Progesterone receptors in human meningiomas
and their relation with proteins involved in apoptosis

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Progesterone receptors in human meningiomas
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Progesteron receptoren in humane meningeomen
en hun relatie met eiwitten betrokken bij apoptose
(met een samenvatting in het Nederlands)

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Voor Ilonka
Voor mijn ouders

Contents

Chapter 1

General introduction

(Adapted from Steroids *2000, **65:** 795-800)*

Progesterone is a steroid hormone involved in female fertility, preparing the uterus for implantation of fertilized oocytes, and in the maintenance of pregnancy. Because of its contribution to reproduction it has been named the "steroid hormone of reproduction" [1]. Besides these specific effects of progesterone on the endometrium, it can also exert some anti-estrogenic and thus antiproliferative effects on tissues. Because of these effects on proliferation it is not surprising that progesterone, synthetic agonists, mesoprogestins (mixed agonists-antagonists) or pure antagonists may be used in cancer prevention and treatment. Progesterone acts via the progesterone receptor (PR). Knowledge of the function and regulation of PRs is important to determine clinical significance of involvement of PR in tumor growth regulation.

This thesis focusses on the function and regulation of PR in meningiomas, tumors of the central nervous system (CNS), that are partly under the influence of hormones. To understand more about the PR, the mechanism of its activation and function are described in this introduction. Thereafter, a short overview is given of apoptosis, particularly of the bcl-2 family of proteins, some of which are regulated by progesterone. The final part of this introduction concerns the scope of this thesis.

Pathology and epidemiology of human meningiomas

Meningiomas are the most common tumors (up to 20%) of the CNS that are derived from the arachnoidal cells of the meninges, the membranes covering brain and spinal-cord [2-4]. Meningiomas occur at

a rate of about 2 in 100,000 subjects per year [5]. The development of meningiomas may be attributable to different factors like head traumas, viruses, ionizing radiation, and neurofibromatosis 2 (NF-2). Meningiomas are benign neoplasms and are usually well demarcated from the adjacent brain tissue. Therefore, surgical resection is the primary treatment and subsequently radiation therapy may be used when necessary [6]. Meningiomas show a relatively high recurrence rate (20%) and are sometimes unresectable. For this group of patients a secondary adjuvant therapy would be very helpful. However, such a treatment is not readily available yet, since meningiomas are relatively insensitive to chemotherapy.

Meningiomas have attracted attention as possibly being hormone-sensitive tumors due to (i) higher incidence rate in women than in men (see Table 1-1; ratio 2.5 : 1) [3], (ii) the epidemiological association of meningiomas and breast cancer [7-11], and (iii) the reversible aggravation of the symptoms during periods of relative progesterone excess, such as during pregnancy and in the luteal phase of the menstrual cycle [12]. During pregnancy, however, the increase in the size of the tumor might be the result of cellular edema [13].

	Males *(n=104)*	***Females*** *(n=237)*	***Sex unknown*** *(n=63)*	***All*** *(n=404)*
Phenotype				
ER– / PR–	32.7%	19.0%	23.8%	23.4%
ER– / PR+	55.8%	67.5%	61.9%	63.4%
ER+/ PR–	3.8%	1.7%	3.2%	2.5%
ER+/ PR+	7.7%	11.8%	11.1%	10.7%
Receptor level[a]				
ER (mean)	4.5	4.6	3.3	4.4
S.E.M.	1.7	0.8	0.7	0.6
median	0	0	0	0
PR (mean)	118.8	172.9*	150.9	156
S.E.M.	18.5	19.6	63	13.2
median	57	70.5	25.3	66
age				
mean	57	58		58
median	59	61		61
range	15-84	20-89		15-89

Table 1-1. Occurrence and concentration of ER and PR in human meningiomas as determined by ligand-binding assay, update 2001 [70]. PR expression in meningiomas from female patients is statistically higher than in those from male patients. [a]*fmol/mg;*P<0.02*.

Occurrence of progesterone receptors in meningiomas

Following the first report by Donnell *et al.*,[14] on the presence of ER in meningioma tissue, a large number of papers have appeared dealing with this subject. Some authors reported the occurrence of both ER and PR, whereas others established an ER-negative (ER–) and PR-positive (PR+) phenotype. The differences appeared to be caused by methodological differences because large numbers of apparently ER positive tumors were found to be positive only by inadequate single-point binding assays and not by more reliable Scatchard plot analysis, enzyme immuno-analysis, or immunohistochemistry. Although the discussion has settled in favor of the ER–/PR+ phenotype, small amounts of ER can be found using appropriate techniques in a number of meningiomas.

The cumulative results of steroid receptor measurements performed by this laboratory are shown in Table 1-1. These results illustrate that the majority (87%) of 404 meningiomas was devoid of ER (<10 fmol/mg cytosol protein) and confirm the predominant ER–/PR+ phenotype for meningiomas. Figure 1-1 shows that in our series of cytosols from meningiomas and breast cancers, the predominant steroid receptor phenotype for meningiomas is ER–/PR+ and for breast cancer ER+/PR+. Although the presence of ER in meningiomas seems to be slightly increased as compared to our previous updates [15,16], it should be noted that even if ER could be demonstrated in meningiomas, it is present at a very low level as indicated in Table 1-1. With the extension

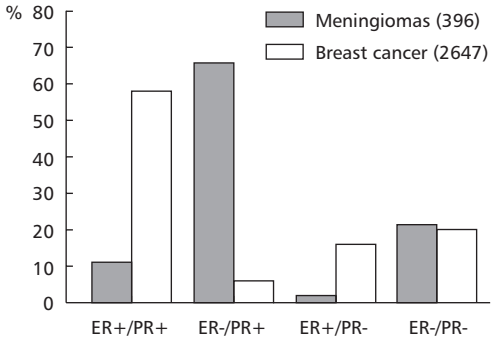

Figure 1-1. Incidence of estrogen and progesterone receptors in human meningiomas and breast cancer cytosol. Update 1999 [72]. *Abbreviations: ER, estrogen receptor; PR, progesteron receptor.*

of our series, the difference in PR level between men and women has become more pronounced, and this difference is statistically significant ($P < 0.02$). It remains to be seen whether this difference has any biological significance.

Structure and mechanism of action of the progesterone receptor

The genomic effects of progesterone are mediated via the PR. The PR is a member of a superfamily of transcription factors, which includes receptors for steroid hormones, thyroid hormones, retinoic acid, and orphan receptors. These proteins are characterized by an organization into specific functional domains that are conserved between species and family members (see Figure 1-2) [17]. PR is localized in the nucleus, most likely as a result of a continuous active transport into the nucleus, counter-balanced by diffusion into the cytoplasm [18]. This active transport

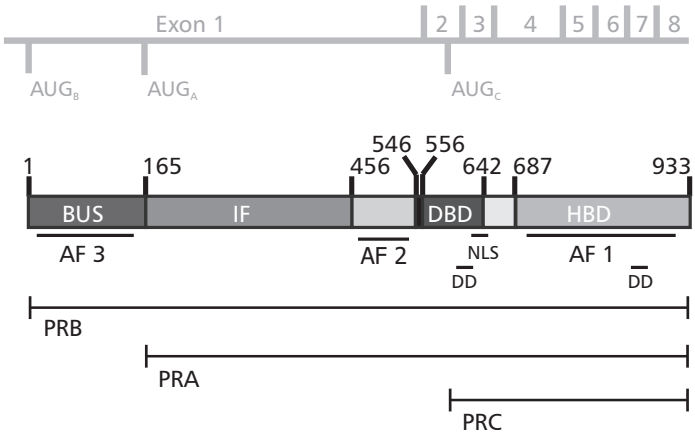

Figure 1-2. Schematic illustration of the structure and function domains of the human progesterone receptor (PR). The PR mRNA is depicted showing three different translation initiation start sites (AUG). In the middle, the PR protein structure is illustrated. The three major PR isoforms are also shown, PR-A, PR-B, and PR-C (adapted from references 21, 24, 37). *Abbreviations: BUS, B Unique Segment; IF, inhibitory function; AF, activation function; NLS, nuclear localization signal; DD, dimerization domain; DBD, DNA binding domain; HBD, hormone binding domain.*

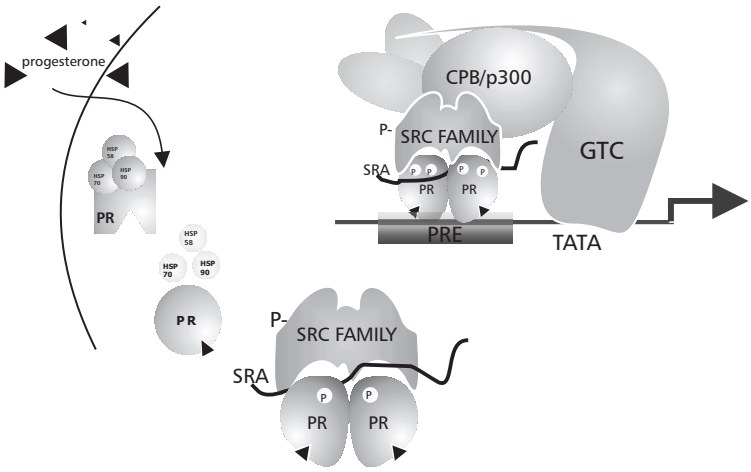

Figure 1-3. Model for activation mechanism of the human progesterone receptor (PR). Unliganded PR is inactive and bound to heat shock proteins (HSP). Upon ligand binding, HSP dissociate and PR dimerizes and is phosphorylated. By dimerization PR adopts a conformation capable of binding to a member of the SRC-co-factor family. The complex is then capable of binding to progesterone responsive elements (PRE), located in the promoter regions of PR target genes. Transcription initiation can take place after binding of components of the general transcription complex (GTC) machinery and additional co-factors (adapted from reference 71).

requires the so-called nuclear localization signal (NLS) [19]. The most highly conserved region of the PR is the center of the protein that corresponds to a two 'zinc finger' DNA binding motif (DBD). The carboxy-terminal domain serves as the ligand-binding domain (LBD) and is less conserved compared to the DBD. This domain contains a region called the activation function 2 (AF-2), this AF-2 is ligand dependent. The amino-terminal domain of PR contains a ligand independent activation function (AF-1). Both AF-1 and AF-2 activate transcription in an unique promoter- and celltype specific fashion [20]; they work in a synergistic manner and are required for full receptor activity in most cellular contexts. A third strong autonomous activation domain (AF-3) appears to function only in a restricted cell and promoter context [21].

The unliganded PR is in an inactive conformation and bound to other proteins like heat shock protein 90 [22,23] (see Figure 1-3). Binding of the ligand to the receptor elicits a series of events: dissociation from the heat shock proteins, change of the tertiary structure of the protein, activation of PR by phosphorylation, dimerization, and high affinity binding to Progesterone Responsive Elements (PRE) in the DNA. The activated PR interacts in a specific manner with the transcription machinery complex, thereby initiating transcription of progesterone responsive genes. Apart from proteins from the basal transcription complex, transcriptional activation requires additional regulatory proteins (co-factors) and will be discussed in the next paragraph.

In human, two distinct promoters have been identified that are regulated independently and give rise to different PR-isoforms named PR-A and PR-B [24]. The PR-A and PR-B receptor isoforms are almost identical, execept for an additional stretch of 164 amino acids in the PR-B isoform, called the B Unique/Upstream Sequence/Segment (BUS), which is located at the amino terminus of the receptor. This BUS region encodes the AF-1 that is specific to the PR-B isoform and plays an essential role in specifying target genes that can be activated by the B but not the A receptor isoform [21]. When expressed in equimolar ratios in cells, PR-A and PR-B can dimerize and bind to DNA in three different ways: A:A or B:B homodimers or A:B heterodimers. The specific contribution of each of these dimers to mediate the regulatory effects of progesterone will depend on the differential transactivation properties contributed to these complexes by the B-specific domain.

Both A and B isoforms of PR are capable of binding progesterone, dimerizing and interacting with PRE, as well as activating the transcriptional machinery to regulate PR-target gene expression. While both PR-B and PR-A have similar ligand and DNA binding affinities [25], in most cells the homodimers of both isoforms appear to exhibit different regulating properties [26]. Thus, PR-A and PR-B with different transactivation capacities may regulate different physiological target genes in response to progesterone. In certain cell and promoter contexts, PR-A is capable of repressing transactivation of PR-B and of other steroid hormone receptors

like ER [27,28]. Thus, PR-A has the ability to diminish overall progesterone responsiveness of specific target genes in specific tissues. Therefore, differential expression of the PR isoforms might be of clinical importance in the treatment of benign breast disease with progestins. In breast tumors, a higher expression of PR-A than PR-B has been reported [29]. Unfortunately, all large clinical trials concerning breast cancer did only include total PR expression and not the differential expression of PR isoforms. Furthermore, PR-A and PR-B respond differently to antiprogestins. While antagonist bound PR-A is inactive, antagonist bound PR-B can be converted to a strong active transcription factor by modulating intracellular phosphorylation pathways in PR-B containing cells [30]. In order to understand the response of tumors like meningiomas, to treatment with antiprogestins, knowledge of the differential expression of PR isoforms might be very helpful.

Similar to other steroid receptors, PR is a phosphoprotein and its function and role is still under intensive study. Phosphorylation sites of steroid receptors are generally mapped to serine residues in the N-terminus upstream of the DNA binding domain. There may be as many as 14 phosphorylation sites and phosphorylation is a highly complex multistage process [31-34]. The PR exhibits basal phos- phorylation and this phosphorylation is enhanced in response to hormone treatment suggesting that phosphorylation plays a role in receptor activation, for instance by increasing its DNA binding [35]. A slower round of phosphorylation that requires DNA binding but does not contribute to the net activation follows basal phosphorylation. The BUS region of PR-B, which contains the strong autonomous activation function (AF-3), is heavily phosphorylated [21,35]. Mutations in these phosphorylation sites which do not result in the loss of PR function, however, indicate that phosphorylation is not required for transcription activation [35]. The differences in the specific phosphorylation sites between PR-A and PR-B may attribute to their differences in biological behavior. For instance, a strong preferential hormone-dependent phosphorylation of Ser 294 on PR-B and not on PR-A has been reported [36]. The flanking sequences on both proteins are identical, indi- cating that conformational differences between both proteins are important for phosphorylation [36]. In summary, phosphorylation of the PR may play a major role in the function of the receptor, however, phosphorylation may have subtle effects on transcription activation and may also subserve other functions.

Besides the two major PR isoforms, additional PR like proteins have been described. A PR-C isoform has been described which was not able to bind DNA, however, this isoform was still capable of ligand binding and dimerization [37,38]. PR-C could interfere with the activation of progesterone responsive target genes [37-39]. In breast cancers, a smaller 78 kDa protein was found capable of progesterone binding [29,40]. The origin and biological significance of this PR-78 are still not known.

Progesterone receptor co-regulators

Nuclear receptor co-regulators consist of two groups called co-activators and co-repressors. Either a transcriptionally permissive or non-permissive environment is created at the promoter site, due to a combined effort of the receptor and co-regulaters, which in turn communicate with the general transcription complex (GTC) and RNA polymerase II. The action of most co-regulators is, by definition, rate limiting for nuclear receptor activation and repression, but does not significantly alter basal transcription.

Numerous co-activators have been identified that are recruited by the liganded PR and enhance receptor dependent transactivation. The best-known co-activator family is the SCR family. SCR-1 was the first co-activator identified involved in PR activation, using a yeast two-hybrid screen of a human lymphocyte cDNA library with the hormone bound PR-LBD as bait [41]. Later, the homologous proteins SRC-2 and SRC-3 were described [42]. SRC-1 was found to interact with the PR in a ligand dependent manner and antagonist RU-486 prevented this interaction of SRC-1. Interaction of the PR occurs through the AF-1 and AF-2 domains of the PR [43]. The activation domains of SRC-1 are important for interaction with other co-regulators or the basal transcription machinery and SRC-1, has a role in chromatin remodeling [43,44]. After interaction between PR and SCR-1, this complex is thought to recruit CREB Binding Protein (CBP) and other co-factors [45]. Other factors that have been added to the list of co-activators include (i) Ubiquitin protein ligases, (ii) L7/SPA, (iii) HMG-1, and (iv) SRA [42]. This illustrates the diversity of proteins recruited to the PR promoter and eventually determines the expression of PR target genes.

Besides these co-activators of nuclear receptors, other families of proteins called co-repressors are also known. Using a yeast two-hybrid screen, Jackson *et al.* have described that the nuclear corepressor (NCoR) interacted with antagonist-bound PR-Ligand Binding Domain (PR-LBD) [46]. In addition, overex pression of NCoR and SMRT (Silencing Mediator for Retinoid and Thyroid hormone receptor) markedly suppressed RU-486 partial agonist activity. This effect was reversible in the case of PR by overexpression of the PR-LBD [46]. In addition, NCoR and SMRT preferentially associate with antagonist-bound PR whereas the partial agonist activity of RU-486 bound to PR is ablated by overexpression of NCoR and SMRT [47]. Other co-repressors that have been identified are TRUP, SUNCoR, and NURD [48].

Data of co-factor expression in human meningioma is very limited. Carroll *et al.* have reported expression of SRC-1, "Amplified In Breast cancer" protein (AIB-1), and transcriptional intermediary factor 2 (TIF2) in respectively 81, 76, and 76% of the meningiomas by immunoblot and immunohistochemistry analysis. The expression of SRC-1 and TIF2 was significantly related to the concentration of progesterone receptors [49]. Co-factors are also involved in the differential transactivation capacity of the two major PR isoforms, since PR-A and PR-B have been

reported to have different binding affinities to several co-factors [50].

Antiprogestins

Antiprogestins have a great therapeutic potential, such as interruption of early pregnancy, post-coital contraception, induction of labor, and treatment of hormone-dependent tumors. Studies in animals have suggested that antiprogestins could be used in the treatment of gliomas and ovarian, prostate, and endometrial cancer [51]. Because of the presence of PR in meningiomas and the epidemiological data linking aggravation of meningioma symptoms to periods of high progesterone availability, the use of antiprogestins for the treatment of meningiomas has been suggested. Pilot studies with limited numbers of patients [52-54] and case reports have fostered the optimism with respect to the clinical application of antiprogestins. A randomized double blind placebo controlled phase 3 trial is currently underway to study the activity of the antiprogestin mifepristone in unresectable meningiomas [52].

The apoptotic protein family Bcl-2

Besides proliferative actions of steroids in tissues of the reproductive organs, estrogens and progesterone are considered to be involved in the control mechanism of proteins involved in the regulation of programmed cell death (or apoptosis). In contrast to necrosis, apoptosis is regulated by several sets of genes, the best characterized of which are members of the Bcl-2 family [55]. The Bcl-2 family of proteins comprises anti-apoptotic as well as pro-apoptotic proteins and can be classified in the following subfamilies based on bcl-2 homology and function of each protein [56] (see Fig. 1-4): (i) a subfamily including bcl-2 itself, bcl-x_l and bcl-w, all of which exert anti-cell death activity and share sequence homology particularly within four bcl-2 homology (BH) regions, BH-1 through BH-4; and (ii) a subfamily represented by bax and bak that share sequence homology at BH-1,2 and 3 but not at BH-4. All these proteins exert pro-apoptotic activity; (iii) a subfamily of pro-apoptotic proteins that share sequence homology only within BH-3 and therefore are referred to as BH3-proteins. The overall balance between cell death agonists and antagonists in a cell determines the susceptibility to apoptosis.

One of the features of the Bcl-2 protein family is the ability of members to form homo and heterodimers [57]. Heterodimerization between anti-apoptotic and pro-apoptotic members of this family is considered to inhibit the biological activity of each of the partners [57,58]. This effect is mediated by the insertion of the BH-3 region of a pro-apoptotic protein into a hydrophobic pocket composed of BH-1, BH-2 and BH-3 of an anti-apoptotic protein [59].

Bcl-2 and bcl-x_l were shown to prevent apoptosis induced by various stimuli, including serum deprivation, heat shock, and chemotherapeutic agents, suggesting the ability of both proteins to prevent apoptosis down-stream of a common pathway. Both proteins have been shown to have a mitochondrial localization [60-62]. The

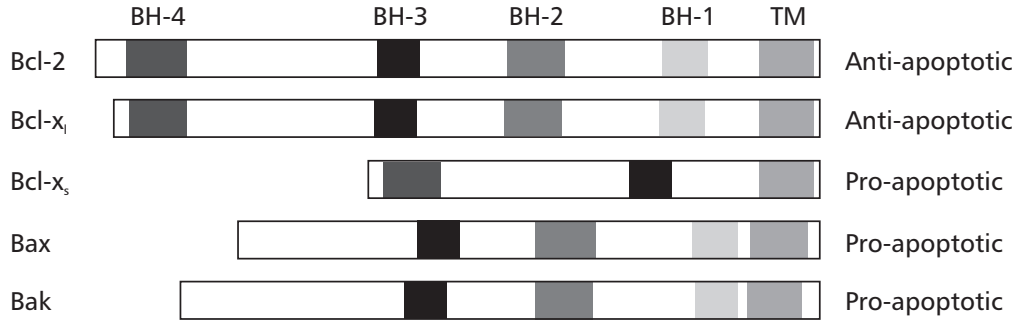

Figure 1-4. Structure of the Bcl-2 family proteins (adapted from reference 72). *Abbreviations: BH, Bcl-2 Homology domain; TM, trans membrane domain.*

regulatory function of bcl-2 has been ascribed to: (i) its ability to interact with other proteins that participate in cell-death regulation; (ii) channel activity that directly or indirectly influences mitochondrial permeability and prevents the release of cytochrome c from the mitochondria and caspase activity [63]. It is known that the bcl-2 protein is phosphorylated in response to different stimuli of cell death [64], however, phosphorylation has been related to both inactivation and activation of the anti-apoptotic function [65]. Besides a role for bcl-2 and bcl-x$_l$ in apoptosis, studies have indicated that Bcl-2 family members might also regulate cell cycle progression [66-69].

Bax, bcl-2 associated protein x, was isolated as the gene encoding a protein that binds bcl-2 [57]. Bax acts in opposition to bcl-2 and overexpression of bax allows apoptosis to proceed, as for instance after interleukin 3 (IL3) withdrawal from IL3 dependent cells, even in the presence of upregulated expression of bcl-2 [57]. The tumor suppressor p53, implicated in induction of both growth arrest and apoptosis following DNA damage, is a positive regulator of bax expression and a negative regulator of bcl-2 expression [73,74]. Bax expression is found diffusely distributed throughout the cytosol, however, upon induction of apoptosis, bax moves intracellularly to a distribution co-localized with the mitochondria [60]. When bax molecules have migrated to the mitochondrial membrane, bax is capable of homodimerization and thereby channeling the release of cytochrome C [75]. After cytochrome C release, caspases are strongly activated that are capable of further inducing apoptosis [76-78].

The human *bak* gene was isolated by amplifying sequences similar to coding

sequences for both BH-1 and BH-2 related proteins. Bak is expressed in a wide range of tissues among which several types of tumors [79,80].

Since several apoptotic proteins have been reported to be involved in tumor development, and their expression is related to tumor grade, recurrence and resistance to chemotherapy, knowledge of the regulation of these proteins in tumors might be of clinical importance. The fact that some of the proteins of the Bcl-2 family are under control of steroid hormones suggests that these steroids might be involved in the homeostasis of these tumors. Not much is known, however, about the expression or regulation of apoptotic proteins in human meningiomas. Since several of these proteins are regulated by progesterone in other tissues, the expression of PR might be involved in the regulation of these proteins in meningiomas as well.

The aim of the thesis

The aim of this thesis is to obtain a better understanding of the effect of the presence of progesterone receptors in human meningiomas. Special emphasis will be on the following questions:

1. Which PR-forms are expressed in meningiomas and are the various PR isoforms expressed equally in meningiomas?

2. Is PR related to the presence and occurrence of apoptotic proteins and thereby to apoptotic processes?

3. Are ER and/or ER splice-variants involved in the regulation of PR expression in meningiomas?

4. What is known about the growth hormone and insuline like growth factor axis in human meningiomas?

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Chapter 2

Progesterone receptor expression in human meningiomas: a cell culture study

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Summary

Meningiomas are benign intracranial tumors derived from the meninges. In general, these tumors have a progesterone receptor (PR) positive phenotype. Meningiomas cultured as monolayer quickly lose their ability to express PR. The loss of PR expression and a high proliferation rate in meningiomas cultured as monolayer indicate that the cells might dedifferentiate when cultured. The aim of this study was to develop a meningioma cell culture model with maintained PR expression high enough for quantitative analysis with a ligand binding assay (LBA). This model could then be used for investigation of the role and regulation of PR in meningiomas.

In this study a monolayer, a spheroid and an *ex vivo* tissue culture method were tested. None of the culture models tested was appropriate for meningioma cells to maintain their PR expression *in vitro* after prolonged periods of culturing. Both in monolayer and in spheroid cultures, after three days of culture, PR levels drop to below the lowest detection limit of the LBA. Spheroids obtained from breast cancer cell lines, T47D and MCF-7, showed comparable amounts of PR expression as found for monolayer cultures of these cell lines indicating that our experimental setup was valid. In the *ex vivo* method, meningioma tissue fragments expressed only 28% of PR in the native tumor after three days of culture. Still, this method is recommended since the tissue fragments maintain their organostructure and stimulation of the cells shortly after excision, when most of the PR is still present, is possible. Because of the rapid decline in PR in cultured meningiomas, all data described in literature concerning stimulation of cultured meningioma cells via the PR should be interpreted carefully.

In preparation

Introduction

Meningiomas are intracranial and intraspinal tumors that arise from arachnoidal cells in the meninges, especially in areas of the arachnoid villi. The great majority of meningiomas are benign neoplasias that can occur virtually anywhere in the central nervous system. At the moment the main therapy is surgical resection with a recurrence rate of 20% [1-3].

Adjuvant hormone therapy could be very helpful in this group of unresectable or recurred tumors. It is known that meningiomas respond to changes in circulating levels of progesterone. Patients suffering from a meningioma show a reversible aggravation of symptoms during periods of relative progesterone excess, such as during pregnancy and the luteal phase of the menstrual cycle [4]. The incidence of meningiomas in female patients is more than twice as high as in males [5]. The incidence of meningiomas is also higher in individuals with other hormone-modulated tumors such as cancer of the breast, ovary and endometrium [6].

This apparent sensitivity for progesterone could be explained by the presence of a functional progesterone receptor (PR) expressed in meningioma cells. Indeed, more than 75% of the meningiomas express high levels of PR in the virtual absence of the estrogen receptor (ER) [7]. Estrogen independent expression of PR in meningiomas is in contrast with PR regulation in other endocrine regulated tumors, like breast cancer.

In order to understand the regulation and function of PR with respect to proliferation, differentiation and development of meningiomas, many studies have been done the last decades. The most used *in vitro* model for stimulation experiments with hormones or growth factors is meningioma cells grown as monolayer. Of these studies, the outcome was contradictory and non-conclusive [8,9]. This discrepancy could be explained by the fact that cultured meningioma cells easily loose their ability to express the progesterone receptor. In cultured meningiomas, as soon as the second day of monolayer culture, the PR levels decrease to 20% of the native tissue [10]. We suggest that data derived from experiments using meningiomas cultured as monolayer need therefore to be interpreted with caution.

With the intention to develop a meningioma cell culture model that maintained PR expression, a new meningioma model was developed by Tonn *et al.* [11]. These authors described immunohistochemical data of PR expression in meningiomas cultured as tri-dimensional structures, so called spheroids, which were obtained by preventing attachment of the cells to the bottom surface of the culture flask, by using an agar coating [11-13]. Spheroids can be obtained from re-aggregation of cells grown as monolayer (referred to as aggregated-spheroids) or by immediately culturing tissue fragments (referred to as fragment-spheroids).

In addition, Camby *et al.* used an *ex vivo* culture model in which tissue of meningiomas was cut in small fragments of approximately 2 mm^3. This method may be superior to others since the original structure of the native tumor is secured [14]. Whether this method results in maintenance of the progesterone receptor expression was not reported.

In order to develop a meningioma cell culture model, appropriate for investment of the role of PR in meningiomas, spheroid- and *ex vivo*- culture methods were optimized. One of the standards for success was that PR levels have to be measurable with a ligand binding assay (LBA) in order to quantify PR expression levels.

Material and Methods

Tissues

Human meningioma tissue was placed in ice-cold Medium (MEM supplemented with 10% FCS, 10mM essential amino acids, 2mM glutamine, 100U/mL penicilin, 100U/L streptomycin, Gibco & Life Technology, Breda, The Netherlands), immediately after removal from the patient. Part of the tumor was stored at –80°C until further use for cytosol preparation. Another part was used for the different culture experiments.

Cytosol preparation

Before harvesting tissue fragments, monolayer and spheroid cultures of meningiomas were cultured in medium containing 10% dextran coated charcoal pretreated serum (DCC-FCS) for one day. Meningioma tissue, culture specimen of meningioma, MCF-7, and T47D cells were chilled in liquid nitrogen, pulverized with a micro- dismembrator (Braun, Melsungen, FRG) and extracted with 10 mM phosphate buffer at pH 7.5 containing, 1.5 mM EDTA, 3 mM sodium azide, 10 mM 1-mono- thioglycerol and 10% (v/v) glycerol. The resulting homogenate was centrifuged at 0-4°C for 30 minutes at 100,000 × *g* yielding a clear cytosol.

