Adrenomedullin Blockade Suppresses Growth of Human Hormone–Independent Prostate Tumor Xenograft in Mice

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Abstract

Purpose: To study the role of the adrenomedullin system [adrenomedullin and its receptors (AMR), CLR, RAMP2, and RAMP3] in prostate cancer androgen-independent growth.

Experimental Design: Androgen-dependent and -independent prostate cancer models were used to investigate the role and mechanisms of adrenomedullin in prostate cancer hormone-independent growth and tumor-associated angiogenesis and lymphangiogenesis.

Results: Adrenomedullin and AMR were immunohistochemically localized in the carcinomatous epithelial compartment of prostate cancer specimens of high grade (Gleason score >7), suggesting a role of the adrenomedullin system in prostate cancer growth. We used the androgen-independent Du145 cells, for which we demonstrate that adrenomedullin stimulated cell proliferation in vitro through the cAMP/CRAF/MEK/ERK pathway. The proliferation of Du145 and PC3 cells is decreased by anti-adrenomedullin antibody (αAM), supporting the fact that adrenomedullin may function as a potent autocrine/paracrine growth factor for prostate cancer androgen-independent cells. In vivo, αAM therapy inhibits the growth of Du145 androgen-independent xenografts and interestingly of LNCaP androgen-dependent xenografts only in castrated animals, suggesting strongly that adrenomedullin might play an important role in tumor regrowth following androgen ablation. Histologic examination of αAM-treated tumors showed evidence of disruption of tumor vascularity, with depletion of vascular as well as lymphatic endothelial cells and pericytes, and increased lymphatic endothelial cell apoptosis. Importantly, αAM potently blocks tumor-associated lymphangiogenesis, but does not affect established vasculature and lymphatic vessels in normal adult mice.

Conclusions: We conclude that expression of adrenomedullin upon androgen ablation in prostate cancer plays an important role in hormone-independent tumor growth and in neovascularization by supplying/amplifying signals essential for pathologic neangiogenesis and lymphangiogenesis. Clin Cancer Res; 19(22); 6138–50. © 2013 AACR.

Introduction

Prostate cancer is the most diagnosed malignant growth in men and is the second leading cause of male cancer-related deaths in the majority of Western countries. The cancerous gland usually contains multiple independent and genetically distinct lesions, demonstrating heterogeneity of the disease (1, 2). Because pathologic growth of the prostate is controlled largely by steroid androgens, treatment of locally advanced or metastatic disease relies heavily on hormonal therapies that target the androgen receptor. A major limitation of hormonal therapy, however, is that it offers only temporary relief; the cancer eventually reappears as an androgen-independent lesion characterized by aggressive growth and invasion of distal organs, predominantly the bone (3). Whether it is clonal expansion with adaptation of the substitutive pathway, development of androgen-independent prostate cancer shows clearly that factors other than, or together with, low levels of androgen must exist to provide survival and growth instructions to the androgen-independent cells.

Adrenomedullin is a multifunctional peptide with properties ranging from inducing vasorelaxation to acting as a regulator of cellular growth and angiogenesis (4–7). Adrenomedullin binds and mediates its activity through the
Adrenomedullin and adrenomedullin receptors were immunohistochemically localized in the carcinomatous epithelial compartment of high-grade adenocarcinomas (Gleason score >7) of prostate cancer specimens, suggesting a role of the adrenomedullin system in the growth of hormone-independent prostate cancer. Adrenomedullin functions as an autocrine/paracrine growth factor to stimulate proliferation of androgen-independent Du145 and PC3 cells, whose effect is inhibited by a neutralizing anti-adrenomedullin antibody causing growth cessation in vitro. The in vivo study highlights the significance of adrenomedullin as an important factor to promote androgen-independent prostate cancer tumor growth and to affect the tumor microenvironment by inducing pathologic neoangiogenesis and lymphangiogenesis. Targeting the adrenomedullin system may provide a rational basis for future therapeutic modalities upon androgen ablation in prostate cancer.

**Translational Relevance**

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**Materials and Methods**

**Human prostate specimens**

We analyzed human prostate specimens from 8 patients with prostate cancer of high-grade adenocarcinomas (Gleason score >7) at the Department of Urology (AP-HM, Marseille, France). Paraffin-embedded tumor specimens were collected from consenting patients, assigned a deidentifying number, and provided by the AP-HM Tumor Tissue Bank (ISO 9001:2008) in accordance with the protocol approved by the relevant institutional committees (Aix-Marseille Université). Sections of paraffin-embedded samples (4 μm) of human prostate cancer specimens were analyzed for adrenomedullin, CLR, RAMP2, RAMP3, and neuron-specific enolase (NSE) proteins as described previously (11). Optimal dilution for anti-adrenomedullin and anti-CLR antibodies was 1/2,000, anti-RAMP2 antibody was 1/750, anti-RAMP3 antibody was 1/1,500, and anti-NSE antibody (Dako) was 1/200. As a control for immunostaining, the antibodies preabsorbed by human synthetic adrenomedullin peptide (50 μmol/L; Bachem), CLR, RAMP2, and RAMP3 peptides (50 μmol/L, CROPS laboratory) were used instead of the primary antibodies.

