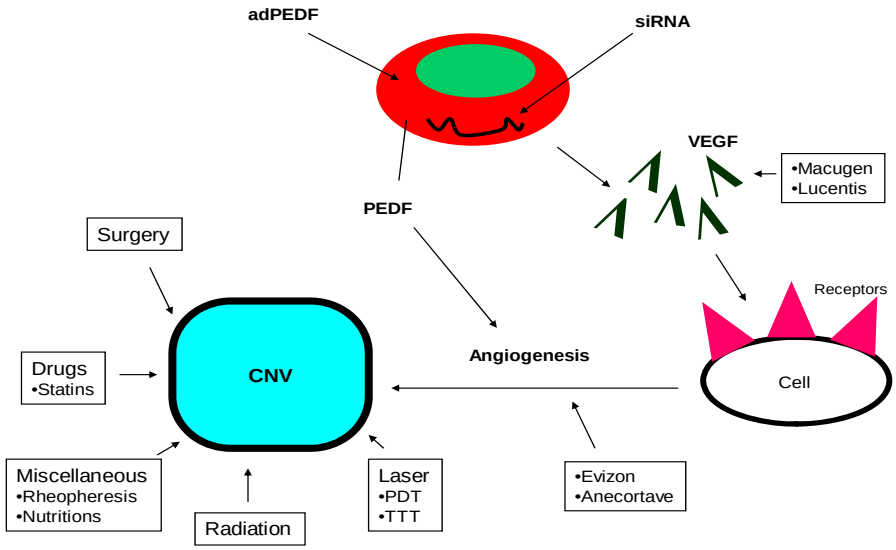

Fig 1. Past, current and possible future treatments of AMD.

Ranibizumab is a humanized anti-VEGF-A recombinant Fab fragment that has been affinity-matured to increase its binding affinity for VEGF-A. Two randomized, double-masked, pivotal phase III clinical trials have demonstrated that monthly intravitreal injection of ranibizumab is effective treatment for subfoveal CNVs in AMD patients, resulting in FDA approval last year. (Rosenfeld 2006) (Brown 2006) Also bevacizumab, a glycosylated, humanized Fab fragment, approved for metastatic colon cancer and non-small cell lung cancer has showed similar efficacy as ranibizumab in different retrospective studies (Avery 2006) (Spaide 2006) and one prospective non-randomized study (Costa 2006)

The IGF-1R

Structure and function

The type 1 insulin-like growth factor receptor (IGF-1R) is a tyrosine kinase (TK) receptor with a 70% homology to the insulin receptor (IR). It was originally considered a redundant receptor used by cells only when signaling from the IR was absent or defective. Both IGF-1R and IR are preformed dimeric TK receptors made up by two extracellular -subunits and two -subunits involving a small extracellular domain, an intramembraneous one as well as an intracellular domain. (Adams 2000) The latter includes the juxtamembraneous domain, the

TK domain and the C-terminal domain. The IGF-1R and IR are highly homologous, especially in the TK domain in which they share 84% amino-acid identities. However, despite these similarities, the function between IGF-1R and IR differs considerably.
Upon ligand binding, a conformational change induces activation of the kinase. Several docking proteins, such as Src homology 2 domain-containing (Shc) protein and insulin receptor substrates (IRS1–4), are subsequently recruited to the phosphorylation sites in the cytoplasmic domain. Then the signal is propagated via the phosphatidylinositol-3-kinase (PI 3-kinase)/Akt and mitogen-activated protein (MAP) kinase pathways resulting in cell proliferation and inhibition of apoptosis. IGF-1R signalling can also induce differentiation, malignant transformation and regulate cell–cell adhesion. A dynamic downstream signalling network of different phosphorylation sites of the receptor and cell-context specific recruitment and activation of signalling molecules regulates these different functions. (Baserga 1999)

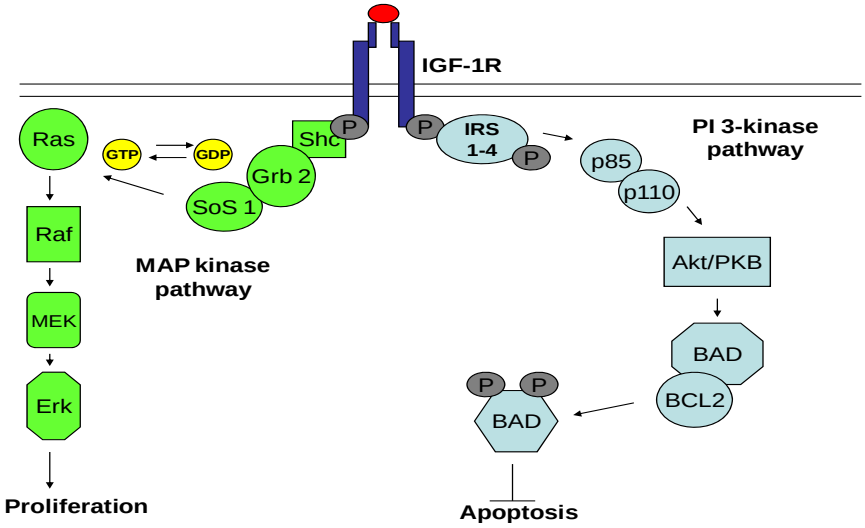

Fig. 2. Simplified scheme of the signalling pathway of the IGF-1R.

