Progesterone receptor A and B expression and progestagen treatment in growth and spread of endometrial cancer cells in nude mice

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Abstract

In endometrial cancer, decreased expression of progesterone receptor (PR) isotypes A and B (PRA and PRB) is a feature of poorly differentiated tumours. In distant metastases, PRB is the predominantly expressed isotype and endometrial cancer cells that express PRB have been observed to be more invasive. Furthermore, PRB-associated in vitro invasion is markedly inhibited by progestagens.

In the present study, ovariectomized mice were injected intraperitoneally with Ishikawa endometrial cancer cells that express only PRA, only PRB, both PRA and PRB, or no PR. Half of the mice were substituted with medroxyprogesterone acetate (MPA). After ten weeks, growth and spread of the cancer cells were examined macroscopically, microscopically, and by PCR detection. Without MPA substitution, cells that express only PRB were found to be the most proliferative and migrative, while cells that express only PRA, both receptor isotypes, or no PR, showed minimal growth and spread. MPA appeared to inhibit growth and spread of PR-positive cells. Surprisingly, when mice that were inoculated with PR-negative cells were substituted with MPA, this resulted in massive abdominal tumour growth.

These results provide further evidence that over-expression of PRB in endometrial cancer contributes to the development of a more aggressive phenotype. MPA inhibits tumour growth and spread of PR-positive cells, but can also have an indirectly stimulating effect on PR-negative tumour cells, probably through a host-mediated response.

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Introduction

In normal human endometrium, the growth-stimulating effects of oestrogens are counterbalanced by progestagens, which exert a differentiating and growth-inhibiting effect. Growth of early, non-invasive stages of endometrial cancer can be inhibited by progesterone treatment, but generally this does not cure the patient (Creaseman et al. 2001, Southcott 2001).

Progesterone exerts its effect through the progesterone receptors (PR) A and B (PRA and PRB), which can act as transcription factors upon activation by ligand. Although both progesterone receptor isotypes have similar DNA- and ligand-binding affinities, PRA and PRB do exhibit different activating properties (Giangrande & McDonnell 1999), and mediate transcription of a different set of genes in endometrial cancer cells (Smid-Koopman et al. 2003).

During progression of endometrial cancer, the expression of PR is decreased (Arnett-Mansfield et al. 2001, Fujimoto et al. 1995, 1997, Fukuda et al. 1998, Kumar et al. 1998, Sakamoto et al. 1999). Although it remains unclear whether this is a consequence of down-regulation of only one PR isotype or both, several studies suggest that relative over-expression of PRB is associated with more aggressive tumour growth. Fujimoto et al. (1995, 1997) reported that in advanced tumours, PRA was lost, and that in distant metastases of endometrial cancer, PRB was predominantly expressed. De Vivo et al. (2002)
described a polymorphism in the promoter of the PR gene that selectively increased the expression of PRB, which was found to be associated with a twofold higher risk for endometrial cancer. In contrast, Sasaki et al. (2001) reported inactivation of only PRB alleles through hypermethylation in endometrial cancer tissue.

Several studies report that in addition to growth inhibition, progesteragens have an effect on tumour integrity in endometrial cancer. Progestagens have been shown to inhibit oestrogen-induced suppression of cell-to-cell aggregation of well-differentiated endometrial cancer cells (Fujimoto et al. 1996), and progestagens have also been shown to inhibit anchorage-independent growth of poorly differentiated HeLa50 endometrial cancer cells (Dai et al. 2001). Our group recently reported that medroxyprogesterone acetate (MPA) inhibits expression of several metastasis-related genes in a set of endometrial cancer sub-cell lines expressing different PR isotypes (Hanekamp et al. 2003). Additionally, MPA has been shown to inhibit in vitro invasion of endometrial cancer cells (Ueda et al. 1996, Dai et al. 2002).