**Cell culture**

The human prostate cancer androgen-dependent cell line LNCaP [American Type Culture Collection (ATCC); CRL-1740] and androgen-independent cell lines Du145 (ATCC; HTB-81) and PC3 (ATCC; CRL-1435), originally authenticated by short tandem repeat (STR) analysis by ATCC, were obtained from ATCC and cultured in RPMI 1640 (Invitrogen Life Technologies Inc.) as described previously (11). Lymphatic endothelial cells (LEC) were obtained from Lonza and cultured in endothelial basal medium 2 (EBM-2) medium supplemented with 2% FBS under moist 5% CO₂/95% air atmosphere.

**Development and characterization of anti-human adrenomedullin antibody**

The adrenomedullin polyclonal antibody was developed against the AM1–52 peptide (Bachem) as reported previously (13) and characterized as described (Supplementary Fig. S1). All purified immunoglobulin G (IgG) of anti-adrenomedullin antibody (αAM) and preimmune serum (rabbit control IgG) were affinity purified on rProtein A Sepharose.
Fast Flow columns (VWR) and tested for endotoxin using the Pyrogent plus Limulus Amebocyte Lysate Kit (Lonza). All antibody preparations used in animal studies contained less than 1.25 endotoxin U/mL.

**Western blot analysis**

Cell extracts were prepared and immunoblotted for CLR, RAMP2, and RAMP3 as described previously (22). Immunoblotting of phospho-CRAF (pCRAF), pMEK1/2, pERK1/2, and ERK1/2 were performed using the MAPK-phospho-ERK1/2 pathway sampler Kit (Cell Signaling Technology, Inc.). Signals were revealed using an enhanced chemiluminescence kit (ECL kit; Invitrogen Life Technologies Inc.).

**Cyclic AMP assay**

Du145 cells (3 × 10^4 cells/mL) treated with adenomedullin (10^{-7} to 10^{-9} mol/L) in the presence of L-methyl-3-isobutylxanthine (IBMX; 10^{-4} mol/L) were prepared to measure the intracellular amount of cyclic AMP (cAMP) using the cAMP enzyme immunoassay Biotrak (EIA) System according to the supplier’s protocol (GE Healthcare).

**Transwell migration assays**

Chemoinvasion of Du145 cells (15 × 10^4) using the filter coated with a layer of Matrigel (0.5 mg/mL; Becton Dickinson) in a modified Boyden chamber assay was performed as described in refs. (7, 23). Human umbilical venous smooth muscle cells (HUvSMC; ScienCell Research Laboratories, Clinciences,) were cultured in smooth muscle cell (SMC) growth medium M199 with 20% FBS to confluence. Confluent HUvSMC were serum deprived in EBM-2 growth medium M199 with 20% FBS to confluence.

**Cell proliferation assay**

The effects of adenomedullin (10^{-7}, 10^{-8}, and 10^{-9} mol/L), αAM (10, 30, and 70 μg/mL), and control IgG (70 μg/mL) on cell proliferation were examined at the indicated time points by cell counting or MTT assay (Z1 series Coulter Counter, Beckman Coulter Inc.) as described in ref. 23.

**RNA preparation and quantitative RT-PCR**

Total RNA was extracted from Du145 cells, PC3 cells, and LECs as well as Du145 and LNCaP xenografts, reverse transcribed to cDNA, and analyzed for the expression of adenomedullin, CLR, RAMP2, RAMP3, GAPDH mRNAs, and 18S rRNA as described in refs. 13 and 22.

**Peptide extraction and RIA**

Protein extracts from Du145 and LNCaP xenografts were prepared for RIA of immunoreactive adenomedullin (i-AM) as previously described (22).

**Animal study**

Animal work was carried out in the animal facility of the School of Medicine according to the institutional animal welfare guidelines. Athymic NMRI (nu/nu) nude mice (Harlan) were maintained in a sterile environment with a daily 12-hour light/12-hour dark cycle. The subcutaneous and orthotopic models were developed for Du145 cells. The subcutaneous tumors were generated by injection of Du145 cells (5 × 10^6) in the right flank of male athymic (NMRI; nu/nu) nude mice (n = 30; Harlan). Tumors were measured with a dial-caliper, and volumes were determined using the formula width × length × height × 0.5236 (for ellipsoid form; ref. 13). At a tumor volume of approximately 600 ± 100 mm^3, animals were randomly divided into two groups. One group (n = 20) received intraperitoneal injection of the αAM (350 μg of purified IgG equivalent to 12 mg/kg) every 3 days. The amount of αAM was determined on the basis of the data of preliminary experiments in which increasing amounts of αAM (100, 200, 350, 500, and 800 μg) were used to determine the best concentration of αAM that inhibits xenograft growth in vivo. As control, one group (n = 10) received a rabbit control IgG (350 μg equivalent to 12 mg/kg) of irrelevant specificity. Mice were sacrificed at the indicated time. Tumor size and general clinical status were recorded every 3 days. For the orthotopic Du145 xenografts, Du145 cells (1 × 10^6) were implanted orthotopically (n = 20) in the dorsal prostate in nude mice. One month later and after tumor palpation, mice were randomized into two groups and treated as above.