Ligand binding assay

The PR levels of the tumors and culture specimen were measured, as described previously [7], by the ligand-binding assay and scatchard plot analysis, according to the guidelines of the EORTC, Breast Cancer Cooperative Group [15]. The protein content of the cytosol was estimated with the method of Bradford, using reagents from Bio-Rad (Richmond, CA, USA) and serum albumin (Kabi, Diagnostica, Stockholm, Sweden) as a standard [16]. The lowest detection limit for PR was 10 fmol/mg protein. The in-between reproducibility for these assay was; PR: 11.3% at 333 fmol/mg protein, and protein: 5.8% at 3.4 mg/ml.

Primary cultures of meningiomas

***Ex vivo* culture:** tissue fragments were rinsed with PBS, cut into pieces of approximately 2 mm^3, and seeded in culture flasks (T175) with a medium overlayer. Tissue fragment were harvested after 3,4 or 6 days of culturing.

Monolayer culture: Tissue fragments were rinsed in PBS and minced using scalpels. To further dissociate any remaining fragments, the tissue was treated with dispase (2.4 units/g tissue) at 37°C for one hour. After centrifugation the pellet was resuspended in medium and seeded in flasks in 30 ml medium. Medium was refreshed the next day.

Monolayer spheroid culture: Spheroids were obtained from cells already cultured as monolayer for one week. Cells were harvested in 10 ml PBS by scraping and centrifuged at 1500 rpm for 10 minutes. The pellet was resuspended in medium and 5 μl was used for cell counting with a Türker cell counting chamber, using tryphan blue for discriminating between intact and disrupted cells. Five million cells were seeded in flasks (T175) with an agar-coated bottom. Within one day, beginning of spheroid aggregation was seen.

In order to quantify growth of spheroids the diameter of several spheroids were measured, using an inverted microscope and a computer assisted measuring program. To validate our experimental culture conditions, spheroids were also generated from two classical breast cancer cell lines, MCF-7 and T47D, both expressing PR.

Results

Generation of monolayer and spheroid cultures of meningiomas

Meningioma cells easily migrate from dissociated meningioma fragments and a confluent monolayer is achieved within one week culturing (Figure 2-1A). Meningiomas grown as monolayers, in contrast to the *in vivo* situation, showed a high proliferation rate. For meningiomas cultured longer than 10 weeks, growth stops and tumor cells were sometimes overgrown with fibroblast like cells. Only meningioma culures without fibroblast overgrowth were used.

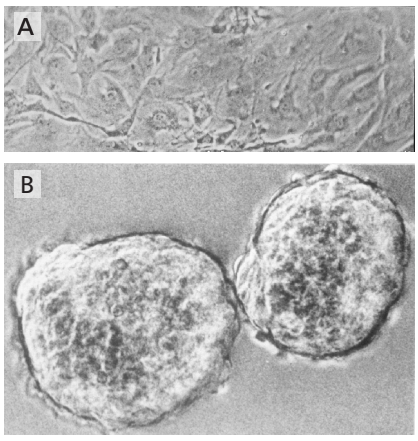

Figure 2-1. Meningioma cells cultured as confluent monolayer (**A**) and re-aggregated spheroids (**B**). *See text for details.*

When a single cell suspension from meningioma cells was seeded on agar, cells attached and gave rise to spheroids (Figure 2-1B). Growth was measured by monitoring the diameter of the spheroid (Figure 2-2). Spheroids continued to grow for at least a period of 10 days.

Progesterone receptor expression in primary cultures of meningiomas

Table 2-1 shows an overview of PR expression in meningiomas cultured as spheroids or as tissue fragment (*ex vivo* culture). All, but two, native tumors were PR positive. Meningioma cells, grown as monolayer, lost the PR already before the monolayer reached confluence. Spheroids generated from monolayer cells did not re-express the PR at measurable levels for the LBA (>10 fmol/mg protein). Also in spheroids directly derived of disaggregated tissue fragments, no PR could be detected one week after surgery.

Progesterone expression in meningioma tissue fragments

To limit culturing time, meningiomas were not dissociated but cultured as tissue fragments referred to as *ex vivo* culturing. An additional advantage of this method is that the architecture of the original tumor is preserved. PR expression was measured in native tumor tissue and fragments after 3, 4 and 6 days of *ex vivo* culturing PR expression was measured. Figure 2-3 shows the PR expression levels of 17 meningiomas. Already after three days of *ex vivo* culturing PR expression declines to 25 ± 28% (mean ± standard deviation) of the levels in the native tissue.

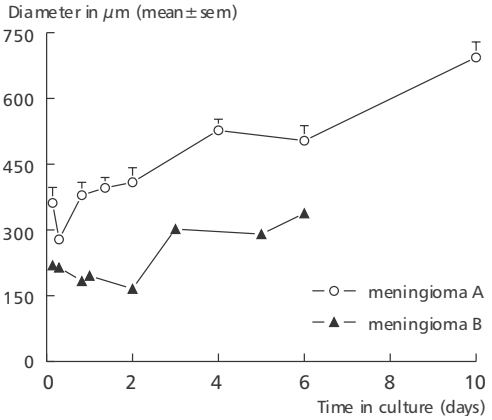

Figure 2-2. Growth kinetics of meningioma cells, cultured as re-aggregated spheroids. *See text for details.*

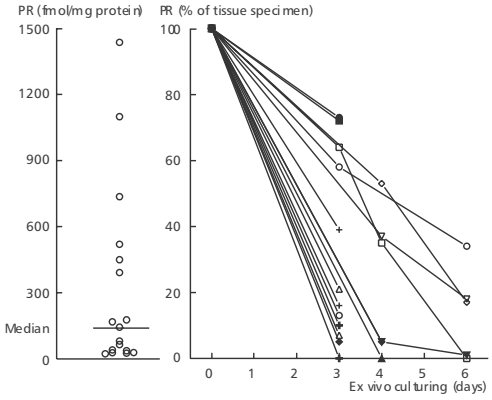

Figure 2-3. Progesterone receptor (PR) content in primary meningiomas. The left panel illustrates the PR expression in fmol/mg protein in the native tissue specimen. The right panel shows the decline of PR expression during time of culturing as *ex vivo* tissue fragments.

PR expression in spheroids from MCF-7 and T47D breast cancer cell lines

As a control for our experimental setup, PR expression levels in monolayer and spheroids obtained from two different breast cancer cell lines, T47D and MCF-7, were measured with a LBA. No significant difference in PR expression in both cell lines could be found between monolayer and spheroid culturing (Table 2-2). For these cells, culturing as tri-dimensional spheroids is an appropriate culture model, and gave sufficient protein amounts in order to measure PR with a LBA.

	monolayer		spheroids		
	PR (fmol/mg)	n	PR (fmol/mg)	n	*P*
MCF-7[a]	106 ± 61	5	163 ± 84	5	ns
T47D[a]	2258 ± 904	6	1866 ± 1284	3	ns

Table 2-2. Progesterone receptor expression in MCF-7 and T47D cells cultured as monolayer and as spheroids. Abbreviation: ns, not significant; [a] *mean ± S.D.*

meningiomas			PR (fmol/mg protein)				Protein (μg/μl)			
nr.	Culture[a] method	sex[b]	tissue	mono[c]	*Ex vivo*[d]	Spheroids[e]	tissue	mono[c]	*Ex vivo*[d]	spheroids[e]
1	a	f	26	<10	-	<10	2.9	0.4	-	1.3
2	a	m	<10	<10	-	<10	1.9	2.8	-	1.1
3	a	f	421	<10	-	<10	1.4	0.6	-	0.1
4	a	f	<10	<10	-	<10	2.0	0.9	-	0.4
5	a	m	23	<10	-	<10	2.5	1.9	-	3.2
6	a	f	160	<10	-	<10	2.9	-	-	1.9
7	a	f	128	-	-	<10	2.1	-	-	0.3
8	a	f	15	-	-	<10	3.2	-	-	0.8
9	a	m	60	-	-	<10	2.2	-	-	0.5
10	b	f	144	-	15	-	3.1	-	2.2	-
11	b	f	492	-	60	-	2.2	-	0.5	-
12	b	f	392	-	27	-	1.8	-	0.7	-
13	b	m	1436	-	223	-	4.4	-	6.3	-
14	b	f	23	-	<10	-	1.7	-	1.3	-
15	b	m	29	-	21	-	2.1	-	1.0	-
16	b	f	41	-	<10	-	4.9	-	5.7	-
17	b	f	168	-	66	-	1.7	-	2.2	-
18	b	f	508	-	109	-	0.8	-	1.4	-
19	b	m	177	-	103	-	2.1	-	0.6	-

Table 2-1. Overview of the progesterone receptor expression in meningioma tissues and cell cultures. [a] *Culture method: a, cells first cultured as monolayer and then spheroids were obtained by using an agar coated culture flask; b, ex vivo method – meningioma tissue was cut in small pieces and immediately seeded in agar coated flasks.* [b] *Patients' age at time of operation.* [c] *Cells harvested 1 week after excision.* [d] *cells harvested 3 days after excision.* [e] *Cells harvested 2 weeks after excision.*

Discussion

It can be argued that progesterone, via its receptor, is involved in the regulation of growth and differentiation of meningiomas. The proliferation rate of meningioma increases when there are high plasma progesterone levels as seen during pregnancy and in the luteal phase of the menstrual cycle [4]. There is also a predominance for female patients [5]. Grunberg *et al.* reviewed the responses seen in several trials treating patients with the progesterone receptor antagonist, mifepristone (RU 486) or Medroxy Progesterone Acetate (MPA), a synthetic progesterone agonist [17].

Meningiomas show a typical sex steroid hormone receptor phenotype. In contrast to breast cancer, which show a low percentage of ER–/PR+ phenotype, more than 75% of the meningiomas express PRs in the virtual absence of the estrogen receptor [18]. That the PR can be expressed and be functional in the absence of ER is demonstrated in the estrogen receptor knock mouse (ERKO) [19]. Regulation of PR is tissue specific and may be regulated via an estrogen independent pathway, however, exact regulation mechanisms of PR synthesis are not known. Meningiomas are an interesting cell model for investigation of the estrogen independent regulation of PR and its role in these tumors.

To investigate the function and the ER unrelated regulation of expression of the PR in meningiomas, there is a need for a good cell culture model. The aim of this study was to achieve a cell culture method, in which meningioma cells, after prolonged culturing, still express the PR at levels high enough for quantitative analysis with a LBA. Three different culture methods were tested, monolayer, spheroids, and *ex vivo* tissue culture. Progesterone receptor expression was found in 24 of the 26 meningiomas used for the experiments (Table 2-1). The meningioma monolayer cell model is a cell system in which not only intracellular matrix components are missing, but also the cell proliferation is extremely high when compared to the growth rate *in vivo*. We and several other authors found that despite the PR positive phenotype of the native tissue, meningiomas cultured as monolayers very rapidly lose the ability to synthesize PR [10,20,21] (table 2-1). The loss of PR and the increased proliferation suggest that meningioma cells dedifferentiate when grown as monolayer cultures. Culturing of meningiomas as monolayer is an inappropriate cell culture model for PR research and there is a need for other culture models.

Another culture method, described by Tonn *et al.*, is meningioma cells cultured in a three-dimensional conformation as re-aggregated or fragment spheroids [13]. Based on immunohistochemistry data, cells located in the non-proliferative regions may express PRs [11-13]. These authors, however, did not find PR expression in all the spheroids, despite the fact that they were obtained from PR positive tumors. When PR expression was found, it was in variable amounts [12]. In the present study, none of the spheroids obtained from their native PR positive

tumor tissue showed any PR expression based on a LBA (Table 2-1). The amount of PR expressed in a number of PR positive cells, as reported by Tonn *et al.* based on IHC-data, may be under the lowest detection limit of the LBA [12]. The method of spheroid generation and PR measurement were proven valid by our results with the T47D and MCF-7 cell lines, which showed in both monolayer and spheroid cultures equal amounts of PR expression (Table 2-2).

Since prolonged culturing as monolayer or as spheroids of meningioma cells resulted in a decrease of PR expression, the *ex vivo* culture method was tested. Freshly resected meningiomas were cut into small tissue fragments and cultured for not longer than a week. The rationale of this method is that the organotypical structure of the tissue is maintained. A disadvantage, however, might be that the inner cells may become ischaemic. It is thought that dedifferentiation could be the cause of the decline of PR in meningioma cells cultured as monolayer. Even in this *ex vivo* culture model, in which dedifferentiation of cells in such a short notice and without disturbing the organo structure is not very likely, PR expression could not be maintained.

Despite the efforts to maintain PR expression in these tissue fragments, the conclusion is that after several days of culturing the PR is declined (Figure 2-3). Tumors that express high levels of PRs at the moment of excision, expressed less than 25% of the PRs found in native tissue after three days of culturing. The maintenance of the infrastructure of the tissue, cell-cell interactions and matrix components, could not prevent a decline of PR expression. Apparently, components of blood, which are missing in all the culture models, may be important in the fine regulation of PR transcription in meningiomas.

When key factors responsible for the activation of the PR gene are missing, a decline in PR mRNA could be expected. Indeed Tonn *et al.* showed that monolayer of meningiomas cells expressed less mRNA than their spheroid counterparts in which they detected PR protein [12]. The possibility of an active degradation, however, could not be ruled out. Leclercq *et al.*, described the down-regulation of the estrogen receptors by degradation of receptor proteins by proteosomes [22]. Whether this may be a mechanism of PR degradation in cultured meningiomas need to be sorted out.

Thus far, we do not known whether the loss of PR in meningiomas cell cultures is a tissue specific and/or a hormone receptor specific process. It may be that not only the PR, but also several other proteins are limited in their expression under *in vitro* conditions. Authors, who described stimulation experiments with tumor cell models, rarely take the effort to establish the level of the target protein expression after the period of stimulation of the cells. Not much is known about the presence of other steroid hormone receptors as well at the beginning of the experiments as afterwards. Zava *et al.* described the presence of glucocorticoid receptor (GR) and androgen receptors (AR) in meningiomas cultured for at least three passages. The authors, however, did not describe the values of AR and GR in the native tissue [23].

Gibelli determined the AR and GR in fresh frozen tissue sections and in meningiomas cultured as monolayer for approximately five days [24]. In their study, the receptor concentration for frozen sections was expressed as fmol/mg protein and for the cell culture specimen as fmol/mg DNA. Although not directly comparable, the authors evaluate that all the GR and AR positive tumors also showed GR and AR positive phenotype in the cell culture subset [24]. In conclusion, these data may suggest that the decline of progesterone receptors in cell culture models of meningiomas may be a PR specific event.

Meningiomas transplanted into nude mice may be the only model left which maintains PR expression. Several authors described a successful transplantation of meningioma tissue or cultured cells into the renal capsule, subcutaneously or intracranially of athymic mice [25-28]. None of these studies concerned PR expression in the meningioma transplant. Only Olson *et al.* focused on the effects of PR antagonists. Meningioma implanted subcutaneously in athymic mice showed a growth inhibition by RU-38486, an antiprogestational agent. PR expression levels in the transplant after stimulation, however, were not reported in the transplant [28]. Besides the xenograft model, two meningioma cell lines were described, IOMM-Lee and KT21-mg1, but there is no report of PR expression in these cells [29].

In summary, monolayer, spheroids and *ex vivo* tissue culture methods were tested, in order to develop a culture method in which meningiomas did not lose the ability to express progesterone receptors. None of the tested culture models were appropriate for meningioma cells to maintain their PR expression *in vitro*. The *ex vivo* method is recommended since the tissue fragments hold their organostructure and stimulation of the cells shortly after excision, when most of the PR is still there, is possible. All data described in literature concerning stimulation of cultured meningioma cells via the PR should, therefore, be interpreted carefully.

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Chapter 3

Progesterone receptor isoform expression in human meningiomas

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Abstract

The majority of meningiomas expresses the progesterone receptor (PR), and show progesterone responsiveness. In addition, an association has been reported between PR and prognosis. At least two PR isoforms exist, PR-B (116-120 kDa) and PR-A (81 kDa), each of which are likely to have different biological functions. Knowledge of the differential expression of both isoforms is necessary to understand the effects of progesterone on meningioma growth. Therefore, in this study, PR-A and PR-B expression levels were determined in 61 human meningiomas by immunoblotting. Total PR expression levels were determined with a ligand binding assay (total PR^{LBA}).

Both PR isoforms and an additional PR 78kDa protein (PR-78) were expressed in the meningiomas. Meningiomas expressing more PR-A than PR-B had significantly higher total PR^{LBA} levels ($P<0.001$). The PR-78 band intensity was negatively associated with that of PR-B ($r_s=-0.76$, $P<0.0001$). PR-78 may represent an endogenous degradation product, but a similar regulation pathway in the biogenesis of both PR-B and PR-78 is not excluded.

Meningiomas contain both PR isoforms, but in highly variable ratios and this variability may have some biological significance. Most meningiomas express more PR-A than PR-B. Therefore in meningiomas, assuming that PR-B is more transcriptional active than PR-A, progesterone responsiveness could be based on transrepression rather than on transactivation of target genes.

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Introduction

Progesterone may play a role in the development and proliferation of hormone dependent tumors, like breast cancer and meningiomas. Progesterone action is mediated via the progesterone receptor (PR), which can be found in 75% of the human meningiomas [1,2]. Meningiomas expressing high levels of the PR have a better prognosis and a higher survival rate [3-5]. PR might therefore serve as target for endocrine therapy [6]. To determine the biological significance of PR expression in meningiomas, the regulation of PR in meningiomas needs to be elucidated.

The progesterone receptor (PR) belongs to the family of ligand activated transcription factors. The PR is expressed as at least two isoforms, a full size protein (PR-B) and a 164 amino acid N-terminally truncated version of the B form, called PR-A [7,8]. The two isoforms are transcribed from two different translation initiation sites located on the same gene [9].

Besides PR-A and PR-B, an additional progesterone binding protein of 78 kDa (referred to as PR-78) was revealed by immunoblot analysis of breast cancer cytosols [10,11]. Yeates *et al.* reported that PR-78 is most likely not an artificial degradation, nor a phosphorylation product of PR-B or PR-A [11]. The origin and biological function of PR-78 remains to be elucidated.

While both PR-A and PR-B have similar DNA and ligand binding affinities [12], in most cell and promoter contexts both isoforms appear to exhibit different regulating properties. *In vitro* studies reveal that PR-B is a strong transactivator of progesterone-responsive genes, whereas PR-A is considered to be a dominant negative transrepressor of PR-B and other steroid hormone receptors [13-15]. The precise mechanism underlying the differential activities of PR-A and PR-B is not fully understood. PR-A, however, is missing the N-terminally located 'B Upstream Sequence' (BUS), resulting in a possible change in conformation and loss of phosphorylation sites [16]. The PR isoforms may have different affinities for co-factors and other proteins of the transcription machinery [17]. Because of the differential activities of PR-B and PR-A, the ratio of expression of both isoforms determines the final overall response of cells upon progesterone stimulation, and may be important for the regulation of growth and differentiation of endocrine related tumors.

Meningiomas are tumors which are thought to be hormonally regulated [18]. They are generally benign intracranial tumors derived from arachnoidal cells in the meninges, the membranes covering brain and spinal cord. Meningiomas are the most common intracranial tumors with a 20% recurrence rate following current standard therapy, surgical resection with subsequent radiation therapy when necessary [19,20]. Growth of meningiomas is increased during periods with high progesterone levels in the circulation [21]. Meningiomas express high levels of total PR, as measured by a ligand binding assay (LBA) [1]. There is an association between

total PR expression in the tumor and prognosis for the patient [3-5]. Several experiments, *in vitro* and *in vivo*, have been performed to inhibit cell growth using PR antagonists [6,18,22,23]. For development of endocrine therapy, however, the regulation and function of PR expression in meningiomas needs to be known.

In several previous publications, the presence of PRs in meningioma, based on a LBA, was reported [1,2]. The expression pattern of the PR isoforms in meningiomas, however, is not known. Both isoforms differentially mediate progesterone or PR antagonist signaling. Therefore, the differential expression of both PR isoforms in meningiomas is of great interest. This study was conducted to determine PR-A and PR-B expression levels with an immunoblot analysis. Results were compared with total PR expression, determined with a LBA (referred to as total PR^{LBA}), and tumor grade.

Patients and Methods

Tissues

Sixty-one PR positive human meningioma tissues were collected from 40 female and 21 male patients, the mean age at the time of surgery was 57 years (median: 60 years; range 45-81 years), operated at the University Medical Center Utrecht. The study was conducted in accordance with the guidelines of the local ethical committee. Tissues specimens were placed on ice immediately after removal from the patient. Representative specimens were frozen at -80°C until used for cytosol preparation.

Cytosol preparation

The tissue (250-500 mg) was chilled in liquid nitrogen, pulverised with a microdismembrator (Braun, Melsungen, FRG) and suspended in 2ml of 10 mM phosphate buffer containing, 1.5 mM EDTA, 3 mM sodium azide, 10 mM 1-monothioglycerol and 10% (v/v) glycerol, at pH 7.5. The resulting homogenate was centrifuged at 0-4°C for 30 min at 100,000 × *g* to yield a clear cytosol. Total PR was measured by LBA immediately after cytosol preparation.

Receptor assay

Total tumor PR levels (total PR^{LBA}) were measured, as previously described, by the LBA and scatchard plot analysis, according to the guidelines of the European Organization for Research and treatment of Cancer (EORTC), Breast Cancer Cooperative Group [1,24]. The protein content of the cytosol was estimated with the method of Bradford, using reagents from Bio-Rad (Richmond, CA, USA) and serum albumin (Kabi, Diagnostica, Stockholm, Sweden) as a standard [25]. The lower cut-off level for PR positivity was set to be 10 fmol/mg protein. The between-assay variability of these assays is PR (n=31): 11.3% at 333 fmol/mg protein and for the protein assay (n=31): 5.8% at 3.4 mg/ml.

Protein electrophoresis and immunoblot analysis

Sixty-one cytosols with PR^{LBA} >10 fmol/mg protein were used for the immunoblot analysis. PR isoforms were separated by electrophoresis through 6.5% polyacrylamide (30% acrylamide/bis solution, 37.5:1) resolving gel and a 2.6% stacking gel both containing 0.10% Sodium Docedyl Sulphate (SDS), using a Mini Protean II apparatus (Bio-Rad, Richmond, CA). The cytosol of each sample loaded on gel contained 10 fmol PR, and was diluted to 80μl with sample buffer. For cytosol samples with very low PR levels (<40 fmol/mg protein), the maximum amount of cytosol possible was used. Samples were heated for 8 min at 99°C and then loaded onto gel. After electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore, Bedford, GB) at 125 V for 1.5h at 4°C in 25 mM tris, 192 mM glycine, 20% (v/v) methanol as described by Towbin *et al.* [26]. The conditions used for transfer were chosen after pilot experiments which showed that shorter transfer times significantly altered the ratio of PR-A:PR-B in favour of PR-A (the lower molecular weight form). Increasing the transfer times beyond 1.5 h did not alter the observed ratio indicating that transfer was complete at this time.

After transfer, the membrane was blocked with 1.5 mM phosphate-buffered saline (PBS, pH 7.4) containing 5% low fat milk powder (Protifar; N.V. Nutricia, Zoetermeer, The Netherlands), and incubated with a specific antibody (AB) against human PR, monoclonal antibody Ab-4 (clone hPR a4, Neomarkers, Fremont, USA) at a 1:1000 dilution [27]. PR-A migrates as a single band whereas PR-B migrates as a triplet, for analysis the sum of the triplet was used.

The specificity of hPR4a was verified using other antibodies against both isoforms (hPRa1, Neomarkers) and against only PR-B (clone hPR a6, Neomarkers). In order to establish the inter-assay reproducibility of each experiment, on each gel at least two identical amounts of cytosol of human myometrium were used as PR positive controls. The molecular weight of each PR isoform was established with a molecular weight standard (BioRad, Richmond, CA). After incubation with the PR antibodies, blots were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody at a final dilution of 1:2500 (Dako A/S, Denmark). Specific bands were visualized by using enhanced chemiluminescence detection substrates (0.01% (v/v) 250 mM luminol in DMSO, 0.004% (v/v) 90 mM p-loumaric acid in DMSO, 0.002% (v/v) H_2O_2 in Tris 0.1 M, pH 8.5) and blots were exposed to Kodak film (Biomax-ML, Kodak, Rochester, NY, USA). A control immunoblot, with and without a specific hPR4a PR antibody, of T47D (PR=1800 fmol/mg protein), LNCaP (PR<10fmol/mg protein) and human myometrium cytosol revealed that the bands visualised were specific for PR.

Multiple exposures of each immunoblot were made, and results were used only from those where PR-A and PR-78 bands could be analyzed separately, and that fell within the linear range of the film. Otherwise, a new assay was run using more diluted samples. Band intensities

were measured densitometrically (Sharp JX330, Japan). The linear range of detection of PR on immunoblot was established by a standard curve, made using increasing concentrations of control cytosol, and analysis by densitometry. A linear relationship between total PR concentration (based on LBA) and densitometric detection by immunoblot analysis could be established. The between-assay variability of PR in cytosols of human myometrium was 10.1% (n=31).

Statistical evaluation

For each sample the mean of the PR isoform expression was calculated from at least three independent immunoblot assays. Spearman rank sum tests (r_s) were used to compare total PR^{LBA}, PR-A, PR-B and 78-kDa band expression. Mann-Whitney non-parametric tests were performed to compare protein expression between the subgroups. P values of <0.05 were considered statistically significant.

Results

Total progesterone receptor (PR) expression in human meningiomas based on ligand binding assay (LBA)

The PR concentration of 61 PR positive meningiomas is depicted in Table 3-1. The average PR concentration of the cytosols used was 181±24 fmol/mg protein (mean ± s.e.m., n=61). The total PR^{LBA} concentration fell in the range 10-755 fmol/mg protein with the median at 98 fmol/mg protein. PR-A expression was considered to be higher than PR-B expression when the total optical density of the band was higher (Table 3-1, PR-A>PR-B) and visa versa for PR-B>PR-A. No significant differences could be found between

Meningioma subtype	n	Total PR^{LBA} [a] (fmol/mg protein)	PR-A > PR-B [b]	PR-B > PR-A [b]
- Syncitial	16	183 ± 49	9	7
- Transitional	4	82 ± 20	4	0
- Atypical	3	66 ± 11	2	1
- Malignant	1	23	1	0
- Unknown	37	221 ± 36	24	13
Total	**61**	**181 ± 24**	**40**	**21**

Table 3-1. Progesterone receptor (PR) isoform expression in human meningiomas. *Abbreviations: Total PR^{LBA}, progesterone receptor determined with a LBA.* [a] Data are presented as the mean ± standard error of the mean (s.e.m.). [b] PR-A or PR-B is considered to be higher than PR-B or PR-A expression when the optical density of the specific band was higher.

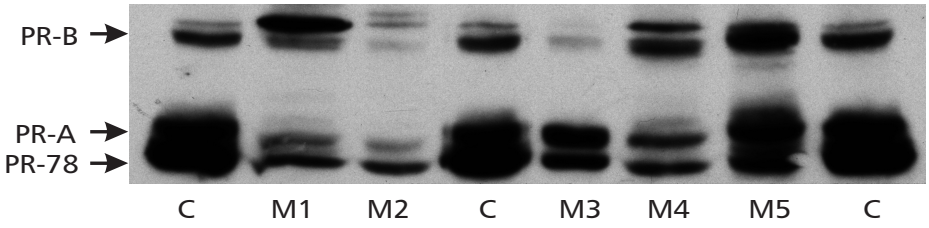

Figure 3-1. Immunoblot analysis of the progesterone receptor (PR) isoforms in human meningioma cytosols. For each of the vife samples depicted as M1 to M5, three control myometrium cytosols were used (C). Each lane contains equal amounts (10 fmol) of PR established with a ligand binding assay (LBA). PR-B (116-120 kDa) shows multiple bands depending on phosphorylation. Just below PR-A (81 kDa) a 78 kDa band migrates.

histological meningioma subgroups and total PR^{LBA} concentration.

PR isoforms

Figure 3-1 shows a representative immunoblot of the two PR isoforms, PR-A (81 kDa) and PR-B (116-120 kDa) and an additional band PR-78. The PR-B isoform migration pattern depends on the phosphorylation status and can be seen as multiple bands. Sixty-six per cent of the tumors express more PR-A than PR-B (Table 3-1). No significant differences in the distribution of the two isoforms among the histological subtypes of meningiomas could be found. Figure 3-1 also shows that there is a high variation (coefficient of variation of 30%) between PR immunoblot band density (PR-A + PR-B + PR-78) and the calculated total PR^{LBA} levels of 10 fmol/mg protein. This was unexpected since the between-assay variability of the immunoblot assay and of the LBA were 10.1% (at 79.5 % PR-A, n=31) and 11.3% (at 333 fmol/mg protein, n=31), respectively.

The PR-78 isoform

An additional 78 kDa band migrating just below of PR-A was detected in all 61 meningiomas. To analyze whether this 78kDa band is associated with PR-A or PR-B, Spearman rank correlation analysis was performed for all the PR isoforms. Figure 3-2 shows that PR-B and PR-78 are significantly negatively associated (r_s=-0.76, P<0.0001). No association was found between PR-A and PR-78 (r_s=-0.20, P>0.5). Besides PR-A and PR-B, because of its capability of ligand binding, PR-78 has also been included in the comparison with the total PR^{LBA}.