Furthermore, the IGF-1R can interact with steroid hormones and their receptors, other peptide growth factor receptors, extracellular matrix proteins, integrin receptors and cytokines, such as transforming growth factor-β. (Jones 1995)
In normal physiology, IGF-1R stimulates linear body growth, promotes neuronal survival and myelination, postnatal mammary development and lactation, and is implicated in bone formation and renal function. (Jones 1995) The IGF-1R plays a central role in integrating

signals of nutrition and stress into energy shifts from energy-expensive anabolic processes, such as growth and reproduction, to preserving responses under catabolic circumstances (Niedernhofer 2000)

The role of IGF-1R in cancer

The involvement of the IGF-1R in malignant transformation was first recognised in fibroblasts derived from homozygous IGF-1R null mice embryos. (Sell 1993) Mouse embryo fibroblasts are prone to transformation; however, in the absence of IGF-1R they become resistant to malignant transformation by a number of oncogenes (e.g. Simian Virus 40T antigen (SV40 T) Ewing Sarcoma fusion protein). Re-expression of the IGF-1R restored susceptibility to transformation in these cells. In population studies, high serum levels of IGF-1 have been associated with an increased risk on prostate cancer and premenopausal breast cancer. (Renehan 2004) Increased incidence of colorectal adenomas and cancer is seen in acromegaly, in which hypersecretion of GH is accompanied by elevated IGF-1 levels. (Jenkins 2006)

In vivo overexpression of IGF-1R accelerated the development of tumors in a mouse model of cancer (RIP1-TAG2). (Lopez 2002) In this mouse model, expression of oncoprotein SV40 T in pancreatic islet β cells leads to tumor formation, which is characteristically accompanied by IGF-2 upregulation, providing an activating ligand for the IGF-1R. Remarkably, IGF-1R overexpressing RIP1-TAG2 mice developed more invasive tumours with an increased amount of distant metastases than parental mice. Recently, transgenic expression of a constitutively active IGF-1R fusion protein in mice resulted in spontaneous development of invasive adenocarcinomas of salivary and mammary glands. (Carboni 2005)

The above-mentioned studies provide proof of the principle that active IGF-1R signalling facilitates malignant transformation, drives growth and progression of established tumors and enhances capability to invade and metastasise.

The tumor promoting functions of IGF-1R are embedded in the multi-dimensional process of cancer development and progression. Increased IGF-1R activation, by GH/IGF-1 status or other mechanisms, might create an anti-apoptotic environment thereby favoring cell survival and malignant transformation. Oncogenes, such as the hepatitis B virus oncoprotein (HBx) or Ewing sarcoma fusion proteins, recruit and activate the IGF-1R signalling pathway by increasing transcription of the IGF-1R gene, while loss of tumor suppressor genes, such as p53, BRCA1 or WT1, results in IGF-1R overexpression by loss of transcriptional control. Overexpression of IGF-1R in tumour compared to normal tissue is shown in a number of studies with polymerase chain reaction (PCR) detection of IGF-1R mRNA. (Werner 2000) (Weber 2002) Amplification of the IGF-1R gene, however, is infrequent, as shown in breast tumors (<2%) (Almeida 1994) and (Berns 1992) and sarcomas. (Sekyi-Out 1995) Activating mutations of the receptor have not been described yet. Numerous other molecular mechanisms that modulate IGF-1R signalling in cancer, such as loss of imprinting of IGF-2, altered glycosylation and constitutive activation of downstream proteins, have been described. (Samani 2007) Up-regulation of IGF-1R signalling has recently been implicated in the development of resistance to anti-cancer therapy, such as radiotherapy, hormonal therapy and human epidermal growth factor receptor 2 (HER2) targeting. (Nahta 2006) (Milano 2006)

Targeting IGF-1R in cancer

The vast expression of IGF-1R in neoplastic cells and tissues combined with its crucial roles in cancer cell growth makes this tyrosine receptor an attractive target to combat malignant diseases.
A variety of approaches aimed at targeting IGF-1R has been utilised to prove the concept, or are being developed as potential anticancer therapies. Targeting IGF-1R to block its signalling may be obtained by interference with ligand/receptor interactions, receptor synthesis and expression, receptor TK activity, or combinations of these strategies.
Strategies aimed to block the ligand-receptor interaction involve receptor neutralising antibodies. (Kalebic 1994) Among those most studied is the monoclonal antibody -IR3, which competes with IGF-1 for binding to the receptor and blocks receptor activation (Van Wyk 1985). Antibody blockade of IGF-1R has been attempted in breast cancer model systems. However, the large size of the therapeutic molecule restricts its access to tumor cells, particularly in the central regions of solid tumors (Russell 1992). Smaller fragments are currently being studied as a substitute for whole antibodies in an effort to improve access and uptake. Sachdev et al (2003) used a single-chain antibody directed against IGF-1R (IGF-1R scFv-Fc) to examine the effects on IGF-1R signalling. In vivo treatment of mice bearing MCF-7 xenograft tumours with scFv-Fc resulted in near complete downregulation of IGF-1R.
Antisense techniques are another way to inactivate the IGF-1R. Resnicoff et al (1994) used antisense RNA to IGF-1R by introducing it into cells by either addition of oligodeoxynucleotides or by transfection with plasmids expressing antisense RNA to IGF-1R RNA. Injection of glioblastoma cells (C6) IGF-1R antisense cells into rats carrying an established wild-type C6 tumor caused complete regression of the tumors. This indicate that practical applications may be developed to target IGF-1R.
A direct strategy to interfere with IGF-1R activity is to induce selective inhibition of its TK by developing selective small-molecular inhibitors. The major advantage of this approach is that small molecules have a considerable higher bioavailability compared to antibodies, dominant-negative receptors and antisense oligonucleotides. However, TK inhibitors face the problem that IGF-1R and IR are so similar. Actually many of the hitherto developed IGF-1R TK inhibitors have also caused substantial inhibition of the IR. Such cross reaction would probably cause diabetic reactions in patients and can therefore not be accepted. On the other hand, IR-A dependent tumors would not be affected by a fully selective IGF-1R inhibitor.

