Angiogenesis and screening in uveal melanoma

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CONCLUSION

To the best of our knowledge, this is the first proteomic analysis of anterior chamber aqueous fluid. We found two proteins that could distinguish control eyes from uveal melanoma eyes. The combined use of bioinformatics tools and proteomic profiling might provide an approach for the screening for potential tumour markers in the near future. However, the high rate of samples in which no protein spectrum could be measured due to the protein content being too low is a drawback with this technique. In addition, protein sequencing is necessary to identify the actual protein.
Future studies are necessary to confirm the results of this pilot study and identify the proteins involved.

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Summary and discussion
Samenvatting

SUMMARY AND DISCUSSION

In the last thirty years, extensive research has been performed to understand the aetiology and growth of uveal melanoma and to improve the treatment of patients with this tumour. Although the primary tumour can often be treated adequately, patients with a large uveal melanoma still have their eye enucleated and have a poor chance of survival. Research on the treatment of primary tumours has focused on the prevention of blindness and preservation of the eye (e.g. TTT and TSTT research), but while many authors have studied metastasis formation, no effective therapy has as yet been found for prevention of uveal melanoma metastasis. In this respect, recent reports on e.g. isolated liver perfusion and chemotherapy seem promising.

We still do not know why tumours develop, but we start to realise how important blood vessels are in the malignant growth process, as well as for metastasis formation. In uveal melanoma, haematogenous spread is even more important as lymphatic vessels are absent and spread is therefore limited to the haematogeneous route. Only large ciliary body tumours have been reported to spread through lymphatic vessels. The first objective of this thesis is to develop a better understanding of the role of angiogenesis and angiogenic factors in uveal melanoma and their influence on the development of metastases.

Although curative therapies are lacking, some systemic therapies and liver-focused therapies can already extend the patients' life. In this context, a timely detection of metastases would be of great benefit. Until now, screening protocols consist mainly of liver ultrasound and blood liver function tests, but these only detect large metastases. New serum tests may be an answer, and perhaps new candidate serum markers will be identified by studying proteomic profiles of tumour material, eye fluids and blood of uveal melanoma patients. This is discussed in the second part of this thesis.

Angiogenesis in uveal melanoma

A uveal melanoma develops in one of the most vascular tissues of the human body. Nevertheless we showed that in large uveal melanomas, more than 50% of tumours had internal necrosis (**Chapter 2**), suggesting a lack of oxygen supply, perhaps due to insufficient angiogenesis. One of the inducing factors of angiogenesis is ischemia. We showed (preliminary results) that there is expression of Hypoxia-inducible factor-1 (Hif-1α) in uveal melanoma. Hypoxia and Hif-1α induce the expression of several angiogenic factors, such as the members of the Vascular Endothelial Growth Factor (VEGF) family. In the first part of this thesis, we report on the expression of the different members of the VEGF-family in uveal melanoma tissue, in vivo and in vitro, and in ocular fluid (**Chapters 3-4**). Our results show that VEGF-A, at this moment the best known angiogenetic factor, is expressed by uveal melanoma cells and secondly that this VEGF-A is active in a murine bone-model (**Chapter 4**). One can imagine that a growing

tumour becomes too big to be supported by its own blood supply and goes into necrosis, a hypothesis that was confirmed by necrosis presence in most large tumours. As a result, we think that hypoxia-induced factors such as Hif-1α are expressed in uveal melanoma, which enhances the production of vaso-active factors in the tumour.

A growing tumour not only grows into the sclera (with a choroidal excavation on ultrasound as consequence), but also into the vessels and capillaries of the choroid (which facilitates metastases), and into the overlying retina. As a result, large tumours not only grow through Bruch's membrane, but also cause detachment and subsequent hypoxia in the retina. We demonstrated that the retina on top of the tumour produced a large amount of VEGF-A, secondary to hypoxia (**Chapter 3**). The production of VEGF-A induces a break-down of the blood-retina barrier, and changes the electrolyte balance within the eye. This also facilitates the development of a retinal detachment, as seen in most eyes containing a large tumour.