Association between PR isoforms and total PR

Clinical studies that have compared PR concentration in meningiomas with prognosis or recurrence rate, have found some relation between these factors and total PR determined by a LBA (in this study referred to as total PR^{LBA}). The differential expression of PR isoforms may play an important role in these

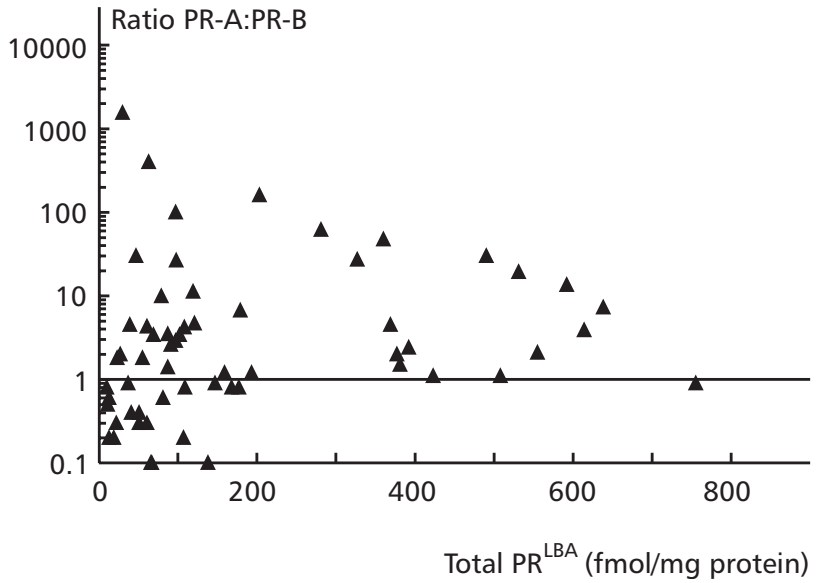

Figure 3-2. The PR-A to PR-B ratio and the total PR^{LBA} expression in human meningiomas. The number of tumors that express more PR-A than PR-B is higher in meningiomas expressing more than 200 fmol/mg protein. In the group with a low total PR^{LBA}, both PR-A>PR-B and PR-B>PR-A expression ratios are observed.

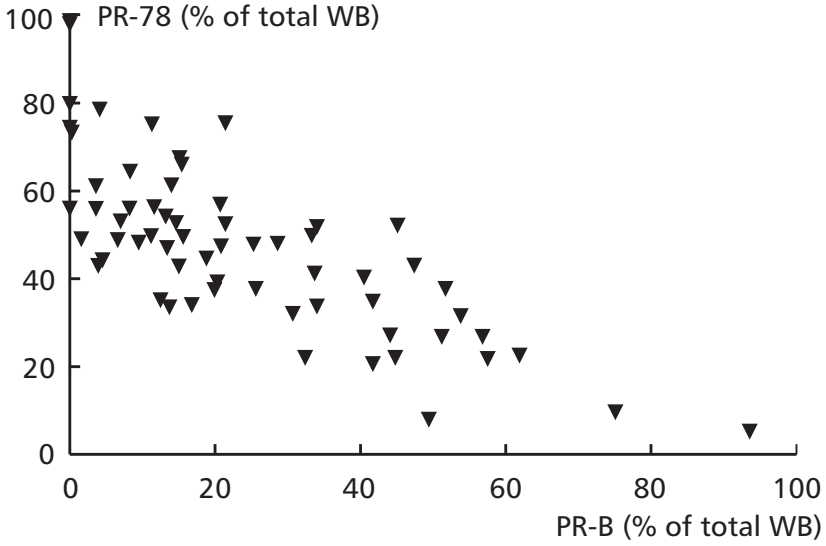

Figure 3-3. The association between the band intensities of PR-B and PR-78. The densities of the PR-B band and PR-78 band are strongly negatively-associated. No association was found between the density of PR-A and PR-78 bands. *Abbreviation: WB, Western immunoblot*.

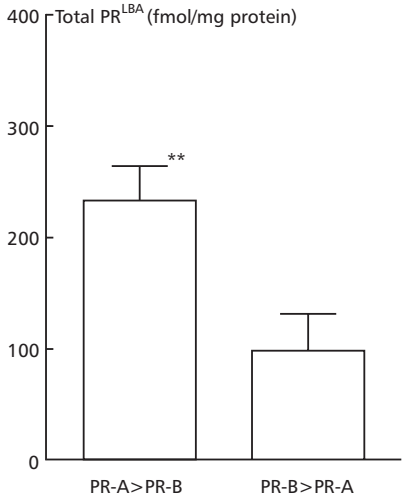

Figure 3-4. Total PR^{LBA} expression in meningiomas with differing ratios of PR-A and PR-B. The group of meningiomas with a higher PR-A than PR-B band intensity (PR-A>PR-B, $n=39$) expressed significantly more total PR^{LBA} than the group with a higher PR-B than PR-A band intensity (PR-B>PR-A, $n=22$). ***(P<0.001)*.

observations, therefore the association between total PR^{LBA} and de differential expression of the PR isoforms expression was determined.

PR-A and total PR^{LBA} were found to be significantly associated ($r_s=0.45$; $P<0.001$). Figure 3-3 shows the relationship between the ratio PR-A:PR-B and the total PR^{LBA}. High expression levels of PR-A with respect to PR-B were found in all the tumors with a high total PR^{LBA} expression (>200 fmol/mg protein). The group of meningiomas expressing less PR-A than PR-B, expressed significantly lower amounts of total PR^{LBA} than the group with more PR-A than PR-B (Figure 3-4; $P<0.001$). An association was found between PR-78 and total PR^{LBA} of $r_s=0.3$ ($P=0.03$). Meningiomas with a high total PR^{LBA} expression (>200 fmol/mg protein) tended to express more PR-78 than PR-B.

Discussion

In this study, the distribution of progesterone receptor isoforms was determined in human meningioma cytosols and compared with total PR levels determined with a LBA (total PR^{LBA}). The findings of this study showed that more than 65% of the tumors expressed more PR-A than PR-B. In addition, PR-A was positively associated with total PR^{LBA} levels. The differential expression of PR isoforms in meningiomas may have important implications for the clinical aspects of the disease.

Progesterone is thought to play a crucial role in meningioma development and proliferation. Follow up studies of patients with meningioma disease showed that the PR contents of meningiomas may have clinical significance as a prognostic

parameter for survival and recurrence. Hsu *et al.* and Brandis *et al.*, showed that the presence of PR is a favourable prognostic factor for meningiomas [3,4]. In agreement with Hsu, Fewings *et al.*, in a study of 62 meningiomas, found that PR positive benign meningiomas were less likely to recur [5]. Since PR isoforms exhibit differential transcriptional activity, the overall response to progesterone or PR antagonists may depend on PR isoform distribution.

In all our meningioma samples and the myometrium control, the presence of a PR-78 band migrating just below the PR-A isoform was detected. Graham *et al.* also reported the presence of this PR-78 band in a proportion of human breast cancer cytosols [10]. These authors found that the PR-78 band accounted for up to 20% of the total PR concentration. No association was found between PR-78 and PR-A or PR-B in these breast cancer cytosols. The origin of the PR-78 band has been reported not to be an artifact of the cytosol preparation, since deliberate degradation of the breast cancer cytosol samples did not lead to more PR-78 [10,11]. Yeates *et al.* performed additional experiments to characterize the origin of the PR-78 band. In breast cancer they ruled out the possibility that PR-78 could be considered to be a phosphorylated product of PR-A and PR-B. They also showed that PR transcripts, including splice variants, are unlikely to be implicated in the formation of PR-78 isoform. The PR-78 is, therefore, most likely not a direct product of translation of the PR gene. Photo-affinity labeling studies demonstrated specific binding of ligand to PR-78 [11].

In contrast to these findings, the results of our study showed a significant negative correlation between the expression of PR-B and the PR-78 isoform in meningiomas (Figure 3-2). The absence of transcripts encoding for PR-78, and the negative association with PR-B and not PR-A, are consistent with the idea that PR-B endogenously degraded into PR-78 in meningiomas, but the data do not exclude that there maybe a similar regulation pathway in the biogenesis of PR-B and PR-78. In addition, most of the antibodies used in literature were, apparently, not able to detect PR-78 in various other tissues. This may indicate that although PR-78 is capable of progesterone binding, PR-78 might miss some other epitopes besides the B upstream sequence. Nevertheless, PR-78 protein is capable of ligand binding, therefore, it may have a biological function in mediating progesterone signaling. Experiments are underway to further address the origin of PR-78.

Our immunoblot data, in which equal amounts of PR based on ligand binding assay were used, showed a high within-assay variability of PR-A and PR-B band intensities (see Figure 3-1). Several possible explanations were ruled out by performing the appropriate control experiments, such as equalizing the total protein levels. The amount of blood contamination also did not alter these high differences. The presence of endogenous ligands in meningiomas might not be important since all tumors were from post-menopausal women, or from men. When the amount of PR-78 band is taken into account, this discrepancy decreases by 20%. Other PR

proteins that bind ligand in the LBA, for instance PR-C, which could not be detected in our experimental subset, might explain a part of the remaining discrepancy.

The existence of PR-like proteins that are capable of binding progesterone might have important implications. If these isoforms, PR-78 and possible others, do not have a biological function the total PR concentration used for clinical purposes is overestimated. If the PR-78 isoform interferes in the progesterone-signalling pathway, for instance by binding to cofactors, it might act as a transrepressor. In addition, PR-like proteins may interfere with the prognostic significance of steroid hormones in hormone-responsive cancers. In conclusion, PR-like proteins are present in tumor cytosols and their precise function and role in progesterone signalling needs to be ellucidated.

The PR is a phosphoprotein and contains at least seven phosphorylation sites. PR-B contains three more phosphorylation sites than PR-A located in the PR-B upstream region [28-30]. Slower electrophoretic mobility on SDS-gels has been reported to be associated with increased phosphorylation [28,31]. In addition, in our immunoblot analysis of meningioma cytosol samples, variable differential expression of three closely migrating PR-B bands was observed. The biological relevance of this variability in PR-B sub-band expression is not clear. Whether phosphorylation of PR has a functional role PR remains poorly defined. Phosphorylation may not alter transcriptional activity of PR-B, but it could have other functions [29].

Growth of meningiomas seems to be influenced by progesterone levels and a lot of studies have achieved tumor growth remission by using progesterone agonists, progesterone depletion and, above all, progesterone receptor blockade by progesterone antagonists. Olson *et al.*, reported inhibition of meningioma growth *in vitro* of three meningiomas after treatment with mifepristone (PR antagonist), ranging from 18% to 36% after 28 days in culture [32]. We have shown that in meningiomas of 13 patients cultured for 8 days, the thymidine-labelling index significantly fell when increased concentrations of mifepristone were added[18]. Other authors, however, could not confirm these findings [33]. In clinical studies responses were seen in patients with unresectable meningiomas treated with mifepristone 200 mg orally daily for 6-24 months. Grunberg *et al.* reported objective responses seen in 8 out of 20 patients accompanied by subjective improvement in 5 patients [34]. Lamberts *et al.* noted regression in 3 out of 10 patients while headaches were improved in five patients [35]. Haak *et al.* also reported a case in which mifepristone was used successfully [36]. Whereas elevated levels of progestin in meningioma patients seems to stimulate tumor growth, high dose of medroxy-progesterone acetate (MPA) resulted in a decrease of tumor size and PR expression in a study reported by Markwalder *et al.* [23,37]. However, Jääskeläinen could not confirm this in a study of 5 meningioma patients using the same dose of MPA [38]. Although most studies that used anti-progestins for treating meningioma have reported some responses, more than half of the treated patients did not show a

response at all. Thus, data from both *in vivo* and *in vitro* reports are not yet conclusive.

In gynecological cancers progestins are used as therapeutic agents as reviewed by Gadducci et al. [39]. In metastatic breast cancer, progestins are used as third line therapy. Progestins are also used in patients with advanced or recurrent endometrial cancer and regression and stabilisation of advanced recurrent low-grade endometrial stromal carcinomas. The theory behind the success is that progestins have anti-estrogenic effects like down regulation of the oestrogen receptor (ER) and stimulation of the conversion of oestradiol to the less oestrogenic oestrone. In addition, progestins seem to downregulate the anti-apoptotic factor bcl-2, and to suppress estrogen induced expression of vascular endothelial growth factor (VEGF)-subtypes.

It has been reported that for breast cancer PR-A expression is higher than that of PR-B [10]. In leiomyomas, both PR forms are also expressed and there was a predominance of PR-A over PR-B [40]. For endometrial cancers, 5 out of 11 cases seemed to have a predominant expression of PR-B mRNA. However, it is not yet known whether this is translated to the protein level [41]. The different response of meningiomas and gynaecological cancers to progestin administration could up to now not be clarified by PR isoform distribution. Firstly, not much is known about the ratio PR-A:B from clinical trials, and secondly in most of the gynaecological tumors PR-A expression also seems to be higher. The anti-oestrogenic effect of progestins, however, will not play a role in meningioma since the majority of the tumors do not express appreciable levels of ER.

We suggest that progesterone blockade may only be effective in certain subsets of meningiomas. Meningiomas with a clear prevalence for PR-A or PR-B will respond in a different way to endocrine agents. If in meningioma PR-B is the more transcriptionally active of the two PR-forms, it could be anticipated that meningiomas with a prevalence for PR-A may be at least likely to respond to endocrine agents. The prognostic value of PR and the better outcome of meningiomas that express high levels of PR, may be due to transrepression activity of the prevalent PR-A isoform, and thus via a transrepression instead of a transactivation mechanism. Our initial idea: progesterone action via the PR activates progesterone-responsive genes in meningioma, needs to be reconsidered. Since there is no (pre-)clinical evidence that fully addresses the significance of PR isoform distribution and the response to endocrine therapy, further clinical investigations are needed.

In summary, meningiomas express both PR-A and PR-B isoforms and an additional PR-78 product. The ratio of PR-A to the PR-B protein expression levels is highly variable and higher in the meningiomas with a high total PR^{LBA} expression. Perhaps this variability has some clinical and biological significance in meningioma disease.

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Chapter 4

Progesterone receptor, bcl-2 and bax expression in human meningiomas

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Abstract

Meningiomas are generally benign central nervous system neoplasms, which frequently express progesterone receptor (PR) and only rarely express the estrogen receptor (ER). For breast cancer a relation between steroid hormone receptors and proteins involved in the apoptotic process has been described. For meningioma the exact relation between PR and these proteins is not known. In this study ER, PR, bcl-2 and bax expression levels were determined in cytosol from meningiomas. As a reference for our experimental conditions we also determined these proteins in cytosol from breast cancer. PR and ER were determined with a ligand binding assay and scatchard-plot analysis. The expression levels of the anti- and pro- apoptotic proteins, bcl-2 and bax respectively, were determined by immunoblotting.

In 65% of the meningiomas bcl-2 expression was found in variable amounts. In contrast to breast cancer, a significant negative association between PR and bcl-2 was found ($P < 0.01$). Bax expression in meningiomas appeared constitutive, not related to PR, and 2.6 times higher than in breast cancer. As both PR and bcl-2 appear positively associated with prognosis, the negative relationship between bcl-2 and PR found in this study might have some biological and clinical significance.

Submitted for publication

Introduction

Meningiomas are generally benign intracranial tumors that arise from meningothelial cells found in the arachnoid villi of the meninges, the membrane covering brain and spinal cord [1]. Meningiomas are one of the most common brain tumors in adults. The standard treatment is surgical resection and, when necessary, additional radiation therapy is used. Meningiomas recur in more than 20% of the cases, and are sometimes inoperable due to their relatively inaccessible locations. Standard therapy alone is then insufficient and there is a need for adjuvant therapy [1,2]. There are particular grounds for assuming that meningiomas are depending on hormones for their growth and differentiation. Intervention of tumor growth via steroid hormone receptors could be helpful for treatment of unresectable meningiomas [3].

Hormone-dependent tissues, such as uterus and breast cancer, express both estrogen receptors (ER) and progesterone receptors (PR) to regulate their hormone dependent proliferation. PR expression is, at least partly, under estrogen control. Meningiomas, which express relatively high levels of PR, seldom express ER [4,5]. An extensive overview of literature from the last two decades has showed that PRs are expressed in almost 2/3 of the tumors investigated (see also Chapter 1). ER positive meningiomas are relatively rare and have very low expression levels of ER [4,5]. In meningiomas, PRs are most likely functional and progesterone may play a role in the growth of the tumor. The presence of PRs may also serve as a favorable prognostic indicator of recurrence [6,7].

In breast cancer a relation has been described between PR and several apoptotic proteins, like bcl-2 [8]. Bcl-2 seems under progesterone and estrogen control in *in vitro* experiments [9]. Bcl-2 is a member of the Bcl-2 proto-oncogene family. Bcl-2 protein itself (bcl-2) is an anti-apoptotic protein and acts after a converging point in the apoptotic cascade. Bcl-2 forms heterodimers with bcl-2-associated X protein (bax), which is the pro-apoptotic counterpart of the bcl-2 protein. The ratio between bcl-2 and bax appears to determine the sensitivity of cells for apoptosis. However exact working mechanisms are still unclear.

In meningiomas bcl-2 expression has been reported. Mosnier *et al.* showed that bcl-2 expression in 82% (32/39) of the tumors they investigated by immunoblot analysis of meningioma cytosols [10]. In addition, tumors with high bcl-2 expression levels appeared to have a favorable prognosis [11,12]. Knowledge of the exact relation of bcl-2 and PR in meningiomas is limited, though may have some clinical significance since both proteins have been described to be prognostic markers.

In this study, both bcl-2 and bax protein levels were quantified in meningioma cytosols, using immunoblot analysis. ER and PR expression levels were quantified to establish the relationship between bcl-2 and bax expression. For comparative

purposes the same experiments were done with breast cancer specimens, of which hormonal regulation of bcl-2 and bax expression already has extensively been studied.

Patients and Methods

Tissues

Human breast cancer or meningioma tissue was placed on ice immediately after removal from the patient. Representative specimens were frozen at -80°C until they were used for cytosol preparation.

Cytosol preparation from tissue

The tissue (250 mg) was chilled in liquid nitrogen, pulverized with a microdismembrator (Braun, Melsungen, FRG) and extracted with 10 mM phosphate buffer containing, 1.5 mM EDTA, 3 mM sodium azide, 10 mM 1-monothioglycerol and 10% (v/v) glycerol, at pH 7.5. The resulting homogenate was centrifuged at 0-4°C, at 100,000 × *g* for 20 min, to yield a clear cytosol. The ER and PR were measured immediately after cytosol preparation. Aliquots for assessment of bcl-2 and bax were stored at -80°C.

Receptor assay

The ER and PR levels of the tumors were measured, as described previously, by the ligand-binding assay and scatchard plot analysis, according to the guidelines of the European Organization for Research and Treatment of Cancer (EORTC), Breast Cancer Cooperative Group [13,14]. The protein content of the cytosol was estimated with the method of Bradford, using reagents from Bio-Rad (Richmond, CA, USA) and serum albumin (Kabi, Diagnostica, Stockholm, Sweden) was used as a standard [15]. The lower cut-off level for ER and PR positivity was set to be 10 fmol/mg cytosol protein. The between-assay reproducibility for the assays were: ER(n=31): 10.6% at 58 fmol/mg protein, PR (n=31): 11.3% at 333 fmol/mg protein, and protein (n=31): 5.8% at 3.4 mg/ml.

Protein Electrophoresis and Immunoblot analysis

Tumor cytosol proteins (40 μg/lane) were separated by electrophoresis through 12.5% polyacrylamide (30% Acrylamide/Bis Solution, 37.5:1) resolving gel and a 2.6% stacking gel both containing 0.10% SDS, using a Mini Protean II apparatus (Bio-Rad, Richmond, CA, USA). Proteins were transferred to Immobilon-P membrane (Millipore,GB) (100V, 1hr, 4°C) in 25 mM tris, 192 mM glycine, 20% (v/v) methanol as described by Towbin et al. [16]. After transfer, unbound sites remaining in the membrane were blocked in 5% Protifar (Protifar; N.V.-Nutricia, The Netherlands) in PBS buffer, (Phosphate buffered saline, pH 7.4) and incubated

with specific antibodies (AB) against bcl-2 and Bax proteins. Bcl-2 was detected using specific anti-bcl-2 mouse AB at a final dilution of 1:1000 (Clone 124, Dako A/S, Denmark) and bax with anti-bax rabbit AB at a final dilution of 1:10,000 (polyclonal N-20, Santa Cruz Biotechnology, USA). Blots were incubated with a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody at a final dilution of 1:5000 (Dako A/S, Denmark). For both bcl-2 and bax specificity of the first antibody was confirmed with control experiments, in which the first antibody was left out of the assay. Specific bands were visualized by using enhanced chemiluminescence detection substrates (200 μl 250 mM luminol in DMSO, 88μl 90 mM p-Loumaric acid in DMSO, 12 μl 30% H_2O_2 in 20 ml Tris 0.1 M, pH 8.5) and blots were exposed to an auto-radiograph. Molecular weight was determined with biotinylated SDS-PAGE standards broad molecular weight marker and avidin HRP (Bio-Rad, USA). Three controls of myometrium tissue, positive for bcl-2 and bax, were included on each gel containing 5 samples.

Band intensities were measured densitometrically (Sharp JX330, Japan). The linear range of detection of bcl-2 and bax on immunoblot was established by a standard curve made using increasing concentrations of control cytosol. This was analyzed by densitometry and established the relationship between bcl-2 and bax concentration and linearity of detection by this method. Multiple exposures were always made from each immunoblot of tumor cytosols, and results were used only from those that fell within this linear range. Densities of specimens were referenced to those of the controls. The interassay reproducibility for this assay was 12.1%.

Statistical evaluation

All meningioma and breast cancer cytotols were ordered by increasing levels of PR or ER and divided in three equal groups, resulting in tertiles 1 to 3 with low, moderate, and high PR or ER expression, respectively. Mann-Whitney non-parametric tests were performed to compare protein expression between the tertiles. Spearman rank sum tests (r_s) were used to compare bcl-2, bax expression results with ER or PR expression. *P* values of less than 0.05 were considered statistically significant.

Results

Steroid receptor expression and apoptotic proteins in meningiomas

The results of steroid hormone receptor analysis in meningioma cytosols are shown in Table 4-1. In 15% of the meningiomas ER expression was found in a concentration of 14 ± 2 fmol/mg protein (mean ± s.e.m.). PR expression was found in 86% of the meningiomas, and expression levels were 205 ± 31 fmol/mg protein. At least 75% of the meningiomas expressed PR without the presence of ER,

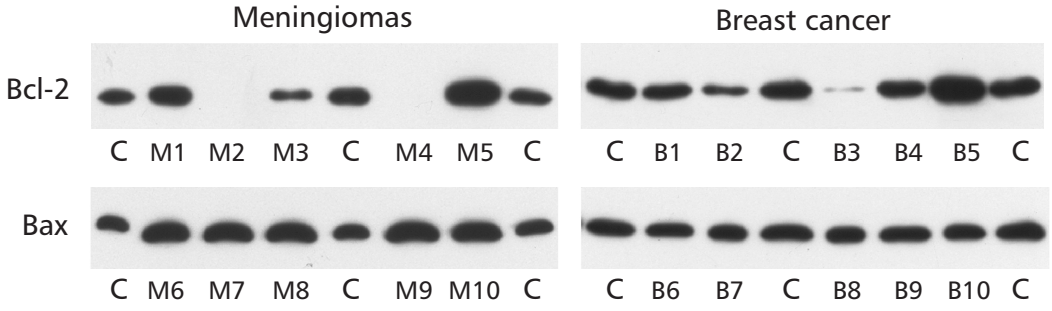

Figure 4-1. Immunoblot analysis of bcl-2 and bax expression in breast cancer and meningioma cytosols. Bcl-2 is variably expressed in meningioma as well as in breast cancer cytosols. Bax is constitutively expressed in both tissues. Bax expression levels are higher in meningiomas than in breast cancer. Meningioma samples are depicted as M1 to M10 and breast cancer samples as B1 to B10. Together with 5 samples, 3 control cytosols (C) were measured and used as an internal standard.

in agreement with previous results, indicating the representativity of the specimens. Examples of results of bcl-2 (Mr 26,000) and bax (Mr 21,000) immunoblots are shown in Figure 4-1 (left panel). Expression of bcl-2 was found in 31/48 (65%) meningiomas. Bax expression was found in almost all meningioma specimens. Bax protein expression levels were 2.6 times (266% against 104%) higher in meningiomas than in breast cancer tissue, as shown in Figure 4-2 ($P<0.0001$). A summary of the statistical analysis of the relationship between steroid hormone receptors and the apoptotic proteins bcl-2 and bax is given in Table 4-2. In meningiomas, we found a significant negative association between PR and bcl-2 ($P<0.01$). Meningiomas with high levels of PR showed a significant decrease in bcl-2 expression levels (Figure 4-3, black bars). Bax appeared to be expressed constitutively, and was not associated with the PR in meningiomas (as shown in Figure 4-2). Moreover, little biological variation was found in bax expression between the tissues.

Receptor phenotype		Meningiomas (n=47)	Breast Cancer (n=81)
ER	PR		
–	–	11 %	9 %
+	+	11 %	81 %
–	+	75 %	6 %
+	–	4 %	5 %
Receptor expression			
ER positivity		7	70
[ER] [a,b]		14 ± 2 [c]	130 ± 16 [c]
Median		11	81
PR positivity		40	69
[PR] [a,b]		205 ± 31 [c]	331 ± 50 [c]
Median		119	156

Table 4-1. Progesterone and estrogen receptor expression in meningiomas and breast cancer cytosols. [a] *positive only;* [b] *fmol/mg protein;* [c] *mean ± s.e.m.*

Steroid receptor expression and apoptotic proteins in breast cancer cytosol

Since in breast cancer bcl-2 appeared to be positively associated with PR and under progesterone control, the negative association found in meningioma was unexpected. In order to verify our experimental conditions, we performed the same experiments with breast cancer cytosols. In breast cancer cytosol ER expression was found in 86% of the tumors and the average expression level was 130 ± 16 fmol/mg protein. PR expression was found in 87%, 331 ± 50 fmol/mg protein. A predominance of the ER+/PR+ phenotype with 81% was found (Table 4-1). The Spearman correlation between the ER and PR was r_s=0.34, with a $P < 0.01$ (Table 4-2). Examples of bcl-2 and bax immunoblot assay are shown in Fig. 1 (right panel). Bcl-2 was expressed in 71/82 (87%) of the breast cancer cytosols. Bax expression was found in almost all breast cancers. Bcl-2 was significantly positively associated with ER and PR. Higher levels of steroid hormone receptors are associated with significantly higher levels of bcl-2 expression, as represented in Figures 3 and 4 for PR and ER, respectively. Bax was not associated with PR or ER.

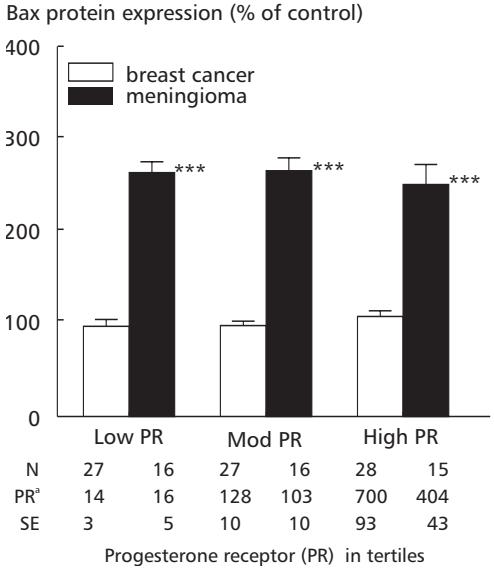

Figure 4-2. Bax protein levels in meningioma and breast cancer cytosols expressed as a percentage of control, and divided in three subgroups with low, moderate and high PR expression. Bar and error bar represent mean± sem, respectively. The mean of PR expression is shown below each corresponding bar. Bax is constitutively expressed in both meningioma and breast cancer cytosols. Bax expression in meningiomas is significantly higher than in breast cancer. Neither in meningiomas nor in breast cancer an association between bax and PR was found. *Abbreviations: PR progesterone receptor;* [1] *mean (fmol/mg protein); *** P<0.0001 vs. Breast cancer.*

	Meningiomas			
Breast cancer	ER	PR	bcl-2	bax
ER	-	nd	ns	ns
PR	0.34*	-	-0.37*	ns
bcl-2	0.41***	0.50***	-	ns
bax	ns	ns	ns	-

Table 4-2. Summary of the spearman rank correlation analysis in the comparison of bcl-2, bax and steroid hormone receptor expression in meningiomas and breast cancer (r_s). *Abbreviations: ns not significant, nd not determined. * P<0.01; *** P<0.0001.*

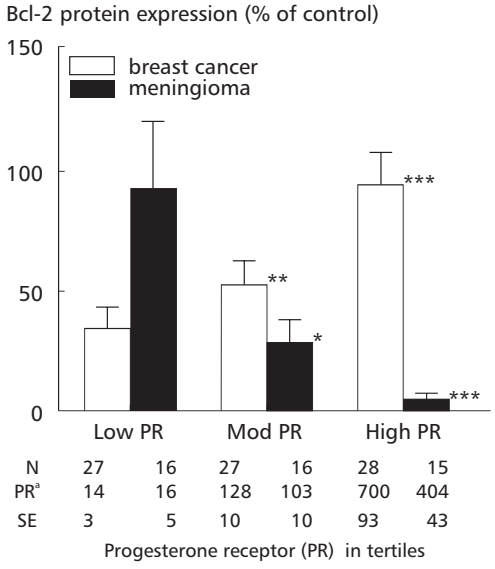

Figure 4-3. Bcl-2 expression in meningioma and breast cancer cytosols expressed as a percentage of control, and divided in three subgroups with low, moderate and high PR expression. Bar and error, bar represent mean ± sem, respectively. The mean of PR expression is shown below each corresponding bar. The group with high PR has a significantly lower bcl-2 expression in meningiomas and a significantly higher bcl-2 expression in breast cancer versus their corresponding low PR group. *Abbreviations: [1] mean (fmol/mg protein); *** P<0.0001 vs. corresponding low PR group; ** P<0.02 vs. corresponding low PR group; * P<0.05 vs. corresponding high PR group; PR, progesterone receptor.*

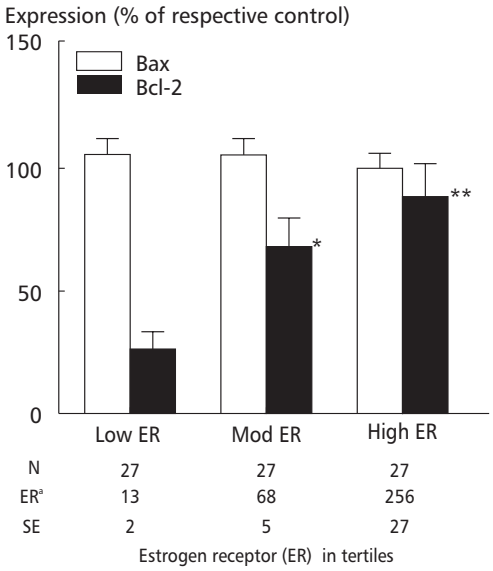

Figure 4-4. Bcl-2 and bax protein levels in breast cancer expressed as a percentage of control, and divided in three subgroups with low, moderate and high ER expression. Bar and error bar represent mean ± sem, respectively. The mean of ER expression is shown below each corresponding bar. Bcl-2 but not bax expression is significantly increased with higher ER expression levels in breast cancer cytosols. *Abbreviations: [1] mean (fmol/mg protein); ** P<0.001 vs. corresponding low ER group; * P<0.01 vs. corresponding low ER group; ER, estrogen receptor.*

Discussion

In this study, we found that most of the meningiomas had an ER negative and PR positive phenotype, this was a confirmation of our previous finding [4,14]. In most other hormonally regulated tissues, PR is regulated via the ER pathway. Genes that are under strict estrogen control may not come to expression in the absence of ER. Koehorst *et al.* described the absence of pS2 and the low CatD expression, both estrogen inducible proteins, in 46 meningiomas [17]. Other genes, like the PR gene,

appear to have at least a second transcription initiation pathway. How transcription of the PR gene is regulated in meningiomas is still unknown.