Recently, we observed (E E Hanekamp, S C J Gielen, P E de Ruiter, S Chadha-Ajwani, F J Huikeshoven, C W Burger, J A Groote good & L J Blok, unpublished observations) that endometrial cancer cells that express PRB are more invasive in vitro than cells that do not express PRB. In the present study, we set out to investigate the in vivo metastatic potential of these cells in comparison with PR-negative cells and with cells expressing only PRA or both PRA and PRB, as well as to examine the effect of progestagens on in vivo metastasis.

Materials and methods

Animals

Female athymic NMRI nude/nude mice (Harlan, Horst, The Netherlands) were used for this study. The animals were housed at the Erasmus MC animal facility (EDC) in individually ventilated cage units (five animals per unit). Food and water were provided ad libitum.

Cells

Ishikawa clone 3H12 was transfected to stably express high levels of human PRA, PRB, or both PRA and PRB (Blok et al. 2003, Hanekamp et al. 2003b, Smid-Koopman et al. 2003). This resulted in the sub-cell lines PRA-1, PRB-1 and PRAB-36. A sub-cell line stably transfected with empty vector (EV3) was used as control. These cell lines do not express oestrogen receptors. Cells were routinely maintained in DMEM-F12 culture medium supplemented with 10% v/v fetal calf serum (GibcoBRL/LifeTechnologies, Carlsbad, CA, USA), penicillin/streptomycin, neomycin (500 μg/ml, ICN Biomedicals BV, Zoetermeer, The Netherlands) and hygromycin (250 μg/ml, Invitrogen Corporation, Carlsbad, CA, USA).

Western blot

Cells were lysed as described (Hanekamp et al. 2003b). Equal amounts of protein were separated on a polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking in 5% non-fat milk powder in PBS, the membrane was incubated with a mouse monoclonal antibody recognizing PRA and PRB (hPRα8; Labvision Neomarkers, Fremont, CA, USA) and a secondary peroxidase-labelled goat-anti-mouse antibody (Sigma). Bands were visualized using Western Lightning ECL reagent (Pierce, Rockford, IL, USA).

Experimental design

The experiments were performed according to protocol 124-01-08, as described by Hanekamp et al. (2003b). The mice (n = 40) were bilaterally ovariectomized, and half of them were substituted with a subcutaneously placed 90-day time-release MPA pellet. After 1 week, the mice were exposed to 5 Gy of total body irradiation 6h prior to injection with tumour cells. Irradiation was performed to improve the attachment of tumour cells to peritoneal surfaces (Strobel et al. 1997). One mouse had died before this procedure. At t = 0, mice were intraperitoneally injected with 5 × 10⁶ EV3, PRA-1, PRB-1 or PRAB-36 cells (n = 8 for each group, of which 4 had MPA substitution) in a volume of 300 μl PBS. Seven animals were injected with 300 μl PBS only (of which 4 had MPA substitution). Two mice died without any obvious reason, one at week 6 (EV3, no MPA substitution) and one at week 7 (AB-36, no MPA substitution) and were lost for further analyses. Also at week 7, one mouse was euthanized because of discomfort (PRAB-36 no substitution). This mouse was examined, but no signs could be found that its discomfort was caused by the experimental procedures. This animal was included in the final analyses. At 10 weeks, the remaining animals (n = 36) were killed. Upon decapitation, blood samples were taken. Subsequently, the peritoneal cavity was opened and examined macroscopically. Samples of all abdominal organs, as well as samples of the lungs, heart, brain and upper hind leg muscle were snap frozen in liquid nitrogen for DNA extraction. The remaining tissue was formalin-fixed and processed for routine histological examination (haematoxylin-eosin staining) and immunohistochemistry.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples of internal organs were sliced into 8 μm sections. Every tenth section...
was routinely stained with haematoxylin and eosin, and 
examined microscopically. When a tumour mass 
was detected, consecutive sections were fixed on polylisin-
coated slides for immunohistochemical staining. Endo-
genous peroxidase activity was inhibited and a 10 mM 
citric acid/microwave pre-treatment was applied. PBS 
containing 10% normal swine serum (DAKO, Glostrup, 
Denmark) was used as a blocking buffer, followed by an 
additional avidin/biotin blocking step, according to the 
manufacturer’s protocol. The primary antibodies used 
were directed against PR (hPRα8; LabVision Neomar-
kers, Fremont, CA, USA) and against CD44 (DF1485; 
Santa Cruz Biotechnology, Santa Cruz, CA, USA), 
diluted 1:200 in 1% blocking buffer. Negative control 
slides were incubated with 1% blocking buffer only. 
Antibodies were detected indirectly with biotin-labelled 
swine Multilink antibodies 1:400 in blocking buffer, 
followed by StreptABComplex (DAKO, Glostrup, Den-
mark), according to the manufacturer’s protocol. Staining 
was developed with DAB/concentrated metal complex 
(Pierce). Slides were counterstained with haematoxylin.

