Adrenomedullin blockade suppresses growth of human hormone-independent prostate tumor xenograft in mice

Caroline Berenguer-Daizé1,2, Françoise Boudouresque1,2, Cyrille Bastide3, Asma Tounsi1,2, Zohra Benyahia1,2, Julie Acunzo1,2, Nadège Dussault1,2, Christine Delfino1,2, Nathalie Baeza1,2, Laurent Daniel1,2, Mylène Cayol1,2, Dominique Rossi3, Assou EL Battari1,2, Denis Bertin4, Kamel Mabrouk4, Pierre-Marie Martin1,2,5, L’Houcine Ouafik1,2,5.

Affiliation:

1Aix-Marseille Université, CRO2 UMR 911, 13005, Marseille, France ;
2Inserm, CRO2 UMR 911, 13005, Marseille, France ;
3AP-HM, CHU Nord, Service Urologie, 13015, Marseille, France ;
4Aix-Marseille Université, LCP UMR 6264, CROPS, 13397, Marseille, France ;
5AP-HM, CHU Nord, Service de Transfert d’Oncologie Biologique, 13015, Marseille, France

Key words: Adrenomedullin; Prostate cancer hormone-independent; invasion; tumor growth; tumor-associated neoangiogenesis and lymphangiogenesis.

Abbreviated Title: αAM therapy represses hormone-independent CaP tumors

Conflict of interest: The authors have no potential conflict of interest to disclose

Corresponding author: Pr. L’Houcine Ouafik
Inserm UMR 911 – CRO2
Faculty of Medicine
27 Boulevard Jean MOULIN
13385 MARSEILLE CEDEX 05
FRANCE.
Voice: (33) 491324447
Fax: (33) 491254232
E-mail: lhoucine.ouafik@univ-amu.fr
Abstract

Purpose: To study the role of Adrenomedullin (AM) system (AM and its receptors “AMR; CLR, RAMP2 and RAMP3”) in cancer of prostate (CaP) androgen-independent growth.

Experimental design: Androgen-dependent and independent CaP models were used to investigate the role and mechanisms of AM in CaP hormone-independent growth and tumor-associated angiogenesis and lymphangiogenesis.

Results: AM and AMR were immunohistochemically localized in the carcinomatous epithelial compartment of CaP specimens of high-grade (Gleason score >7) suggesting a role of the AM system in the CaP growth. We used the androgen-independent Du145 cells for which we demonstrate that AM stimulated cell proliferation in vitro through the cAMP/CRAF/MEK/ERK pathway. The proliferation of Du145 and PC3 cells is decreased by anti-AM antibody (αAM) supporting that AM may function as a potent autocrine/paracrine growth factor for CaP androgen-independent cells. In vivo, αAM therapy inhibits Du145 androgen-independent xenografts growth and interestingly LNCaP androgen-dependent xenografts growth only in castrated animals suggesting strongly that AM might play an important role in tumor regrowth following androgen ablation. Histological 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.

Conclusion: We conclude that expression of AM upon androgen ablation in CaP plays an important role in hormone-independent tumor growth and in neovascularization by supplying/amplifying signals essential for pathological neoangiogenesis and lymphangiogenesis.
**Translational significance**

Adrenomedullin (AM) and AM receptors were immunohistochemically localized in the carcinomatous epithelial compartment of high-grade adenocarcinomas (Gleason score >7) of cancer of prostate (CaP) specimens, suggesting a role of the AM system in the growth of human hormone independent CaP. AM 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-AM antibody causing growth cessation *in vitro*. The *in vivo* study highlights the significance of AM as an important factor to promote androgen-independent CaP tumor growth and to affect the tumor microenvironment by inducing pathological neoangiogenesis and lymphangiogenesis. Targeting AM system may provide a rational basis for future therapeutic modalities upon androgen ablation in CaP.
Introduction

Prostate cancer (CaP) is the most-diagnosed malignant growth in men and is the second-leading cause of male cancer 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 (AI) lesion characterized by aggressive growth and invasion of distal organs, predominantly the bone (3). Whether it is clonal expansion with adaptation of substitutive pathway, development of AI prostate cancer shows clearly those factors other than, or together with, low levels of androgen must exist to provide survival and growth instructions to the AI cells.

Adrenomedullin (AM) is a multifunctional peptide with properties ranging from inducing vasorelaxation to acting as a regulator of cellular growth and angiogenesis (4-7). AM binds to and mediates its activity through the G protein-coupled receptor calcitonin receptor-like receptor (CLR), with specificity for AM being conferred by the receptor activity modifying protein -2 (RAMP2) and -3 (RAMP3) (8). The ability of CLR/RAMP2 and CLR/RAMP3 to respond with high affinity to AM implies the existence of two molecularly distinct AM receptors respectively, referred as AM1 and AM2 receptors (9). AM is widely expressed in a variety of tumor types (10) and was shown to be mitogenic for many human cancer cell lines in vitro (4,11-15). Several in vivo studies have shown a reduction of tumor angiogenesis and growth upon the treatment with neutralizing AM antibodies (13), AM receptor antagonist (16, 17), or AM receptor interference (14).

Others and we have reported the expression of AM in human prostate carcinoma and prostate cancer cell lines (11, 18-20). The expression of AM increased in hormone independent manner after a primary response to castration in LuCaP model in vivo (21). AM is an angiogenic factor that is induced in the absence of androgens (22), promotes prostate cancer cell growth in vitro and in vivo (22) and is required for maintenance of the neuroendocrine phenotype in LNCaP cells (22). In the
absence of androgen, administration of AM to LNCaP xenografts bearing-animals promotes tumor growth suggesting a pivotal role of AM for tumor resurgence following primary response to androgen ablation (22).