There are some arguments for thinking that PRs play a role in meningioma growth. For instance a predominance of female patients was found [18]. The size of meningiomas increased when there are high progesterone levels as seen during pregnancy and in the luteal phase of the menstrual cycle [19]. Grunberg *et al.* reviewed responses seen in several trials treating patients with the progesterone receptor antagonist, mifepristone (RU 486) or Medroxy Progesterone Acetate (MPA), a synthetic progesterone agonist [3]. Markwalder *et al.* described another argument, they treated patients with MPA prior to surgical resection and found that the treated group had a significantly higher level of PR [20]. Several *in vitro* experiments have described hormone dependence of meningiomas grown in cell culture experiments [21].

PR expression in meningiomas might have some clinical significance since Brandis *et al.* found that PR was absent more frequently from non-benign meningiomas [22]. Moreover, they have found that the PR status in meningiomas is related to tumor differentiation and thus may be a prognostic factor [22]. In addition, Fewings *et al.* found that meningiomas that are PR positive were less likely to recur [6,7].

Bcl-2 immunostaining of meningiomas has been described by several authors [10-12,23,24]. In addition, Mosnier *et al.* confirmed their own immunohistochemtry (IHC) findings by immunoblotting and found 32/39 tumors expressing measurable levels of bcl-2, however, in contrast to own findings, they could not find a relation with the PR based on IHC data [10]. Since our results differ from Mosnier *et al.* and the positive relation that is found between PR and bcl-2 in breast cancer, we validated our experimental conditions by measuring these proteins in breast cancer cytosols. Our findings confirmed the positive relation between PR, ER and bcl-2, and therefore, validate our experiments. The discrepancy with the findings of Mosnier *et al.* may be caused by differences in technique.

Bcl-2 expression in meningiomas may have clinical significance since Karamitopoulou *et al.* found that bcl-2 protein expression was associated with unfavourable prognosis of meningiomas [12]. The negative association between PR and bcl-2 expression levels found in the present study links the findings of these authors.

Hara *et al.* reported the only study of bax expression in untreated meningiomas [11]. They found very weak staining of bax proteins in meningiomas using IHC. This is in contrast to our own findings, in which we observed constitutively high expression levels of bax in meningiomas compared to breast cancer. High levels of bax may indicate that meningiomas may be sensitive for apoptosis induction. Indeed, apoptosis is described in meningiomas, however, uncommonly for low grade meningiomas [23,25].

In the present study, where we paid particular attention to proper quantification of the results by introducing a common specimen in all experiments (*see* "*patients and methods*" *section*), neither in

breast cancer nor in meningiomas an association was found between bax protein levels and the steroid hormone receptors ER and PR. Kandouz *et al.* found that bax seems to be constitutively expressed in breast cancer cell lines independent of hormone stimulation [26]. A direct association between PR and bax in ovarian tissue was reported [27]. It seems that steroid hormones, at least in meningioma and breast cancer, may not regulate bax expression levels. The hormonal regulation of bax is most likely tissue-specific and remains to be elucidated.

The ratio between pro- and anti-apoptotic proteins determines the overall sensitivity for cells to apoptosis. In meningiomas that express high levels of the pro-apoptotic bax in contrast to low levels of the anti-apoptotic bcl-2, you might expect the involvement of other anti-apoptotic proteins to make tumor growth possible. Work is currently underway to address the question whether the anti-apoptotic protein Bcl-x_l and/or insulin like growth factor II are involved in maintaining growth in meningiomas.

In summary, a significant negative relation exists between PR and bcl-2 in meningiomas. Bax expression was high in meningiomas and might be constitutive. Bcl-2, bax and PR may be valuable in predicting success of anti cancer endocrine therapy and for other prognostic purposes. The precise regulation of PR and bcl-2 proteins, in an estrogen independent pathway, remains to be elucidated.

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Chapter 5

Expression of progesterone receptor, bcl-x_l and bak proteins in human meningiomas

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Abstract

Meningiomas are generally benign neoplasias that arise from the meninges and comprise 20% of all intracranial tumors. These tumors frequently express the progesterone receptor (PR) and meningioma growth is affected by treatment with antiprogestins as shown by *in vitro* and clinical studies. In addition, the PR is associated with recurrence and survival rate. Progesterone could also be important in the regulation of apoptosis since a negative correlation exists between PR and bcl-2. Bcl-2 is an anti-apoptotic protein that is a prognostic factor in meningiomas. The PR exists in at least two isoforms, PR-A and PR-B each of which are likely to have different biological functions depending on cell and promoter context.

This study was conducted to investigate the expression of other apoptotic proteins than bcl-2, and their relationship with the PR. Thus far, nothing is known about the expression of the anti-apoptotic protein bcl-x_l and the pro-apoptotic protein bak in human meningiomas and this issue was addressed in the present study by using immunoblot analysis. Bcl-x_l expression was found in 87/99 meningiomas and PR negative meningiomas expressed significantly less bcl-x_l protein. A positive correlation was found between bcl-x_l and PR-B protein expression ($r_s = 0.34$, $P < 0.02$). Bak was expressed in 52/60 meningiomas and appeared to be unrelated to bak and PR expression levels. Bcl-x_l and bak were significantly associated ($r_s = 0.42$, $P < 0.001$).

In conclusion, both bcl-x_l and bak are variably expressed in human meningiomas. PR expression might be involved in regulating the apoptotic balance in meningioma, since bcl-x_l was associated with PR-B, and this may have some biological and clinical significance.

Submitted for publication

Introduction

Meningiomas arise from arachnoidal cells present in the meninges, the membranes covering brain and spinal cord, and comprise as many as 20% of all intracranial tumors [1]. They are generally benign neoplasms that frequently express the progesterone receptor (PR), despite the fact that estrogen receptor expression is rarely found by ligand binding assay (LBA) [2]. The PR belongs to the superfamily of ligand activated transcription factors. Clinical studies have shown that PR expression might be clinically important since the PR is a marker of recurrence and survival rate of meningioma disease [3-5]. The exact biological function of PR expression in meningiomas is unknown.

The PR exists as at least two different isoforms PR-A and PR-B. Both isoforms have distinct transcriptional capacities and may exert different biological functions [6,7]. Meningiomas express both PR isoforms in variable amounts, however, PR-A expression is higher in tumors with high total PR expression [8]. Since PR is thought to be involved in the proliferation of meningiomas, antiprogestins have been tested for their anti-tumor activity in several *in vitro* and clinical studies [9].

In breast cancer, steroid hormone receptors do not only play a role in proliferation but also in cell death or apoptosis. The process of apoptosis is controlled by external signals in combination with an autonomous genetic program. Hormone-dependency has been described of members of the Bcl-2 proto-oncogene family involved in apoptosis [10]. Expression of one of these proteins, bcl-2 itself, is one of the major anti-apoptotic proteins and regulated by estrogen and progesterone [10]. Through the anti-apoptotic function of bcl-2, it is involved in cancer chemotherapy-resistance and associated with prognosis, recurrence and sensitivity for adjuvant therapy of several types of tumors [11].

In meningiomas, bcl-2 expression has also been demonstrated. In contrast to breast cancer, however, meningiomas showed a negative association between bcl-2 and total PR expression levels. The pro-apoptotic counterpart of bcl-2, bax, is also expressed in meningiomas. In general, the ratio bcl-2 to bax expression levels is thought to determine apoptotic sensitivity of cells [12]. Several meningiomas show a high ratio bax to bcl-2, indicating that at least other anti apoptotic proteins are involved in maintaining meningioma growth.

Knowledge of expression levels of other members of the bcl-2 proto-oncogene family in meningiomas is limited. Another major anti-apoptotic protein besides bcl-2 is bcl-x_l. Bcl-x_l prevents bax induced apoptosis and is described in other hormone dependent malignancies, like glioma and neuroblastoma [13-15]. Expression of bcl-x_l seems to be hormonally regulated by sex steroids, as was revealed by breast cancer studies [16-19]. The expression of bcl-x_l has been reported to be involved in tumor progression. In addition, bcl-x_l might serve as therapeutic target in antisense anticancer therapy. Therefore, knowledge of the expression level of this

protein in meningiomas is of clinical importance.

Another protein from the bcl-2 family is the pro-apoptotic protein bak. Bak expression has been reported in normal and malignant breast tissue and other malignancies [20,21], however, thus far, nothing is known about the expression in meningiomas. The hormone dependency of bak expression in breast cancer seems less than was found for bcl-2 and bcl-x_l. Although one study reported that bak expression was decreased in ER-α negative breast cancers [21].

Total PR expression in meningiomas is associated with recurrence and survival rate, but the exact function of this receptor in these tumors is unknown. PR might be involved in the regulation of the apoptotic process, illustrated by the negative association between total PR and bcl-2. This study was conducted to determine the expression of other apoptotic proteins, bcl-x_l and bak, and their relation with PR in human meningiomas.

Patients and methods

Tissues

Human breast cancer or meningioma tissue was placed on ice immediately after removal from the patient. Representative specimens were frozen at -80°C until they were used for cytosol preparation.

Cytosol preparation

The tissue was chilled in liquid nitrogen, pulverized with a micro dismembrator (Braun, Melsungen, FRG) and extracted with 10 mM phosphate buffer containing 1.5 mM EDTA, 3 mM sodium azide, 10 mM 1-monothioglycerol and 10% (v/v) glycerol, at pH 7.5. The resulting homogenate was centrifuged at 0-4°C for 30 min at 100.000 × *g* to yield a clear cytosol. The protein content of cytosols was estimated with the method of Bradford using reagents from BioRad (Richmond, CA, USA) and human serum albumin (Kabi Diagnostica, Stockholm, Sweden) as a standard [22]. The PR was measured immediately after cytosol preparation. Aliquots for assessment of bcl-x_l and bak were stored at -80°C.

Receptor assay

Meningiomas. PR levels were measured by a ligand-binding assay (LBA) and scatchard plot analysis, according to the guidelines of the European Organization for Research and Treatment of Cancer (EORTC), Breast Cancer Cooperative Group as described previously [23,24]. The lower cut-off level for PR positivity was set to be 10 fmol/mg cytosol protein. The between-assay variability for thess assays was: PR (n=31): CV=11.3% at 333 fmol/mg protein, and protein (n=31): 5.8% at 3.4 mg/ml.

Breast cancer. For breast cancer cytosol samples, total PR was assayed in duplicate by enzyme immunoassay according to the instructions of the manufacturer (Abbott Laboratories, Chicago, IL, USA). Day-to-day performance of the kits was monitored by assaying control samples provided with the kits and a myometrium control cytosol prepared at our laboratory. For the kit controls, the found/target ratio for PR was 0.99 ± 0.13 (n=8; CV=12.7%). The myometrium in-house control specimen read 717 ± 127 (n=8; CV=17.7%) fmol/ml in the PR assay. Samples were considered to be receptor positive when the assay result exceeded 20 fmol/mg protein.

Protein Electrophoresis and Immunoblot analysis

Tumor cytosol proteins (50 μg/lane) were separated by electrophoresis through 12.5% polyacrylamide (30% Acrylamide/Bis Solution, 37.5:1) resolving gel and a 3.9% stacking gel both containing 0.10% SDS, using a Mini Protean II apparatus (Bio-Rad, Richmond, CA, USA). Proteins were transferred to Immobilon-P membrane (Millipore, GB, 125V, 1hr, 4°C) in 25 mM tris, 192 mM glycine, 20% (v/v) methanol as described by Towbin et al. [25]. After transfer, unbound sites remaining in the membrane were blocked with 10% Protifar (Protifar; N.V.-Nutricia, The Netherlands) in PBS buffer, (Phosphate buffered saline, pH 7.4) and incubated with specific antibodies (AB) against bcl-x_l proteins and bak. Bcl-x_l was detected using specific anti-bcl-x_l mouse AB at a final dilution of 1:750 (Bcl-x_l (Ab-2), Calbiochem) and bak with anti-bak rabbit Ab at a final dilution of 1:1000 (Bak, Santa Cruz Biotechnology). Blots were incubated with a horseradish peroxidase-conjugated goat anti-mouse (Dako A/S) or donkey anti-rabbit (Amersham) secondary antibody at a final dilution of 1:2500 or 1:10000, respectively. Visualization using ECL reagents (Amersham) was according instructions of the manufacturer. Blots were exposed to an autoradiograph. Molecular weight was determined with biotinylated SDS-PAGE standards broad molecular weight marker and avidin HRP (Bio-Rad, USA). Three controls of myometrium tissue, positive for bcl-x_l and bak, were included on each gel containing 5 samples.

Band intensities were measured densitometrically (Sharp JX330, Japan). The linear range of detection of bcl-x_l on immunoblot was established by a standard curve made using increasing concentrations of control cytosol and analyzed by densitometry. A linear relationship between bcl-x_l and bak concentrations and densitometry could be established. Multiple exposures were always made from each immunoblot of cytosol samples, and results were used only from those that fell within the linear range. Densities of specimens were referenced to those of the controls. The between-assay variability of bcl-x_l and bak protein levels in cytosols of human myometrium were 11.6 % (n=31) and 14.2% (n=73), respectively.

Statistical evaluation:

Meningioma and breast cancer cytosol samples were ordered by increasing levels of PR and divided in four groups,

resulting in quartiles, with total PR negative, low, moderate, and high total PR expression, respectively. Mann-Whitney non-parametric tests were performed to compare protein expression between the subgroups. Spearman rank sum tests were used to compare PR isoforms, bcl-2, bax, bcl-x_l and bak expression with each other, and Spearman's correlation coefficient (r_s) was used to evaluate the relationship between the expression levels of the various proteins. *P* values of <0.05 were considered statistically significant.

Results

Progesterone receptor (PR) expression in meningiomas

Expression levels of total PR in meningiomas were determined with a ligand-binding assay (LBA). In the total PR positive tumors the PR isoforms, PR-A and PR-B, were determined by immunoblotting (example is shown in Figure 5-1). In 67% (66/99) of the meningiomas PR was expressed with an average expression level of 174±23 fmol/mg protein (median: 94 fmol/mg protein). In 66% of the total PR positives overexpression of PR-A to PR-B was found, median ratio of PR-A to PR-B was 1,7. No differences in total PR and PR isoform expression levels in meningiomas could be found between genders.

Expression of apoptotic proteins in meningioma

Up to now, nothing was known about the expression levels of bcl-x_l and bak proteins in human meningiomas, both proteins belong to the bcl-2 proto-oncogene family. Therefore, immunoblot analysis was performed to analyze expression of these proteins in meningiomas. Representative immunoblots are shown in Figure 5-2. The bcl-x_l immunoblot revealed a doublet band at 32.5kDa and overall analysis showed variable expression of bcl-x_l in 87 out of 99 meningiomas. Bak immunoblot analysis showed also variable expression of bak in 52 out of 60 meningiomas. Some cytosol samples showed an additional bak band that turned out to be a degradation product of bak full-length protein (data

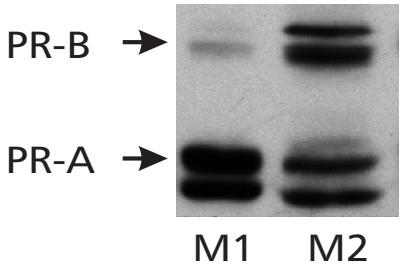

Figure 5-1. Example of immunoblot analysis of the progesterone receptor isoform expression in meningioma cytosol. PR-B (116-120 kDa) appeared as multiple bands depending on phosphorylation. PR-A shows a single band (81 kDa) and just ahead of PR-A a 78 kDa PR like protein migrated as described by Graham *et al.*[8,35] Both isoforms were expressed in meningiomas at different levels.

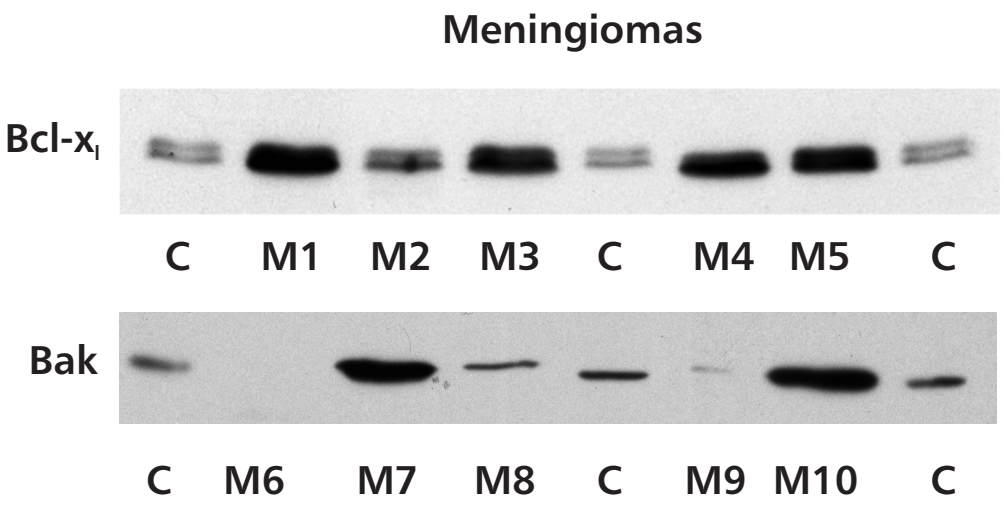

Figure 5-2. Example of immunoblot analysis of bcl-x_l and bak expression in meningioma cytosols. Both bcl-x_l and bak were variably expressed in meningioma cytosols. Meningioma samples were depicted as M1 to M10. Together with 5 samples, 3 control cytosols (C) were measured, as an internal standard.

not shown), therefore those samples were not used for analysis. Bak expression was significantly lower in meningiomas from female than from male patients, as shown in Figure 5-3 ($P<0.01$; 260% and 695%, respectively).

Previous studies have shown that bcl-2 expression was negatively associated with total PR protein expression level, therefore progesterone could play a role in the regulation of apoptosis in meningioma. In this study, therefore, the association of total PR with other apoptotic proteins was investigated. Figure 5-4 shows the bcl-x_l and bak expression levels in groups with different expression levels of total PR; PR negative, low, moderate and high. In total PR negative

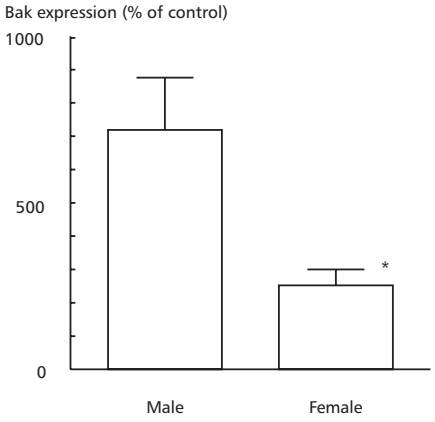

Figure 5-3. Bak expression in meningioma cytosol samples from male ($n=19$) and female ($n=37$) patients. Results are expressed as means ± sem. *$P<0.01$.

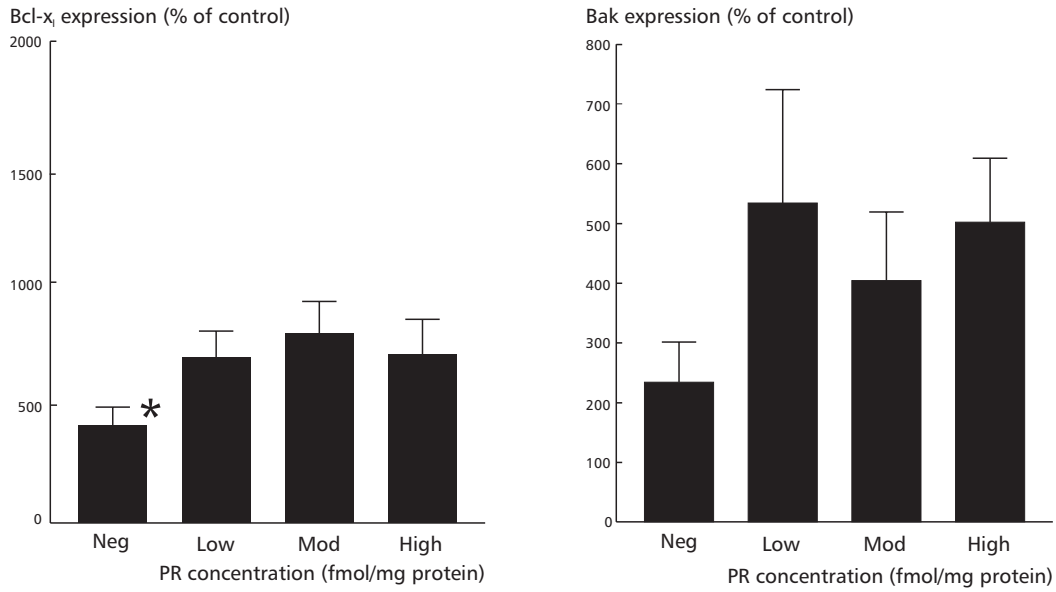

Figure 5-4. Means of bcl-x_l and bak expression in meningioma cytosols expressed as a percentage of control, and divided in three subgroups with low, moderate and high PR expression. Bars and error bars represent mean ± s.e.m., respectively. The left panel shows that PR negative meningiomas express significantly less bcl-x_l ($P<0.01$). *Abbreviations: PR, progesterone receptor; *P<0.01.*

meningiomas, bcl-x_l expression levels were significantly lower ($P<0.01$). In all other PR subgroups, bcl-x_l and bak expression were not significantly different.

Spearman rank correlation analysis revealed no significant association between total PR and both bcl-x_l and bak. This, however, could be masked by the differential PR isoform expression. Therefore, correlation analysis was performed with PR-B and bcl-x_l and bak expression levels. Figure 5-5 shows that PR-B and bcl-x_l were positively associated, $r_s=0.34$ and $P<0.02$. Bak expression was not significantly associated with PR-A or PR-B.

Inter apoptotic protein analysis

For a large number of meningiomas used in this study, bcl-2 and bax expression levels were determined in a previous study, therefore, multiple correlation analysis could be performed between the different apoptotic proteins. A significant

association was found between bcl-x_l and bak expression levels (r_s=0.42; P<0.002). Bcl-2 was not associated with bcl-x_l or with bak expression levels.

Breast cancer cytosols

In breast cancer, bcl-x_l is known to be positively associated with the expression of total PR. Since under our conditions such a positive association could not be found in meningioma cytosol, breast cancer cytosols were analyzed as a reference for our experimental conditions. Indeed western immunoblot and Spearman rank correlation analysis revealed a positive association between total PR and bcl-x_l of r_s=0.54 and P<0.01.

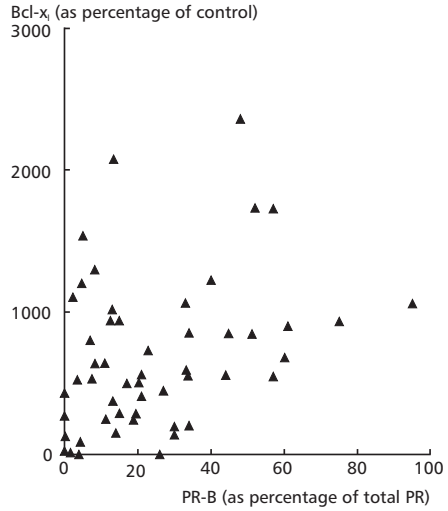

Figure 5-5. Association between expression levels of PR-B and bcl-x_l in meningiomas. The expression levels are positively associated (r_s=0.34, P<0.02).

Discussion

The aim of this study was to elucidate the involvement of the PR in the regulation of proteins from the apoptotic cascade in human meningiomas. Expression levels of the apoptotic proteins bcl-x_l and bak were determined in meningioma cytosols samples by immunoblotting. These protein values were then compared with expression levels of total PR and PR isoforms. This study showed that both bcl-x_l and bak were variably expressed in meningioma cytosol samples. In addition, bcl-x_l was associated with PR-B and significantly lower in PR negative meningiomas. Bak expression seemed not to be regulated by progesterone or to be associated with the PR.

The *bcl-x* gene is a bcl-2-related gene. Due to alternative splicing, different mRNA products are formed. A large bcl-x form, bcl-x_l, shows close similarity with bcl-2. Bcl-x_l has been reported as a potent inhibitor of apoptotic cell death in various cell types [26]. It exhibits regulated expression during lymphoid development, and is expressed in developing B cells as shown by immunoblot analysis [27]. Bcl-x_l expression has also been reported in various types of cancers; such as gynecological tumors, like ovarian tumors, breast cancer and in breast cancer cell lines [14 28,29]; lymphomas [30]; and in prostate cancer [31]. Bcl-x_l expression levels have been reported to be clinical important. Transfection of bcl-x_l in

human ovarian carcinoma cells resulted in highly resistance of the cells to chemotherapeutic agents [29]. In addition, promising results were obtained in studies in which cell proliferation of breast cancer cells was inhibited by bcl-x_l-bispecific antisense oligonucleotide treatment of these cells [32,33].

The other protein invloved in apoptosis and investigated in this study was bak. Bak is a pro-apoptotic protein and expressed in normal breast tissue, as well as in breast cancer, malignant glioma cells, gastric ad colorectal cancer [20,21]. Regulation of bcl-x_l and bak by sex steroids has been described in several hormonally regulated tissues. Marone *et al.* established a negative correlation between PR and bcl-x_l in neoplastic ovarian tissue [14]. In addition, progestins increase the ratio of bcl-x_l to bcl-x_s in a rat endometrial cell line [17]. Less is known about the hormone sensitivity of bak expression. Gompel *et al.* findings showed that estrogen and progesterone did not affect bak expression levels [10]. In contrast, Leung *et al.* reported inhibition of a time dependent increase of bak mRNA in MCF-7 cell line [34]. In addition, a decrease in bak expression has been described in ER-α negative breast cancers [21].

Previous investigations have showed a negative association between Bcl-2 and total PR expression levels in meningioma cytosols. Treatment of meningioma tissue fragments with the progesterone agonist org2058 altered the bcl-2 expression levels (data not shown) suggesting a direct effect of progesterone on bcl-2 expression regulation. This study showed that bak seems not associated with PR. In contrast, bcl-x_l seems positively associated with PR-B. These findings suggest that bcl-2 expression is most likely regulated differently than bcl-x_l or bak.

For comparative purposes, the relation between bcl-x_l and total PR as analyzed in breast cancer cytosol samples. For breast cancer, a positive correlation between PR and bcl-x_l based on IHC data has already been described in literature. Breast cancer cytosol analysis revealed a positive association between bcl-x_l and PR in PR positive tumors. This result further validates our experimental method and showed that the hormonal regulation of *bcl-x* gene seems tissue specific.

For bcl-2, bax and bcl-x_l no difference between male and female patients could be detected. Bak expression, however, was significantly lower in meningiomas of female patients. Involvement of sex steroids in the origin of this observation is not very likely. First, this study showed that PR was not involved in bak expression regulation. Second, the group of meningioma patients in this study mainly consisted out of man or post-menopausal woman, thus with low estrogen levels. The biological importance of lower bcl-x_l levels in woman is not known.

In summary, estrogen and progestin dependency has been reported for bcl-2, bcl-x_l and bak in other tissues. Also, correlation between ER, PR and these apoptotic

proteins has been found. In meningioma, only PR is abundantly expressed and only negatively associated with bcl-2 expression levels. Bcl-x_l was positively associated with PR-B and bak seemed not regulated by progesterone in meningiomas. The hormone dependent regulation of bcl-2 seems distinct from the regulation of that of Bcl-x_l and bak. This is confirmed by the absence of a correlation between bcl-2 with the latter two proteins. In contrast is the association between bcl-x_l and bak, indicating that they may share a common progesterone independent regulation pathway in meningioma.

