N-cadherin increases after androgen deprivation and is associated with metastasis in prostate cancer

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Short title: Androgen deprivation upregulates N-cadherin

Key words: Prostate cancer; N-cadherin; castration-resistant; castration; LNCaP-19; E-cadherin
ABSTRACT

Androgen deprivation therapy (ADT) is standard treatment for metastatic prostate cancer. One factor that has been implicated in the metastatic process is the cell adhesion molecule N-cadherin. In this study we investigated if expression of N-cadherin was influenced by androgen deprivation and associated with metastasis in prostate cancer. The effect of androgen deprivation on N-cadherin expression was initially studied in androgen-dependent (AD) LNCaP and androgen-independent (AI) LNCaP-19 and PC-3 prostate cancer cell lines. Expression of N-cadherin increased in absence of androgens in AI LNCaP-19 primary tumors and metastases and also in vitro, but not in AI PC-3, indicating a possible involvement of the androgen receptor in the regulation of N-cadherin. N-cadherin was absent in AD LNCaP. No clear associations between N-cadherin and factors related with epithelial to mesenchymal transition (EMT) or neuroendocrine (NE) differentiation could be established. In addition, N-cadherin was evaluated by immunohistochemistry in human prostate tumors. Expression of N-cadherin was more frequently found in tumors from patients treated with ADT than in tumors from patients with no prior hormonal treatment. N-cadherin expression was also associated with metastasis and Gleason score. Further, increased N-cadherin was detected in prostate cancer biopsies already three months after initiation of ADT when tumors were in a regressed state. The results indicate that androgen deprivation induces N-cadherin in prostate tumors. Moreover, N-cadherin was increased in castration-resistant tumors in patients with established metastases. This might indicate that castration induces molecular alterations in the tumor cells, resulting in a more invasive and metastatic phenotype.
INTRODUCTION

Most cancer deaths are due to metastatic disease. The metastatic process involves several steps, including detachment from the primary tumor, migration, intravasation into vessels and establishment at a new site (Chambers et al. 2002). N-cadherin is one factor that has been implicated in the metastatic process. In normal tissue, N-cadherin is highly expressed in the nervous system but it is also found in the vascular endothelium and myocardium (Hatta et al. 1987; Salomon et al. 1992). It is located to adherens junctions and with its extracellular domain it mediates a dynamic contact between cells and between cells and matrix (Hazan et al. 1997). In addition, the cytoplasmic domain is involved in multiple intracellular signaling pathways (Derycke and Bracke 2004). In several human cancers, expression of N-cadherin is found to be elevated (Derycke and Bracke 2004). Increased expression of N-cadherin is, together with loss of the tumor suppressor E-cadherin, one feature of epithelial to mesenchymal transition (EMT) that takes place during cancer progression, and this “cadherin switching” plays an essential role for the motility of cancer cells (Maeda et al. 2005). The EMT process is also clearly of importance for metastasis of prostate cancer (Xie et al. 2010). In contrast to N-cadherin, E-cadherin mediates homotypic interactions between cells, which maintain the integrity of epithelial tissues (Giehl and Menke 2008). Functional studies show that N-cadherin makes tumor cells more motile and promotes invasiveness and metastasis in experimental models of cancer (Hazan et al. 1997; Hazan et al. 2000; Li et al. 2001). The most possible mechanism by which N-cadherin renders tumor cells more motile is through the homophilic adhesion to other cells. Firstly, N-cadherin mediates a dynamic cell adhesion resulting in weaker interactions between adjacent cells than E-cadherin (Chu et al. 2004). Thus, it allows the dissociation of single cells from the primary tumor. Secondly, homophilic interactions between tumor cells and N-cadherin expressing tissues, such as the stroma and vasculature facilitate the transit through the tissue and survival of tumor cells in distant organs (Sandig et al. 1997). Moreover, the invasive
capacity of N-cadherin is in part due to an interaction with the fibroblast growth factor receptor 1 (FGFR-1) (Suyama et al. 2002). In addition to the prometastatic properties of N-cadherin, aberrant expression in cancer has also been shown to inhibit apoptosis (Li et al. 2001; Tran et al. 2002) and promote angiogenesis (Derycke et al. 2006a; Derycke et al. 2006b). An N-cadherin antagonist, ADH-1 (Exherin™) has been evaluated in experimental models of cancer and is at present in clinical trials for treatment of solid tumors (Augustine et al. 2008; Beasley et al. 2009; Perotti et al. 2009; Shintani et al. 2008).