The subcutaneous LNCaP xenograft tumors were generated by injection of LNCaP cells (8 × 10^6) mixed at a 1:1 dilution with Matrigel (BD Biosciences) as above. At a tumor volume of approximately 500 mm^3, mice (n = 20) were randomized into two groups, a group of animals (n = 10) was castrated and separated into two groups that received an intraperitoneal injection of 12 mg/kg of αAM (n = 5) or control IgG (n = 5) every 3 days for 4 weeks. The same paradigm was applied to noncastrated animals.

**Immunohistochemical analysis**

Thin (6 μm) sections were incubated with anti-vWF antibody (diluted 1:400; Dako) and anti-αDNA antibody (diluted 1:80; Dako) to assess tumor vascularity, or a goat polyclonal anti-LYVE-1 antibody (diluted 1:100; R&D Systems) to assess the lymphatic vessels, and anti-PDGF-β antibody (diluted 1:100; ebioscience) to assess mural cells. To assess programmed cell death, tissue sections were evaluated using mAb F7-26 to detect single-strand DNA (ssDNA; Eurobio AbCys). Staining was carried out as detailed previously (17, 23).

**Statistical analysis**

Data are expressed as mean ± SEM from at least three independent experiments. One-way ANOVA or Fisher PLSD test (Statview 512; Brain Power Inc.) was used for statistical analysis. Differences were considered significant at values of P < 0.05.
Results

Expression of adrenomedullin, CLR, RAMP2, RAMP3, and NSE proteins in human prostate cancer specimens

Serial sections of human prostate cancer specimens (Gleason score >7; Fig. 1) were labeled with antibodies revealing NSE, adrenomedullin, CLR, RAMP2, and RAMP3 proteins (Fig. 1). Overt NSE labeling of stromal and epithelial cells can be observed, suggesting a neuroendocrine differentiation of cancer specimen (Fig. 1). Carcinomatous epithelia displayed overt and strong labeling for adrenomedullin, CLR, RAMP2, and RAMP3 (Fig. 1). Dispersed among the stromal collagen septa, numerous clusters of labeled stromal cells for adrenomedullin, CLR, RAMP2, and RAMP3 can be observed (Fig. 1). Illustrating the complexity of its localization, the adrenomedullin protein was localized to either the nucleus and/or the cytoplasm (×400). AM immunoreactivity is completely canceled by the antibody preabsorbed with 50 μmol/L AM peptide.

Other human prostate cancer tissues (n = 7) present the same staining paradigm for adrenomedullin, CLR, RAMP2, and RAMP3 as shown in Fig. 1. Furthermore, the expression of adrenomedullin was analyzed with tissue microarray in serial prostate sections from 72 patients with adenocarcinoma. More than 79% (57/72) of the biopsies were strongly positive for adrenomedullin. Together, these data indicate that the adrenomedullin system is well expressed in prostate cancer tissues and might be involved in tumor cell growth in vitro and in vivo.

Exogenous adrenomedullin stimulates Du145 cell growth, cAMP activity, and invasion in vitro

The effects of treatment with adrenomedullin were studied in vitro on androgen-independent Du145 cells that demonstrate expression of CLR, RAMP2, and RAMP3 mRNAs (Supplementary Fig. S2A). By Western blot analysis, Du145 cells produced a CLR as a distinct band of 48 kDa and multimer, presumably heterodimers CLR/RAMP2 (AM1 receptor) or CLR/RAMP3 (AM2 receptor), at 73 to 76 kDa (Supplementary Fig. S2B, lane 1). RAMP2 and RAMP3 were seen as a monomer of 28 kDa and multimer, presumably homodimer.
at 50 kDa and heterodimer at 73 to 76 kDa (Supplementary Fig. S2B, lanes 3 and 5). These data suggest that Du145 cells could be sensitive to adrenomedullin stimulus.

We next examined whether adrenomedullin increases intracellular cAMP, the major second messenger of adrenomedullin (25, 26) in cultured Du145 cells. Adrenomedullin increased the cAMP level in a dose-dependent manner with a peak at 5 to 20 minutes for the higher concentrations and then a sudden decrease at 30 minutes, probably due to receptor desensitization (Fig. 2A). The cAMP