PCR detection

PCR detection of tumour cells is based on amplification 
of the hygromycin resistance (HR) gene, which is present 
on the vector that was transfected into the parental 
Ishikawa 3H12 clone, and was performed as described 
(Hanekamp et al. 2003b). The HR gene was amplified by 
nested PCR (forward primer: GTGGATATGTCCTGC-
GGTAA, reverse primer: TCACTGCAATTCTAGTTG- 
TG; nested forward primer: CGCAAGGAATCGGT-
TCAATACA, nested reverse primer: TCGTCCGATCC-
GGAGGAGCC). PCR detection was statistically ana-
lysed (Student’s t-test) using SPSS software (Chicago, IL, 
USA). P values <0.05 were considered significant.

Results

Cell lines

Western blot confirmed the PR status of the cell lines that 
were used, at the time of injection (Fig. 1). EV3 does not 
express PR, PRA-1 expresses only PRA, PRB-1 only 
PRB, and PRAB-36 expresses both PR isotypes (more 
PRB than PRA).

Macroscopic and microscopic detection of 
tumour mass

Upon macroscopic investigation of mice injected with the 
different cell lines, PRB-1 cells clearly produced the 
highest tumour load in the absence of MPA substitution 
(Fig. 2C, Table 1). Most tumour spots had a diameter of 
approximately 1 mm, but on the diaphragm of one mouse 
a tumour mass of approximately 4 mm was found. When 
PRB-1-injected mice were substituted with progestagens, 
tumour could only be detected in two animals (Fig. 2D, 
Table 1). When mice were injected with PRA-1 or PRAB-
36 cells, a distinct tumour mass could be detected in only 
one animal. Substitution with MPA did not have a 
marked effect (Fig. 2, Table 1).

For mice that were injected with EV3 cells (which do 
not express PR) in the absence of progestagens, tumour 
load was virtually undetectable (Fig. 2G, Table 1), while 
substitution with MPA dramatically increased tumour 
burden (Fig. 2H, Table 1). The EV3 tumours were larger 
than those in the other groups, with a diameter of 
approximately 3 mm on average. Also, these tumours 
appeared to be cystic, while PRA-1, PRB-1 and PRAB-36 
tumours had a solid appearance (Fig. 3A, inserts).

Histological findings

In general, tumour masses were detected growing loosely 
connected by stalk-like structures (Fig. 3B, panels I, II, 
IV) and attached to abdominal surfaces (Fig. 3B, panels 
II–VI). All tumours consisted of a non-differentiated mass 
(Fig. 3A), with occasional regions that appeared more 
organized; virtually all tumours (originating from all four 
cell lines) contained blood vessels and lymphocytic 
infiltrations. The EV3 and PRB-1 tumours were found 
to be capable of invading into the pancreas (Fig. 3C) and 
most EV3-tumours showed necrosis, leading to the 
formation of fluid-filled cavities (Fig. 3A), which give 
these tumours a cystic appearance.