In a previous study, we reported that a switch from E-cadherin to N-cadherin was associated with transition of androgen-dependent (AD) LNCaP into androgen-independent (AI) LNCaP-19 prostate cancer cells (Jennbacken et al. 2006). This switch has also been reported to occur in other AI cell lines (Bussemakers et al. 2000; Tran et al. 1999). In human prostate cancer, N-cadherin expression has been found in poorly differentiated areas (Bussemakers et al. 2000; Tomita et al. 2000) and its expression correlated with Gleason score (Jaggi et al. 2006). In a recent study it was shown that N-cadherin expression in human prostate tumors was associated with pelvic lymph node infiltration and shorter time to skeletal metastasis (Gravdal et al. 2007).

The established treatment of locally advanced and metastatic prostate cancer is androgen deprivation therapy (ADT). ADT is initially successful, resulting in reduced tumor burden that could last for several months or even years. However, eventually an androgen-independent tumor (i.e. castration-resistant tumor) with an aggressive and metastatic phenotype relapses. Treatment in the castration-resistant stage is today never curative, only palliative, and includes continued hormonal therapy, radiation or cytotoxic treatment (Damber and Aus 2008). Transition into androgen-independency is not clearly understood but several mechanisms involving the androgen receptor (AR) have been suggested to be of importance (Gregory et al. 2001; Linja et al. 2001; Marcelli et al. 2000). To
improve treatment in the castration-resistant stage there is a need to identify regulators of aggressive and metastatic prostate cancer and clarify which molecular alterations that are induced by ADT. In contrast to E-cadherin, the expression of N-cadherin in prostate cancer is not extensively studied and there is no study addressing the association of N-cadherin to tumor progression after ADT. The objective of the present study was to investigate how the expression of N-cadherin was influenced by androgen deprivation, both in prostate cancer cell lines and in human prostate tumors.
MATERIALS AND METHODS

Cell lines and culture conditions

LNCaP cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and its subline LNCaP-19 was previously established in our laboratory (Gustavsson et al. 2005). PC-3 cells were obtained from European Collection of Cell Cultures (ECCC, Wiltshire, UK). Cells were maintained as previously described (Tesan et al. 2008).

For RNA analyses, cells were cultured for 10 days in 10% DCC-FBS (steroid-depleted serum), without or with addition of the synthetic androgen R1881 (NEN Life Science Products, Inc., Boston, MA, USA) in different concentrations (0.1 nM and 1 nM). In addition, a proportion of the LNCaP-19 cells were first cultured in absence of androgens for 10 days followed by androgen stimulation (0.1 nM or 1 nM) for 7 days. The experiment was repeated three times.

Animals and implantation of tumor cells

Male athymic BALB/c nude mice, 8 weeks old, were purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA) and housed in a controlled environment. The use of animals was approved by the animal ethical committee in Gothenburg.

For subcutaneous implantation, two million tumor cells suspended in equal volumes of medium and matrigel (BD Bioscience, Bedford, MA, USA) were inoculated on the flank of the mice. Castration or sham-operation was performed via a scrotal incision under anesthesia prior to cell injection. Orthotopic implantation of tumor cells was performed as previously described (Jennbacken et al. 2009). Briefly, one million tumor cells suspended in 7 µl matrigel (BD Bioscience) were injected into the dorsolateral lobe of the prostate. In experiments involving castrated mice, castration was
performed prior to cell injection, via the abdominal incision. The animal experiments were discontinued after 9 weeks. Tumors were harvested and one part was fixed in formalin for paraffin-embedding and the other part was frozen in liquid nitrogen and stored at -80 °C prior to protein and RNA preparation.