Figure 2. Adrenomedullin (AM) stimulates Du145 cell proliferation and invasion in vitro. A, AM induced cAMP formation in cultured Du145 cells. Cells were treated with AM for the indicated times. Bars represent SEM of three independent experiments (**, P < 0.01; *** P < 0.001). B, opposite effects of AM and αAM on the growth of Du145 cells (2 x 10⁵) in vitro. Bars represent SEM of four independent experiments (**, P < 0.001). C, AM stimulates Du145 cells invasion in vitro. The bottom wells of all the chambers were filled with RPMI 1640 containing 2% FBS in the presence of control buffer (control), AM (10⁻⁷, 10⁻⁸, and 10⁻⁹ mol/L), αAM (70 μg/mL) in the presence of AM (10⁻⁷ mol/L), or bFGF (10⁻⁵ mol/L). Du145 cells (15 x 10⁵) were placed in the upper chamber and then incubated for 12 hours at 37°C. The migrated cells were stained with 4', 6'-diamidino-2-phenylindole (DAPI) and counted at x200 magnification using a microscope. Bars represent the mean ± SEM of three independent experiments (**, P < 0.01; *** P < 0.001). D, the intracellular signaling pathway induced by AM in Du145 cells. Du145 cells treated with AM (10⁻⁷ mol/L) for the indicated times and immunoblotted for pCRAF, pMEK1/2, pERK1/2, and ERK1/2. EGF was used as positive control known to stimulate phosphorylation of CRAF, MEK1/2, and ERK1/2. Preincubation of Du145 cells with αAM or αAMR for 30 minutes inhibits AM-induced phosphorylation of ERK1/2. Preincubation of Du145 cells with αAM without additional AM decreases pERK1/2. β-Actin was used as a loading control.
accretion by adrenomedullin could be mediated by G protein, probably Gαs, which is involved in many systems of receptor-operated cAMP increase, or by other mechanisms, such as facilitating the interaction between activated Gαs and adenyl cyclase as demonstrated for other factors. The production of cAMP suggests that adrenomedullin might be involved in the growth of Du145 cells. Accordingly, adrenomedullin significantly stimulates the proliferation of Du145 cells in a dose-dependent manner by 8 days of treatment (Fig. 2B). To determine whether aAM can inhibit cell growth in vitro, Du145 cells were exposed to increasing concentrations of aAM at 10, 30, and 70 μg/mL, and the effect on proliferation was assessed by MTT assay. aAM inhibits Du145 cell proliferation in a dose-dependent manner, reaching 15% (P < 0.05), 28% (P < 0.01), and 52% (P < 0.001), respectively, by 8 days of treatment (Fig. 2B). In contrast, 70 μg/mL of the control IgG of irrelevant specificity showed no inhibition of cell growth (Fig. 2B).

We next analyzed the effect of adrenomedullin on Du145 cells invasion. The addition of adrenomedullin (10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/L) to the bottom wells increased the number of invading cells in a dose-dependent manner after 12-hour incubation, reaching 95% ± 15% (P < 0.001), 60% ± 18% (P < 0.001), and 10% ± 3% (P < 0.01), respectively (Fig. 2C). The induced effect of adrenomedullin on invasion was inhibited when cells were preincubated with aAM, suggesting that the endogenous adrenomedullin secreted by Du145 cells might be involved in the invasion process, presumably by autocrine/paracrine manner (Fig. 2C). The effect of adrenomedullin on invasion was similar to that observed with basic fibroblast growth factor (bFGF; 10⁻⁶ mol/L; Fig. 2C). Interestingly, prostate hormone-independent PC3 cells demonstrate the expression of adrenomedullin and its receptors (Supplementary Fig. S3A), inhibition of cell growth reaching 50% upon incubation with aAM (Supplementary Fig. S3B), induction of migration (Supplementary Fig. S3C), and invasion (Supplementary Fig. S3C) by adrenomedullin. Together, these data suggest that adrenomedullin acts as an autocrine/paracrine growth factor to regulate many functions of Du145 and PC3 cells in vitro.

**Adrenomedullin mediates phosphorylation of MAPK**

Extracellular signal-regulated kinase (ERK) and Akt are known to regulate cell proliferation and this signaling pathway was reported to function downstream of the adrenomedullin/cAMP pathway (27, 28). Therefore, we investigated the activation of different pathways and found that adrenomedullin (10⁻⁷ mol/L) increased CRAF phosphorylation as early as 5 minutes and declines to reach the control levels by 2-hour treatment (Fig. 2D). To investigate mitogen–activated protein kinase (MAPK) activity, we measured the phosphorylation of MAP–ERK kinase (MEK1/2), ERK1 (p44MAPK), and ERK2 (p42MAPK). The levels of pCRAF, pMEK1/2, and pERK1/2 in Du145 cells were increased as early as 5 minutes, respectively, after adrenomedullin (10⁻⁷ mol/L) treatment (Fig. 2D). These effects sustained to be higher than control levels for up to 1 hour and decline to control levels by 2-hour treatment (Fig. 2D).