Most of the IGF-1R TK inhibitors produced so far have served as competitive ATP inhibitors. Since the region of the TK domain covering the ATP binding site is identical to that of the IR, such cross-inhibitions are not unexpected. However, there is a recent interesting exception. Garcia-Echeverria et al (2004) presented a new compound (a pyrrolo[2,3-d] pyrimidine) that although inhibiting the IGF-1R and IR TK equipotently in cell-free systems, exhibited several-fold selectivity for the IGF-1R in a cellular context and reduced the growth of IGF-1R positive fibrosarcomas in vivo.

Blum et al (2003) presented a new family of bioisostere inhibitors, based on the structure of AG 538, a tyrphostin inhibiting the IGF-1R TK at the substrate level and not at the ATP binding site (Blum et al, 2000). These AG 538 bioisosteres possessed similar but weaker biological properties to AG 538 but are more stable and blocked the formation of colonies of prostate and breast cancer cells in soft agar systems (Blum et al, 2003).

Recently, we demonstrated that the cyclolignan PPP inhibited phosphorylation of IGF-1R without interfering with insulin receptor activity (Girnita et al, 2004), as well as it reduced

phosphorylated Akt, (Girnita 2004) (Girnita 2006) (Vasilcanu 2004) (Colon 2007) caused apoptosis and induced tumour regression in xenografted mice. PPP did not compete with ATP but interfered with phosphorylation in the activation loop of the kinase domain, in which it blocked phosphorylation of the tyrosine (Y 1136) residue, while sparing the two others (Y1131 and Y1135). Since an IGF-1R construct, in which the tyrosine at position 1136 was replaced by a phenylalanine, also led to a strong inhibition of phosphorylated Akt in transfected cells, it was suggested that this mechanism may be responsible for the apoptotic effect of PPP (Vasilcanu et al, 2004).

The IGF-1R has been shown to play a central role in transformation and tumorigenesis. Targeting of IGF-1R signaling by many different approaches has caused a reversal of the transformed phenotype and has induced apoptosis in vitro and in vivo. Moreover, cancer cells are sensitized to conventional chemotherapeutic treatment and irradiation. Therefore, the IGF-1R is a promising target for a specific and selective tumor therapy.

AIMS OF THE STUDY

To determine whether c-Met and IGF-1R could be used as prognostic markers for uveal melanoma using immunohistochemistry.

To investigate whether targeting of IGF-1R with an inhibitor like PPP, could affect the metastatic meccanisms of uveal melanoma.

To evaluate the efficacy of oral PPP in uveal melanoma in vivo and look for synergism of PPP with different anti-tumor agents.

To investigate the effect of PPP on a animal CNV model and its effect on VEGF secretion from RPE cells.

MATERIALS AND METHODS

Clinical material

Primary uveal melanoma specimens, fixed in formaldehyde and paraffin embedded, from 152 consecutive patients that undergone enucleation were available for this study. Twenty lesions were deemed extensively necrotic (defined as >50% of cells necrotic) and excluded from further evaluation, leaving 132 lesions to be immunostained as outlined herein. These lesions were from 55 female and 77 male patients (average age, 63 years; range, 25–85).

Antibodies

In paper I, a rabbit polyclonal antibody directed to the human IGF-1R (N-20) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and a mouse monoclonal antibody directed to human c-Met (NCL-cMET) was provided by Immunkemi (Novocastra Ltd., Newcastle-upon-Tyne, UK). In paper II, III and IV the phosphotyrosine (PY99) and polyclonal antibodies to the β-subunit of IGF-1R (H-60) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), while in paper IV polyclonal antibodies to to IGF-1(H-70), to VEGF (VG-1) and to GAPDH (FL-335) were from Santa-Cruz Biotechnology Inc. (Santa Cruz, CA).

Reagents

PPP was synthesized as described (Girnita et al 2004) and was dissolved in DMSO (0.5 mmol/L) before addition to cell cultures.
PPP/food mixture was prepared as follows: One hundred and eighty mg of PPP was first dissolved in 50 ml acetone. The solution was then added to a mixture of 500 mg of food powder (Lactamin, Kimstad, Sweden) and acetone: sterile water (1:1) to make the food semi-liquid. The final solution was then mixed thoroughly with a mixer and dried overnight at 40°C.
Imatinib-mesylate (Gleevec) was a gift from Novartis Pharmaceuticals (Basel, Switzerland).
Cisplatin, doxorubicin and 5-FU was a kind gift from Professor Linder. (CCK, Karolinska Institute, Stockholm)

Cell cultures

Four cell lines obtained from human primary uveal melanomas (OCM-1, OCM-3, OCM-8 and 92-1) were previously described (All-Ericsson 2004). R+ cells (overexpressing the human IGF-IR) were from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA). R+ cell line was cultured in the presence of G-418 (Promega, Madison, WI). R -v-src were from Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA).
The R -v-src fibroblasts are R- cells transfected with the v-src (being the only single oncogene that can bypass the requirement for a functional IGF-1 receptor in anchorage-independent growth), and have IR substrate-1 and Shc constitutively tyrosine phosphorylated.
ARPE-19 is a non-transformed human diploid RPE cell line that displays many differentiated properties typical of RPE in vivo. (Dunn 1996) ARPE-19 cells were plated at subconfluency and maintained in culture at 37°C in 5% CO2. All ARPE-19 cultures were maintained at 37°C in 5% CO2 in Dulbecco's modified Eagle's medium (DMEM): nutrient mixture F12 plus 10% fetal bovine serum.