For the metastasis experiment, male SCID CB17 mice, 7 weeks old, were used (Charles River Laboratories International, Inc.). LNCaP-19 cells were implanted in the prostate in intact and castrated mice as described above. After 15 weeks, mice were sacrificed and examined for metastasis. Primary tumors and macroscopic lymph node metastases were collected and tissues were treated as described above.

Patient characteristics and tissue specimens

Formalin-fixed and paraffin-embedded tissues were obtained from patients with prostate cancer from the Department of Urology, Sahlgrenska University Hospital, Gothenburg, Sweden. The studies with human material were conducted with ethical approval of the local research ethical committee.

TURP samples: Tissue specimens were obtained from 54 patients by transurethral resection of the prostate (TURP). 28 patients had TURP-diagnosed untreated prostate cancer in stage T1b and 25 patients had recurrent castration-resistant prostate cancer after ADT. One of the patients in the castration-resistant group underwent TURP at two different occasions and therefore the tissue specimens in this group were 26. In the hormone naïve T1b group mean age was 77 years (range 60-90) and mean Gleason score was 6.6 (range 5-9). In the castration-resistant group, mean age was 79 years (range 65-88) and mean Gleason score was 9.1 (range 7-10).

Biopsy samples: Tissue specimens were obtained from 28 patients by needle biopsies. Biopsies were obtained sequentially during prostate cancer progression and prostate specific antigen (PSA) was measured in serum at the same time points. Biopsy 1: sampled at the time of prostate cancer
diagnosis prior to ADT. Biopsy 2: sampled approximately three months after initiation of ADT. The androgen deprivation therapy consisted of medical or surgical castration. Biopsy 3: sampled when the tumor relapsed as indicated by a rise in PSA or when the patient had symptomatic progression. The third biopsy was sampled from 10 of the 28 patients. Mean Gleason score in the groups were 7.3, 7.2 and 8.1 respectively. Mean age at the time of diagnosis was 73 years (range 55-86).

**RNA preparation and real-time RT-PCR**

Total RNA from tumors was extracted as previously described (Jennbacken et al. 2009) and total RNA from cultured cells was extracted by using the RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany). RNA quality and concentration were measured using a bioanalyzer (Agilent 2100, Agilent Technologies Inc., Santa Clara, CA, USA) and nanodrop, respectively. The RNA was reversely transcribed into cDNA as previously described (Tesan et al. 2008).

Real-time RT-PCR was performed with the ABI Prism 7500 Fast Sequence Detector (Applied Biosystems, Applera Corporation, Foster City, CA, USA). PCR primers and TaqMan MGB probes targeting N-cadherin (Hs00983062_m1), E-cadherin (Hs00170423_m1), Vimentin (Hs00958116_m1), Snail (Hs00195591_m1), Slug (Hs00161904_m1), Twist1 (Hs00361186_m1), ZEB1 (Hs00232783_m1) and endogenous control 18S rRNA (Hs99999901) were purchased as TaqMan Gene expression Assays (Applied Biosystems). PCR parameters were according to the manufacturer’s protocol and the ∆∆Ct method was used for relative mRNA quantification. PCR reactions for target genes and control were performed in duplicates for all samples and repeated twice.
Protein preparation and western blotting

Total protein was prepared from tumor tissue by homogenization and sonication in presence of protease inhibitors (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). Protein concentrations were measured using the BCA Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer’s protocol. Western blotting was performed as previously described (Jennbacken et al. 2006). Primary antibodies used were N-cadherin (1/500 #610921, BD Biosciences) and E-cadherin (1/1000, #610182, BD Biosciences). Actin (1:2000, A2066, Sigma–Aldrich, St Louis, MO, USA) was used as loading control. As positive control for the antibodies, rat brain was used for N-cadherin and ovcar-3 cell lysate was used for E-cadherin (Jennbacken et al. 2006). Chemiluminescent signals were visualized by a LAS-4000 CCD camera (Fujifilm, Tokyo, Japan).