Inhibition of MEK, an immediate upstream activator of ERK1/2, with U0126 (10 μM/L, 30 minutes) prevented adrenomedullin’s activation of ERK1/2 (Fig. 2D). Preincubation of Du145 cells with aAM or aAMR inhibits the stimulus of adrenomedullin on pERK1/2, suggesting that the effect of adrenomedullin is specific and is one of the factors involved in the activation of the MAPK pathway via CLR/RAMP2 and/or CLR/RAMP3 receptors (Fig. 2D). Interestingly, the effects of aAM without additional adrenomedullin demonstrate a decrease of pERK1/2 as early as 5 minutes to barely detectable levels by 2-hour treatment as compared with control cells, suggesting that adrenomedullin is one of the Du145 cell-derived factors involved in the activation of the MAPK pathway in an autocrine/paracrine manner (Fig. 2D). Dose–response studies demonstrate that adrenomedullin (10⁻⁶ to 10⁻⁴ mol/L) induced a strong phosphorylation of CRAF, MEK1/2, and ERK1/2 by 10-minute treatment (Supplementary Fig. S4). These results suggest that adrenomedullin-induced cell proliferation is mediated at least in part by the cAMP/CRAF/MEK/ERK pathway.

**Expression of adrenomedullin and its receptors in Du145 and LNCaP xenografts**

To assess the steady-state levels of adrenomedullin mRNA and AMR mRNAs, total RNA was prepared from subcutaneous and orthotopic Du145 tumors and LNCaP tumors from castrated and noncastrated animals. Quantification of adrenomedullin mRNA transcripts shows equivalent expression in subcutaneous and orthotopic Du145 xenografts (Fig. 3A). In LNCaP xenografts, the data show a clear increase of adrenomedullin mRNA by approximately 5.5 ± 0.3-fold (n = 10; mean ± SEM) in castrated animals as compared with noncastrated animals (P < 0.01; Fig. 3A) as previously reported (22). Quantitative PCR of CLR, RAMP2, and RAMP3 mRNAs demonstrate the expression of these transcripts in all xenografts analyzed (Fig. 3A). Interestingly, the expression of these transcripts is not regulated by androgen in LNCaP xenografts in vivo, and the same finding was observed in LNCaP cells in vitro (22).

RIA demonstrates that castration increased ir-AM levels by 3.5 ± 0.4-fold (n = 10; mean ± SEM), being 230 ± 15 pg/μg protein in castrated animals and 65 ± 5 pg/μg protein in noncastrated animals (P < 0.01; Fig. 3B). The amount of ir-AM determined in both types of Du145 xenografts showed an average of 170 pg/μg protein (Fig. 3B). Taken together, these data demonstrate that the adrenomedullin system is expressed in both xenografts and that ir-AM, the target of aAM, is well expressed in Du145 and LNCaP xenografts in castrated animals.

**aAM inhibits growth of androgen-independent prostate cancer tumor xenografts**

On the basis of our observations on the effects of adrenomedullin on Du145 cell growth and invasion in vitro, we further wished to analyze the effect of adrenomedullin on tumors developed in vivo in immunodeficient mice. We sought to determine if adrenomedullin is just a classic...
growth factor involved in tumor cell proliferation in vivo, or if adrenomedullin has a complex role to sustain tumor growth by performing a stable angiogenesis and lymphangiogenesis leading to functional blood and lymphatic vessels. To this end, we performed a series of experiments in which Du145 subcutaneous xenograft tumor–bearing mice were treated with αAM and control IgG. In the first series of experiments, tumor cells were injected subcutaneously in the flanks of athymic mice (nu/nu). Once tumors reached a size of 600 ± 100 mm³, mice started to receive intraperitoneal injection of 12 mg/kg of αAM or control IgG every 3 days for 5 consecutive weeks. No signs of toxicity, such
as weight loss in response to αAM treatment, were observed. A clear regression of tumor growth was observed in αAM-treated tumors as compared with control IgG–treated tumors (∼0.001; Fig. 3C). At day 22, tumors in the αAM-treated mice reached a mean size of approximately 421 ± 179 mm³, whereas the tumors in the control group exhibited a mean size of approximately 2,612 ± 300 mm³. At this time of treatment, a group of animals was sacrificed; tumor weights were taken and tumor tissues were saved to assess vascularity. The mean tumor weights in the control and the αAM-treated animals were 2.5 and 0.6 g, respectively.

Because the stromal environment can affect the tumor growth, we next tested the effect of αAM on tumor growth using the Du145 orthotypic model. Mice received intraperitoneal injection of 12 mg/kg of αAM or control IgG three times a week until sacrifice for analysis (Fig. 3C). Ten weeks after orthotopic tumor-cell implantation, mice in the control group seemed sickly and cachectic, characterized by sluggishness, an unkempt appearance. At the same time point, antibody-treated mice seemed active and maintained normal grooming behavior. After 6 weeks of treatment, animals were sacrificed and tumor burden was assessed. Nine of 10 mice treated with control IgG developed fulminant disease (Fig. 3C). In contrast, 6 of 10 αAM-treated animals showed a dramatically decreased response, exhibiting only small tumors upon gross inspection (∼0.001; Fig. 3C). Tumor weights were significantly lower in αAM-treated animals than in control IgG–treated animals, further documenting tumor suppression (∼0.001; Fig. 3C). Pathologic examination of tissues from control IgG and αAM-treated animals showed the presence of metastasis in lung, kidney, and spleen only in control orthotopic animals. No metastasis can be observed in αAM-treated animals.