Immunohistochemistry

In paper I immunostaining of tissue sections was performed by using the standard avidin-biotin complex (ABC) technique (Vector Laboratories, Burlingame, CA). Briefly, 4-µm tissue sections were cut from each of the selected 132 paraffin-embedded tumor specimens. Tissue sections were deparaffinized and rehydrated. The samples were bleached with hydrogen peroxide-disodiumhydrogenphosphate at room temperature — IGF-1R samples overnight and c-Met 3 hours only, because they were subjected to antigen retrieval according to the manufacturer's instructions.
After antigen retrieval (c-Met) tissue sections were rinsed in Tris-buffered saline (TBS, pH 7.6) and incubated with blocking serum (1% bovine serum albumin) for 20 minutes at room temperature followed by an overnight incubation at 8°C with an excess of anti c-Met antibody or anti-IGF-1R antibody. Biotinylated anti-mouse monoclonal IgG and anti-rabbit polyclonal IgG antibodies were added for c-Met and IGF-1R, respectively, and incubation was continued for an additional 30 minutes at room temperature, followed by application of the ABC complex. The peroxidase reaction was developed for 6 minutes at room temperature with 0.6 mg/mL 3'3-diaminobenzidine tetrahydrochloride (DAB) with 0.03% hydrogen peroxide. Counterstaining was performed with Mayer's hematoxylin. TBS was used for rinsing between the different steps. Appropriate positive and negative controls were included.

Staining assessment

In paper I, all stained cells were considered positive, irrespective of staining intensity. We scored the results of c-Met and IGF-1R immunoexpression as negative when no staining was present, low when less than 10% of cells were stained, moderate when 10% to 50% of cells were stained, and high when more than 50% of cells were immunoreactive. At a later stage, and without knowledge of the initial result, the same observer (ME) repeated the assessment for a random sample of slides from 30 uveal melanoma specimens. These slides were also independently assessed by an experienced ophthalmic pathologist (SS) using the same grading system. The interobserver reproducibility according to the -test was 0.73 (95% CI, 0.54–0.92), and the intraobserver reproducibility was 0.69 (95% CI, 0.49–0.89). Both observers were masked to results from earlier assessments and to survival data.

Immunoprecipitation and Western blotting

In paper II, cells were cultured to subconfluency in 6-cm plates. After one day the cells were serum-depleted for 24 hours before addition of PPP, and then stimulated by IGF1 for 5 min. For determination of IGF1 phosphorylation, the cells were then lysed and subjected to immunoprecipitation by adding 20 µl of resuspended volume of the sepharose conjugate (Protein G-Sepharose) and incubation at 4°C with the anti-IGF-1R β antibody H-60. Immunoprecipitants were analysed by Western blotting. Antibodies to IGF-1R β-unit were used as loading control.
In paper III, tumor samples obtained from the in vivo experiments with OCM-1 and OCM-8 cells were analysed. Samples from fresh-frozen tumors from drug- and solvent-treated mice were cut in pieces and suspended in freshly prepared homogenization buffer as described (Girnita 2004). After centrifugation at 14,000 x g for 10 min at 4°C the supernatants were immunoprecipitated for IGF-1R and IR, and determination of IGF-1R and IR

phosphorylation, after indicated treatments, was completed. Fifteen l Protein G Plus-A/G agarose and 1µg antibody were added to 1 mg of protein material. After overnight incubation at 4°C on a rocker platform, the immunoprecipitates were collected by centrifugation in a microcentrifuge at 2,500 rpm for 2 min. The supernatant was discarded, whereupon the pellet was washed and then dissolved in a sample buffer for SDS-PAGE.
Twenty micrograms of protein per sample were electrophoresed in a 10% Tris–Glycin gel (Novex, invitrogen, Carlsbad, CA). Following electrophoresis the proteins were transferred overnight to nitrocellulose membranes (Amersham, Uppsala, Sweden) and then blocked for 1 h at room temperature in a solution of 5% (w/v) skimmed milk powder and 0.02% (w/v) Tween 20 in PBS, pH 7.5. Incubation with appropriate primary antibodies for IGF-1R, IR and VEGF was performed for 1 h at room temperature, or overnight at 4°C. This was followed by washes with PBS and incubation with either a HRP-labeled or a biotinylated secondary antibody (Amersham) for 1 h. Following the biotinylated secondary antibody, incubation with streptavidin-labeled horse peroxidase was performed. The detection was made with either ECL, Amersham or by Supersignal West Pico reagents (Pierce). The films were scanned by Fluor-S (BioRad).
In paper IV the vitreous and retina were removed from the krypton laser treated mice and the choroid and RPE layer were lyzed for 30 min on ice in buffer (1% NP-40, 0.5% Deoxycholate, 1% SDS, 150 mmol/L NaCl, 50mmol/L Tris–HCl [pH=8]) supplemented with a mixture of protease inhibitors (Sigma). The samples were cleared by centrifugation (14000rpm, 30 minutes, 4 Cº) and assessed for protein concentration (Bradford assay, Bio Rad laboratories, München, Germany). Twenty micrograms of protein per sample were electrophoresed in a 10% Tris–Glycin gel (Novex, Invitrogen, Carlsbad, CA).
IGF-1R and VEGF protein expression levels from choroidal-RPE samples from C57BL/6J mice of all 3 groups were normalized to the corresponding expression of GAPDH control protein and expressed as arbitrary units.

Cell survival assay

Cell viability determinations were done using the Cell Proliferation Kit II (Roche, Inc., Indianapolis, IN), which is based on colorimetric change of the yellow tetrazolium salt 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt in orange formazan dye by the respiratory chain of viable cells (Girnita 2005). All standards and experiments were performed in triplicates.