Because of hypoxia in the eye, and in the retina in particular, neovascularizationis formed in tumour eyes particularly after irradiation, which may lead to the formation of neovascular glaucoma and secondary enucleation of the eye. We demonstrated a very high expression of VEGF-A in irradiated eyes, especially after proton beam irradiation. The application of anti-VEGF-A therapy may reduce the incidence of radiation retinopathy or the number of complications caused by neovascularization in tumour eyes. Eventually, it may prevent secondary enucleation in some eyes (although the number of secondary enucleations is limited, **Chapter 2**).

Vascularization is crucial for the primary tumour, and is also important in determining the prognosis of the patient. Microvascular density, measured in hot spots of uveal melanoma specimens, has been inversely correlated with patient survival. We could demonstrate that the expression of VEGF-receptors was also correlated with microvascular density (preliminary observations). This means that large tumours have more vessels, and in consequence have more expression of VEGF-receptors. This not necessarily means that these factors are also important in the process of metastases formation. It is of course so that a large tumour has more vessels, has more expression of VEGF-receptors and has more chance to metastasize, but the exact role of angiogenesis in survival is still unknown. To support its importance, we demonstrated that tumours with intravascular invasion have indeed a worse outcome (**Chapter 2**). Nevertheless, it remains difficult to estimate the precise impact of these factors in the metastatic process. The current trials on the use of anti-angiogenesis agents in metastatic tumour patients have not shown the results anticipated. It will be necessary to increase our knowledge of angiogenesis and angiogenic factors in the normal body before we can fully adapt this process to conquer tumours. This thesis has tried to do this for uveal melanoma.

Tumour vaccination

Since the time of Jenner and Pasteur, patients are vaccinated against all sorts of viruses and bacteria, starting with smallpox in 1798. The last decades, after the discovery of tumour-specific antigens and increased understanding of the immune system, the hypothesis of tumour therapy by vaccination has been developed. It has been proven in animals that animals that were injected first with tumour extracts, could resist the injection of this tumour later on, but not other tumours. This means that tumour-specific rejection is possible. In that manner vaccination based on melanoma-associated antigens has been applied to cutaneous melanoma with variable success.

The lack of effect may be due to the fact that we are not able to elicit a strong enough immune response against cutaneous or uveal melanoma cells. This would of course change if we could improve the presentation of tumour antigens to the immune system.

Heat shock proteins are interesting in this view, for two reasons. First, they have a function in coupling tumour antigens to the HLA-MHC class I molecules, in this way thus improving the presentation of antigens. Secondly, they are chaperones for tumour-specific antigens. By using the heat shock proteins and the tumour-specific antigens attached to them to vaccinate the patient, we could induce a patient-specific tumour response. In **Chapter 5** we explore whether uveal melanomas express these HSP proteins, and whether their expression would be high enough to enable vaccination in the clinical setting.

Screening for metastatic uveal melanoma

Until now the effort to screen for metastatic uveal melanoma is limited as no hopeful therapies are available. In recent years, the efforts to treat metastatic uveal melanoma have been multiplied. It is nevertheless important to study the metastatic behaviour of this tumour. In 50 to 90% of cases the first metastases is located the liver. Liver metastases carry a bad prognosis. If we could detect micrometastases earlier, a necessary step for early treatment of liver metastases, we may prolong patient survival. The surgical excision of a limited number of small liver metastases and the use of isolated liver perfusion is only possible if there are no diffuse metastases in the liver.