Immunohistochemistry

Tissue sections, 4 µm, were deparaffinized and rehydrated. Immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) as previously described (Jennbacken et al. 2006). Primary antibodies were against N-cadherin (1/100 for the TURP specimens and 1/150 for the biopsies, #M3613, DAKO, Glostrup, Denmark), E-cadherin (1/300 for the xenografts and 1/10000 for the human biopsies, #610182, BD Biosciences), Chromogranin A (1/500 #Ab-1 LK2H10, Neomarkers, Freemont, CA, USA) and Neuro specific enolase (NSE) (1/5000 #Ab-1, clone E27, Neomarkers). Negative controls were performed by omitting primary antibodies. Pancreas was used as positive control for Chromogranin A and NSE.
Evaluation of N-cadherin and E-cadherin immunostaining in human samples

N-cadherin was evaluated as the proportion of positive tumors cells in the whole section. There was no large variation in staining intensity between tumors and therefore intensity was not taken into consideration. Proportion of positive cells was scored as 0 = no positive cells; 1 = <25% positive cells; 2 = 25-50% positive cells; 3 = 50-75% positive cells; 4 = >75% positive cells.

For E-cadherin, intensity varied considerably between tumors, and E-cadherin was therefore evaluated by combining the proportion of positive tumor cells with staining intensity. Proportion of positive cells was scored as 0 = no positive cells; 1 = <1/3 positive cells; 2 = 1/3-2/3 positive cells; 3 = >2/3 positive cells. Intensity was scored as 0 = no detectable signal; 1 = weak staining; 2 = moderate staining; 3 = strong staining. The total score was obtained by multiplying the intensity score with the positive number score. Sections were evaluated in a blinded fashion in 200 x magnification in a light microscopy.

Statistical analyses

Mann-Whitney U test was used to analyze differences between independent groups and Wilcoxon signed rank test was used to analyze differences between paired groups (i.e. human biopsy material). Spearman’s rank correlation test was used to analyze correlations. Comparison of the proportion of positive N-cadherin cases in different groups was analyzed with Chi-square test. Data are presented as mean ±SEM. A P-value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 16.0 software for Windows.
RESULTS

N-cadherin increased by castration in AI LNCaP-19 xenografts

In this study, we investigated whether N-cadherin was affected by androgen deprivation, which is commonly used to treat advanced stage prostate cancer. We found that castration increased N-cadherin protein levels in the AI LNCaP-19 tumors. The negative regulation of N-cadherin by androgens was observed both in subcutaneous and orthotopic tumors (fig. 1a, b). The difference was also observed at mRNA levels (data not shown). The AI PC-3, which in contrast to LNCaP-19 does not express AR, also displayed N-cadherin but no androgen regulation was found. In contrast, N-cadherin was absent in LNCaP and the expression was not induced by castration (fig. 1a, b).

Inoculation of LNCaP-19 cells in the prostate of SCID mice resulted in formation of macroscopic metastases to the lymph nodes (sacral, lumbar and renal). Similarly to the primary tumors, higher expression of N-cadherin was detected in metastases from castrated mice than from intact mice. No major differences in N-cadherin expression between primary tumors and metastases in the intact or castrated group were found (fig. 1c).

N-cadherin increased by androgen deprivation in AI LNCaP-19 in vitro

To investigate if androgens affected N-cadherin directly or via the surrounding tumor environment, in vitro experiments were performed. Corresponding to the in vivo experiments, androgen deprivation significantly increased N-cadherin mRNA expression in the LNCaP-19 cells. The expression increased about three times in comparison to cultures in presence of androgens (0.1 nM and 1 nM R1881). Furthermore, the upregulation of N-cadherin in LNCaP-19 could be reversed if
synthetic androgens were added and there was a negative association between N-cadherin expression and androgen concentration (fig. 2).