Integrin expression profile

Expression profile of collagen-specific integrins (1, 2, 3, v, ß1, ß3, and vß3) was assessed by fluorescence-activated cell sorting using antibodies in the Chemicon Investigator Kit (Chemicon Europe, Hampshire, United Kingdom).

ECM adhesion assays

Adhesion of uveal melanoma cells to extracellular collagen matrix (ECM) proteins (collagen type I, collagen type IV, fibronectin, laminin, and vitronectin) was assessed using a CytoMatrix screening kit (Chemicon International, Temecula, CA). Cells were nonenzymatically disaggregated from culture flasks using cell dissociation solution (Sigma-Aldrich, Stockholm, Sweden) and resuspended at a concentration of 5 x 105/mL in RPMI 1640 with 0.1% bovine serum albumin. Cell suspension (100 µL) was added to each well and the plate was incubated at 37°C for 1 hour. After washings and staining (0.2% crystal violet in

10% ethanol for 5 minutes), the levels of adhesion were determined by measuring the absorbance at 540 nm on a microplate reader. Triplicate wells were assessed for each treatment; experiments were repeated thrice and the mean value was calculated. In all cases, adhesion to wells coated with bovine serum albumin acted as negative controls and levels of adhesion to ECM substrates were calculated relatively to the controls.

Gelatin zymography

The gelatinolytic activity of MMP-2 was analyzed by zymography as previously described (Zhang 2004). Unheated aliquots of conditioned media (50x concentrated) were electrophoresed on a 7.5% SDS polyacrylamide gel containing 1 mg/mL gelatin (Bio-Rad, Stockholm, Sweden) before and after 2-hour treatment with the MMP-2 activator p-aminophenylmercuric acetate (APMA; 1 mmol/L). Recombinant MMP-2 (Chemicon International) was used as control. Molecular weight standards were run simultaneously. The gels were stained with Coomassie blue and destained with acetic acid-methanol until the desired color intensity was obtained. Gelatinolytic enzymes were detected as transparent bands on the background of Coomassie blue–stained gelatin.

Assessment of cell migration and invasion *in vitro*

To assay the effect of PPP on migration and invasion of uveal melanoma cells, BD BioCoat Matrigel chambers were used following the instructions of the manufacturer (BD Biosciences, San Diego, CA). In brief, cells (5 x 104/mL) treated with PPP or solvent for 1 hour were washed and put into chambers containing an 8-µm pore size PET membrane without (for assay of migration) or with a thin layer of Matrigel serving as a reconstituted basement membrane (for assay of basement membrane invasion) or with basement membrane covered by confluent human endothelial human umbilical vascular endothelial cells (for assay of endothelial invasion) for overnight incubation (Qiang 2004). Medium containing 10% serum or IGF-I (as a chemoattractant) is added to the well outside of the chambers.

VEGF protein detection with ELISA

Experiments were performed with ARPE-19 cells, a non-transformed human diploid RPE cell line that displays many differentiated properties typical of RPE in vivo. Culture medium was changed to 1% FBS 12 hours prior to treatments. The cells were treated at subconfluency for 25 hours with PPP and/or IGF-I (100ng/ml) for 24 hours. Conditioned medium was collected and the VEGF ELISA was performed according to the manufacturers instructions (R&D Systems, Minneapolis, MN, United States), except that the conditioned medium was not diluted. VEGF protein amount was correlated to total protein determined with the Bradford reagent (Sigma-Aldrich, St Louis, MO, United States).

Transfection with VEGF promoter-driven luciferase construct

The construct was a kind gift from Dr. Dieter Manne, Institute of Molecular Oncology, Tumor Biology Center, Freiburg, Germany, and its use has been described previously. (Finkenzeller 1995) In brief, cells were cultured in 96-well Kinovate fast transfection plate (Kinovate Life Sciences, Oceanside, CA) and transfected using and 0.5µg of DNA/10000 cells/ per well, in a serum free media, according to the manufacturer's instructions. After 24 h the cells were stimulated with 100ng/ml IGF-1, in the presence or absence of 1 uM PPP for another 24

hours. To enable the levels of transcripts to be quantified, standard curves were generated using serial dilutions of ARPE-19 cells. Negative controls consisting of cells transfected with empty vector were included. All transfections were setup in triplicate. The firefly luciferase activities were measured using the Luciferase Assay System Kit (Promega) and correlated to cell number using an XTT cell survival assay (Roche Diagnostics).

In vivo experiments

In papers II and III ten-week-old pathogen-free severe combined immunodeficient mice were used and housed within plastic isolators in a sterile facility. OCM-1, OCM-3, OCM-8 or R-v-src (IGF-1R negative) cells were injected s.c. at 10^7 cells per mice in a 0.2-mL volume of sterile saline solution. Experimental treatments with PPP were done (20mg/kg/12h) were performed by daily intraperitoneal injections of the compound in 10µl volume of DMSO: vegetable oil, 10:1 (v/v) for 7-14 days or with PPP/food mixture. Control mice were treated with the vehicle only or with normal food.
In paper IV male C57BL/6 mice between 6-10 weeks old were purchased from Charles River, Sulzfeld, Germany. Animals were given food and water ad libitum, maintained in pathogen-free, 12-hour light–dark conditions. For all procedures, anesthesia was achieved by subcutaneous injections of ketamin (10%; Ratiopharm, Germany)/xylazine (2%; Bayer, Germany) (Ratio 16:1) and the pupils were dilated with phenylephrine HCl (0.25%)–tropicamide (0.05%).
All of the experiments were done according to the ethical guidelines for laboratory animal use and approved by the institutional ethical committee.