The expression of PR was conserved in the PRA-1, 
PRB-1 and PRAB-36 tumours (Fig. 4 panels B, E, H),
Figure 2  Macroscopic anatomy of mice representative of their group. (A and B) PRA-1; (C and D) PRB-1; (E and F) PRAB-36; (G and H) EV3. Mice without MPA substitution are shown on the left (A, C, E, G), mice with MPA substitution are shown on the right (B, D, F, H). The massive abdominal tumour growth in the EV3 mice that were substituted with MPA is clearly visible (H, arrows). Small tumour spots are also visible in the PRB-1 without MPA mice (C, arrows). No tumour is visible in PRA-1 and PRAB-36 mice (A, B, E, F).
Table 1 Mouse tissues in which tumours were detected. Tumours were detected on macroscopical investigation or by PCR detection. Numbers in brackets indicate in how many of the animals tumours were found in that tissue, out of the total number of animals in that group. Singel spots are defined as small (<1 mm) but distinct tumour patches on the serosa covering intraperitoneal organs. Clusters of tumours are defined as a collection of multiple interconnected tumours (on average 3 mm in diameter). These were found only in mice injected with EV3 cells which were treated with MPA (see Fig. 2H, white arrows).

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1 Single spots and clusters of tumours do not qualify as mouse tissue. However, to complete the overview of tumour growth, these macroscopically detected single spots and tumour clusters have also been included in the table.

2 These tumour masses were detected only after microscopy. In the corresponding tissue that was processed for PCR detection, no further tumour mass was detected.
Figure 3  Histological comparison of tumour spots.  (A)  Haematoxylin-eosin stained sections of tumour spots originating from the different cell lines. Small inserts show the macroscopic appearance of the spot, microscopic pictures are haematoxylin-eosin stained sections. The black bar represents 0.05 mm.  (B)  Tumours were found growing from stalk-like structures (I and II, EV3 tumours), and attached to abdominal surfaces: III, EV3 tumour attached to fatty tissue; IV, EV3 tumour growing on ventral abdominal wall; V, PRB-1 tumour growing on ventral abdominal wall; VI, PRB-1 tumour growing on the diaphragm. The black bar represents 0.05 mm.  (C)  Tumours originating from EV3 (upper panel) and PRB-1 (lower panel) cells were found to grow invasively into the pancreas. The black bar represents 0.05 mm.
but was not homogeneous. This is illustrated in Fig. 5, where three tumours from the same mouse (PRB-1, no MPA) were stained to detect expression of PR. Some tumours expressed PR in all cells (Fig. 5A), but heterogeneous staining throughout the tumour (Fig. 5C), or regions of cells expressing PR next to regions that did not express PR (Fig. 5E) were also observed. All tumours expressed CD44 (Fig. 4C, F, I, L), which was used to make a distinction between mouse and human cells (using the current antibody, CD44 could be detected in the four human cell lines but not in any mouse tissues).

Detection of circulating tumour cells

Detection of tumour cells by PCR was possible in all groups (Fig. 6). Tumour cells could be detected predominantly in tissues of mice injected with PRB-1 cells without MPA substitution, and in mice injected with EV3 cells with MPA substitution. The number of tissue samples in which tumour cells could be detected was significantly higher in the group of animals injected with PRB-1 cells ($P < 0.05$), Fig. 6E). While macroscopically and microscopically virtually no tumour mass could be detected in PRA-1 or PRAB-36 mice, tumour cells could
be detected by PCR. Not surprisingly, tumour cells could be detected mostly in the abdominal tissues (A and B), but also outside the abdomen (C). In contrast, no tumour growth outside the abdominal cavity was observed macroscopically. Tumour cells were detected most often in samples of the diaphragm and the pancreas, but also in the lower intestine, the stomach and the mesentery, and less frequently in the spleen, upper intestine, uterus and even kidney. The different cell lines could generally be detected in the same tissues, with no targeting of a specific tissue by specific cell lines.