In PC-3, N-cadherin mRNA could be detected, but its expression was not altered by removal of androgens. The LNCaP cells did not express detectable levels of N-cadherin mRNA in cultures, neither in presence or absence of androgen (fig. 2).

E-cadherin decreased after castration in orthotopic AI LNCaP-19 xenografts

Increased levels of N-cadherin are often accompanied by a concomitant decrease in E-cadherin. Therefore, we investigated if E-cadherin decreased by androgen deprivation in LNCaP-19. In subcutaneous LNCaP-19 tumors, castration did not alter the E-cadherin levels (fig. 3a). In contrast, castration reduced E-cadherin level in the orthotopic tumors (fig. 3b). This result was confirmed by immunohistochemistry. E-cadherin was uniformly distributed at the surface of the tumor cells in orthotopic LNCaP-19 from intact mice (fig. 3c). However, in the tumors from castrated mice, the staining was weaker, more scattered and not present on every cell (fig. 3d).

Androgens did not downregulate E-cadherin in the LNCaP-19 cells in vitro, showing the importance of the prostatic environment for this process (data not shown).

Expression of transcription factors related to EMT and neuroendocrine markers

To address if upregulation of N-cadherin was related to EMT, expression of the mesenchymal marker Vimentin and of transcription factors known to activate N-cadherin during induction of EMT, was investigated with real-time PCR in the animal model. However, there were no apparent differences in the mRNA levels of Twist1, Snail, Slug, ZEB1 or Vimentin that could explain the increased levels of N-cadherin observed after castration (data not shown). To investigate whether N-cadherin was part of the neuroendocrine (NE) differentiation often seen in prostate cancer after ADT,
markers for NE was studied by immunohistochemistry in the animal model. No association between N-cadherin and Chromogranin A or NSE was detected (data not shown). These results indicate that the upregulation of N-cadherin was not associated with EMT or NE.

N-cadherin increased in castration-resistant prostate cancer and was associated with metastasis

N-cadherin was evaluated in hormone-naïve and castration-resistant TURP prostate cancer samples by immunohistochemistry. N-cadherin staining was mainly located to the plasma membrane of the tumor cells, but some areas also showed cytoplasmic staining (fig. 4a). Benign prostate epithelial cells were negative (fig. 4b). In addition to the tumor cells, macrophages showed positive reactions and served as a convenient internal positive control. In the hormone-naïve group, 50% (14/28) of the tumors were positive for N-cadherin, compared to 81% (21/26) in the castration-resistant group ($P = 0.018$). In addition, the castration-resistant tumors had a significantly higher N-cadherin score, than the hormone-naïve tumors (1.42 vs. 0.79 respectively, $P = 0.023$) (fig. 4c). There was also a positive correlation between the N-cadherin score and the Gleason score (Spearman’s correlation coefficient $= 0.485$, $P < 0.001$) (data not shown). Further, the castration-resistant group was divided into two groups based on the metastasis status of the patients. In tumors from patients with established metastases, 93% (14/15) were N-cadherin positive, compared to 56% (5/9) of the tumors from patients without known metastases ($P = 0.027$). Tumors from patients with metastases (M1) also displayed a significantly higher N-cadherin score compared to tumors from patients without metastases (M0) (1.73 vs. 0.78 respectively, $P = 0.048$) (fig. 4d).
N-cadherin increased after initiation of ADT in human prostate cancer

To evaluate the direct effect of ADT on N-cadherin expression in human prostate cancer, N-cadherin was studied in prostate biopsies taken sequentially during tumor progression. N-cadherin was found in 46% (13/28) of the biopsies sampled at diagnosis (biopsy 1) and the mean N-cadherin score was 0.7. Three months after initiation of ADT (biopsy 2), 82% (23/28) of the biopsies displayed N-cadherin staining and the mean score had increased to 1.9, which was statistically significant from biopsy 1 ($P < 0.001$). In the relapsed castration-resistant group (biopsy 3), 70% (7/10) of the biopsies were positive and the mean N-cadherin score was 2.4, which was also statistically significant from biopsy 1 ($P = 0.038$) (fig. 5a).