In conclusion, the apoptotic proteins bcl-x_l and bak are expressed in meningiomas at variable amounts and this might be clinically important. Our hypothesis is that the PR may regulate the balance between pro- and anti-apoptotic proteins by differentially regulating the anti-apoptotic proteins bcl-2 and bcl-x_l.

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Chapter 6

p53 Mutations in human meningiomas
With an overview of literature

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Abstract

Overexpression of p53 has been reported to play a role in neoplasms of the central nervous system. Meningiomas are generally benign intracranial tumors derived from the meninges. Overexpression of the p53 protein in meningioma and a relation with histological type and recurrence has been reported. Mutation of the p53 gene leads to a more stable p53 protein in quantities high enough for detection by immunohistochemistry. In the search for these mutations the core domain of the p53 gene of meningioma has been analyzed thus far. Only a very low incidence of mutations was reported. The apparent discordance between overexpression of p53 protein and p53 gene mutations may be explained by mutations located outside the core domain. This issue was addressed in the present study. All 11 exons of p52 of 17 meningiomas were analyzed for alterations of DNA by PCR single-strand conformation polymorphism (PCR-SSCP) analysis with subsequent sequencing.

PCR-SSCP analysis showed a various number of band shifts and nucleotide alterations, caused either by alterations in the flanking introns or common polymorphism (codon 36 and 72). The allele frequencies of the polymorphisms found in this small population of tumors resemble that of frequencies found in literature. In addition, three nucleotide changes located in intron 2, 3 and 7 were found in 11, 3 and, 4 respectively out of 17 specimens. Based on this study and reports by others, we conclude that it is not very likely that p53 mutations are involved in the etiology of meningiomas.

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Introduction

The p53 tumor suppressor gene is located on the short arm of chromosome 17 and encodes a 53 kDa nuclear phosphoprotein involved in the regulation of proliferation, DNA repair, and induction of apoptosis [1,2]. Abnormalities of the p53 gene have been reported to occur frequently in a wide spectrum of human cancers [3]. The wild type p53 product suppresses transformation, whereas the mutated p53 protein may inactivate the wild type p53 function, allowing DNA replication on a damaged template and therefore resulting in cell transformation [4].

P53 has been reported to play a role in neoplasms of the central nervous system (CNS) [5]. Meningiomas, generally benign intracranial tumors derived from the meninges, account for at least 15% of all CNS tumors [6]. Overexpression of the p53 protein in a substantial number of meningiomas has been reported by several authors and there may be a relation between p53 expression and histological subtype and recurrence [7-11]. One of the main causes of p53 overexpression is a mutation in the p53 gene. In the search for these mutations in meningiomas, research has focused on the core domain of the p53 gene and only a small percentage of mutations was found [12-14]. This discordance may be explained by mutations located outside the core domain, of which is known that they also may contribute to p53 protein over-expression [15]. To ellucidate a role of p53 gene mutations in the etiology of meningiomas, knowledge about the mutation frequency of exons not included in the core domain is required.

In this study we used single-strand conformation polymorphism (SSCP) analysis of polymerase chain reaction (PCR) products, with subsequent sequencing, to analyze mutations in exon 1-11 of the p53 gene of 17 meningiomas of various histological types (see Table 6-1).

Material and Methods

Tissues

Seventeen patients with meningeal tumors diagnosed at the De Wever Hospital (Heerlen, The Netherlands) between 1997 and 2000 were included in our study. The study was conducted in accordance with the guidelines of the local ethical committee. The characteristics of the tissue are shown in Table 6-1.

DNA extraction and PCR analysis

Meningiomas were stored at –80°C until used for DNA isolation. DNA was isolated according to standard experimental procedures using a DNA isolation kit (Qiagen). The coding regions for p53 gene were amplified by Polymerase Chain Reaction (PCR). The sequences of the p53 primers, the predicted sizes of the

nr.	histological type	age[a]	sex	exon 4 codon 36	exon codon 72	exon 4 intron 3	exon 2-3 intron 2	exon 7 intron 7
1	fibroblastic	33	f		x		x	x
2	meningothelial	34	f		x		x	
3	maligne	38	m				x	x
4	syncytial	49	f		x		x	
5	not specified							
6	haemangio-pericytoom	25	m	x	x		x	
7	atypical	46	f		x	x	x	
8	meningothelial	74	f		x		x	
9	atypical	69	m					
10	syncytial	64	f					
11	fibroblastic	41	m		x			
12	meningothelial	51	f					
13	transitional	45	m		x		x	x
14	unknown	29	m			x	x	x
15	fibroblastic	44	m	x	x		x	
16	fibroblastic	48	f		x	x	x	
17	fibroblastic	70	f					

Table 6-1. Patients characteristics and PCR-SSCP data of 17 human meningiomas. [a] *age at operation.*

amplified products and restriction enzymes used are depicted in Table 6-2. PCR primers were adopted from Kropveld *et al.* [16]. The PCR reaction was performed in a 50-μl reaction mixture containing 100 ng of genomic DNA, 10 mM dNTP, 200 ng of each of the primers, 1.0-2.5 mM $MgCl_2$ (see Table 2), Mg^{2+}-free PCR buffer (10 mM Tris and 50 mM KCl, pH 8.3) and 1.25 U AmpliTaqGold (Roche Diagnostics). After a first step of denaturation (10 min, 95°C), 35 cycles of PCR amplification 30s at 94°C, 30s at annealing temperature as shown in table 1, 45 sec at 72°C, were carried out, followed by 10 min at 72°C final extension in a geneamp PCR system 2400 thermal cycler (Perkin Elmer), Amplified fragments were purified using a PCR purification spin kit (JETQuick) PCR products were digested with restriction enzymes according to standard experimental procedures using restriction enzymes and SuRE/Cut buffer (Boehringer Mannheim) depicted in Table 6-2.

Single Strand Conformational Polymorphism (SSCP) Analysis and Sequencing

Amplified and purified fragments were analyzed with SSCP using a GeneGel Excel 12.5/24 kit (Pharmacia Biotech) at 5, 15 and 30 °C. For most exons, excellent results were obtained at 15°C (600 V, 30W,

Exon	DNA-primer	(DNA) position	$MgCl_2$ (mM)/ T(°C)	Restr. enzyme (Buffer)	Fragments (bp)
Exon 1	5′ TTC TTC CTT CCA CCC TTC A	519-537	1.5/58	Cfo I (L)	300+342
	3′ GTC CTA ACA TCC CCA TCA TCT	1140-1160			
Exon 2-3	5′ GCT TGG GTT GTG GTG AAA CA	11506-11525	1.0/58	Hpa II (L)	200+252
	3′ TCC AGG TCC CCA GCC CAA C	11940-11957			
Exon 4	5′ GGG GCT GAG GAC CTG GT	11974-11990	1.5/58	Eco 57 (V)	114+264
	3′ ATA CGG CCA GGC ATT GAA GT	12332-12351			
Exon 5-6	5′ GCA GGA GGT GCT TAC ACA TG	12949-12968	1.0/56	Hpa II (L)	190+348
	3′ CAC TGA CAA CCA CCC TTA AC	13467-13486			
Exon 7	5′ CTC TTG GGC CTG TGT TA	13972-13991	2.5/55	nd	275
	3′ ATT CCC GCC GGA AAT GTG AT	14227-14246			
Exon 8-9	5′ GGG TGG TTG GGA GTA GAT	14355-14372	1.5/50	Cfo I (L)	163+298
	3′ CGG CAT TTT GAG TGT TAG A	14797-14815			
Exon 10	5′ TCC GTC ATA AAG TCA AAC AAT	17480-17500	2.0/51	Stu I (L)	138+250
	3′ CGT GGA GGC AAG AAT G	17852-17867			
Exon 11.1	5′ GAA AAG GGG CAC AGA CC	18533-18549	1.0/53	Hpa II (L)	289+247
	3′ TAT GGG CCT TGA AGT TAG AGA A	19047-19068			
Exon 11.4	5′ GTG AGG GAT GTT TGG GAG AT	18918-18937	2.0/57	Mva I (H)	103+333
	3′ GGG GTG AGA TTT CCT TTT AGG T	19332-19353			
Exon 11.3	5′ GCC AAA CCC TGT CTG ACA ACC T	19294-19315	1.0/55	Hae III (M)	316+318

Table 6-2. DNA primers and restriction enzymes used for SSCP based mutation analysis of the p53 gene. Primers were derived from Kropveld *et al.* with exeption of exon 11 [16] . *Abbreviation: nd, not digested*.

30 mA) and a running time of 2-5.5 hours. PCR products of each exon containing a single mutation in the 5' primer region were made and used as a positive control (see figure 6-1). Following SSCP analysis, suspect PCR products were used for direct sequencing. Double strand PCR products were sequenced by dideoxy-chain-termination method [17] using the Dye Primer Cycle Sequencing Ready Reaction Kit with Taq FS (ABI). The PCR primers were used as sequencing primers (3.2 pmol) for direct sequencing of the amplified exon in both orientations. After cycle sequencing, sequence products were separated on a 6% denaturating polyacrylamide gel using an automated DNA sequencer (373A, ABI). Mutations were manually identified by multi sequence analysis to validate the distinction between background and mutations, by comparing sequence profiles of many samples.

Results

The characteristics of the patients and histological type of the meningiomas and PCR-SSCP and sequence data are shown in Table 6-1. All exons of the p53 gene were amplified with PCR using primers located in flanking introns (Table 6-2). When the total PCR product length was more than 300 base pairs, the products were digested enzymatically to assure optimal mutation detection sensitivity

PCR-SSCP analysis

Of the seventeen meningiomas analyzed no band shifts were observed in SSCP analysis of exon 1 and 8 to 11. Although all positive controls of these exons did show a mobility shift.

Of PCR products of exon 1-11, exon 2 to 7 showed multiple band mobility changes. Of the 17 meningiomas examined 12 showed a band shift in SSCP analysis of the PCR product of a combination of exon 2 and 3 (Table 6-1). From 17 meningiomas 10 showed three different migration patterns of exon 4 PCR-SSCP products, possibly indicating different nucleotide

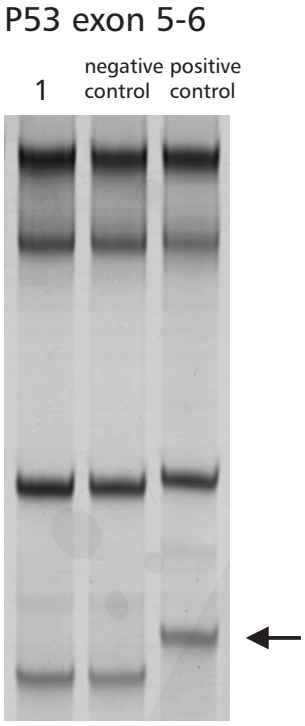

Figure 6-1. An example of PCR-SSCP analysis of exon 5-6 showing one negative, one positive control and one meningioma (1) sample. All PCR controls were checked by sequence analysis.

	codon	type	Freq. (A)	Freq. (a)	description	reference
Intron 2	38bp3'exon 2	point			G>C	(41)
Exon 4	36	point	94 CCG	6 CCA*	silent	(26,27,28)
	72	point	71 CGC	29CCC*	Arg>Pro	
Intron 3	65bp3'exon 3	point			C>A	(38)
Intron 7	72bp3'exon7	point			C>T	
	92bp3'exon7	point			T>G	

Table 6-3. P53 nucleotide alterations found in 17 meningioma specimen. **17 individuals*.

alterations. Four of the meningiomas showed combinations of band shifts of exon 4 (see #1-4 in Figure 6-1, left panel). In 3 out of 17 meningiomas, SSCP analysis revealed band shifts for the combination of exon 5 and 6 PCR products. In 4/17 meningiomas also mobility changes were found for the PCR products of exon 7. All suspected PCR-products, the wild type and the positive controls were directly analyzed by sequencing and compared with the p53 database sequences.

Direct DNA sequence

To characterize the nucleotide sequence alterations detected by mobility shifts in PCR-SSCP analysis, the PCR products were sequenced with the same primers as were used for PCR-SSCP analysis. Overall results are shown in Table 6-3. An example of sequence analysis of exon 4 is shown in Figur 6-2 (right panel). The mobility shifts of exon 4 PCR products appeared to be caused by two common polymorphisms and an additional mutation in intron 3 (Figure 6-2; specimen #2). The first polymorphism is a point mutation in codon 72, CGC to CCC resulting in an amino acid change of ARG to PRO. The allele frequencies found in 17 meningiomas were 0.71 for CGC and 0.29 for CCC. The second polymorphism in exon 4 was a point mutation codon 36, CCG to CCA and allele frequencies were 0.94 for CCG and 0.06 for CCA. This nucleotide

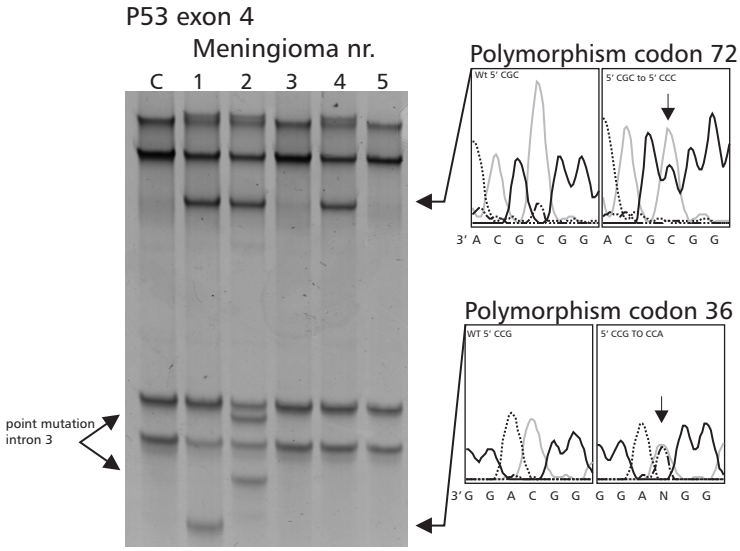

Figure 6-2. Example of PCR-SSCP analysis of exon 4 of the p53 gene in meningiomas is shown. Multiple band shifts were observed. Lane 1 resembles the wild type phenotype. The right panel shows sequence analysis of two polymorphisms at codon 36 and 72.

author	reference	year	p53 mutation	remark
Nagashima *et al.*	(22)	1999	0/7	High p53 expression
Janowska *et al.*	(42)	1998	0/3	Angiomatous meningiomas
Ohkoudo *et al.*	(9)	1998	0/4	Recurrent meningiomas, high p53 PI
Tan *et al.*	(43)	1997	1/13	From abstract, article in Chinese
Ono *et al.*	(44)	1996	0/27	
Chent *et al.*	(12)	1995	0/22 (benign) 1/5 (malignant)	
Wang *et al.*	(13)	1995	1/5	IHC p53 positive
Ellison *et al.*	(20)	1995	0/9	IHC p53 positive
Albrecht *et al.*	(45)	1994	0/1	Malignant
Ohgaki *et al.*	(14)	1993	0/22	Of which 7 atypical
Mashiyama *et al.*	(25)	1991	1/8	Exon 2-11

Table 6-4. Summary of published data on p53 mutations in human meningiomas. *Abbreviations: IHC immunohistochemistry; PI, proliferation index.*

alteration does not lead to an amino acid change. Besides two polymorphism of exon 4, three meningiomas also showed a point mutation in intron 3 (65bp 3'exon3). The mobility shift of exon 2-3 PCR products was explained by a point mutation, 38 bp of the 3' side of exon 2, G>C with allele frequencies of 0.3 and 0.7 respectively. No further mutations were found in the coding region. The mobility shift of exon 7 PCR products was explained by two point mutations in intron 7 (72 and 97 bp 3'exon7, respectively). The mobility shift of exon 5-6 PCR products could not be explained by mutations within the coding region.

Discussion

P53 gene alterations play a role in the pathogenesis of many different types of tumors. Wild type p53 induces cell cycle arrest, preventing DNA replication errors and allowing time for DNA repair. It has also been linked to programmed cell death or apoptosis of cell types containing critical levels of DNA damage [18]. Dysfunction of p53 caused either by mutation of the gene or by inactivation of the wild type protein, may therefore promote neogenesis by perpetuating errors in

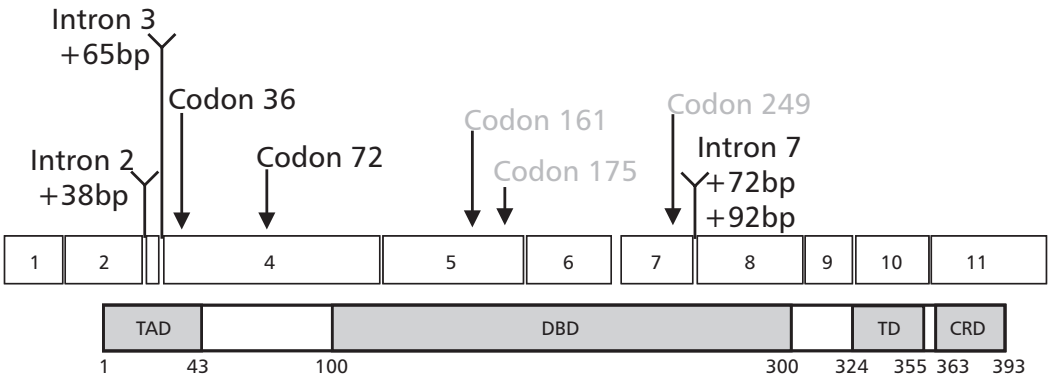

Figure 6-3. Overview of point mutations of the *p53* gene in human meningiomas derived from the present study (black arrows) and from literature (gray arrows)[12,13,25]. All p53 exons are depicted with the functional and structural domains of the p53 protein. Figure adapted from [40]. *Abbreviations: TAD, transactivation domain; DBD, DNA binding domain; TD, tetradimerization domain; CRD, c-terminus regulatory domain.*

DNA. [4,19]. In normal cells, wild type p53 is present only in minute quantities. Mutations in the p53 gene may lead to a more stable mutated p53 protein resulting in quantities high enough for detection by immunohistochemistry (IHC) [13,20].

Several authors have described p53 expression in several subtypes of meningioma with IHC [8,9,20,21]. Ohkouda *et al.* and other authors reported clinical significance for p53 expression in meningioma since an association was found between p53 immuno-reactivity and histological type or recurrence [9-11,22]. Wang *et al.* found p53 expression in 4 out of 5 atypical meningiomas, but in non of the 19 benign meningiomas investigated. Therefore they conclude that p53 could be considered as a marker for malignant transformation [13]. However, other authors could not confirm these observations [20,23].

Although overexpression of p53 protein was found in approximately 36% of the meningiomas, p53 gene alterations were reported in only 4/126 specimens (see Table 6-4). This suggests that p53 mutations are not related to positive immunohistochemistry results and, are not likely to play a major role in the etiology of meningiomas. All reports, however, focused on just half of the p53 exons, i.e. 5-9. This could have led to an underestimation of the actual p53 mutation frequencies in meningiomas. Kropveld *et al.* reported mutations located outside the hotspot region that revealed 33% of the mutations found in head and neck squamous cell carcinoma [15]. This indicates that all the P53 exons should be analyzed before a role of p53 mutations in meningioma could be excluded.

In the present study we have used PCR-SSCP analysis for detecting

mutations in all the coding regions of the human p53 gene in 17 meningiomas specimen. The effectiveness in detecting mutations with SSCP-PCR is reportedly high [24,25]. PCR products with a single mutation were used as positive controls. Several exons showed differential band migration by SSCP analysis and direct sequencing of the suspected PCR products revealed several nucleotide alterations. In exon 4 of the p53 gene two types of polymorphism were present. A polymorphism at codon 72 results in a translation to either Arginine or Proline. The allelic frequencies, 0.71p53Arg and 0.29p53Pro, found in the tissues of our meningioma patients corresponded with those described for other tissues [26-28]. This polymorphism has been reported to be associated with the susceptibility to several types of cancer. The Arginine phenotype may confer protection against development of human papillomavirus- associated vulval neoplasia as shown in a UK woman population [29]. Codon 72 polymorphism also seems to affect the risk of lung cancer related or unrelated to smoking [30-33]. Several other population studies done among patients with cervical cancer in UK and Taiwan, in smoking related non-small cell lung cancer patients, a Chinese population suffering from several types of cancer, however, revealed no association with codon 72 polymorphism [33-37]. Although no healthy subjects were included, the allele frequencies found in the present study, which are comparable to those in published control populations [26-28], do not point to a major role for codon 72 polymorphism in etiology of meningiomas.

The second polymorphism observed was a nucleotide change in codon 36 which does not lead to an amino acid change but eliminate a FinI restriction site and creates a BccI site. The allelic frequencies found in meningiomas also corresponded with those found for other populations [38]. To our knowledge, the allele frequencies of these two polymorphisms of exon 4 were not described previously for meningiomas.

No nucleotide alterations could be detected in eleven exons of the p53 gene that could reveal a role for p53 mutations in the etiology of meningiomas. Other possibilities, like mdm-2 expression, may contribute more to the p53 overexpression found in meningiomas. Mdm-2 is involved in the stabilization of p53 resulting in a possible accumulation of the wild type protein [39]. Mdm-2 expression has been reported in meningiomas with p53 expression [9].

In summary, then, in the present study we analyzed DNA of 17 meningiomas for possible mutations located in exon 1 to exon 11. Although SSCP analysis showed a various number of band shifts in several exons, only nucleotide alterations could be found that were caused by alterations in the flanking introns or caused by known polymorphisms. Based on this study and reports by others, the conclusion is that p53 mutations are not very likely to be involved in the development of meningiomas.

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Chapter 7

Alternatively spliced estrogen receptor beta mRNA expression in human meningiomas

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Abstract

The abundant expression of progesterone receptors (PR) in human meningiomas is well established. It is unknown, however, how PR expression is regulated, especially since the estrogen receptors (ER) are seldom found and only very low concentrations in these tumors. At the mRNA level, ER-α splice variants occurring in meningioma have been described, however, these variants appear not to be involved in the apparently autonomous PR expression. Wild type ER-β mRNA has already been demonstrated in meningiomas, but was not correlated with PR expression. However, ER-β splice variants could still play a role in PR regulation in meningiomas. In normal breast tissue, breast cancer and other tissues these ER-β variants have already been demonstrated. This study was conducted to reveal a possible role for ER-β in the regulation of PR expression in meningiomas. At mRNA level, the presence of wild type (wt) and splice variants of ER-β were analyzed with rt-PCR and southern blot analysis, the total PR protein expression levels were determined with a ligand binding assay. Meningiomas contained wt, Δ2 in 11 out of 14, Δ4 in 1 of 10, and Δ5 ER-β mRNA in 5 out of 8 tumors. However, a relationship with PR expression could not be found, both PR negative and PR positive meningiomas contained wt and splice variants of ER-β mRNA. Based on previous findings concerning ER-α, and this study, we conclude that ER is not likely involved in the apparently autonomous regulation of PR expression in meningiomas.

Submitted for publication

Introduction

The autonomous expression of progesterone receptors (PR) in human meningiomas has been established for more than two decades now. The parallel observation that, in spite of PR expression, meningiomas essentially lack estrogen receptors (ER) has presented a challenge for the same period of time. The ER and PR are members of the steroid/thyroid/retinoid receptor gene superfamily [1]. Meningiomas are regularly benign lesions and one of the most common tumors of the central nervous system. Standard therapy is surgical resection with subsequent radiation therapy when necessary [2,3]. Meningiomas have attracted attention as being possible hormone sensitive tumors due to their higher incidence in woman than in men [4]; the epidemiological association of meningiomas and breast cancer [5]; and the reversible aggravation of the symptoms during pregnancy and in the luteal phase of the menstrual cycle [6].

The estrogen independent expression of PR has led to the hypothesis that in meningiomas, ER forms may prevail which do not bind the ligand, and hence are not detected with a Ligand Binding Assay (LBA) and immunoassays employing antibodies that recognize the hormone binding domain of the ER. These ER-splice variants could be capable, however, of binding to an estrogen responsive element (ERE) and triggering PR expression. The ER-α splice variants have been demonstrated in meningiomas, however,

ER-α variant mRNA	tissue / cell line	ER-β variant mRNA	tissue / cell line
Δ2	HPA,, T47D, PRL, GPH, HOSE [9,15,16,26,27]	Δ 2	HPA [15]
Δ3	HPA, T47D, RP [9,26,28]	Δ 3	RO, RP [16]
Δ4	HPA, RB, M, RP, HOSE, VSMC, BT, MCF7 [8,15,16,26,27]	Δ 4	BGC [31]
Δ5	HPA, M, BT, PRL, GPH, HOSE [8,15,16,26,27]	Δ 5	MDA-MB-231, MCF7, BT, OT, NHB [13,14,32,33]
Δ6	BT [34]	Δ 6	OT, BT [14,32]
Δ7	HPA, M, T47D, HOSE [8,9,15,26,27]	Δ 7	
Δ3/4	RP, VSMC [28,30]	Δ 5/6	OT, BT [14,32]

Table 7-1. ER mRNA variants identified in tissues and cell lines. *Abbreviations: HPA, human pituitary adenoma; representative, rat pituitary; RB, rat brain; T47D/ MDA-MB-231/ MCF7, breast cancer cell line; HT, human testis; PRL, prolactinomas; GPH, gonadotroph tumors; BT, breast tumor; OT, ovarian tumor; M, meningiomas; MB, mouse breast; MO, mouse ovary; MU, mouse uterus; RO, rat ovary; BGC, bovine granulosa cells; NHB, normal human breast; HOSE, human ovarian surface epithelial cells; VSMC, vascular smooth muscle cells.*

they are most likely not responsible for the initiation of PR expression. ER-α/Δ4 was unable to bind ligand as well as an ERE and is not transcriptionally active [7]. ER-α/Δ5 is present in both PR positive and negative meningiomas [8]. ER-α/Δ7, finally, is a dominant negative repressor [9]. Ten years after the cloning of ER-α, a second ER-form was cloned known as ER-β, initially from rodent and later from human tissue [10-12]. Of the ER-β also several splice variants have been reported. Table 7-1 gives an overview of the expression of splice variants in several types of tissues. For these splice variants a biological significance has been reported by several authors [13-16]. In a significant proportion of meningiomas, wild type ER-ß mRNA has been demonstrated by Carroll *et al.* [17]. Although ER-α splice variants are not involved in PR regulation in meningiomas, splice variants of the novel ER-β have not yet been studied in meningiomas. This study was conducted to reveal the presence of ER-β splice variants and to compare the outcome with the PR phenotype of meningiomas. ER-β splice variants were determined with a sensitive PCR-Southern blot analysis and PR expression levels were measured with an LBA.

Materials and methods

Tissues

Human meningioma tissues were placed on ice immediately after removal from the patients. Specimens were frozen at –80°C until they were used for RNA extraction. For southern blot analysis, 17 human meningioma tissues were collected from 9 female and 8 male patients, the mean age at the time of surgery was 62 years (median: 67 years; range 25-75), operated at the University Medical Center Utrecht, The Netherlands. The study was conducted in accordance with the guidelines of the local ethical committee.

RNA Isolation

Total RNA was prepared from 100 mg of frozen meningioma tissue sections, a testis sample and a myoma, using Trizol (GibcoBRL) reagent according to the manufacturer's instructions and quantified spectrophotometrically. Its integrity was assessed by gel electrophoresis.

cDNA synthesis

Total RNA (1.5 μg) and 100 ng random hexamers were denatured at 70°C for 10 minutes. cDNA synthesis was carried out in 20 μl 50 mM Tris-HCl (pH 8.3) containing 75 mM KCl, 8 mM $MgCl_2$, 10 mM dNTP, 20 U RNAse inhibitor (Boehringer, Mannheim, Germany) and 200 U of M-MLV Reverse Transcriptase (Promega madison, WI, USA). After denaturation of the RNA the reverse transcription was performed in one cycle: 50 minutes of incubation at 42°C and 15 minutes enzyme inactivation at 70°C. Reactions without M-MLV RT were also carried out as a

negative control during cDNA synthesis, and also amplified with PCR

PCR amplification

In the PCR reactions, 5 µl of cDNA product was used. PCR amplification was carried out in 50 µl 15 mM Tris-HCl pH 8.3 containing 57.5 mM KCl, 1.8 mM $MgCl_2$, 250 ng primers, 0.5 mM dNTP and 1 U Ampli Taq Gold™. The nucleotide sequences of primers and probes used are presented in Table 7-2. Primers 1 to 10 were used during the 35 cycles of amplification. Each cycle consisted of a denaturation step at 94°C for 1 min, annealing step at 60°C for 1 min, and 2 minutes of extension at 72°C. To control for extraneous contamination of genomic DNA or cDNA, PCR mixture with no template was included.

Southern hybridization

PCR products (18 μl) were applied to a 2% agarose gel and electrophoresed for 1.5 hours at 100 V. After denaturation in 0.5 M NaOH for 30 minutes, the gel was neutralized in 0.5 M Tris-HCl (pH 7.2) containing 1.5 M NaCl and 0.001 M EDTA (pH 8.0) for 30 minutes. PCR products were transferred under vacuum to a Hybond™-N membrane (Amersham, Little Chalfont, UK). The membrane was prehybridized for 2 hours at 55°C in 5x SSPE, 5x Dennhardt's solution, 0.5 % SDS and 50 μg/ml of sheared salmon sperm DNA. The probes were end-labeled in 10 mM Tris-HCl pH 7.6 containing 10 mM $MgCl_2$, 5 mM DTT and 100 ng probe (Table 7-2) using T4 Polynucleotide kinase and [α-^{32}P]ATP. After purification of the ^{32}P end labeled probes using ProbeQuant™ G-50 Micro columns according to the manufacturer's instructions, hybridization was performed overnight at 50°C. After hybridization the membrane was washed in SSPE 2x, 0.1% SDS at 60°C and exposed to X-ray films. Meningioma samples not containing a wild-type ER-ß signal were not taken into account since this was most likely due to a PCR failure.