Figure 4. αAM-treated tumors are depleted of endothelial cells and pericytes. A and B, tumor sections from subcutaneous (A) or orthotopic (B) xenografts were evaluated by immunofluorescence for vWF (red) and αSMA (green). Tissue sections were counterstained with 4’,6-diamidino-2-phenylindole (DAPI; blue). Scale bar = 50 μm. C and D, quantitative assessment of cells that stained positive for vWF in subcutaneous (C) and orthotopic (D) xenografts was conducted through a microscope. MBF Image J 1.43U software was used for analysis. Values ± SEM; n = 6; ***, P < 0.001.

αAM represses LNCaP tumor xenograft growth in vivo only in castrated animals

Treatment of noncastrated animals with αAM showed no inhibition of tumor growth when compared with the control IgG group (Fig. 3D). Following castration, LNCaP xenografts present a hormone-independent growth pattern comparable to one observed for hormone-independent cells such as Du145 cells. To further investigate whether the endogenous adrenomedullin expressed upon castration (Fig. 3A) might be involved in tumor growth, intraperitoneal administration of αAM or control IgG was given to castrated and noncastrated animals. Interestingly, the growth of xenografts was significantly decreased by αAM treatment in castrated animals when compared with the control IgG group, suggesting that adrenomedullin might play an important role in the tumor regrowth process after androgen ablation (Fig. 3D).

Adrenomedullin blockade depletes endothelial cells and pericytes in tumors

Immunostaining of subcutaneous and orthotopic Du145 tumors with anti-vWF antibody demonstrates that αAM-treated Du145 tumors were significantly less vascularized than Du145 control tumors (Fig. 4A and B). Costaining with anti-vWF and anti-αSMA antibodies demonstrates that both cell types are sparse, and the vascularization is deeply disrupted (Fig. 4A and B; inset). Quantification of vWF-stained endothelial cells and αSMA-stained pericytes demonstrates a clear decrease of both cell types in αAM-treated tumors when compared with control IgG–treated Du145 tumors in both xenograft models (∼0.001; Fig. 4C and D). In contrast, control IgG–treated Du145 tumors showed a well-organized vasculatization. Importantly, the vascularization in normal tissues was not disrupted by αAM treatment (Supplementary Fig. S5).
Figure 5. αAM disrupts tumor-associated lymphangiogenesis in orthotopic Du145 prostate tumor xenografts. Du145 cells (1 × 10^6) were implanted with orthotopic injection into dorsal prostate of athymic mice that were randomly divided into two groups and treated with αAM or control IgG. A, representative images of tumors from control and αAM-treated animals. Tumor sections were stained with anti-LYVE-1, or costained with anti-LYVE-1 and anti-αSMA or anti-PDGFR-β to reveal αSMA or PDGFR-β expressing cells in green and LECs in red, respectively. DAPI-stained nuclei are in blue. Pros, prostate; T, tumor tissue. B and C, quantitative assessment of cell density of cells that stained positive for LYVE-1 (B) or PDGFR-β (C) was conducted through a microscope. MBF Image J 1.43U software was used for analysis. Values are mean ± SEM; n = 8; ***, P < 0.001. D, migration of HUVSMC toward LEC-conditioned medium (CM) + function-blocking antibody against adrenomedullin (AM). αAM and IgG control were used at 70 μg/mL, AM (10^{-7} mol/L), and PDGF-BB (5 ng/mL) was used as positive control. The migrated cells were stained with DAPI and counted using microscope. Bars represent the mean ± SEM of three independent experiments (**, P < 0.01; ***, P < 0.001).
αAM blocks the development of tumor-associated lymphangiogenesis

To determine the effect of αAM treatment on tumor-associated lymphangiogenesis, Du145 orthotopic tumor treated with αAM or control IgG were evaluated for tumor-associated lymphatic vessels by immunostaining for LYVE-1 (lymphatic vessel endothelial receptor 1; Fig. 5). αAM-treated tumors showed marked disruption and reduction of the lymphatic tumor vasculature compared with control IgG–treated tumors (Fig. 5A). Importantly, LYVE-1–positive lymphatic vessels detected in the normal mice tissue adjacent to the tumor periphery remained unaffected by the αAM treatment (Fig. 5A). Furthermore, the smooth muscle cells surrounding the collecting vessels in tumor tissue were eradicated in αAM-treated tumors compared with control IgG–treated tumors (Fig. 5A). The immunostaining for PDGFR-β, a marker of mature periendothelial support cells (29), revealed expression of PDGFR-β in perivascular cells that are in close contact with LECs (Fig. 5A). The same finding is observed with αSMa, consistent with the identification of the PDGFR-β⁺ cells in these tumors as a class of pericyte. A very few lymphatic vessels were found without periendothelial support cells (not shown). Quantitative evaluation of the number of lymphatic vessels revealed a significant reduction specifically in αAM-treated tumors (P < 0.001; Fig. 5B). No significant difference can be observed for the number of lymphatic vessels in peritumoral tissues between control IgG and αAM-treated animals, suggesting that αAM treatment does not impede preexisting or non–tumor associated lymphatic vessels (Fig. 5B). Quantitative evaluation of the number of PDGFR-β positive cells revealed an overall reduction of mural cells of 61% ± 2.5% specifically in αAM-treated tumors compared with control IgG–treated tumors (P < 0.01; Fig. 5C). Together, these data are in agreement with the recently published work that implicates SMCs responsible for lymphatic vessel morphogenesis and function (30). To strengthen our findings, we therefore tested the possibility that adrenomedullin secreted by LECs might be involved in the mural cells/pericytes recruitment. Adrenomedullin receptors are expressed in cultured primary HUVSMCs (not shown). This suggests that SMC could therefore respond to adrenomedullin secreted by LECs to regulate SMC recruitment during collecting vessel formation. The migration assay demonstrates that LEC-conditioned medium promoted SMC migration in Transwell assay; this effect was significantly inhibited by a function-blocking antibody to adrenomedullin (Fig. 5D). These data strongly suggest that adrenomedullin must be one of the LEC-derived factors responsible for SMC recruitment.