Immunostaining of E-cadherin was also evaluated in the biopsy material. E-cadherin was located to the cell membrane of benign epithelial cells and tumor cells. A majority of the cases in each group were positive for E-cadherin and there was no major difference in the E-cadherin score between biopsy 1, 2 and 3 (4.1 vs. 3.1 vs. 4.7) (fig 5b).

The patients’ PSA value was measured at the time when biopsies were collected. The PSA values declined ten fold after initiation of ADT, showing that the tumors responded to the treatment. At tumor relapse, the PSA value increased approximately four fold compared to the low level at the time of biopsy 2 (fig. 5c).
DISCUSSION

In this study, we showed that N-cadherin was induced by androgen deprivation in experimental AI prostate cancer as well as in human prostate tumors. Moreover, expression of N-cadherin in human samples was associated with metastasis and Gleason score.

To our knowledge, the downregulation of N-cadherin by androgens in prostate cancer has not previously been reported. In contrast, androgenic upregulation of N-cadherin has earlier been described in motoneurons (Monks and Watson 2001). The N-cadherin gene contains a cluster of androgen response elements (AREs) in its intron 1 (Takayama et al. 2007), and it is possible that the repressed N-cadherin gene expression is attributed to a direct inhibition by binding of activated AR to this site. Even though the main effect by androgens on prostate cells through the AR is stimulatory, inducing proliferation and anti-apoptotic responses, negative regulation by androgens has also been reported (Li et al. 2005; Terry et al. 2009; Wikstrom et al. 1999). In this study, expression of N-cadherin was found in the AI cell lines LNCaP-19 and PC-3. However, it was only in the AR positive LNCaP-19 that N-cadherin levels could be elevated further by androgen deprivation, thus demonstrating the importance of AR in the regulation of N-cadherin. Exactly how AR and androgens regulate N-cadherin are at present unknown and further studies are needed to be able to address this issue.

In general, the regulation of E- and N-cadherin is believed to be tightly connected, resulting in a switch from one to another. In this study we observed different E-cadherin responses to castration, depending on the location of the tumor. In the orthotopic LNCaP-19 tumors, the upregulation of N-cadherin after castration was accompanied with decreased E-cadherin levels, as expected. However, this response could not be observed by androgen deprivation in vitro or in subcutaneous LNCaP-19 tumors. The reduction in E-cadherin in the orthotopic tumors from castrated mice was therefore probably an indirect effect mediated by the surrounding prostatic environment. Further, there was no
obvious increase in the mesenchymal marker Vimentin or the transcription factors Twist, Snail, Slug or ZEB1 in the animal model that accompanied the increased N-cadherin levels. The results presented here therefore indicate a distinct regulatory mechanism of N-cadherin, which is not connected to the EMT and E-cadherin regulatory mechanisms.

In this study, N-cadherin was more frequently expressed in castration-resistant tumors compared to early stage T1b prostate tumors, which is not unexpected, since it has been reported that N-cadherin increases in poorly differentiated prostate cancer and correlates to Gleason grade (Bussemakers et al. 2000; Jaggi et al. 2006; Tomita et al. 2000). Moreover, a switch from E-cadherin to N-cadherin has been correlated to the progression of prostate cancer in hormone naïve tumors and expression of N-cadherin was associated with pelvic lymph node infiltration and shorter time to skeletal metastases (Gravdal et al. 2007). Whether the increase in N-cadherin observed after initiation of ADT really leads to a poor outcome for the patients and if it influences the propensity for tumor cells to form metastases remains to be clarified. However, a possible clue could be that patients with established metastases in this study more frequently expressed N-cadherin than patients without known metastases. The finding that N-cadherin was increased already three months after initiation of ADT (biopsy 2) and not only in the relapsed castration-resistant cases (biopsy 3), indicates that it could be a direct consequence of androgen deprivation and not of transition into the castration-resistant stage.