Murine model of laser-induced CNV

In order to induce choroidal neovascularization krypton laser-induced ruptures of Bruch's membrane were created as described previously (Mori 2001). Laser photocoagulation (110 mW, 100 ms, 50 µm; Argon, Coherent Novus 2000; Carl Zeiss Meditec, Oberkochen, Germany) was performed in the right eye of each animal on day 0. Three laser spots were applied in a standardized fashion around the optic nerve using a slit lamp delivery system and a coverslip as a contact lens. Formation of evaporation blebs at the site of laser exposure indicated the effective rupture of Bruch's membrane, and only burns in which a bubble was produced were included in the study.

Labeling and quantification of CNV

Eyes were enucleated at the indicated times after krypton laser and PPP or vehicle treatment, fixed in 4% paraformaldehyde for 30 min, the cornea and lens were removed, and the entire retina was carefully removed. The RPE-choroid-sclera eyecups were rinsed in PBS, permeabilized in 0.1% Triton X-100, and blocked with 1% goat serum in PBS/Triton X-100. The eyecups were then incubated with 0.5% FITC isolectin B4 (lectin from Griffonia simplicifolia; Sigma, St. Louis, MO, USA) overnight at 4◦C, and the eyecups were flattened and mounted with antifade medium (Vectashield Mounting Medium, Vector Laboratories) with the sclera facing down and the choroid facing up. Flat mounts were examined with a fluorescence microscope (Axioskop 2; Carl Zeiss) and images were captured with a digital camera (Carl Zeiss) and further analyzed using the AxioVision LE software (Carl Zeiss),

Each lesion was manually measured in a masked fashion, and the total area of hyperfluorescence associated with each burn was recorded. The average CNV lesion area (μm^2) of each treated eye was treated as a single statistical point.

Histomorphology of PPP-treated and control CNV lesions

In two groups of C57BL/6J mice, in which we induced experimental CNV in the right eye as described above, were treated with either 20 mg/kg/12hr of PPP i.p, or the vehicle for 2 weeks. At the end of the experiment mice were sacrificed and the eyes were enucleated.
Formalin fixed eyes were embedded in paraffin and serial sections (4 µm thick) were cut throughout the entire extent of each laser burn and stained with hematoxylin-eosin. To evaluate the effect of PPP on CNV membranes, hematoxylin-eosin stained serial sections were examined at 200x magnification with a light microscope (Axioscope; Carl Zeiss Meditec) and a digital color camera (Axiomat; Carl Zeiss Meditec), as previously described. (Berglin 2003)

Statistical Analysis

In paper I the survival time from the date of enucleation to death or to the end of 1992 was considered censored if the patient was alive at the end of the study or if the patient had died of any cause that was not melanoma related. Kaplan-Meier survival curves were plotted for each semiquantitatively assessed group of immunoexpression of c-Met and IGF-1R. Univariate and multivariate Cox regression models were used to assess the prognostic value of the covariates. The statistical significance level was set at 0.05. All calculations were performed on computer (Statistica, ver. 5.5; Statsoft Inc., Tulsa, OK; MedCalc software; MedCalc Inc., Mariakerke, Belgium).
In papers II, III and IV all results are expressed as the mean ± standard deviation (SD). The results were compared using analysis of variance (ANOVA) and Student´s t-test. Statistical significance was set at $p<0.05$..

Results

Paper I

We investigated the possible relationship between cell surface receptors for two growth factors that are synthesized in the liver: the c-Met proto-oncogene, which constitutes the receptor for hepatocyte growth factor/scatter factor (HGF/SF), and the insulin-like growth factor 1 receptor (IGF-1R) by analysing paraffin-embedded tumor specimens from 132 patients with primary uveal melanoma using immunohistochemistry. The intercorrelation of receptor expression and association with melanoma-related survival of patients were determined by univariate and multivariate analyses. Their role as a prognostic factor was also clarified. Whereas the expression of both IGF-1R and c-Met was significantly associated with melanoma-specific mortality by univariate analysis ($P = 0.004$ and $P = 0.007$, respectively) only IGF-1R showed independent prognostic value by multivariate analysis, $P = 0.004$. The prognostic value of IGF-1R was stronger than such currently used prognostic parameters as tumor cell type and tumor diameter ($P = 0.021$ and $P = 0.026$, respectively). The expression patterns of the two growth factors receptors were weakly intercorrelated.

Paper II

In this study we investigated the effect of picropodophyllin (PPP), an inhibitor of the IGF-1R, on uveal melanoma in vivo and how it affects the mechanisms involved in invasion of uveal melanoma cells. We found that PPP efficiently blocks growth and viability of uveal melanoma cells in cultures with the IC50 values being between 50 and 500 nmol/L and that PPP causes tumor regression in xenografted mice ($P < 0.005$). In addition, treatment with PPP inhibited several mechanisms involved in metastasis, including tumor cell adhesion to ECM proteins, activity and expression of matrix metalloproteinase 2, and cell migration as well as invasion through basement membranes and endothelial cell layers. Furthermore, PPP significantly delayed establishment of uveal melanoma tumors ($P < 0.001$) and drastically reduced the incidence of liver metastasis in mice. ($P < 0.00001$)