The reduction of lymphatic vessels upon αAM treatment indicates that adrenomedullin is necessary for LECs’ survival, suggesting that αAM treatment may induce LECs’ death by apoptosis. The apoptotic cells, as revealed by immunostaining of ssDNA with monoclonal antibody (mAb) F7-26 antibody (Fig. 6A; inset), were predominantly located within the vascular lining and costaining with LYVE-1 antibody identified them as LECs (Fig. 6B; inset). The apoptotic index of the αAM-treated tumors reached an approximately 6-fold increase when compared with control IgG–treated tumors (P < 0.001; Fig. 6C). Together, these findings indicate that αAM treatment can (i) destroy specifically tumor-associated lymphatic vessels and (ii) prevent the tumor from inducing lymphangiogenesis.

Discussion

Progression of prostate cancer toward androgen-independent status is an oncologic challenge. The mechanisms responsible for the tumor progression in androgen-independent manner are not well understood. Recently, we demonstrated that adrenomedullin expression is induced by androgen withdrawal, suggesting that its production may be important for tumor resurgence following androgen ablation (22). To determine the role of the adrenomedullin system in androgen-independent tumor growth of prostate cancer, we used in the current study the androgen-independent Du145 and PC3 cells. Our data showed that adrenomedullin significantly increased Du145 cell proliferation, invasiveness, stimulation of cAMP, and the activation of the CRAF/MEK/ERK/MAPK pathway. We have also shown that...
adrenomedullin is involved in the proliferation, migration, and invasion of PC3 cells. These data indicate that hormone-independent prostate cancer cells are able to respond to adrenomedullin in ways that would be expected to further the aggressiveness of androgen-independent prostate cancer. We demonstrated that αAM inhibited the basal levels of prostate cancer androgen-independent cell proliferation and invasion in vitro, supporting the conclusion that adrenomedullin can act in an autocrine manner in androgen-independent prostate cancer. The presence of autocrine loop suggests that foci of adrenomedullin-producing cells in a tumor could stimulate cells expressing adrenomedullin receptors via autocrine/paracrine mechanisms.

The expression of adrenomedullin and adrenomedullin receptors by prostate cancer cells and stromal cells suggests that the adrenomedullin system may play an important role in situ. The role of adrenomedullin in tumor progression by stimulation of tumor cell proliferation, inhibition of apoptosis, and stabilization of angiogenesis has been well established (7, 12, 13, 17, 31), and all these activities may be relevant in prostate cancer. Our data demonstrate that αAM could be efficiently delivered in vivo and significantly suppress the growth of established Du145 xenografts. The immunostaining of αAM-treated tumor sections with anti-vWF antibody demonstrated that more than 84% of the vessels disappeared with a clear depletion of the endothelial cells and pericytes, suggesting strongly that the adrenomedullin system must be involved in neovascularization and/or vessel stabilization in hormone-independent prostate cancer. Because the Du145 cell proliferation is inhibited in vitro by αAM, the inhibition of Du145 xenografts growth by αAM could be a result of combined effects on tumor cell growth as well as on tumor neoangiogenesis. Importantly, the physiologic vascularization in normal tissues that has a long acting doubling-time (about 3 years) could not be disrupted by αAM treatment, suggesting that the adrenomedullin system must be highly activated in tumor neoangiogenesis in which far shorter doubling-time is observed (few days; ref. 32).

We have reported that in castrated animals (absence of androgen in vivo), intraperitoneal injection of adrenomedullin stimulates the growth of LNCaP xenografts, suggesting that adrenomedullin might be involved in tumor resurgence following androgen ablation (22). The inhibition of LNCaP tumor growth by αAM treatment only in the castrated animals confirms our hypothesis in agreement with the data obtained with Du145 xenografts. These data brought strong evidence that the production of ir-AM stimulated by androgen ablation must participate in prostate cancer tumor growth. Interestingly, in intact animals or in the presence of androgen, αAM could not inhibit tumor growth, suggesting that in noncastrated animals, LNCaP xenograft growth is adrenomedullin independent in agreement with the barely detectable levels of adrenomedullin in xenografts developed in noncastrated animals (22). Recently, adrenomedullin was found to be over-expressed in AR-E231G prostate and shown to act as novel effector of androgen receptor–mediated prostate tumorigenesis by promoting cell proliferation and survival (33). Collectively, these results highlight the role of adrenomedullin as a major factor that affects the tumor microenvironment to promote neoangiogenesis leading the nutrient and oxygen supply and hormone-independent prostate cancer tumor growth.