In the literature, N-cadherin is mostly described as a factor that promotes migration and metastasis of tumor cells. However, N-cadherin has also been reported to be involved in survival of tumor cells, via induction of anti-apoptotic pathways (Hazan et al. 2000; Tran et al. 2002). Consequently, induction of N-cadherin after ADT can promote a survival advantage for tumor cells at low androgen levels.

ADT is standard treatment for patients with locally advanced or metastatic prostate cancer. The benefits for the patients are indisputable and it has even been suggested that ADT should be initiated
in an early phase of the disease to extend patient survival (Anderson 1999). However, conflicting results are reported in the literature, regarding the effects of androgen deprivation on the cellular level. Disruption of the androgen signaling pathway by ADT may result in deregulation of the cell control, which could contribute to the carcinogenic process. Early initiation of ADT might therefore speed the development of castration-resistant disease. In addition, it has been suggested that treatment with anti-androgens such as bicalutamide could induce alterations in the prostatic environment that promote emergence of castration-resistant tumors (Lee and Tenniswood 2004). Studies have shown that an intact androgen signaling pathway in prostate tumor cells decreased invasion and metastasis in animal models (Cinar et al. 2001; Niu et al. 2008). In contrast, there are also reports showing that testosterone signaling via AR promotes invasion of prostate tumor cells \textit{in vitro} (Hara et al. 2008). Because of the contradicting data in the literature, further studies emphasizing the molecular effects of androgen deprivation on prostate cancer cells are clearly needed.

In the present study we show that the proinvasive factor N-cadherin was increased by androgen deprivation and correspondingly, there are other reports in the literature supporting the possibility that androgen deprivation could induce the metastatic machinery in the cells. Nestin, which is an intermediate filament protein that has a role in metastasis of tumor cells, and the lymphatic growth factor Vascular Endothelial Growth Factor C (VEGF-C) are induced in response to androgen withdrawal (Kleeberger et al. 2007; Li et al. 2005; Rinaldo et al. 2007). Recently, there was also a report of augmented levels of class III \(\beta\)-tubulin after androgen ablation and in castration-resistant prostate tumors, which could explain resistance to therapy with taxanes in this disease stage (Terry et al. 2009). With these results in hand it is tempting to speculate that ADT induces molecular alterations in the tumor cells that will give the them an advantage in forming metastases in the relapsed castration-resistant stage.
Increased levels of N-cadherin in poorly differentiated and castration-resistant prostate cancer specimens open for the possibility to use N-cadherin antagonists as second-line therapy for castration-resistant prostate cancer. At present, an anti-N-cadherin peptide, ADH-1 (Exherin™) is being evaluated in clinical trials against N-cadherin expressing tumors. Our results warrant further investigations of the role of N-cadherin in aggressive prostate cancer. Targeted therapy using an N-cadherin antagonist in combination with chemotherapy or other targeted therapies could be a novel approach also for treating metastatic and castration-resistant prostate cancer.

Taken together, this study demonstrates that N-cadherin, a potentially important factor for formation of metastases, was induced by androgen deprivation in experimental AI prostate cancer and in human prostate cancer. Further, expression of N-cadherin showed a positive association with Gleason score and metastases. The results might indicate that ADT directly influences the prostate cancer cells to acquire properties associated with the metastatic phenotype observed in castration-resistant prostate cancer.
DECLARATIONS OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

FUNDING

This work was supported by the Swedish Cancer Society, Sahlgrenska University Hospital, Swedish Johanniterhjälpen, Swedish Medical Society, Research Foundations of af Jochnick, C. & S. Hagströmer, A. Gabrielsson, M. Bergvall, Å. Wiberg, L. Hierta, A. & B. Björnsson, W. & M. Lundgren, M. & G. Ågren and H. Fries.