Regarding clinical applicability, we especially focused on finding serologic markers that indicate early metastasis. For this purpose we looked for S100 and Melanoma Inhibitory Activity (MIA) in serum of uveal melanoma patients. These proteins have been shown to be of use in cutaneous melanoma, not only for detection of the primary tumour, but also for detecting early metastases and subsequent monitoring of different treatments of metastatic melanoma. As uveal melanoma metastases are very small at the time of enucleation, they may not produce enough proteins to cause the presence of markers in blood. Indeed, we could not find an increased level of S100, S100 dimers or MIA in the blood of uveal melanoma patients at the time of enucleation (**Chapter 6** and preliminary results). However, these proteins could be detected in patients with metastases. This may render these proteins interesting in screening for metastatic melanoma, also, because serum tests are less time-consuming than liver ultrasound. Another advantage of the screening for these proteins, which are produced by the metastases, is that they are increased independent of

metastasis localisation. As such, they also could detect early metastases outside the liver.

In our study, S100 was a better marker than the routinely performed liver function tests. However, this has to be investigated in a much larger metastatic melanoma group. The use of these proteins in (half)-yearly screening is still under investigation. It has to be verified whether S100 or other serum proteins will effectively be better screening tools than liver ultrasound.

Perhaps, S100 may also be useful in monitoring the effect of different uveal melanoma metastasis treatments.

Proteomics: time for a revolution in oncology?

he search for a metastasis marker is basically a search to find a tumour-specific protein. In essence this is proteomics: the study of the proteome of the cell and the (human) body. In the last five to ten years, more tools have become available to determine the proteome of fluids and tissues, e.g. SELDI- and MALDI-TOFs.

The SELDI-TOF, used in this thesis, can compare different sample groups with regard to their proteome (**Chapter 7**). With this technique, we could detect differences in the proteome of aqueous humour of uveal melanoma patients compared to control persons. It is expected that in the near future, the proteome of different uveal melanoma cell lines, melanoma tissue, ocular fluids and blood of uveal melanoma patients will be compared to controls, and differences between patients with and without metastates will be found. The difficulties of these techniques are exact protein identification, and sometimes low sensitivity,which make identical harvesting and conservation conditions of all samples essential.

The proteomic era will have an impact on the detection and treatment of tumours. One will be able to detect more specific melanoma-proteins (not only the melanoma degradation proteins and associated proteins like S100 and MIA we know today). Probably, future treatment will be focused on inhibiting or modulating these proteins.

Conclusion : on the threshold of hope ?

This thesis describes different subjects in current ophthalmologic oncology research. Although we still are not able to promise our patients a life without metastases, we nevertheless can detect some improvements. Genomic and proteomic results, now still in the research setting, are bound to give some clinically useful results in the future. At the same time, new treatments, both anti-angiogenic and immunologic, are being developed to conquer this tumour. The current explosion of articles on screening and different surgical and chemo-therapeutic treatments for uveal melanoma, expresses the growing interest and success of ophthalmologists and oncologists in this field. Further studies, both on angiogenesis and screening, are continued at our department, to make this hope become reality.

SAMENVATTING

Het uveamelanoom is de meest voorkomende oogtumor bij volwassenen met een incidentie van zes tot acht per miljoen in de Caucasische bevolking. In de laatste dertig jaar is er veel onderzoek ondernomen om de behandeling van deze tumor te verbeteren, maar ook om de etiologie en de groei van het uveamelanoom beter te begrijpen. Hoewel men tegenwoordig de primaire tumor adequaat kan behandelen, is de uiteindelijke overleving van patiënten met een groot uveamelanoom nog steeds beperkt. Onderzoek naar de behandeling van primaire uveamelanomen richt zich tegenwoordig vooral op het behoud van het gezichtsvermogen, maar tot op heden is er geen effectieve therapie gevonden om de vorming van metastasen te voorkomen of te behandelen.
Dit proefschrift beschrijft de resultaten van onderzoek naar de angiogenese en de cytokines die in angiogenese een rol spelen enerzijds, en de zoektocht naar nieuwe markers voor het uveamelanoom anderzijds.