Exon	Primers	Nucleotide	Probe		Nucleotide
2-3	5' ggtccatcgccagttatcac 3'	368- 387	ex2 S	5' caggttcaaagagggatgctc 3'	517- 537
	5' gccacatcagccccatcatt 3'	779- 800	ex3 AS	5' accattcccacttcgtaacacttc 3'	719- 742
4	5' tgccaggcctgccgacttc 3'	699- 717	ex3 AS	5' caggttcaaagagggatgctc 3'	719- 742
	5' gccacatcagccccatcatt 3'	112-1134	ex2 S	5' accattcccacttcgtaacacttc 3'	996-1019
5	5' ggccaagagaagtggcggccacg 3'	830- 852	ex4 S	5' aagttggccgacaaggagttggta 3'	996-1019
	5' aaaccttgaagtagttgccaggagc 3'	1235-1259	ex5 AS	5' gccacatcagccccatcatt3'	1115-1134
6	5' accaagtgcggctctt 3'	1075-1090	ex5 AS	5' gccacatcagccccatcatt 3'	1115-1134
	5' ggtcacggcgttcagcaag 3'	1382-1400	ex6 S	5' gctcctggcaactacttcaaggtt 3'	1235-1258
7	5' gctcctggcaactacttcaaggtt 3'	1235-1258	ex6 AS	5' gatcatggccttgacacaga 3'	1291-1310
	5' gatcatggccttgacacaga 3'	1644-1667			

Table 7-2. Primer and probe sequences used for southern blot analysis of ER-β.

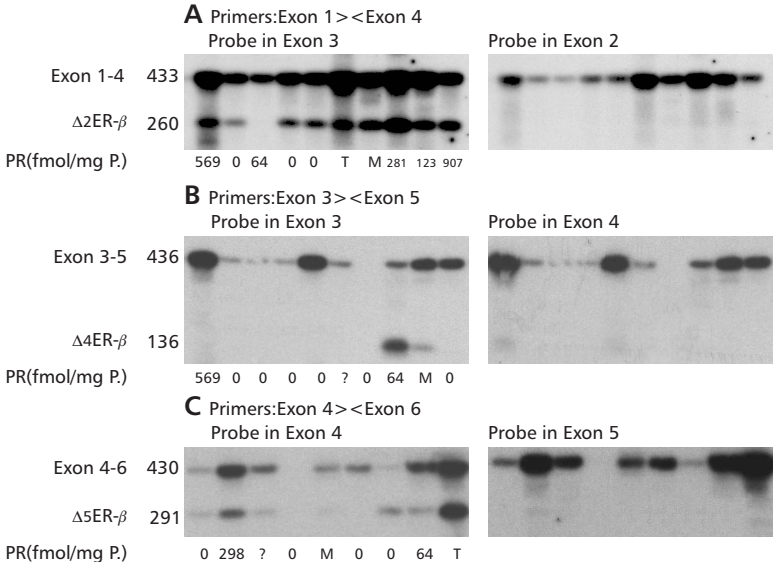

Figure 7-1. Example of southern blot hybridization of PCR products revealed ER-β deletion variants (see Table 7-2 for primers and probes). **A.** Hybridization was performed with a probe in exon 1 and 4 (left panel) and revealed two bands, the wild type product and an additional ER-β/Δ2 splice variant as confirmed by hybridization with a probe in exon 2 (right panel). **B.** Hybridization was performed with a probe in exon 3 and 5 (left panel) and revealed two bands in one meningioma and myoma control, the wild-type product and an additional ER-β/Δ4 splice-variant, as was confirmed by hybridization with a probe in exon 4 (right panel). **C.** Hybridization was performed with a probe in exon 4 and 6 (left panel) and revealed two bands, the wild type product and an additional ER-β/Δ5 splice variant was confirmed by hybridization with a probe in exon 5 (right panel).

Sequence analysis

PCR products were purified with A JETQuick PCR purification spin kit. The PCR primers were used as sequencing primers (3.2 pmol) for direct sequencing of the amplified exon in both orientations. After cycle sequencing, sequence products were separated on a 6% denaturating polyacrylamide gel using an automated DNA sequencer (373A, ABI).

Results

Southern blot analysis of ER-β

In order to reveal the presence of ER-β splice variants in meningiomas, we performed PCR and southern blot analysis of exons 2-7 of the ER-β gene in human meningiomas. Full length mRNA of the ER-β wild type was detected in all meningiomas as well as in myoma and testis controls. To detect ER-β splice variants, PCR was performed with exon flanking

primers and southernblot was performed with radio labeled probes in and outside the specific exons (see Table 7-2).

Besides the wild type ER-β mRNA, several additional bands with a different mobility were detected. To establish the exact character of the bands, southern blot analyses was performed with probes located in the specific exon. In those blots only wild type ER-β mRNA remained visible (Figure 7-1). In addition, the extra bands were isolated and analyzed by direct sequencing (an example is given in Figure 7-2). Direct sequencing proved that indeed these extra bands were Δ2, Δ4 and Δ5 splice variants of the ER-β gene.

ER-β/Δ2 mRNA was detected in 11 out of 14 meningiomas analyzed and no correlation could be found with ER or PR. ER-β/Δ4 mRNA could only be detected in the myoma control and in one out of 10 meningiomas. The steroid receptor phenotype of this meningioma was ER negative/PR positive. ER-β/Δ5 mRNA was found in 5 out of 8 meningiomas analyzed of which both PR positive and PR negative phenotypes were present.

An ER-β variant with a 54 nucleotide insertion at the exon 5-6 junction found in other tissues, could not be found in 13 meningiomas. The Δ6 and Δ7 splice variants were also not present in 11 and 14 meningiomas, respectively.

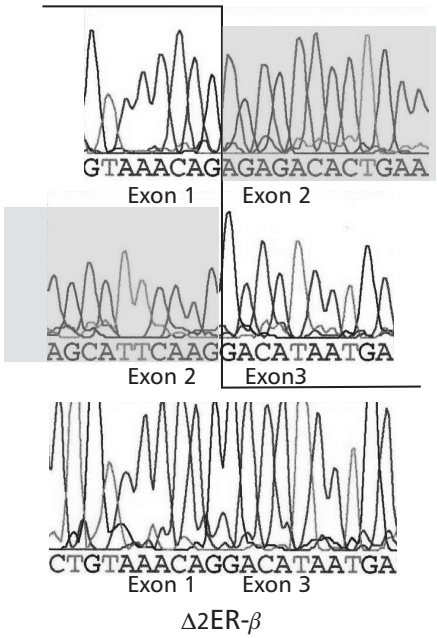

Figure 7-2. Sequence analysis of the wild type ER-β and ER-β/Δ2 amplified with primers in exon 1 and 4. Exon 1-2 transition and exon 2-3 transition are shown in the upper two panels, whereas the junction between exon 1 and exon 3 mRNA is demon- strated in the lower panel.

Discussion

Generally, meningiomas have a progesterone receptor (PR) positive and estrogen receptor (ER) negative phenotype based on a LBA [18]. Even when ER is present it is only expressed at low quantities. In most other hormone sensitive tissues, PR expression is under estrogen control, and hence PR and ER are positively

associated. Clinical studies showed that PR expression in meningiomas might have significance as prognostic marker for survival or recurrence [19-21]. How the PR expression is regulated in meningiomas is still unknown, and knowledge of this regulation could be helpful for understanding the biological role of PR in these tumors.

Although ER protein levels are not detectable with a LBA, mRNA expression of both ER-α and ER-β has been demonstrated by PCR and southernblot analysis [8,17], but no relationship could be found between ER mRNA, neither for α nor for β, and PR expression levels. Besides the wild type ER forms, ER splice-variants of both ER genes have been described in literature. On basis of detailed studies, the ER-α splice-variants were not likely involved in the regulation of PR expression in meningiomas [8]. ER-α splice-variants of the ER-α in meningiomas has not been studied, therefore this study was conducted to reveal whether splice-variants of the ER-β could be involved in PR regulation. The presence of ER-β splice variants in meningiomas was analyzed with PCR-southernblot analysis and the relationship between the presence of ER-β splice variants and PR expression levels was investigated.

In this study besides wild type ER-β mRNA, multiple forms of the ER-β splice variants were detected. The functional consequences of these ER-β forms in meningiomas are unknown. Full length ER-β is comprised of several functional domains important for DNA binding, hormone binding, and maximal transcription activation (see Figure 7-3). Deletion of a specific domain due to alternative mRNA splicing may give rise to a variant receptor with altered ER activity. ER variants may exert different biological effects; first by

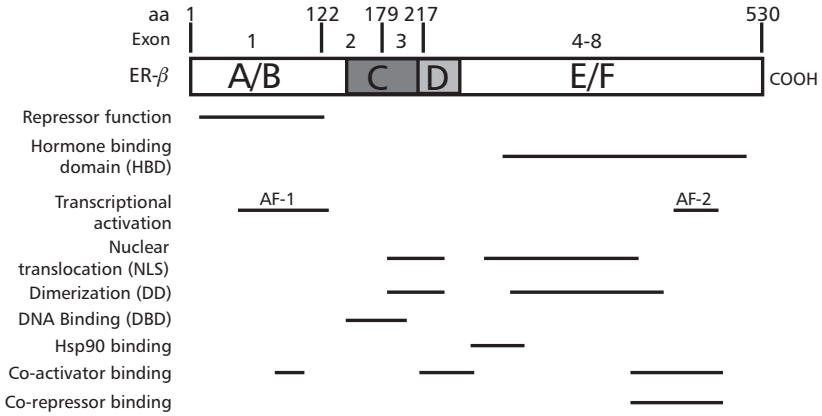

Figure 7-3. Schematic representation of the human ER-β protein. The common and functional domains of the ER-β are indicated. Above the A-F domains the corresponding exons of the ER-β gene are depicted (adapted from reference 25).

competing with the ability of wild type ER-β to bind with high affinity to estrogen; second by affecting the formation of stable ER homodimers after ligand binding; third by altering the transactivation of ER-β at estrogen responsive elements; and fourth by scavenging of co-activators or co-repressors. Therefore, alternatively spliced ER-β variants may give rise to a variety of receptor isoforms that may potentiate diverse actions of estrogen via ERs.

We found that the ER-$\beta/\Delta 2$ mRNA is frequently co-expressed with wild type ER-β in human meningiomas. Shupnik *et al.* observed ER-$\beta/\Delta 2$ in human pituitary tumors [15]. The protein encoded by ER-$\beta/\Delta 2$ mRNA will be severely truncated due to the introduction of a stop codon in exon 3 and misses the ligand binding domain. ER-$\alpha/\Delta 2$ has been shown to be highly effective in repressing Fos-mediated transcription in Hela cells [15,16]. The biological significance of ER-$\beta/\Delta 2$, however, is not known. In meningioma no relationship was found between the presence of this splice variant mRNA expression and the PR protein expression levels.

The ER-$\beta/\Delta 4$ mRNA could only be found in one meningioma which was positive for PR expression. The ER-α variant lacking exon 4 is known to be a silent variant not capable of binding estradiol or binding to an estrogen responsive element (ERE) [7]. Walther *et al.* found ER-$\beta/\Delta 4$ in several types of tissues, but the function of this splice variant could not be clarified [22]. The deletion of exon 4 would induce a frame shift, though exon 5-8 would not be affected. We consider it unlikely that ER-$\beta/\Delta 4$ is responsible for PR expression in meningiomas, since both PR negative and positive meningiomas were ER-$\beta/\Delta 4$ negative.

Jacobs *et al.* recently revealed that ER-$\beta/\Delta 5$ could not be responsible for PR synthesis in meningioma [8]. The ER-$\beta/\Delta 5$ is found in several meningiomas in this study. This splice variant should be expected to encode a c-terminally truncated ER-β protein that is capable of binding ligand. As the DNA binding domain and hinge region remain intact, this splice variant may also be capable of dimerisation and may have some transcriptional activity. Vladusic *et al.* reported co-expression of wild type ER-β and the $\Delta 5$ splice-variant in an estrogen unresponsive breast cancer cell line and in malignant breast tumors. Also, a hormone dependent breast cancer cell line expressed both wild type and ER-$\beta/\Delta 5$ [23]. Since ER-$\beta/\Delta 5$ was found in ER negative and PR negative meningiomas, it could not be responsible for PR expression in these tumors.

An ER-β variant with a 54 nucleotide insertion at the exon 5-6 junction has been described in mouse uteri, ovaries and mammary tissue by Lu *et al.* [14], and in normal rat ovary and pituitary tissue by Petersen *et al.* [24] In ER-β positive breast cancer, however, this variant could not be detected. This ER-β variant is able to ligand binding and ERE binding, but it requires 1000 fold higher concentrations of estradiol compared to the wild type ER-β. This variant is also able to suppress wild type ER-α and ER-β mediated transcription [24]. In our subset of meningiomas this insertion, however, could not be

detected. No ER-β mRNA missing exon 7 has been described up to now. Though the ER-α/Δ7 has been reported to be a dominant negative repressor of transcription activation [9]. The ER-β/Δ7 could not be found in meningiomas.

In conclusion, although several splice variants of the ER-β gene could be detected in human meningioma specimens, not one of the found splice variants is likely to be responsible for PR expression in meningiomas. A role for both ER-α/β wild type and splice variants based on mRNA data in meningiomas is not very likely.

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Chapter 8

Estrogen receptor-alpha expression in human meningiomas

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Abstract

The predominance of meningiomas in women, the association between meningiomas and breast cancer, and their accelerated growth during periods of high progesterone levels in the circulation, have led to a number of studies examining a possible role for the progesterone (PR) and estrogen receptors (ER) in these tumors. Data from previous studies based on reliable ligand binding assays (LBA) shows that meningiomas are rich in PR, whereas ER is seldom found and only in very low concentrations. Because of the discrepancy in literature about the presence of ER in meningiomas and the abundant expression of PR, which is considered to be an estrogen target gene, a sensitive immunoblot assay for further exploring ER protein expression in these tumors was developed.

Despite the initially assumed ER negative and PR positive phenotype of meningiomas, ER-α expression was detected in all 22 meningiomas investigated by immunoblotting. A relation, however, with the level of PR could not be established, since PR negative meningiomas also expressed ER-α. Therefore, it remains to be elucidated whether these minute quantities of ER-α have any transcriptional activity and hence biological significance. This is doubtful since other estrogen target genes, like pS2, appeared also to be downregulated in meningiomas.

In preparation

Introduction

The aberrant expression of progesterone receptors (PR) in human meningiomas has been established for more than two decades now. The observation that, in spite of PR expression, meningiomas essentially lack estrogen receptors (ER) has presented a challenge for the same period of time. Meningiomas are regularly benign lesions and one of the most common tumors of the central nervous system. Standard therapy is surgical resection with subsequent radiation therapy when necessary [1,2]. Meningiomas have attracted attention as being possible hormonally sensitive tumors due to their higher incidence in woman than in men [3]; the epidemiological association of meningiomas and breast cancer [4,5]; and the reversible aggravation of the symptoms during pregnancy and in the luteal phase of the menstrual cycle [6]. Following the first report in 1979 by Donnell *et al.* on the presence of ER in meningioma tissue, a large number of papers have been published dealing with this subject. Some authors reported the occurrence of both ER and PR, whereas others established an ER negative and PR positive phenotype.

The discrepancy could be explained by the use of different techniques with differential sensitivity, like single point ligand binding assay (LBA), multiple point LBA with scatchard plot analysis, enzyme immunoassay, or immunohistochemistry. An overview of LBA data of 400 meningiomas has been reported [7]. These results illustrate that, using the well established cut-off of 10 fmol/mg protein, ER in the majority of the meningiomas were below this level confirming the predominant ER-/PR+ phenotype of meningioma.

Our previous findings of PCR-southern blot analysis of ER-α and ER-α mRNA in meningioma tissue that were negative by the cut-off used for LBA [8], have led to the hypothesis that meningiomas may express ER in minute quantities that are not high enough for detection by a LBA. At least in theory, these low ER levels might be biological active and could be involved in the up-regulation of PR. To evaluate this hypothesis, a sensitive immunoblot assay for detecting ER-α in meningiomas was developed.

Material and methods

Tissues

Human meningioma tissues were placed on ice immediately after removal from the patients. Specimens were frozen at -80° C until they were used for cytosol preparation. For immunoblot analysis, 18 human meningiomas were collected from 11 female and 7 male patients, the mean age at the time of surgery was 68 years (median: 67 years; range 52-95) operated at the University Medical Center Utrecht. The study was conducted in accordance with the guidelines of the local ethical committee.

Cytosol preparation

The tissue (500 mg) was chilled in liquid nitrogen, pulverized with a microdismembrator (Braun, Melsungen, FRG) and suspended in 2 ml of 10 mM phosphate buffer containing, 1.5 mM EDTA, 3 mM sodium azide, 10 mM 1-monothioglycerol and 10% (v/v) glycerol, at pH 7.5. The resulting homogenate was centrifuged at 0-4°C for 30 min at 100,000 × *g* to yield a clear cytosol. Total ER and PR were measured by LBA immediately after cytosol preparation, as previously described [7] and aliquots for protein electrophoresis were stored at –80°C.

Protein electrophoresis and immunoblot analysis

Meningioma cytosol samples (2 to 3 times concentrated as compared to control cytosol from breast cancer and myometrium) were used for immunoblot analysis under denaturating conditions. Total proteins were separated by electrophoresis through a 7.5% polyacrylamide (30% acrylamide/bis solution, 37.5:1) resolving gel and a 2.6% stacking gel both containing 0.10% sodium dodecyl sulphate (SDS), using a Mini Protean II apparatus (Bio-Rad, Richmond, CA). Maximal amounts of cytosol (62.5 μl) were diluted with 12.5 μl Sample buffer. Samples were heated for 8 min at 99°C and then loaded onto the gel. After electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore, Bedford, GB) at 125 V for 2 h at 4 °C in 25 mM tris, 192 mM glycine, 20% (v/v) methanol containing 0.01 % sodium docedyl sulphate (SDS), as described by Towbin *et al.* [9]. After transfer, the membrane was blocked with 1.5 mM phosphate buffered saline (PBS, pH 7.4) containing 5% low fat milk powder (Protifar; N.V. Nutricia) and incubated with a specific antibody (AB) against human ER-α (ER Ab-15 (clone AER611)), mouse monoclonal at a 1:1000 dilution [10].

The molecular weight of each ER form was established with a molecular weight standard (BioRad). As control samples ER-α human recombinant (Panvera) and positive control lysate for ER-α Ab-10 (Neomarkers) were used. After incubation with the ER antibodies, blots were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody at a final dilution of 1:2500 (Dako A/S). Dilutions of cytosol containing less than 0.5 fmol/mg protein (based on LBA) could easily be detected with this immunoblot assay. Specific bands were visualized by using enhanced chemiluminescence detection substrate (Westpico ECL, Amersham) and blots were exposed to Kodak film (Biomax-ML, Kodak). Quantification of these immunoblots is not appropriate and result were only used for establishing the presence of ER-α.

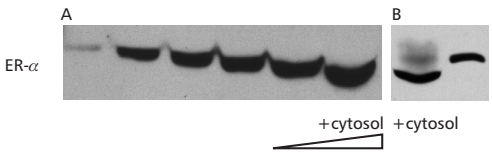

Figure 8-1. Immunoblot analysis of ER-α positive cytosol sample. A) Increasing amounts of cytosol protein resulted in a shift of the ER-α immunoblot signal. B) Addition of albumin to a positive ER-α construct sample resulted in a band shift of the signal.

Results

The concentrated cytosols of meningiomas as used, contained large amounts of albumin that interfered with the migration pattern of the ER protein band. Figure 8-1 shows that increasing amounts of cytosol resulted in a lower migration of the ER-α band. By adding meningioma cytosol to an ER-α positive control the effect of albumin on the migration of ER-α was demonstrated. Although the ER-α band of the positive construct and the ER bands found in the meningioma cytosol samples migrated differently, they are actually of the same size. The use of ER positive breast cancer cytosols, an ER positive cell lysate, and an ER-α construct, showed the specificity of the ER antibodies used. The size of the ER-α was between 60-65 kDa. In addition, experiments (data not shown) were performed to determine that cross-reactivity between both ER forms did not take place. Figure 8-2 shows a representative immunoblot of ER-α expression in concentrated meningioma cytosol samples and unconcentrated breast cancer cytosols. The results are summarized in Table 8-1. The LBA revealed that 13/22 meningiomas showed no ligand binding, indicating that ER protein levels were below 10 fmol/mg protein. Immunoblot analysis, however, revealed that all meningioma cytosol samples showed ER-α expression in variable amounts. Groups of meningiomas with low and high expression of PR expressed ER-α.

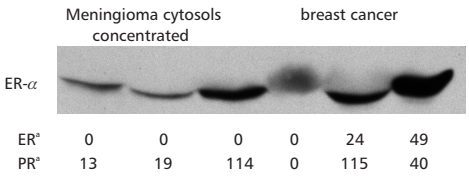

Figure 8-2. Immunoblot analysis of ER-α expression in human meningioma and breast cancer cytosol samples. ER-α expression was found in both meningiomas and breast cancer cytosols. [a] *fmol/mg cytosol protein – Band intensities can not be compared since different amounts of cytosol protein were loaded onto the gel.*

Meningioma	LBA		Imblot
nr.	PR[a]	ER[a]	ER-α
1	382	<10	+
2	19	<10	+
3	15	<10	+
4	107	<10	+
5	137	<10	+
6	114	<10	+
7	74	50	+
8	105	39	+
9	<10	<10	+
10	123	<10	+
11	97	<10	+
12	13	<10	+
13	236	<10	+
14	34	23	+
15	250	11	+
16	340	<10	+
17	215	35	+
18	48	<10	+
19	<10	<10	+
20	<10	<10	+
21	<10	<10	+
22	<10	<10	+

Table 8-1. Estrogen receptor (ER) expression in human meningiomas by ligand binding assay (LBA) and immunoblotting (Imblot).

Discussion

Meningiomas are generally benign lesions of the meninges and show a high recurrence rate of more than 20% [1,3]. For unresectable and recurrent meningiomas adjuvant endocrine therapy could be very helpful. The progesterone receptor has been considered to be a potential target for endocrine therapy using antiprogestins [11]. The PR has also been described as a prognostic factor for recurrence and survival rate for meningioma patients [12-14]. In most other hormone sensitive tissues, PR expression is under estrogen control and the PR is associated with the ER [15]. To unravel the regulation and function of the PR in meningiomas a lot of research has been performed in the last three decades to investigate the presence of ER in these tumors.

There are numerous discrepancies in the literature among the results for ER expression in human meningiomas. Most can be explained by the use of different techniques. Studies done with 16α[^{18}F]-fluoro-17-β-oestradiol ([^{18}F]FES) using positron emission tomography (PET) demonstrated a marked uptake of [^{18}F]FES by the tumor in four of the six patients [16]. In addition, addition of 17-β-estradiol to meningioma cell cultures showed that 2 out of 4 cultures responded to this steroid [17]. Kumar et al. used estrogen bound to fluorescent-iso-thiocyanate (FITC) to demonstrate the presence of ER in 33% of 46 meningiomas, although receptor concentrations in these tumors were not known [18]. In contrast to these findings are the findings of Bozzetti *et al.*, who investigated the occurence of ER in patients with primary untreated meningiomas with a dextran coated charcoal (DCC) method and the results were compared with those of an immunocytochemical assay (ICA). Low concentrations of ER were found in 8 out of 44 samples assayed by DCC, specific staining was never observed in any of the samples tested by ICA [19]. In agreement with this observation, our own data show ER expression could not be found in the vast majority of the meningiomas, based on our own LBA data [20,21].

The specificity of the antibodies used in the immunoblot analysis of the current study were confirmed by the estimated molecular weight of the ER-α band and by positive controls of ER-α positive cell lysate and an ER-α construct. The migration of the ER protein bands was dependent on the amount of albumin in the cytosol samples. Cross reactivity of the ER-α antibody with ER-β was excluded. Thus, a sensitive immunoblot assay was developed for the detection of ER-α protein levels, which could be helpful for investigation of the ratio of ER isoform expression and their role in tumor development.

ER-α mRNA levels were found in most of the meningiomas based on PCR-southernblot analysis, indicating that ER mRNA was present in meningioma cells. Due to amplification by the PCR method, the exact concentration of ER mRNA could not be determined. Our novel hypothesis was that low concentrations of

ER mRNA are present in meningiomas and that these transcripts are translated in to minute ER protein levels. This is in contrast with our initial idea that meningiomas contained appreciable amounts of ER mRNA and that the absence of ER protein levels indicated a post-translation inhibition of ER mRNA.

This study showed that, at least by immunoblotting, ER-α was expressed in meningiomas in low quantities. When presence of a receptor and not its biological activity is the criteria for ER positivity, meningiomas should be considered to have an ER positive and PR positive phenotype. The regulation of PR expression in meningiomas is still not clarified by the presence of the minute ER protein expression levels and remains unknown.

The biological function of these low levels of ER expression in meningiomas is not known. PR expression is considered a marker of ER activity in other hormonally regulated tissues. The correlation between ER and PR expression is tissue specific and in myometrium cytosol samples we found a positive correlation of r_s = 0.80 [22]. Most of the meningiomas in this study expressed both ER and PR. However, ER expression was also found in meningiomas with no or low PR expression. Expression of other ER regulated proteins in meningiomas seems also to be affected. Koehorst et al., have compared expression of the estrogen–modulated proteins Cathepsin-D and pS2 in breast cancer to that of meningiomas [23,24]. By contrast, however, pS2 was not expressed at all in meningiomas and Cathepsin-D protein was found at only half the concentration seen in breast cancer [24]. According to international guidelines for cytosol based assays, ER>10 fmol/mg cytosol protein usually indicates ER activity; ER>100 fmol/mg cytosol protein is considered strongly positive. The expression levels found in meningiomas were far below 10 fmol/mg cytosol protein. This indicates that involvement of ER-related pathways in gene expression in meningiomas is not likely.

In contrast, as long as the agonist-occupied ER has the ability to bind an ERE (or, alternatively, bind to AP-1 or Sp-1 transcription factors), there is no reason why the target gene would not be up-regulated, providing the appropriate coactivators and RNA Polymerase II transcriptional components are available in the nucleus. Although meningiomas should from now on be considered as ER and PR positive tumors, the biological activity of ER in these tumors remains unknown.

In conclusion, a specific and sensitive immunoblot assay was developed, which revealed that, in contrast to the previously existing thoughts, meningiomas are ER-α positive. The minute ER expression levels, however, could not clarify the expression of PR, a marker for recurrence and survival of meningioma patients and a possible target for endocrine adjuvant therapy, since ER-α expression was also found in PR negative meningiomas.

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Chapter 9

The IGF axis in meningiomas

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Introduction

Several decades of research have demonstrated that there is an association between the insulin growth factors (IGFs) and tumor growth. IGFs and their receptors are expressed in human tumors of the central nervous system (CNS) such as meningiomas. Meningiomas are benign neoplasias derived from the meninges. Treatment of these tumors consists out of surgical resection, however, these tumors have a high recurrence rate (20%) and are sometimes unresectable, indicating that there is a need for adjuvant therapies. As meningiomas are relative insensitive to chemotherapy adjuvant therapies have to be searched in hormonal treatments. Large clinical trials using progesterone receptor (PR) antagonist are underway to address this anti-tumor endocrine approach. Quite recently, tumor growth remission was reported by using a growth hormone (GH) receptor antagonist, indicating the possibilities of endocrine therapy in the treatment of tumors. Not much is known about the functional significance of the GH-IGF axis in meningiomas, which could be a target for endocrine therapy. In this manuscript, we review the expression of the GH-IGF axis in meningiomas and the possibility to use the GH-IGF-axis in endocrine therapy.

The IGF-axis components

The IGFs, IGF-I and IGF-II, are potent mitogenic and differentiation-promoting growth factors. They can inhibit apoptosis and also induce differentiation [1]. the IGFs are produced in multiple tissues and can act both in an endocrine and autocrine/paracrine fashion. They both exert their intracellular effects predominantly via the type 1 IGF-R. The type 2 IGF-R is believed to be involved in clearing of the IGFs from the circulation [2]. In the circulation, IGFs are bound to members of high affinity IGF binding proteins (IGFBPs). The six known IGFBPs modulate IGF activity by providing tissue and cell-type specific localization of the IGFs and by directly modulating the interaction of the IGFs with their receptors. They mainly act as autocrine / paracrine factors at or close to their sites of synthesis and may either inhibit or potentiate IGF activity. Furthermore, they differ in their IGF-binding capacity [1].

IGF-I

IGF-I is mainly produced in the liver, where its transcription in controlled by GH. IGF-I mediates many of the growth promoting effects of GH and has a long term impact on cell cycle progression, which is regulated by the type 1 IGF-R [1]. IGF-I stimulates the expression of cyclin D1, which accelerates progression of the cell from G_1 to S phase [3,4], resulting in increased DNA synthesis. A cellular action of the IGF-I complementary to its stimulation of cell proliferation is its capacity to inhibit cell death in certain cell contexts [5]. IGF-I is able to stimulate the expression of Bcl-2 proteins (anti-apoptotic) and to suppress the expression of bax (pro-apoptotic). This results in an increase in the relative amount of bcl-2/bax heterodimer, thereby blocking initiation of the apoptotic pathway [5-7]. IGF-I is thought to be primarily involved in postnatal growth and less inprenatal development. The expression of the *igf-1* gene is developmentally regulated, with levels of IGF-I mRNA in most tissues increasing 10- to 100-fold between birth and adulthood [8] and then serum IGF-I gradually declines [9].