ACKNOWLEDGEMENTS

The authors would like to thank Anita Fae and Karina Jernsand for excellent technical assistance during animal experiments and laboratory work. We also thank Ulric Pedersen for help with the illustrations.
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FIGURE LEGENDS

Fig. 1. N-cadherin expression in tumor xenografts analyzed by western blotting. A) Subcutaneous and B) orthotopic LNCaP, LNCaP-19 and PC-3 tumors grown in intact or castrated mice. Images show two representative samples of at least three in each group. C) N-cadherin expression in orthotopic LNCaP-19 primary tumors and lymph node metastases from intact and castrated mice. Actin was used as a loading control. N-cadherin was detected at a molecular size of 130 kDa and actin at 42 kDa. Int = tumors from intact mice; cast = tumors from castrated mice; p = primary tumor; m = lymph node metastasis.

Fig. 2. N-cadherin expression in vitro was analyzed by real-time RT-PCR. Cells were cultured in presence of androgens (0.1 nM or 1 nM R1881) or in absence of androgens (0 nM R1881) for 10 days and the N-cadherin mRNA was then analyzed (indicated day 10 in the figure). To investigate if the increase in N-cadherin by androgen deprivation could be reversed, a proportion of the LNCaP-19 cells cultured in absence of androgens were continued in culture and androgens were added (0.1 nM or 1 nM). After 7 days, N-cadherin mRNA was analyzed (indicated day 10+7 in the figure). The ∆∆Ct method was used to calculate relative expression and 18S was used as endogenous control. Results are presented as mean ±SEM (n=3).

Fig. 3. Expression of E-cadherin protein in LNCaP-19 tumor xenografts. Western blot images of A) subcutaneous and B) orthotopic LNCaP-19 tumors grown in intact or castrated mice. Images show two representative samples of five in each group. Actin was used as a loading control. E-cadherin was detected at a molecular size of 120 kDa and actin at 42 kDa. The downregulation of E-cadherin in orthotopic tumors from castrated mice was confirmed by immunohistochemistry. Immunostaining
was localized to the cell membrane. C) Tumors from intact mice showed a homogeneous positive E-cadherin reaction from most tumor cells, while E-cadherin expression in D) tumors from castrated mice showed a more scattered staining pattern (arrows) and were not present on every cell. Magnification 400 x.

Fig. 4. Expression of N-cadherin in human prostate cancer TURP specimens was evaluated by immunohistochemistry. A scoring system for the proportion of positive tumor cells in the section was used. A) N-cadherin was located to the plasma membrane of the cancer cells and some areas also showed cytoplasmic staining. B) Benign prostate epithelial cells were negative (arrow). Positive N-cadherin staining from cancer is indicated by an arrowhead. Magnification 200 x. Magnification in insert 400 x. Mean N-cadherin score in C) hormone-naïve (HN) and castration-resistant (CR) tumors and D) in prostate tumors from patients with established metastases (M1) and patients without known metastases (M0). Data are presented as mean ±SEM. * P < 0.05 vs. HN/M0.

Fig. 5. Expression of N-cadherin and E-cadherin in human prostate cancer biopsies was evaluated by immunohistochemistry. For N-cadherin, a scoring system for the proportion of positive tumor cells in the section was used. For E-cadherin, proportion of positive tumor cells was combined with staining intensity. Biopsies were collected sequentially from untreated, hormone-naïve tumors at the time of prostate cancer diagnosis (biopsy 1), three months after initiation of ADT (biopsy 2) and from the recurrent castration-resistant tumors (biopsy 3). A) The N-cadherin score and B) the E-cadherin score in the tumor biopsies. C) Relative PSA values at the time when the prostate cancer biopsies were taken. Data are presented as mean ±SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001 versus biopsy 1.
160x57mm (300 x 300 DPI)
160x113mm (300 x 300 DPI)