Paper III

In this study, we aimed to investigate the efficiency of orally administered PPP on growth of uveal melanoma xenografts. Further, we focused on the effect of PPP on VEGF in vivo as well as evaluated its effects in combination with other established anti-tumor agents in vitro. Oral PPP inhibited uveal melanoma growth in vivo in OCM-3, ($P=0.03$) and OCM-8, ($P=0.01$) injected xenografts and was well tolerated by the animals. Also we demonstrated that oral administration of PPP did not affect tumor growth of established xenografts composed of IGF-1R negative vsrc transformed murine fibroblast (R-vsrc), whereas IGF-1R positive tumors were fully responsive. Also PPP was found to be superior to the other anti-tumor agents (imatinib mesylate, cisplatin, 5-FU and doxorubicin) in killing uveal melanoma cells in four different cell lines (OCM-1, OCM-3, OCM-8, 92-1) with the the IC50s for PPP being lower than 0.05 µM. Tumor samples obtained from the in vivo experiments were analyzed for VEGF and IGF-1R expression by Western blotting. VEGF expression in the PPP groups for both OCM-8 and OCM-1 xenografts was strongly decreased compared to the controls ($P=0.03$ and $P=0.006$ respectively). On the other hand, PPP did not affect VEGF expression in R-vsrc tumors, supporting specificity for the IGF-1R in this context. There was also a decreased expression of IGF-1R, especially in the OCM-1 tumors ($P=0.01$)

Paper IV

In this study, we investigated the anti-angiogenic activity of PPP on a murine CNV model and the effect of PPP on VEGF both in vitro and in vivo. C57BL/6J mice, with krypton laser induced experimental CNV, were treated with PPP, administered intraperitoneally or orally, showed 22-32% ($P=0.02$-0.002) decrease in CNV area on choroidal flat-mount preparations. Also VEGF levels in choroids and retinal pigment epithelial cells (APRE-19) were measured by Western blot or ELISA after PPP treatment. VEGF levels in the choroids were significantly reduced ($P=0.005$) while PPP reduced IGF-1 induced VEGF secretion significantly ($P<0.05$) in APRE-19 cells. Lastly, transcriptional activation of the VEGF promoter was determined by luciferase reporter gene assay. Upon treatment with IGF-1 the activity increased 2.5 fold but when PPP (1 M) was incubated along with IGF-1 the luciferase activity was significantly decreased ($P=0.0001$), as related to the control.

Discussion

During the last 40 years there has been a remarkable improvement in terms of survival for patients with many types of cancer and some 90% of patients with cancer now survive. In UM, new diagnostic and therapeutic tools have improved the eye conservation rate, but improved survival is rarely achieved. Up to half of UM patients develop metastases with a median time of 2.4 years from ocular diagnosis, usually leading to death within a few months. (Gragoudas 1991)
There is a need for identifying new, reliable prognostic markers that could be used to treat patients with metastatic UM or at high risk to develop metastatic UM at an early stage. At the same time there is also a need to improve our therapeutic modalities and find treatments that prolong survival.

The hepatocyte growth factor/scatter factor (HGF/SF) and the insulin-like growth factor (IGF-1) are two growth factors mainly produced in the liver. A high expression of the receptors of these growth factors, c-Met and IGF-1R respectively, has been linked with maintenance of malignant phenotype, promotion of cell growth, proliferation, motility, adhesion, and invasion of tumor cells (Werner 1997) (Baserga 1995) (Cruz 2003) In previous studies it was found in a small number of samples that IGF-1R immunoexpression could potentially be used as a predictor of metastasis (All-Ericsson 2002). Similarly, Hendrix reported that uveal melanoma cells with metastatic phenotype express c-Met. (Hendrix 1998) We have shown in a large sample of patients (n=132) that a high expression of c-Met and IGF-1R was associated with high mortality. More specifically, IGF-1R showed to be a strong independent predictive factor both in a univariate and a multivariate analysis-even stronger than tumor cell type and tumor diameter. This suggests that IGF-1R could be used as a predictive factor in the clinical management of uveal melanoma and that IGF-1R could play a crucial role in tumor dissemination and consequently could be a potential target for uveal melanoma treatment.
The next step was to investigate the effect of a specific IGF-1R inhibitor, the PPP, in vitro and in vivo and see how can affect the different steps of the metastasis process in uveal melanoma.
We showed that PPP is capable to inhibit in vivo ongoing disease – by reducing both tumorigenesis and the size of already established tumors – and to drastically reducing the incidence of liver metastasis. Furthermore, individual steps of the metastatic process such as tumor cell adhesion to ECM proteins, activity and expression of MMP 2, and cell migration as well as invasion through basement membranes and endothelial cell layers were strongly attenuated.
Preventing tumor cells from spreading from the original site of the primary tumor to different organs is a vital step in order to prolong survival for every type of cancer including UM. Finding strategies that not only will target the primary tumor but also will prevent it from spreading elsewhere in the body is the real challenge in the treatment of UM. UM, represent a a paradigm for pure hematogeneous dissemination of cancer, because of the lack of lymphatics within the eye. Therefore, the development of a tumor microcirculation in UM is a rate-limiting step for hematogenous metastases. Furthermore, Maniotis (2007) described a novel process by which tumors develop a highly patterned microcirculation that is independent of angiogenesis in aggressive primary and metastatic melanomas, the tumor cells generate acellular microcirculatory channels composed of extracellular matrix and lined externally by tumor cells. This process, by which aggressive tumor cells generate non-endothelial cell-lined channels delineated by ECM, is called ‘vasculogenic mimicry’. The IGF-1R signalling pathway, through the phosphoinositol-3-kinase branch, has been shown to

play important roles in the interaction with ECM. IGF-1R has been found to control cell migration and integrin-mediated adhesion to ECM proteins. (Leventhal 1997) IGF-1R may also influence adhesion to ECM proteins by either affecting the expression of integrins or by activating specific integrins. (Felding-Habermann 2003) Furthermore, IGF-1R has been shown to affect biosynthesis and activation of MMPs. (Zhang 2003) These findings are consistent with our present results showing that inhibition of IGF-1R, using PPP, significantly decreases UM cell adhesion to the ECM proteins fibronectin, laminin, and collagen IV, as well as down-regulates the expression and activity of MMP-2. Cell adherence to, and digestion of, the basement membrane are necesserary requirements to enable passage into the blood stream. The main components of basement membranes are laminin and collagen IV. Both the adherence of tumor cells to the basement membrane and MMP-2-mediated basement membrane digestion have been shown to be under the control of IGF-1R. Using the Matrigel model, we found that PPP strongly inhibits the basement membrane invasion of UM cells. Last step of the metastatic cascade is for the tumor cells to pass from the circulation into establishment in new tissues. We found that the capability of uveal melanoma cells to produce tumors in distant organs and mainly to the liver of the immunocompromised mice was decreased after use of PPP.