IGF-II

In contrast to IGF-I, IGF-II synthesis appears to be relatively independent of GH [10]. IGF-II mRNA levels in all tissues are high during late fetal and perinatal periods and decline thereafter [11]. After birth, human IGF-II levels remain constant throughout adulthood. IGF-II is suggested to play an essential role in embryonic and fetal growth [12,13], whereas IGF-I is suggested to be mainly involved in postnatal growth. The igf-2 gene contains four separate promoter sites [14] and differential promoter usage regulates the tissue and developmental stage expression of IGF-II [15]. The use of specific promoters has been reported to be upregulated in various types of tumors [15-21]. Besides regulation by different promoters, the *igf2* gene has also parental allele specific expression. In normal cells, IGF-II is maternally imprinted in that it is expressed only from the paternal copy of the gene [22]. Loss of imprinting results in biallelic expression of IGF-II leading to overexpression of this growth factor. Loss of IGF-II imprinting has been reported in a variety of tumors [23-26]. The

above described data indicate that disturbed *igf* gene regulation may result in tumor growth.

The IGF receptors

Type 1 IGF-R protein is a tetramer of two identical α-subunits and two identical β-subunits [27,28]. The actions, mitogenic and anti-apoptotic, of both IGF-I and IGF-II are mediated through the type 1 IGF-R [27,29]. However, IGF-I has a 2-15 fold higher binding affinity than IGF-II [30,31]. IGF-I downregulates its own receptor resulting in a negative feedback mechanism [28,32-34]. Highest levels of type 1 IGF-R mRNA occur during fetal development and in the early postnatal period. Although type 1 IGF-R expression is significantly downregulated in the adult, it is present in most types of tissue [10].

In contrast to type 1 IGF-R, type 2 IGF-R is a single peptide chain and has no tyrosine kinase activity [35]. The type 2 IGF-R is also known as the mannose-6-phosphate receptor. The binding affinity of IGF-II for the type 2 IGF-R is 500 fold higher than that of IGF-I. Binding of IGF-II to the type 2 IGF-R results in degradation of IGF-II and thereby antagonizing its biologic activity [2]. Besides the scavenger function, it is less clear whether any of the biological actions of IGF-II are mediated by this receptor. There is some evidence that the type 2 IGF-R mediates migration stimulation of cells [36]. This capacity to induce motility response in cells has important implications in the invasive and metastatic potential in tumor cells.

IGFBPs

IGFBPs are able to inhibit or enhance the action of IGFs, resulting in either suppression or stimulation of cell proliferation [37,38]. When binding to IGFs, IGFBPs play three major roles: 1) transporting IGFs; 2) protecting IGFs from degradation; and 3) regulating the interaction between IGFs and type 1 IGF-R. Normally, IGFBPs have higher binding affinity for the IGFs than does the type 1 IGF-R, thereby blocking the interaction of the IGFs with the type 1 IGF-R. However, binding of the IGFs by the IGFBPs also protects IGFs from proteolytic degradation, which enhance the action of IGFs by increasing their bio-availability at the local level. The actual impact of IGFBPs on IGFs depends largely on posttranslational modification of IGFBPs by phosphorylation and proteolysis [1,37,38]. Besides IGF dependent functions, IGFBPs also have IGF independent effects. For example, IGFBP-3 has inhibitory effects that are independent of IGF binding, which is probably mediated by cell surface receptors [1].

The IGF-axis in meningiomas

IGF-receptors

First evidence for the presence of IGF binding sites in meningiomas came from Glick *et al.* [39]. They reported a high affinity ^{125}I-IGF-I binding in all 5 meningiomas as measured by competition binding assay. Similar results were obtained by Kurihara *et al.* [40], after Scatchard plot analysis these

authors suggested the presence of a single high class affinity binding site for IGF-I in all eight examined meningiomas. IGF-I binding sites were localized homogeneously in tissue sections derived from fibrous and meningothelial types of meningiomas [40]. Tsutsumi *et al.* [41] also localized ^{125}I-IGF-I binding sites in tissue sections of meningiomas. Binding sites were localized in all eight (2 meningotheliomatous, 4 transitional, 1 angioblastic and 1 malignant) meningiomas. In situ hybridization of 3 meningiomas (2 transitional and 1 syncytial) showed strong type IGF-R expression in all 3 meningiomas [42]. Lichtor *et al.* explored the expression of type 1 IGF-R in 12 meningotheliomatous meningiomas by immunohistochemistry. Four of the 12 tumors showed type 1 IGF-R immunoreactivity. However, none of the type 1 IGF-R positive tumors showed IGF-I immunoreactivity [43]. The staining pattern was very regional and only tumor cells showed immunofluorescence. No immunoreactivity was found in tumor vessels, fibrous components or tumor capsule [43]. In angioblastic types of meningiomas, relatively large numbers of IGF-I binding sites were located in the intratumoral vasculature and the number of binding sites in the stromal component was lower [40]. ^{125}I-IGF-I binding was found in tissue with cell rich components rather than in fibrous components. However, identification of precise cell types expressing these binding sites in meningioma tissue was not feasible with the used quantitative autoradiographic system, because of limitations in resolution of the technique [41]. There are conflicting results whether IGF-I binding sites are also present in normal brain tissue. Three out of four studies could not find expression of the type 1 IGF-R in control tissue [40-43].

Much less is known about the expression of the type 2 IGF-R in meningiomas. Strong type 2 IGF-R mRNA levels were found in meningiomas using *in situ* hybridisation (ISH) in one study and no significant levels could be found in the control pachymeninges [42].

In conclusion, it appears that meningiomas express elevated levels of the type 1 IGF-R predominantly located in the tumor cells of various histological subtypes. Whether there are protein levels of type 2 IGF-R present in meningiomas is not clear, however, mRNA levels of this receptor have been demonstrated. The high expression of the type 1 IGF-R, mediating mitogenic and apoptotic signals, might have biological significance in the development of meningiomas.

IGF-I

IGF-I was shown to stimulate DNA synthesis in a dose-dependent manner of cultured meningothelial cells, cultured angioblastic meningioma [41], primary meningioma subcultures, as shown by 3H-thymidine incorporation and cell counting [40]. It was shown that suramin, antagonist of IGF action via type 1 IGF-R, inhibits ^{125}I-IGF-I binding to meningiomas in a dose dependent manner, indicating that the mitogenic action of IGF-I was mediated via the type 1 IGF-R [41]. Quantitative receptor autoradiography showed that addition of suramin dissociated ^{125}I-IGF-I previously bound to meningioma tissue as

a function of time. Suramin potently inhibited or even blocked the IGF-I induced cell proliferation Presence of IGF-I in meningiomas was shown by immunohistochemistry. Only tumor cells were fairly intense and uniform stained. No staining was found in tumor vessels, fibrous components or tumor capsule. As mentioned before, none of these IGF-I positive tumors was type 1 IGF-R positive [43]. There was no significant IGF-I staining in control pachymeninges.

ISH studies showed the presence of IGF-I mRNA in the tumor cells of meningiomas, indicating local production of IGF-I by meningiomas. IGF-I mRNA expression in non-malignant control pachymeninges was significant lower [42]. However, Detta *et al*. concluded that IGF-I expression was lower as compared to other proto-oncogene expression measured in the same study [44]. In contrast, Glick *et al.* failed to detect IGF-I transcripts in 5 human meningiomas of the same pathological grade using Northern blot analysis [45]. This difference might be due to a difference in sensitivity of the techniques used. Autocrine secretion of IGF-I *in situ*, in tumor cyst fluid, was studied using a specific radioimmunoassay [46]. This assay could measure IGF-I and IGF-II separately, after removal of IGFBPs, measurable levels of IGF-I were detected in the cyst of both meningiomas tested [46]. Autocrine secretion of IGFs by meningiomas *in vitro* was also studied using medium of primary meningioma tumor cultures [47].

In summary, IGF-I mRNA is demonstrated in meningioma cells indicating that these tumors are capable of expressing IGF-I. Furthermore, it appears that IGF-I is secreted into the intratumoral fluid and exerts an auto and/or paracrine activity.

IGF-II

Expression of IGF-II meningioma cells was also shown using ISH [42-44]and northern blot analysis [44,45]. However, not all analyzed meningiomas showed IGF-II expression, and Detta *et al.* concluded that IGF-II expression was lower than other proto-oncogene levels measured in the same study [45]. Several studies have been performed to study IGF-II protein levels in human meningiomas. Immunohistochemistry using immunofluorescence showed IGF-II protein in 8 out of 12 meningiomas analyzed. Another study showed strong staining of IGF-II protein in 2 transitional and 1 syncytial meningiomas [42].

IGF-II expression appeared elevated in meningiomas since there was no significant IGF-II mRNA expression in the control pachymeninges [42]. In addition, no immunoreactive staining was found in normal brain tissue [42,43]. IGF-II expression appeared to be restricted to solely tumor cells. No immunoreactivity was found in tumor vessels, fibrous components or tumor capsules, and endothelial cells lining the blood vessels in the meningioma also were negative for IGF-II.

Glick *et al.* investigated the capability of meningiomas to produce and excrete

IGF-II *in vitro*. All studies were performed either with cells in primary culture or after a single passage, IGF-II was excreted by 6 of 11 meningiomas [47].

In conclusion, meningiomas express elevated levels of IGF-II mRNA and IGF-II protein solely in tumor cells. Since secretion of IGF-II by meningioma cells has been demonstrated *in vitro*, IGF-II might exert an autocrine or paracrine function in the development of meningiomas.

IGFBPs

Although IGFBPs have been reported to play a role in oncogenesis, not much research has been performed on IGFBP expression in meningiomas. Unterman *et al.* [48] demonstrated and characterized IGFBPs in meningioma tissue, primary cultures or after a single passage. Binding activity of IGF-I was detected in conditioned medium from 6 out of 7 meningiomas, even very high levels of binding activity was present in conditioned medium of four meningiomas. The IGFBPs from the media from these meningiomas were further characterized by different techniques. Western ligand blotting using ^{25}I-labled IGF probes revealed the presence of different IGFBPs. Immunoblotting and immunoprecipitatio n revealed the presence of IGFBP-1, 3 and most likely -4. In addition, immunohistochemical studies were performed with several specific mouse monoclonal antibodies against IGFBP-1. Of seven meningiomas that were examined, three stained intensely with each of the monoclonal antibodies against IGFBP-1 tested, while three were weakly positive. Positive staining was primarily present within cells and in the extracellular matrix, although little immunoreactivity was detected in fibrous stroma present in some of the meningiomas [48].

Nordqvist *et al.* [49] studied the relation between IGFBP-2 and IGF-II. In 68 meningiomas IGF-II and IGFBP-2 expression was determined by northern analysis. Variable levels of expression of both IGF-II and IGFBP-2 mRNA levels were observed. In many tumors an inverse correlation between the levels of IGF-II and IGFBP-2 mRNA existed so that high levels IGF-II mRNA were accompanied by very low levels of IGFBP-2 mRNA or vice versa. Screening for a high ratio IGF-II to IGFBP-2 could identify all (8 out of 68) anaplasia and/or atypical meningiomas. The remaining cases were meningiomas with a lower malignancy grade. Fourteen of these tumors showed high IGF-II and high IGFBP-2 mRNA levels without any sign of anaplasia. The 8 meningiomas showing high IGF-II/IGFBP-2 mRNA ratios and 8 with low IGF-II-IGFBP-2 mRNA ratios were subjected to ISH. In particularly, the IGF-II mRNA distribution varied considerably from sample to sample. Some tumors showed quite uniform labeling all through the tissue, whereas others had distinct foci with strong labeling surrounded by areas with no or very low levels of expression. In contrast the pattern of IGFBP-2 expression was more uniform. In tumors with regions that labeled intensely with the IGF-II probe, IGFBP-2 labeling was non-overlapping, with most abundant labeling surrounding the IGF-II foci [49].

Growth hormone receptors (GH-Rs)

Little is known about GH-R and GH in meningiomas. The presence of GH-R mRNA was shown in 14 meningiomas investigated. Blockade of the GH-R with a GH-R antagonist (B2036) reduced serum-induced DNA synthesis in 20% of the meningioma cultures. In addition, increased thymidine incorporation was found in a dose dependent manner after IGF-I stimulation [50].

In follow up experiments, 15 meningioma cultures were xenografted into athymic mice to further explore the modulation of the GH/IGF-I axis. Again they observed significant growth retardation of the tumor in mice treated with a GH-antagonist. Since intratumoral IGF levels were not altered, the authors suggest a direct blockade of the GH-R in the tumors or decreased IGF levels in the circulation causing the tumor size reduction [51]. Since the characteristics of mening iomas grown in culture very rapidly alter, we still have our reservation about these findings.

Apoptosis and the IGF axis

Meningiomas show a relative low apoptotic index in combination with a low proliferation rate, resulting in a low growth rate of these tumors. Meningiomas express relative high levels of the pro-apoptotic proteins bax and bak compared to breast cancer. The anti-apoptotic proteins from the same Bcl-2 proto-oncogene family, bcl-2 and bcl-x_l, are variably expressed in meningiomas. A relation between members of the Bcl-2 family and the type 1 IGF-R has been described for several cell types. IGF-I appeared to be a strong inhibitor of apoptosis induction. The fact that meningiomas express elevated levels of the type 1 IGF-R and show low levels of apoptosis have led to the hypothesis that the type 1 IGF-R might be involved in the regulation of apoptosis in meningiomas.

Singleton *et al.* described a relation between activation of the type 1 IGF-R and apoptotic proteins in neurons. Short-term activation of the type 1 IGF-R by both IGF-I and IGF-II blocked hyperosmotic-coupled apoptosis and inhibited apoptosis-associated changes in bcl-2 cellular concentration [52]. Later these findings were confirmed by several others. Wang *et al*. defined the effect of IGF-I on bcl-2, bax, caspase 3 expression in H9C2 cells. IGF-I partially inhibited bax induction, caspase 3 activation and DNA fragmentation and enhanced cell survival. IGF-I stimulation also resulted in decreased induction of bcl-2 [53]. Baker *et al.* performed studies done with two transgenic mice models overexpressing either bcl-2 or IGF-I proteins in olfactory bulb or cerebellar neurons, respectively. These studies indicated that in the developing brain, IGF-I modulates expression of IGFBP-2 as well as the bcl-2 protein [54]. Leri *et al.* reported a markedly down regulation of bax by IGF-I via the induction of mdm2 and the formation of mdm2/p53 complexes in isolated rat myocytes. The complex formation resulted in a downregulation of p53 function [55]. Not only bcl-2 and bax appeared to be affected by the type 1 IGF-R but also other members of the Bcl-2 protein family, like bcl-x_l and bad. Parrizas *et al*. reported a relation between IGF-I and

bcl-x_l in differentiated PC12 cells. Incubation with IGF-I resulted in a significant increase of bcl-x mRNA after 3-6 h incubation and a doubling of bcl-x_l protein levels by 24h incubation [56].

The main signalling pathway for type 1 IGF-R mediated protection against apoptosis has been recently ellucidated. First, IGF-I induces "a cAMP-response element (CRE) site containing" bcl-2 promoter through a phosphatidylinositol-3-kinase pathway (PI-3) [57]. The type 1 IGF-R mediated rescue from apoptosis is blocked by PI-3 kinase inhibitors [58]. Second, the serine threonine kinase AKT/protein kinase B is a target of IGF-I signalling and has been shown to activate CRE-binding protein (CREB). Enhanced CREB activity by AKT signalling leads to increased bcl-2 promoter activity in a rat PC12 cell line [59]. Third, IGF-I prevents potassium deprivation induced bad dephosphorylation in a wortmannin sensitive manner of cerebellar granule neurons [60]. In addition, Type 1 IGF-R activates alternative pathways for protecting against apoptosis induced by withdrawal of interleukin 3 in a murine hemopoeitic cell line devoid of insulin receptor substrate 1. All these pathways, however, appeared to result in bad phosphorylation [61].

The direct involvement of the IGF system in the regulation of expression of bcl-2 protein members and hence sensitivity for apoptosis justifies investigation of anti-tumor endocrine therapeutic approaches interfering with the IGF-axis components. Both IGFs, IGF receptors and bcl-2 protein families are expressed in meningiomas, however, of the relation between the IGF system and bcl-2 familie members nothing is known thus far. Knowledge of this interaction will be of major clinical and biological importance.

Therapeutic approaches using the IGF system

The IGF-system has an important mitogenic and anti-apoptotic function in cell growth and development. For that reason the IGF system could be used as a potential therapeutic target to suppress cellular proliferation. Different approaches are employed to target this system, including antisense strategies, monoclonal antibody technology, peptide analogs, chimeric toxic proteins, dominant negative mutants and suramin.

Antisense strategies for tumor growth remission involve the inhibition of growth factor or growth factor receptor gene expression by introducing a source of nucleotide sequences complementary to a given endogenous mRNA species resulting in translational shutdown of protein synthesis. Insertion of an antisense IGF-I into IGF-I producing glial tumor cells substantially inhibits IGF-I production. The injection of these genetically altered cells into animals results in regression of both altered and wild type tumor cells. Although the mechanism of the regression is unclear, antisense blocking of IGF-I expression may reverse a phenotype that allows the glial tumor cells to evade the immune system [61,62]. The type 1 IGF-R has also been target of antisense gene technology in brain tumors. Antisense type 1 IGF-R RNA inhibited IGF-I mediated growth in monolayers and clonogenicity in soft agar.

When wild type cells were injected in animals, tumors developed, whereas type 1 IGF-R antisense RNA transfected cells were non-tumorigenic. Moreover, transfected cells prevented wild type glial tumor cells completely from tumor formation. Furthermore the transfected cells caused complete regression of established glial tumors [63]. One of the mechanisms by which type 1 IGF-R antisense strategies has been postulated to cause its anti-tumor effects is via the induction of apoptosis. A decrease in the level of type 1 IGF-R may cause massive apoptosis in tumor cells. On normal cells, this decrease has much less effect, while they can take refuge in the G_o (resting) state of the cell cycle [64]. A second mechanism of antisense strategy might be an immune response, since at the place of tumor regression a lymphocytic infiltration occured. The antisense therapy for IGF-II has not yet been tested on brain tumor tissue yet. However, on other cultured tumor cells (pancreatic cancer TC3 and ovarian cancer AO cells) IGF-II antisense strategies inhibited cell proliferation and induced apoptosis [65,66].

The development of monoclonal antibody technology has led to a number of applications for targeted therapy. The use of specific antibodies to inhibit cellular proliferation by blocking the type 1 IGF-R, using αIR3, has been described in a number of malignancies, both *in vivo* and *in vitro* [67]. This antibody binds to the ligand binding domain of the IGF-I receptor and inhibits IGF-I mediated effects [10]. However, the use of this antibody technology in humans will be limited because of anti-idiotype antibodies being rapidly produced. IGF-I peptide analogs that inhibit type 1 IGF-R receptor autophosphorylation have been developed [68]. The growth of three tumor cell lines was inhibited by these peptide analogs of IGF-I [69].

A new class of selective cytotoxic agents has been constructed using gene-splicing techniques in which growth factors, functioning as target agent, are fused to a toxic protein toxin. These IGF-I recombinant constructs [70] were shown to be effective in the specific killing of human glioblastoma cell lines by binding to the type 1 IGF-R and subsequently delivering of the toxin to tumor cells [71]. Although chimeric toxins composed of IGF-I and have extraordinary cell-killing activity, their use *in vivo* may be limited since the type 1 IGF-R is expressed in many major tissues. Systemic administration of IGF-I-toxin fusion proteins may therefore lead to organ toxicity before any therapeutic effect can be achieved. Direct infusion of these toxins in the tumor could be an alternative approach.

Several dominant negative mutant type 1 IGF-R derived from human type 1 IGF-R cDNA have been developed [72]. These dominant negative receptors were transfected into rat glioblastoma cells and they all reduced cell growth, although the levels of inhibition varied. They competed for the ligand with the endogenous receptor, resulting in decreased cell growth [73].

One dominant negative receptor, truncated at residue 952, forms chimerae with wild type endogenous receptors.

Tumor cells expressing this receptor do not form tumors in nude mice, the dominant negative receptor interferes with the endogenous receptor inducing apoptosis [74].

Suramin inhibits the binding of IGFs to the type 1 IGF-R and thereby inhibiting mitogenic signaling of the IGFs via the type 1 IGF-R, resulting in a decrease of growth rate [41,75]. The drug does not affect the receptor itself, but binds directly to the peptide. No *in vivo* studies using suramin on meningiomas have been performed, however the effect of suramin on a human glioma xenografted in a nude mouse considerably reduced tumor growth [76].

In conclusion, targeting the IGF system by a variety of strategies has been shown effective in *in vitro* systems. Before these kind of therapies will be clinical useful multiple difficulties have to be sorted out, like delivery, immune response and specificity.

Conclusion

As a group of essential growth modulators, IGFs play a critical role in regulating cell growth and apoptosis. This important function has led to the speculation concerning their possible role in meningioma development and growth. Several experiments demonstrated biological mitogenic effects of IGF-I via the type 1 IGF-R in meningiomas. IGF-I and IGF-II, type 1 IGF-R and type 2 IGF-R, and several IGFBPs are present in at least a subset of human meningiomas. The IGF components were present in various histological subtypes of meningiomas and mainly localized in the tumor cells. Especially the elevated levels of the type 1 IGF-R compared to normal meninges have attracted our attention since this receptor is directly involved in the regulation of expression of Bcl-2 protein family members. Meningiomas show a high recurrence rate and at the moment no adjuvant therapy is available since these tumors are relatively insensitive to chemotherapeutic agents. We hypothesize that the type 1 IGF-R by regulating bcl-2 and bcl-x_l protein levels might be involved in this chemotherapeutic resistance. The greatest benefit of tumor growth intervention of meningiomas might be expected from upregulation of apoptosis since these tumors have a low proliferation and low apoptotic index. The suspected involvement of IGF components in meningiomas justify investigation of the expression of these components (especially IGFBPs) and the relation between IGFs, IGFBPs, IGF-Rs and members of the bcl-2 proto-oncogene family. The expression of all these proteins has already been described for meningiomas, however, nothing is known about the interaction between these proteins and the possibility to aim at these proteins for adjuvant therapy.

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Chapter 10

General discussion

The present thesis was initiated to improve our understanding of the high concentration of PR in human meningiomas, with a special emphasis on the relation of the PR with proteins involved in apoptosis. This chapter will discuss the outcome of several studies presented in this thesis in conjunction with additional preliminary data.

First, because no cell culture model of meningiomas that actively expresses PR is available or could be developed (chapter 2), our research has mainly been restricted to inventory studies. This general discussion starts with a paragraph about the difficulty of PR investigation in meningiomas. Furthermore, the differential expression of PR isoforms is discussed with special emphasis on the newly discovered PR-78 variant. This is followed by a short discussion on the role for ER in PR synthesis in meningiomas. This paragraph will end with a summary about the use of progestins and antiprogestins in clinical practice.

Second, the present thesis reports about a relation between PR and proteins involved in apoptosis, like bcl-2. To investigate direct effects of progesterone on bcl-2 expression, stimulation experiments were performed using *ex vivo* meningioma tissue fragments. The outcome of these

experiments will be discussed. Furthermore, to demonstrate a direct relation between PR or proteins of the Bcl-2 family with apoptosis in meningiomas, knowledge of actual levels of apoptosis in these tumors is required. Preliminary data from experiments assessing apoptosis by the TUNEL-assay will be presented and discussed.

Third, as shown in chapter 9, knowledge of the GH-IGF axis in meningiomas could be a source for development of novel therapeutic strategies. Our laboratory has started inventory studies to some of these GH-IGF components and preliminary data are shown in this section.

This general discussion will end with our general thoughts and remarks about the significance of PR in meningiomas.

PR expression in cultured meningiomas

The present thesis has focused on the biological role of PR in meningiomas. Studies directly investigating this subject are severely complicated by two components:

First, the PR is very rapidly degraded when meningiomas are cultured, independently of serum, growth factors, hormones or different kinds of culture conditions (chapter 2). Therefore, investigations on the biological role of PR in meningiomas is restricted, unfortunately, to inventory studies. In addition, interpretation of studies in which meningioma cells have been stimulated with progestins or antiprogestins is very difficult. Surprisingly, most studies described in literature ignored this PR degradation in their meningioma cell culture studies, since presence of PR protein levels after stimulation has very rarely been measured. The results obtained from our studies, at least partially, clarify the discrepancy found in literature concerning the response of meningiomas to progestins.

Second, the total PR receptor status of meningiomas, as seen in studies done thus far, over-simplifies the complex mechanism of regulation of PR target genes because of the different PR isoforms with their distinct biological functions. The results described in chapter 3 show that meningiomas express multiple PR isoforms that might have biologically different functions. This indicates that overall, progesterone response of meningiomas to progesterone might be dependent on the differential expression of the PR isoforms.

Progesterone receptor

It is generally accepted that relatively high concentrations of PRs prevail in human meningiomas (chapter 1). The PR exists as multiple isoforms with distinct biological functions, dependent on the cell and promoter context [1]. PR expression in meningiomas has been reported to be associated with clinical parameters as recurrence, histological type and survival rate [2-4]. The exact regulation of transcription of the *PR* gene in meningiomas is still not known. Normal meningothelial tissue also expresses PRs, although, in low amounts [5]. Thus, PR levels appear to be

increased in meningiomas compared to the meninges, however, a role for PR in the etiology of meningiomas remains to be elucidated.

The proliferation rate of meningiomas is low, but tumor size increases during periods of high levels of progesterone in the circulation (chapter 1). In the present thesis, we have shown that both PR-A and PR-B were variably expressed in meningiomas (chapter 3), and that the ratio PR-A to PR-B might have biological significance. Meningiomas that expressed high total PR levels all expressed more PR-A than PR-B. When it is assumed that PR-A is capable of repressing progesterone action via PR-B, as was shown in several *in vitro* experiments in literature (chapter 3), the ratio PR-A to PR-B might determine the response to progestins or antiprogestins.

Meningioma growth is increased during periods with high levels of progesterone in the circulation. To simplify the complex mechanism of PR action a working model was made as is shown in Figure 10-1. Since the transcription activation capacities of PR isoforms is target gene promoter and cell type dependent, several options for PR signaling are shown in Table 10-1. The major PR isoforms are capable of transcription activation, although PR-B is the most potent activator. PR-A, and other PR forms, are capable of repression of the transcriptional activity of PR-B and other steroid hormone receptors (SHR), most likely at the protein level [6]. We hypothesize that the growth

Progestins	
1) PR-A activated	1.1) Growth promoting PR-target genes are upregulated 1.2) PR-B and other SH-R are suppressed and thereby growth inhibiting PR-target genes are downregulated 1.3) PR-A is not suppressing PR-B and PR-B upregulates growth promoting target genes
2) PR-B is activated	2.1) Growth inhibiting PR target genes are upregulated 2.2) see 1.3
Antiprogestins	
3) PR-A is blocked	3.1) Transactivation of PR target genes is blocked 3.2) Transactivation function of PR-B and other SH-R is no longer repressed
4) PR-B is:	
4.1) Blocked	4.1.1) Growth stimulating target genes are blocked 4.1.2) Growth inhibiting PR target genes are no longer suppressed by PR-A
4.2) Activated	4.2.1) Growth inhibiting PR target genes are upregulated

Table 10-1. Working model of the activation mechansim of PR isoforms in human meningiomas.