MPA inhibited the spread of PRB-1 tumour cells \((P = 0.002, \text{Fig. 6E})\), but this was not observed for PRA-1 or PRAB-36 cells. One possible explanation is that the number of positive tissues in the PRA-1- and PRAB-36-injected mice is already very low in the absence of MPA. Substitution of the mice with MPA did not lead to any greater spread of tumour cells to other tissues than when these mice were not substituted with MPA.

In mice injected with EV3 cells, MPA has a significant \((P = 0.001, \text{Fig. 6E})\) tumour cell growth-stimulating effect in the peritoneal cavity: tumour cells could only be detected in the diaphragm of one out of three mice that were not substituted with MPA, while tumour cells could be detected in a total of 12 tissues from three out of four mice substituted with MPA (Fig. 6).

In all mice, no tumour cells could be detected in the blood 10 weeks post injection (Fig. 6D). Previously, we found that tumour cells could be detected in tissues at 3 and 10 weeks post injection, but not at 5 weeks.

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**Figure 5** Variation in homogeneity of PR expression within different tumours in the same mouse. (A) Overall high expression of PR; (C) overall low expression with regions of high expression (arrows indicate regions with high PR expression); (E) only partial expression of PR in tumours taken from one PRB-1 mouse which was not substituted with MPA. Negative control slides are shown on the right (B, D, F). The black bar represents 0.05 mm.
Figure 6 PCR detection of tumour cells in tissues by PCR amplification of the hygromycin resistance gene. The tissues have been divided into different groups as indicated on the left side of the figure: (A) peritoneal membranes; (B) abdominal organs; (C) extraintestinal organs; (D) blood. During the processing of tissues, 6 samples were lost. These are indicated by gaps in the figure. (E) Statistical analysis of the spread of tumour cells as detected by PCR. Numbers of positive tissues per group were compared using Student’s t-test. *P* values are shown of differences between different groups in the absence of MPA treatment, and of the effect of MPA treatment within each group (no MPA vs MPA).

(Hanekamp et al. 2003b). This suggests that early on, the cells are spread through the body but that most of these do not survive. Later, the cells that did survive give rise to a secondary tumour that is large enough to be detected. The absence of circulating tumour cells in the blood confirms that the spread of tumour cells throughout the body is an event that occurs shortly after inoculation. Also, the absence of tumour cells in the blood indicates that the detection of cells in tissues is not due to contamination of these tissues with bloodborne tumour cells.

Table 1 summarizes in which tissues tumour mass was detected macroscopically, microscopically, or by PCR. To complete the overview, macroscopically detected single spots and tumour clusters are also included in Table 1. The macroscopically detected tumour masses were confirmed to be of Ishikawa origin either by microscopical analysis or by PCR detection. Clusters of tumour (as indicated by the arrows in Fig. 2H) were found only in the group of animals injected with EV3 cells which were treated with MPA. In several cases, tumour could be detected by PCR when macroscopically or microscopically...
cally no tumour was found, indicating the higher sensitivity of PCR detection.

**Discussion**

In the present study, we describe how when endometrial cancer cells that express different PR isotypes are injected intraperitoneally into nude mice, cells that express exclusively PRB produce the highest tumour load in the absence of progestagens. Upon substitution with MPA, tumour outgrowth from PRB-expressing cells is inhibited, while PR-negative cells give rise to a massive abdominal tumour load.

Several studies have investigated the effect of progestagens on *in vitro* endometrial cancer cell invasion. It was shown that progestagens stimulated cell-to-cell aggregation (*Fujimoto et al. 1996*) and inhibited anchorage-independent growth (*Dai et al. 2001*) of endometrial cancer cells. *Dai et al. 2002* also showed that MPA could inhibit invasion of Hec50 cells that had been virus-infected to transiently express PRA or PRB. This study did not demonstrate any difference in invasive capacity between cells that expressed different PR isotypes. In contrast, Hanekamp *et al.* (unpublished observations) described how endometrial cancer cells that express PRB are more invasive *in vitro* than cells that express only PRA. In support of this, *Fujimoto et al. 1997* reported that distant metastases of endometrial cancer primarily expressed PRB.