PPP has proven to drastically reduce solid tumor size in vivo in a UM model when administered intraperitoneally. In a recent study, we showed that oral PPP considerably prolongs survival in a model of multiple myeloma. (Menu 2007) We have now showed (paper III) that PPP administrated orally in mice with UM xenografts efficiently blocked melanoma growth. None of the PPP treated mice exhibited any signs of toxicity and they maintained a good appetite throughout the experiments. From a clinical perspective, switching from systemic to oral administration has several advantages. Since PPP has a quite short half life (2-4 h) in vivo, it is likely that treatment of humans would require continous drug infusion during the entire treatment period. Chemotherapy administered orally represents a fundamental change in contemporary oncology practice, driven by pharmacoeconomic issues, patient convenience, and has the potential to improve patient quality of life.
Novel cytostatic therapies that require protracted drug administration periods will also favor an oral formulation. While the use of oral chemotherapy may initially be limited to metastatic disease palliation, demonstration of equivalent efficacy would allow for its subsequent use in adjuvant settings. This efficacy is contingent on circumventing bioavailability limitations and patient noncompliance. (McLeod 1999) Probably a more limited intestinal absorption resulting in a limited bioavailability could be the reason why the per oral PPP strategy resulted only in complete tumor growth inhibition compared to our previous study showing full tumor regression of uveal melanoma xenografts with intraperitoneal administration. We could also show that PPP decreased the levels of VEGF in the uveal melanoma tumor samples. The inhibitory effect that PPP excibites on the important angiogenic factor, the VEGF, could partially explain its antitumor efficacy. Blocking VEGF alone can substantially suppress tumor growth and angiogenesis in several models (Kim 1993) There is now compelling evidence that targeting angiogenesis in general and VEGF signalling in particular is a meaningful approach for the therapy of cancer. A future challenge is establishing optimal dosages and therapeutic regimens. It appears likely that cancer therapy will be in most cases combinatorial. Antiangiogenic agents therefore, will need to be combined with cytotoxic chemotherapy and/or targeted therapies. From our in vitro studies, combining PPP with imatinib mesylate or cytostatics as cisplatin, 5-FU and doxorubicine, we could only detect a week synergistic effect with cisplatin and 5-FU in OCM-8 cells and with doxorubicine in OCM-3. Since PPP is tackling the tumorigenesis in different ways (MMP 2 inhibition, antiangiogenic effect, downstreaming molecules as ERK and Akt) and UM seems to be

especially sensitive to IGF-1R inhibition our study points to the use of PPP for this disease either as a primary or an adjuvant treatment.

Neovascularization plays an important pathogenic role in tumorigenesis but is also associated with ischemic retinal disorders and the wet form of AMD.
In combination with the rapidly growing knowledge about basic mechanisms in angiogenesis, this has led to novel developments in therapeutic strategies resulting in a widening of available treatment options and improved prognostic perspectives. VEGF plays a major role in blood-retinal barrier break-down and pathological intraocular neovascularisation (Ferrara 2003) and currently the two approved antiangiogenic treatments, pegabtanib and ranibizumab are targeting one or more isoforms of VEGF-A. It is also known that IGF-1 participates in ocular neovascularization (Grant 1993) while antagonism of IGF-1R suppresses retinal neovascularization by reducing the retinal endothelial cell response to VEGF. (Smith 1999) We showed in the last study (paper IV) that PPP, administered intraperitoneally or orally, reduced significantly experimental CNV and inhibited the VEGF secretion form retinal pigment epithelial cells. Also the VEGF levels of the choroids treated with PPP were decreased. The ways PPP is affecting the CNV process is directly by inhibiting the endogenous angiogenic effect of IGF-1R signalling, and indirectly via attenuation of VEGF. Also it is possible to decrease neovascularization by blocking MMP 2. MMPs increase the bioavailability of VEGF and especially MMP-2 is playing an important role in promoting choroidal neovascularization in vivo. (Berglin 2003) Also we previously showed that PPP treatment led to decreased phosporylation of the downstream molecules Akt and ERK1/2, two pathways that are involved in all cell responses (production of VEGF, cell migration, and proliferation), and inhibition of this pathway may be a promising method to block RPE cell responses in the CVN process. Another interesting aspect of our data is that PPP reduces experimental CNV when administered orally offering an alternative to invasive treatment, as the currently available treatments are administered intravitreally potentially leading to complications as endophtalmitis, traumatic cataract and is certainly not comfortable for the patient.
All these data taken together supplies evidence that IGF-1R is a reliable prognostic marker in UM, and that targeting this receptor with a specific inhibitor like PPP offers a useful alternative treatment for metastatic uveal melanoma as well as exudative macular degeneration. A randomized controlled trial, possibly in a multicenter setting because of the rarity of UM, , is needed in order to confirm this hypothesis for UM, while additional preclinical studies are needed to further explore the potential therapeutic role of PPP in wet AMD.

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