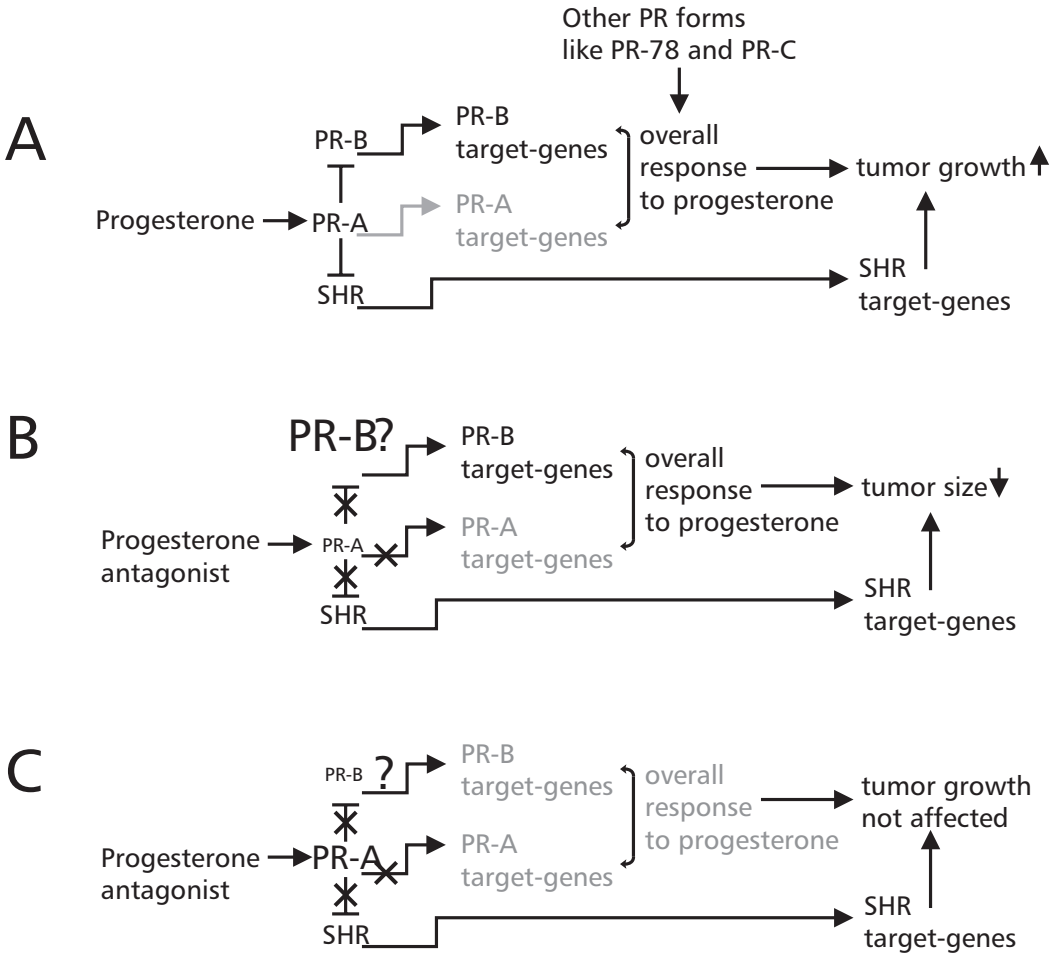

Figure 10-1. a) A schematic hypothetical illustration of the working mechanism of PR action in human meningiomas. Progesterone binds to PR-A, PR-B and other PR forms. The overall progesterone action will be determined by the differential expression of the PR forms in the cells. PR-A is described as a suppressor of PR-B and other steroid receptor action. Progesterone antagonists will block the repression of PR-A and this might result in the (up)regulation of other PR target genes. The overall response will be dependent on the ratio of different PR isoforms (1b, 1c). Meningiomas that express more PR-A than PR-B might already show a repression of progesterone action and these tumors are not likely to benefit from progesterone antagonist treatment (1c).

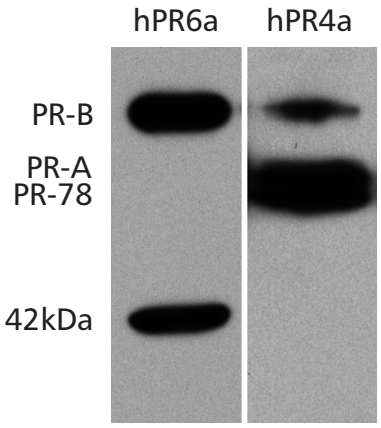

Figure 10-2. Immunoblot example of PR-isoform expression in human meningioma cytosol samples. Two different PR specific antibodies were used to demonstrate a 42 kDa protein that could be arise from the degradation of PR-B in PR-78 and 42kDa residual product. A PR antibody with an N terminal located epitope demonstrates a 42kDa protein band. Because a 42kDa degradation product only contain B unique sequence it should not be recognized by an antibody directed against a c-terminal epitope (hPR4a).

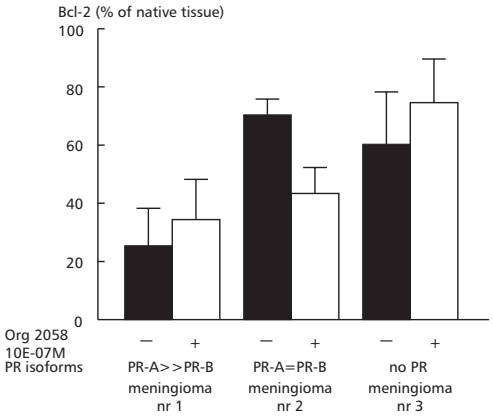

Figure 10-3. Bcl-2 protein levels in tissue fragments of 3 meningioma patients. All three tumors specimens showed different PR isoform expression. Tissue fragments of these tumors were treated with Org 2058, progesterone agonist, for 24h. After stimulation the PR expression was decreased but still present. No differences in bcl-2 expression could be found in treated or not treated tissue fragment. Further experiments are needed to further explore a direct effect of progesterone on the expression of bcl-2.

stimulation by progesteron of meningiomas is caused by the transrepression of growth inhibiting target genes of PR-B or SHR. Antiprogestin treatment will release the repression by antagonist bound PR-A and thereby at least the SHR target genes will be upregulated. The transcriptional activity of PR-B upon antagonist binding is cell type specific and might either be repressed or PR-B target genes might be stimulated, upregulating growth inhibiting genes. We hypothesize that, assuming PR-A is the potent trans-repressor of most PR-target genes, progesterone action in most of the meningiomas is mediated via trans-inhibition of target genes.

A novel PR isoform (PR-78)

Besides the two best known PR isoforms, PR-A and PR-B, other PR like proteins have been described. For instance, Wei *et al.* described an N-terminally truncated PR form referred to as PR-C [7,8]. PR-C is most likely capable of trans-inhibition of the other PR isoforms by competition for co-factors. Graham *et al.* described the existence of a fourth

PR-form capable of ligand binding in breast cancer further referred to as PR-78 [9,10]. We could confirm the presence of such an isoform in meningiomas. The origin of this PR-78, however, is still not known. Our data indicate that PR-78 might be a degradation product of PR-B since there was a negative association found between these two proteins (chapter 3). When it is assumed that PR-B gives rise to two products, PR-78 and a smaller product of 42 kDa, the latter should also be detectable

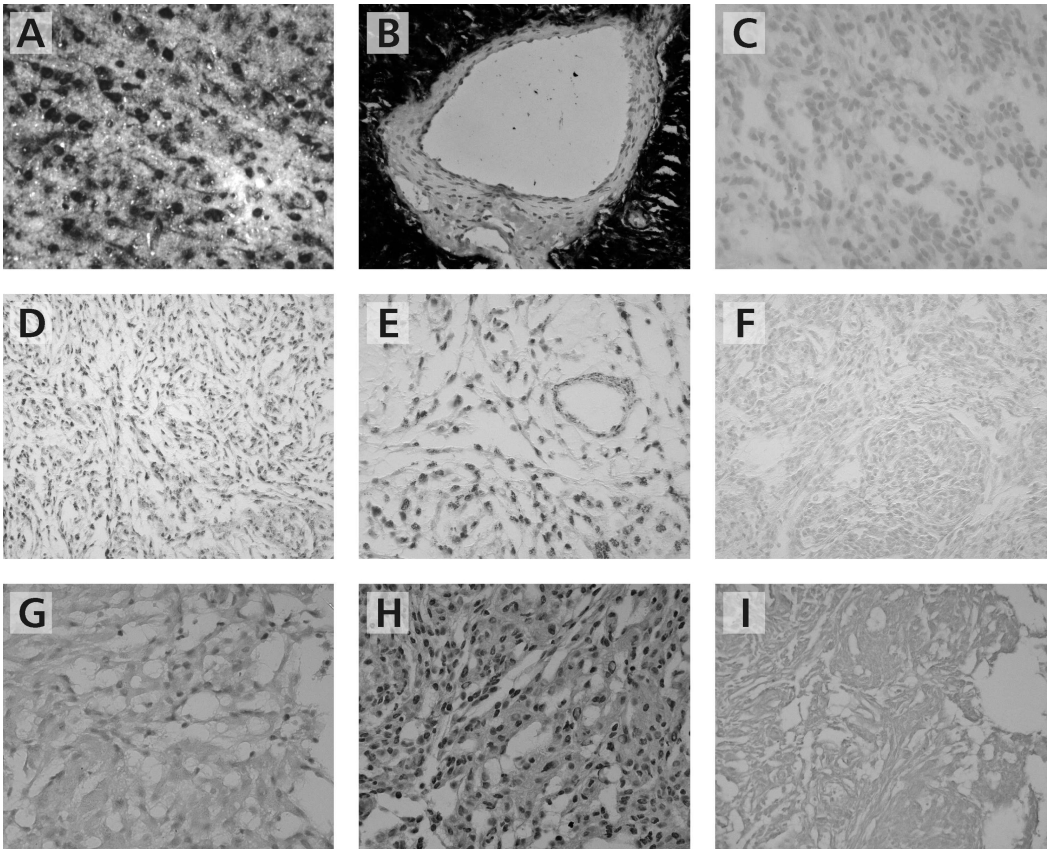

Figure 10-4. A, B, C. In situ hybridization (anti-sense probe in A, B; sense probe in C) analysis clearly demonstrate IGF-II mRNA in nucleus and cytoplasm (to a lesser extend) of most of the meningiomas (19/24). Vascular endothelial cells did not show IGF-II mRNA pointing out that IGF-II transcription is restricted to tumor cells (B). **D, E, F.** Immunohistochemistry study using a specific antibody directed against human IGF-II. Pro-IGF-II protein was found in 12/22 meningiomas. **G, H, I.** TUNEL assay analysis demonstrating apoptotic cells in meningioma tissue samples. Apoptotic cells were found in 9/18 meningiomas. The example shown was a meningioma with a high number of apoptotic cells. ***A color version of this figure is depicted on the cover.*** FOR IGF-II *IN SITU* HYBRIDISATION AND TUNEL ASSAY, E. VAN DEN BORN IS GRATEFULLY ACKNOWLEDGED; FOR IGF-II IHC, N. ROFFERS IS GRATEFULLY ACKNOWLEDGED.

by specific antibodies directed against the N-terminus of PR-B. Therefore, immunoblots were performed to detect a possible residual N-terminal product. Indeed Figure 10-2 shows that cytosols of meningiomas contained a specific band of the expected 42kDa. In addition, a PR antibody directed against the C-terminal part of PR failed to detect this protein. Correlation analysis, however, did not reveal that PR-B and PR-42 are negatively associated. Therefore the hypothesis that PR-B give rise to PR-78 could not be confirmed in this way, and other regulatory pathways behind the origin of PR-78 may not be ruled out at the present.

Since both PR-78 and PR-C are capable of binding progesterone it is obvious that these PR isoforms will be important for understanding the response of meningioma cells to progesterone and antiprogestin stimulation. Because of the existence of several PR-forms, with most likely distinct biological functions and all capable of ligand binding, the use of a LBA in clinical studies should be reconsidered.

Estrogen receptor (ER)

In other hormonally responsive tissues, like breast cancer, PR is under estrogen control. For myometrium cytosol samples a spearman rank correlation between ER and PR of r_s=0.90 was found (unpublished data). Meningiomas contain wild type and splice variants mRNA levels of both ER forms as was demonstrated by rt-PCR and southernblot analysis (chapter 7). The ER splicevariants are not likely to have a regulating role in growth of meningiomas. First, it is most doubtful whether the mRNA of ER splicevariants will be translated into protein in meningiomas. Second, PR is a marker for ER activity in other tissues, however, no relation between ER splice variants mRNA and PR expression could be established in meningiomas (chapter 7).

In literature, by the use of other techniques like immunohistochemistry, ER expression has been reported. The receptor phenotype of meningiomas was considered to be ER negative and PR positive based on ligand binding data (chapter 1). Based on the controversy of apparent absent ER binding activity and high amounts of ER mRNA found in meningiomas, sensitive immunoblot assays have been performed. These experiments revealed that although no ligand binding to ER could be found, these meningiomas expressed minute quantities of ER-α (chapter 8). With this technique ER-α expression was detected in concentrated meningioma cytosol samples that were lower than the cut-off value of the LBA (10 fmol/mg protein) (chapter 8). Immunoblot studies to detect ER-β protein levels are currently underway.

In retrospect, the presence of ER was observed already by Koehorst *et al.* at our laboratory in 1994 [11]. They showed that in bandshift analysis with a synthetic estrogen binding element (ERE) a shift was seen, indicating a protein capable of binding ERE. Further analysis with ER specific antibodies revealed indeed that this protein could be one of the ER forms. Since meningiomas, at that time, were considered ER negative, this ERE binding protein was referred to as ER-like protein.

We conclude that the receptor phenotype of meningiomas is ER and PR positive. Whether these minute ER protein levels found in meningiomas are biologically important remains to be seen. In both PR positive and PR negative meningiomas, ER-α protein was found, this indicates that ER is not likely to play a role in the synthesis of PR in meningiomas.

Progestins and antiprogestins

Progestins have been used for many years as one of the elements in the endocrine therapy of breast cancer. The two main progestins used in clinical practice are medroxy progesterone acetate (MPA) and megestrol acetate (MA). Progestins may decrease the amount of estrogens available to tumor cells, during progestin treatment estradiol (E_2), estrone and androgen levels are decreased. Progestins also have the ability to decrease the amount of estrone sulfate (E_1S) in breast tissue [12]. In addition, progestins can block the conversion of E_1S to E_2 in hormone-dependent breast cells and decrease ER expression levels. In conclusion, the anti-estrogenic action of progestins is important in anti-tumor endocrine therapeutic approaches.

Estrogenic or antiestrogenic actions do not appear to be involved in the development of meningiomas, based on stimulation experiments and the low ER expression levels of far less than 10 fmol/mg protein (see chapter 1 and 8). In other hormonally regulated tissues, like myometrium and breast cancer, ER target genes, i.e. PR, CatD, pS2, c-myc, are upregulated after activation of the ER by estrogens. In meningiomas some, but not all, of these ER target genes are repressed [13,14]. Studies using an estrogen hyper-sensitive cell line have shown that the number of occupied ERs necessary for transcription activation was cell and promoter dependent [15,16]. This may be caused by the number of ERE and AP-1 binding sites on the promoter regions of these target genes. In the case of PR, however, no complete estrogen responsive elements (ERE) in the promoter region of both PR-A and PR-B could be found [17]. When assuming that intra-tumor estrogen levels and ER levels of meningiomas are too low for activating most of the ER-target genes, like PR, the anti-estrogenic effects of progestins, as seen in other types of cancer, will not take place in meningiomas. That means that progestins might only exert their proliferative actions via the relatively high expression of PR. Therefore, several studies have been done to investigate the therapeutic possibilities of anti-progestins. These anti-progestins have been shown to inhibit growth of meningioma cells in culture [18,19] and reduce the size of meningiomas implanted into nude mice [20,21]. In addition, clinical trials in which meningioma patients were treated with anti-progestins found objective responses as shown by reduced tumor size [18]. Overall results, however, are still controversial and non-conclusive and, therefore, the result of large clinical trials is awaited. To further explore and understand the response of meningiomas to progestins and anti-progestins further knowledge of the function and regulation of PR in meningiomas is required.

PR and apoptotic proteins

This present thesis was also focused on the role of PR in the regulation of proteins involved in apoptosis, since members of the Bcl-2 family have been described as being hormonally regulated in breast cancer (chapter 4 and 5). In meningiomas, PR was negatively associated with bcl-2 and positively with bcl-x_l and bak. To find out whether there is a direct effect of progesterone on the regulation of apoptotic proteins stimulation experiments were carefully performed, of course taking notice of the previously described limitations. Meningioma tissue fragments (see *ex vivo* culture in chapter 2) were cultured for 24 hours and stimulated with the progesterone agonist Org 2058 (10E-07 M). The PR isoform expression was determined by immunoblotting at the beginning and at the end of the experiments. Figure 10-3 shows the preliminary results, indicating no difference in bcl-2 levels although no definite conclusion could be drawn of these preliminary data.

It is clear that the differential expression of the PR isoforms will determine the final response of Org-2058 on bcl-2 expression levels, assuming that bcl-2 is under progesterone control in meningiomas. To clarify the association that was found between apoptotic proteins and PR (chapter 4 and 5), the working model (Figure 10-1) might also be helpful. Bcl-2 is negatively associated with total PR. The fact that meningiomas that express high total PR express relatively more PR-A than PR-B this indicates that the transcription of bcl-2 might be repressed by PR-A (Table 1, option 1.2). A second observation was that bcl-x_l was associated with PR-B but not with total PR or PR-A. This indicated that PR-A only interferes in the comparison study with total PR, however, PR-A is not involved in the regulation of bcl-x_l. In the working model this option is represented by option 1.3.

It is concluded that, PR forms are associated with proteins from the Bcl-2 family. The role of the different isoforms in the regulation mechanism, however, is complex and protein dependent. The possible regulation of apoptotic proteins by progesterone needs further elucidation and is clinically important.

Apoptosis in meningioma

Because PR expression levels might be involved in the regulation of apoptotic proteins it might also influence the sensitivity of cells for going into apoptosis. To investigate this, comparative studies were performed in which actual levels of apoptosis in meningiomas were determined by a TUNEL assay and members of the Bcl-2 family by immunoblotting. Of data obtained thus far, correlation analysis were performed to reveal any relation of PR, members of the Bcl-2 family and the presence of apoptotic cells. Figure 10-4G-I shows a representative picture of a meningioma containing apoptotic cells. Thus far, no direct association between PR and apoptosis could be revealed. Interesting was that only a low number of meningiomas were found positive for apoptosis. Apparently, the proteins involved in apoptosis are regulated in such a way that the tumors have a low level of apoptosis. Whether PR isoforms play a

significant role in keeping this balance might be clinically significant and needs to be investigated.

Other endocrine therapeutic approach; the IGF system in meningiomas

The use of anti-progestins as a novel adjuvant therapeutic agent to slow down meningioma growth is not as easily achieved as initially was thought. It is therefore wise to consider other endocrine therapeutic approaches. Two recent publications have reported that the GH-IGF axis is involved in the regulation of proliferation of meningiomas [22,23]. In addition, these authors showed the use of GH-R antagonists could inhibit meningioma cell proliferation. Both IGF-I and II have been reported to have anti-apoptotic functions in tumors. As a basis for further studies chapter nine of this thesis contains a review of IGF components in meningiomas. Meningiomas express relative high levels of type 1 IGF receptors compared to normal meninges. Expression of both IGFs is also demonstrated in meningiomas. Type 1 IGF receptors might be involved in proliverative and apoptotic processes in meningiomas. About the expression of pro-IGF levels in these tumors, described to be involved in some neoplasms, nothing is known up to now. Therefore, our laboratory started pilot experiments to investigate IGF-II and pro-IGF-II. Preliminary results are shown in Figure 10-4. In 55% of the meningiomas demonstrable pro-IGF-II protein levels were found (10-4D-F). IGF-II mRNA was detected in 19/24 meningiomas as shown in Figure 10-4A-C. Correlation studies, however, could this far not reveal a relation between pro-IGF-II or PR and apoptosis. Further investigations of the GH-IGF components are necessary to explore adjuvant therapeutic possibilities in the near future.

Conclusive remarks

The aim of the present thesis was to obtain a better understanding of the presence of PR in human meningiomas. We conclude that meningiomas express multiple forms of the PR. The PR forms were found differentially associated with apoptotic proteins like bcl-2. We hypothesize that PR might be important for the regulation of the sensitivity for apoptosis. In addition, relatively high expression levels of type IGF receptors may contribute to this as well. Both PR and bcl-2 are described as markers for tumor grade, survival and recurrence. The prognostic significance of PR, however, might be due to low expression of bcl-2 levels found in meningiomas with high PR expression levels. The role that PR plays in retaining the balance between pro and anti-apoptotic proteins is clinically important.

Our insight in the expression of estrogen receptors in meningiomas has changed drastically. Initially we thought that meningiomas did not express the ER, however, it turned out that these tumors all contain minute quantities of ER-α. The hypothesis that the expression of PR is estrogen independent, however, still stands. The initial idea that ER splice variants could be responsible for PR regulation has become most unlikely.

Our mechanistic insights in the success change of anti-tumor endocrine approaches are paradoxical. The negative association between PR (therapeutic target) and bcl-2 (involved in therapy resistance) makes that meningiomas expressing high levels of PR should benefit the most of a PR antagonist treatment. In contrast, meningiomas with high PR levels express relatively more PR-A protein. In these tumors PR action might be repressed and PR antagonist treatment will then most likely not be successful. In this case meningiomas with relative more PR-B than PR-A expression will benefit the most of such a treatment.

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Appendices

Appendix A

Summary

Progesterone is a steroid hormone involved in female fertility, preparing the uterus for implantation of fertilized oocytes, and in the maintenance of pregnancy. Progesterone can also exert some anti-estrogenic and anti-proliferative effects on neoplastic tissues. Progesterone acts via its receptor (PR) and knowledge of the function and regulation of PRs is important to determine clinical significance of involvement of PR in tumor growth regulation.

Meningiomas have attracted attention as being progesterone sensitive central nervous system tumors. The aim of this thesis was to obtain a better understanding of the role PR plays in these tumors.

The development of a meningioma cell culture model is discussed in chapter 1. Meningiomas cells cultured as monolayer quickly lose their ability to express PR. The loss of PR expression and a high proliferation rate in meningiomas indicate that the cells might dedifferentiate when cultured. In this chapter a spheroid and an *ex-vivo* culture method were tested, however, both methods failed to maintain PR expression in meningioma cells. Investigation of PR in meningiomas is therefore mainly restricted to inventory studies.

In meningiomas at least two PR isoforms exist, PR-B and PR-A, each of which are likely to have different biological functions. Chapter 3 shows that meningiomas express both PR-A and PR-B, and an additional PR-78 product. The ratio of PR-A to PR-B protein levels is highly variable and higher in the meningiomas with high total PR expression based on a ligand binding assay (LBA). This variability might have some clinical and biological significance in meningioma disease.

Chapters 4 and 5 illustrate that PR expression in meningiomas is related to proteins involved in apoptosis. In contrast to breast cancer, PR and bcl-2 expression in meningiomas is negatively associated. Bcl-x_l was positively associated with PR-B. Bax and bak protein levels

were not associated with PR in meningiomas. This indicates that PR might be involved in regulating the apoptotic balance in meningiomas.

Chapter 6 describes mutation analysis of the gene of another protein involved in the regulation of cell death: p53. Overexpression of p53 caused by mutations of the p53 gene has been reported to play a role in the etiology of nervous system and other neoplasms. In meningiomas a relatively high percentage of p53 protein overexpression is described in literature indicating the presence of mutations in the p53 gene. Based on this thesis and reports by others, however, we conclude that point mutations of the p53 gene are not likely to be involved in the etiology of meningiomas.

The regulation of PR in other endocrine related tissues is under estrogen control. Chapters 7 and 8 report the presence of ER-β splice variants in meningiomas. Some splice variants could fail to bind ligand and hence are not detectable with a LBA, however, could still be capable of transcriptional activation. The experiments described in the present thesis show that it is not very likely that these splice variants are involved in the regulation of PR in meningiomas since PR negative meningiomas also express these variants.

Based on reliable LBA data from previous studies meningiomas are rich in PR whereas ER is seldomly found and only in very low concentrations. ER mRNA analysis (chapter 7) learnt that meningiomas contain ER*wt* mRNA. In addition, some authors reported IHC data of ER expression in meningiomas. Chapter 8 describes the results from a sensitive immunoblot analysis, which reveals that, in contrast to the previously existing thoughts, meningiomas are ER-α positive. These minute ER-α levels alone, however, could not clarify the expression of PR since ER-α was also found in PR negative meningiomas.

Chapter 9 is a review about the relevance of GH-IGF-axis in meningiomas. Several members of the IGF-axis are present in meningiomas. The type 1 IGF-Receptor is present in elevated levels in meningiomas. This is of special interest since this receptor is reported to be involved in the regulation of members of the bcl-2 protein family. This justifies investigation of the use of antagonizing GH-IGF receptors as a possibility for adjuvant therapy.

In summary, we hypothesize that PR might be important for the regulation of proteins involved in apoptosis. Both apoptotic proteins and PR are described as prognostic markers for tumor grade, survival and recurrence in literature. Although minute quantities of ER are present in meningiomas, exact regulation of PR in these tumors is not known and the involvement of estrogens is considered to be not very likely. Meningiomas with high PR expression express relatively more PR-A and this might have consequences for the success of an anti-tumor endocrine therapy.

Appendix B

Samenvatting in het Nederlands

Progesteron is een steroïdhormoon onder andere betrokken bij de regulatie van de zwangerschap. Daarnaast kan progesteron in een aantal tumorweefsels de proliferatie remmen doordat het een anti-oestrogene werking heeft. Progesteron reguleert genen door te binden aan een ligand afhankelijke transcriptiefactor, de progesteron receptor (PR). De regulatie van PR aanmaak en de functie van PR in tumoren zijn biologisch en klinisch zeer interessant, mede doordat het PR niveau de gevoeligheid van tumoren voor progestiva en anti-progestiva bepaalt .

Meningeomen, een van de meest voorkomende tumoren van het centrale zenuwstelsel (CNZ), blijken onder invloed te staan van progesteron. Het doel van het hier beschreven onderzoek is het verkrijgen van een beter inzicht in de functie en regulatie van PR in meningeomen.

Helaas is er op dit moment nog geen goed experimenteel celmodel voor meningeomen. Dit komt doordat als monolaag gekweekte meningeoomcellen de PR expressie verliezen. Dit, naast de hoge proliferatie van gekweekte meningeoomcellen, wijst op een dedifferentiatie van deze cellen *in vitro*. Behalve een monolaag kweekmodel zijn er in hoofdstuk 2 ook een *ex vivo* en een spheroid-kweekmodel getest. Meningeoomcellen waren, echter, in geen van de kweekmodellen in staat om PR tot expressie te blijven brengen.

Er bestaan tenminste twee isovormen van de PR, PR-A en PR-B, die waarschijnlijk verschillende biologische functies hebben. In hoofdstuk 3 hebben we aangetoond dat meningeomen zowel PR-A, PR-B als een extra PR-78 eiwit bevatten. De ratio PR-A tot PR-B was zeer variabel en hoger in meningeomen met een relatief hoge totaal PR expressie. Deze variabiliteit kan klinisch en biologisch van belang zijn.

Hoofstuk 4 en 5 illustreren dat PR niveau in meningeomen gerelateerd is aan dat van eiwitten betrokken bij geprogrammeerde celdood (apoptose). In meningeomen waren de PR en het anti-apoptotische bcl-2 negatief geassocieerd, in tegenstelling tot de positieve associatie gevonden voor borstkanker. Bcl-x_l was positief geassocieerd met alleen PR-B. De expressie van andere pro-apoptotische eiwitten, bax en bak, bleken niet gerelateerd aan PR expressie. Dit alles duidt erop dat de PR een rol zou kunnen spelen in de regulatie van de apoptotische balans in meningeomen.

Een ander eiwit betrokken in de regulatie van apoptose is p53. P53 eiwit kan tot overexpressie komen wanneer het gen gemuteerd. Deze mutaties zijn vaak betrokken bij de ontwikkeling van tumoren. Echter, in hoofdstuk 6 is beschreven dat p53 mutaties niet verantwoordelijk waren voor p53 eiwit overexpressie in meningeomen, en dus geen rol blijken te spelen in het ontstaan van deze tumoren.

In andere hormoonafhankelijke tumoren staat de regulatie van PR vaak onder oestrogene controle. In meningeomen is de ER (gebasseerd op een ligand bindings assay (LBA)) niet of heel laag aanwezig. In hoofstuk 7 wordt beschreven dat er splicevarianten van de ER-β zijn die geen ligand binden, maar nog wel in staat zouden kunnen zijn om transcriptie te activeren. Southernblot analyse toonde echter aan dat noch de splicevarianten van de ER-α noch die van de ER-β betrokken blijken te zijn bij de regulatie van PR. PR negatieve meningeomen bevatten ook mRNA van deze splicevarianten.

ER mRNA analyse leerde ons ook dat meningeomen ER wildtype mRNA bevatten. Hierbij komt dat door sommige onderzoekers in de literatuur gerapporteerd wordt dat ER expressie in meningeomen aantoonbaar is met immunohistochemie. Hoofdstuk 8 laat zien dat een gevoelige immunoblot techniek in staat was om ER-α eiwit aan te tonen in 22 onderzochte meningeomen. Dus, in tegenstelling tot onze eerste aanname, zijn meningeomen ER en PR positief. Het is echter lang niet zeker dat deze lage hoeveelheden biologisch aktief zijn. Het is onwaarschijnlijk dat zij verantwoordelijk zijn voor de activatie van het PR gen, omdat PR negatieve meningeomen ook ER-α eiwit bevatten.

Hoofdstuk 9 is een review over de groeihormoon en IGF as in meningeomen. Veel van de GH-IGF componenten komen tot expressie in meningeomen, maar meest opvallend is de overexpressie van de type 1 IGF-receptor ten opzichte van het gezonde weefsel. Dit is klinisch relevant omdat deze receptor direct betrokken lijkt te zijn bij de regulatie van eiwitten van de Bcl-2 proto-oncogen familie. Het is interessant om de GH-IGF-as te bestuderen omdat daaruit wellicht mogelijkheden voor een nieuwe adjuvante therapie voort komen.

Samengevat, de PR is in meningeomen mogelijk belangrijk is in de regulatie van de eiwitten betrokken bij apoptose. Zowel PR als deze apoptotische eiwitten worden in de literatuur beschreven als prognostische factoren voor recidivering en prognose. Meningeomen zijn, in tegenstelling tot wat tot nu toe algemeen werd aangenomen, zowel ER als PR positief. De biologische betekenis van ER-α in meningeomen is nog niet opgehelderd. Meningeomen die veel PR tot expressie brengen, brengen relatief meer PR-A tot expressie. Dit kan consequenties hebben voor het succes van antiprogestagene endocrine therapie zoals die mogelijk gebruikt kan worden in de toekomst.

Appendix C

Dankwoord

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Appendix D

List of publications

Blankenstein MA, **Verheijen FM**, Jacobs HM, Donker GH, van Duijnhoven MW, Thijssen JH. Occurrence, regulation, and significance of progesterone receptors in human meningiomas. *Steroids*. 2000, **65:** 795-800

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