Based on these findings, we expected tumour cells that express PRB to show more extensive growth and spread *in vivo* than cells that express exclusively PRA or no PR. Cells that express exclusively PRB indeed showed the most extensive macroscopic detectable abdominal tumour outgrowth when injected into ovariectomized nude mice. Upon closer examination of different tissues (*microscopy, PCR detection*), this finding could be further extended. These observations are in agreement with reports that distant metastases of endometrial cancer express predominantly PRB (*Fujimoto et al. 1997*), and with our own observations that PRB-1 cells are highly invasive *in vitro* (*Hanekamp et al., unpublished observations*). As expected, growth and spread of PRB-1 cells was clearly inhibited by MPA substitution. This confirms previous findings in which progestagens inhibit growth and invasion of endometrial cancer cells (*Fujimoto et al. 1996, Ueda et al. 1996, Dai et al. 2001, 2002*). Injection of PRA-1 cells only resulted in minimal tumour growth and spread, which is also consistent with its minimal *in vitro* invasive capacity (*Hanekamp et al., unpublished observations*). The PRAB-36 cell line expresses more PRB than PRA, and has an *in vitro* invasive capacity similar to that of PRB-1 cells. Therefore, we expected that this cell line would behave more like the exclusively PRB-expressing cell line than the exclusively PRA-expressing line. However, injection of PRAB-36 cells did not result in tumour growth and spread similar to PRB-1 cells; rather, it was similar to PRA-1 cells. While this behaviour of PRAB-36 cells was not as predicted, the observed minimal tumour growth and spread does represent the behaviour of such an endometrial cancer in patients: endometrial cancers that express both PRA and PRB are often well-differentiated and stay primarily confined to the uterus.

In the present study, inoculation of PR-negative endometrial cancer cells in MPA-substituted mice resulted in massive abdominal tumour growth. This is in contrast to the *in vitro* situation, where it was observed that these PR-negative cells did not show any response to MPA stimulation. On the basis of this observation, it is thought that MPA acts on the PR-negative cells through the tumour-bearing host. MPA treatment of the mice probably results in a favourable environment for attachment and growth of the injected cells. Because no stimulation of PRA-1, PRB-1 or PRAB-36 cells was observed in the mice, the inhibiting effect of MPA on these cells is most likely dominant over any secondary stimulating effect of MPA through the host. Based on these observations, the following hypothesis is put forward: on the one hand, MPA provides a favourable environment for attachment and growth of endometrial cancer cells via the host, but on the other hand, it inhibits growth of PR-positive tumour cells directly.

An intriguing question arises: how do the present findings relate to the situation in the clinic? Since endometrial cancer is predominantly a disease of postmenopausal women, patients generally have very low endogenous levels of circulating progestagens. In clinical practice many women with advanced or recurrent endometrial cancer are treated with progestagens on the basis that this will inhibit growth of the cancer and will generally improve well-being of the patient. In fact, 20% of patients with advanced or recurrent endometrial cancer do respond to MPA therapy (*Elit & Hirte 2002*). The results of our study lead us to question whether the remaining 80% of these tumours really do show no response, or if tumour growth may in fact be stimulated by progestagen therapy? At this moment, we cannot answer this question. However, if indeed progestagens indirectly stimulate growth of PR-negative endometrial neoplasias, the practice of administering progestagens to endometrial cancer patients regardless of their progesterone receptor status should be re-evaluated.

In summary, the results of this study support the idea that loss of expression of, especially, PRA is associated with a more aggressive type of endometrial cancer. Furthermore, treatment with MPA seems to inhibit the
growth and spread of PR-positive tumours. However, when expression of both PRA and PRB is lost, treatment with MPA may provide a favourable environment for attachment and growth of endometrial cancer cells. Because of possible implications for patient treatment, the dynamics of this phenomenon need to be further investigated, preferably with an in vivo monitoring system.

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