Angiogenese van het uveamelanoom

Het uveamelanoom ontwikkelt zich in een van de meest gevasculariseerde weefsels van het lichaam : de choroidea. Niettemin konden we aantonen dat in meer dan 50% van de uveamelanomen er necrose optreedt (**Hoofdstuk 2**). Men kan zich inbeelden dat een groeiende tumor op een gegeven ogenblik te groot wordt om gevoed te worden door de bestaande bloedvaten. Op dat ogenblik ontstaat een zuurstoftekort en necrose. Het is bekend dat zuurstoftekort cellen aanzet tot de productie van pro-angiogenetische factoren, zoals het Vascular Endothelial Growth Factor (VEGF). In ogen met een uveamelanoom kon dan ook de expressie van VEGF worden aangetoond (**Hoofdstuk 3**). Tevens kon worden aangetoond dat tumoren die voor enucleatie bestraald werden, een hogere expressie van VEGF-A hadden. Dit opent perspectieven voor de behandeling en preventie van radiatie-retinopathieën met de anti-VEGF-therapieën in ogen die bestraald werden omwille van een groot uveamelanoom. De expressie van verschillende pro-angiogenetische cytokines werd onderzocht in **Hoofdstuk 4**. Tevens werd in een botmodel aangetoond dat de VEGF-A isovorm teruggevonden in tumor-ogen inderdaad ook functioneel is.

Tumorvaccinatie

Al twee eeuwen vaccineren we patiënten voor allerlei virussen en bacteries. In de laatste jaren groeit ook het geloof in de mogelijkheid om te ‘vaccineren’ tegen metastasering van tumoren. In dieren werd aangetoond dat wanneer een muis werd gevaccineerd met een

tumorextract, ze later de groei van deze tumor na implantatie kon verhinderen. Tevens kan het verbeteren van de antigen-expressie door de tumor een betere immunologische reactie tegen die tumor teweeg brengen.
Heat shock proteïnen verbeteren de expressie van antigenen door de cel, en hechten zich bovendien specifiek vast aan (tumor)antigenen. Door extractie van deze proteïnen uit de tumor en vaccinatie van de patiënt kan men een specifiek anti-tumor immunologische respons teweeg brengen. In **Hoofdstuk 5** hebben we de expressie van deze proteïnen in het oogmelanoom aangetoond.

Screening voor gemetastaseerd uveamelanoom

Tot nu toe was de interesse in het vroeg opsporen van metastasen van het uveamelanoom beperkt, door het gebrek aan enige therapie. Door de toenemende interesse in dit domein en het besef dat metastasen vooral in een vroeg stadium nog behandelbaar kunnen zijn, zijn we op zoek gegaan naar markers die vroegtijdig de metastasen kunnen aantonen. Het uveamelanoom metastaseert in 50-90% van de patiënten eerst naar de lever. Screening is dan nu ook meestal gefocuseerd op de lever met het aantonen van afwijkende leverfunctietesten in het bloed en ultrasonografie van de lever.

In **Hoofdstuk 6** hebben we de expressie van S100 in het serum van patiënten met een uveamelanoom onderzocht. S100 wordt reeds klinisch gebruikt voor de opsporing van metastasen van het huidmelanoom. Op het ogenblik van de diagnose van het uveamelanoom konden we geen significante verhoging van S100 in het serum van melanoompatiënten aantonen. In het serum van patiënten met een levermetastase konden we echter een verhoging van S100 en MIA (melanoma inhibitory activity) aantonen (preliminaire resultaten).
Het gebruik van deze levertesten kan een waardevolle aanvulling zijn op het huidige screeningsonderzoek.