Like blood vascular angiogenesis, lymphangiogenesis has gained much attention as an important initial step in tumor pathogenesis (34–36). It has been shown that intra- and/or peritumoral lymphangiogenesis increases the risk for metastasis both in animal models and in human tumors (34). To determine whether lymphatic vessels might be impaired by αAM treatment in prostate cancer orthotopic xenografts, we analyzed the lymphatic vessels using a murine LYVE-1 antibody. LYVE-1⁺ lymphatic vessels were observed in the control IgG–treated tumors and were completely devoid within the αAM-treated tumors. Importantly, αAM treatment was not observed to impair preexisting lymphatic vessels detected in the normal tissue. Prolonged inhibition does not affect adult lymphatics, indicating that activation of AM receptors (AM₁ and AM₂) is necessary to induce growth of lymphatics but not required for the maintenance of the lymphatics in adulthood. Accordingly, our data demonstrate that activation of adrenomedullin receptors by adrenomedullin induces proliferation, migration, invasion, and survival of LECs (Supplementary Fig. S6 and S7), suggesting an important role of adrenomedullin to build-up functional lymphatic vessels during tumor growth. Interestingly, we demonstrate that adrenomedullin secreted by LECs participate in SMCs’ recruitment in migration assay, suggesting strongly that adrenomedullin could be involved in SMC recruitment during collecting-vessel formation in vivo. Together, our work provides evidence suggesting that the activation of the adrenomedullin/adrenomedullin receptors signaling pathway upon induction of adrenomedullin expression (22, 33, 37) may not only be important for supporting tumor cell growth and neoangiogenesis, but also for promoting tumor lymphangiogenesis (Supplementary Fig. S8).

Adrenomedullin has been postulated to possess lymphangiogenic properties (38, 39). Interestingly, the genetic loss of AM, Calcrl, or RAMP2 causes preferential reduction in the proliferation of LECs of the jugular lymphatic vessels (38). It has been reported that the loss of adrenomedullin signaling results in severely hypoplastic jugular lymph sacs (38). Systemic administration of adrenomedullin stimulates both lymphangiogenesis and angiogenesis at a site of injury to mouse lymphatic vessels (39). It is well known that tumor cells enter the lymphatic vasculature by invading preexisting lymphatic vessels in the tumor periphery or by eliciting lymphangiogenesis via growth factor production (40, 41). It is conceivable to propose that adrenomedullin produced by tumor cells may stimulate growth and dilation of the peritumoral lymphatic vessels to prevent increases in tumor tissue pressure and to facilitate tumor cell entry through the lymphatic endothelium as it was previously demonstrated for other lymphangiogenic growth factors (42, 43), as
reported for VEGF (44, 45) and platelet-derived growth factor-BB (PDGF-BB; ref. 46).

Many studies have brought the lymphatic system to the forefront as an important route of tumor metastasis (47–49). αAM therapy might be expected to effectively control lymph node and systemic metastases in tumors that metastasize via the vasculature and the lymphatic vessels. Further characterization of adrenomedullin signaling and its pharmacologic modulation via AM₁ and AM₂ receptors might lead to novel therapeutic target to suppress angiogenesis and lymphangiogenesis as well as potential tumor growth in hormone-independent manner in prostate cancer and dissemination. In fact, blocking tumor-induced angiogenesis and lymphangiogenesis is an increasingly important strategy in the design of antitumor drugs. The present study and others identified the adrenomedullin system as a promising target for the development of a neutralizing mAb and/or the design of a nonpeptidic modulator that could be useful for the treatment of cancer. Efforts are underway to develop mono-specific and/or bi-specific mAbs targeting adrenomedullin and/or adrenomedullin receptors as well as the development of an adrenomedullin small-molecule antagonist (13, 16, 17, 24, 50).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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Acknowledgments
The authors thank V. Gagna for her excellent secretarial assistance.

Grant Support
This study was supported by grants from Institut National du Cancer (INCa, grant CaPAM), Institut national de la santé et de la recherche medicale (INSERM), AP-HM, ARTC Sud, and the Association pour la Recherche sur les Tumeurs de la Prostate (ARIP).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked received OnlineFirst October 7, 2013; DOI: 10.1158/1078-0432.CCR-13-0691


www.aacrjournals.org Clin Cancer Res; 19(22) November 15, 2013 6149

Published OnlineFirst October 7, 2013; DOI: 10.1158/1078-0432.CCR-13-0691


Adrenomedullin Blockade Suppresses Growth of Human Hormone-Independent Prostate Tumor Xenograft in Mice

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