Onderzoek van het proteoom – het volledige proteine repertoire van een cel – zal in de toekomst beter onderzoek naar specifieke markers voor het uveamelanoom mogelijk maken. In **Hoofdstuk 7** hebben geprobeerd in het voorste oogkamervocht markers te vinden voor het uveamelanoom, door gebruik te maken van de SELDI-TOF techniek (surface enhanced laser desorption / ionisation - time of flight). Op basis van twee proteïnen kon een onderscheid gemaakt worden tussen het oog met een uveamelanoom en het normale oog. Het proteomics-onderzoek geeft een hoopvol perspectief voor een groeiende kennis en behandeling van het oogmelanoom.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 16 oktober 1976 te Hasselt (België). Het lager en middelbaar onderwijs werd gevolgd aan het St.-Jozefscollege te Hasselt (1981-1993). Vanaf 1993 werd de opleiding Geneeskunde gevolgd aan de Katholieke universiteit te Leuven waar op 10 juli 2001 het diploma van arts werd behaald. Vanaf juli 2001 werd het promotie-onderzoek beschreven in dit proefschrift, uitgevoerd in het laboratorium oogheelkunde van het Leids Universitair Medisch Centrum o.l.v. Prof Dr J.E.E. Keunen en Dr M.J. Jager. Het onderzoek werd mede begeleid door Dr J.M.G. Bonfrer (afdeling klinische biochemie, Nederlands Kanker Instituut, Amsterdam) en Dr R.O. Schlingemann (afdeling oogheelkunde, AMC, Amsterdam). Het onderzoek werd uitgevoerd in samenwerking met afdelingen van de Katholieke Universiteit Leuven, het Medisch Centrum van de Universiteit Utrecht, de Vrije universiteit Amsterdam, het Anthonie van Leeuwenhoekziekenhuis / Nederlands kanker Instituut Amsterdam, Slotervaartziekenhuis Amsterdam en het Academisch Medisch Centrum van de Universiteit Amsterdam. In juli 2004 ontving de auteur de Junior Award van de European Society of Ophthalmic Plastic and Reconstructive Surgery voor het project “Cytotoxic effect of sodium hypochlorite 0.5% on ocular melanoma cells in vitro” onder de begeleiding van Prof Dr R.J.W. De Keizer. De opleiding tot oogarts werd aangevangen op 1 mei 2004 o.l.v. Prof C.C. Sterk.

Verschillende onderzoeksprojecten werden mondeling of via posters verdedigd op talrijke congressen in binnenland (Rotterdam, Amsterdam, Maastricht) en buitenland (Leuven, Brussel, Parijs, Padua, Alicante, Vilamura, Helsinki, Hyderabad en Fort Lauderdale).

In maart 2005 werd een korte stage gevolgd bij de afdeling Oncology van Wills eye hospital (Philadelphia) o.l.v. Prof J Shields en Prof C. Shields. De auteur is lid van de Société Française d’Ophthalmologie, buitengewoon lid van het Nederlands Oogheelkundig Gezelschap en aspirant lid van het Belgisch Oogheelkundig Gezelschap. Hij is tevens lid van ARVO en EVER en sinds 2004 lid van de Ocular Oncology Research Society.

De auteur is reviewer voor Investigative Ophthalmology and Visual Science en Graefe’s Archive for Clinical and Experimental Ophthalmology sinds 2004.

LIST OF PUBLICATIONS

1. **Heat shock protein expression in the eye and in uveal melanoma.**
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   Invest Ophthalmol Vis Sci 2003, July; 44(7): 3059-3065.

2. **Prognostic value of S-100b serum concentration in patients with uveal melanoma.**
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   Arch Ophthalmol 2003; 121: 1117-1119.

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   Submitted

8. **Prognostic value of MIA and S100-isoforms serum concentration in patients with uveal melanoma. A comparison with liver function tests.**
   G.S. MISSOTTEN, C VAN DEHN, N.E.TANG, I.C. NOTTING, C.M.KORSE, J.E.KEUNEN, M.J. JAGER, J.M.G.BONFRER.
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    S. KEIJSER, C.M. VAN LUIJK, G.S. MISSOTTEN, M. VESELIC-CHARVAT, D. DE WOLFF-ROUENDAAL, R.J.W. DE KEIZER.
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11. **Survival in uveal melanoma. A comparison of histopathological prognostic factors.**
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12. **Vascular Endothelial Growth Factor-A in eyes with uveal melanoma.**
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    Submitted