These observations suggest that AM expression upon androgen receptors blockade might be involved in CaP hormone-independent tumor growth. Accordingly, the aim of our study was to investigate the potential role of endogenous AM in the growth of hormone independent CaP by evaluating the effect of anti-AM polyclonal antibody (αAM) on androgen-insensitive CaP cells. We demonstrate that AM plays a significant role in the proliferation and invasion of androgen-insensitive CaP cells via CLR/RAMP2 and CLR/RAMP3 receptors; and that αAM decreases the proliferation and invasion of androgen-independent Du145 and PC3 cells in vitro and inhibits the growth of Du145 xenografts and LNCaP xenografts (only in castrated mice) by targeting tumor cell growth and tumor-associated angiogenesis and lymphangiogenesis.
Materials and Methods

Human prostate specimens
We analyzed human prostate specimens from eight patients with CaP 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 a protocol approved by the relevant institutional committees (Aix-Marseille Université). Sections of paraffin-embedded samples (4 μm) of human CaP specimens were analyzed for AM, CLR, RAMP2, RAMP3 and NSE proteins as described (11). Optimal dilution for anti-AM and anti-CLR antibodies was 1/2000, anti-RAMP2 antibody was 1/750, anti-RAMP3 antibody was 1/1500 and anti-NSE antibody (Dako, Trappes cedex, France) was 1/200. As a control for immunostaining, the antibodies preabsorbed by human synthetic AM peptide (50 μM; Bachem, Switzerland), CLR, RAMP2 and RAMP3 peptides (50 μM, CROPS laboratory) were used instead of the primary antibodies.

Cell culture
The human CaP androgen-dependent cell line LNCaP (ATCC, CRL-1740) and androgen-independent cell lines Du145 (ATCC, HTB-81) and PC3 (ATCC, CRL-1435) originally authenticated by short repeat tandem (STR) analysis by American Type Culture Collection (ATCC), were obtained from ATCC (Rockeville, MD, USA) and cultured in RPMI 1640 (Invitrogen Life Technologies Inc., Paris, France) as described (11). Lymphatic endothelial cells (LECs) were obtained from Lonza (Lonza, Verviers, Belgium) and cultured in EBM-2 medium supplemented with 2% FBS under moist 5%CO2/95% air atmosphere.

Development and characterization of anti-human AM antibody
The AM polyclonal antibody was developed against the AM1-52 peptide (Bachem, Weil am Rhein, Germany) as reported previously (13) and characterized as described (Supplementary Fig. S1). All purified IgG of anti-AM antibody (αAM) and preimmune serum (rabbit control IgG) were affinity
purified on rProtein A Sepharose Fast-Flow columns (VWR, Strasbourg, France) and tested for endotoxin using the Pyrogent plus Limulus ameboycote lysate kit (Lonza). All antibody preparations used in animal studies contained < 1.25 endotoxin U/ml.

**Western blot analysis**

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

**cAMP assay**

Du145 cells (3x10^4 cells/ml) treated with AM (10^-7 to 10^-9 M) in the presence of IBMX (10^-4 M) were prepared to measure the intracellular amount of 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 x 10^5) using the filter coated with a layer of Matrigel (0.5 mg/ml, Becton Dickinson, Paris, France) in a modified Boyden chamber assay was performed as described (7, 23). Human umbilical venous SMCs (HUVSMCs; Science Cell Res laboratories, Clinisciences, Nanterre, France) were cultured in SMC growth medium M199 with 20% FBS to confluence. Confluent HUVSMC were serum deprived in EBM-2 growth medium containing 0.5% FBS overnight. 1 x 10^5 cells were seeded in the upper chamber and allowed to migrate toward EBM-2 supplemented with 0.5% FBS or conditioned medium collected from LECs over 48 h period in presence or absence of a function-blocking antibody to AM or control-IgG for 23 h.
Cell proliferation assay

The effects of AM (10^{-7} M, 10^{-8} M, 10^{-9} M), αAM (10, 30 and 70 μg/ml) and control IgG (70 μg/ml) on cell proliferation was examined at the indicated time points by cell counting or MTT assay (Z1™ series Coulter counter, Beckman coulter, Inc. Fullerton, CA) as described (23).

RNA preparation and real-time 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 AM, CLR, RAMP2, RAMP3, GAPDH mRNAs and 18S rRNA as described (13, 22).

Peptide extraction and radioimmunoassay

Protein extracts from Du145 and LNCaP xenografts were prepared for RIA of immunoreactive AM (ir-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, Gannat, France) were maintained in a sterile environment with a daily 12-h light/12-h dark cycle. The s.c. and orthotopic models were developed for Du145 cells. The s.c. tumors were generated by injection of Du145 cells (5 x 10^6) in the right flank of male athymic (NMRI) (nu/nu) nude mice (n=30) (Harlan, Gannat, France). Tumors were measured with a dial-caliper, and volumes were determined using the formula width x length x height x 0.5236 (for ellipsoid form) (13). At a tumor volume of ~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 three days. The amount of αAM was determined based on the data of preliminary experiments in which increasing amounts of αAM (100 μg, 200 μg, 350 μg, 500 μg, 800 μg) were used to determine the best concentration of
αAM that inhibits xenografts 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 x 10⁶) 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 s.c. LNCaP xenografts tumors were generated by injection of LNCaP cells (8 x 10⁶) mixed at a 1:1 dilution with Matrigel (BD Biosciences Europe, Le Pont de Claix Cedex, France) as above. At a tumor volume of ~500 mm³, mice (n=20) were randomized into two groups, a group of animals (n=10) was castrated and separated into two groups that received an i.p. injection of 12 mg/kg of αAM (n=5) or control-IgG (n=5) every three days for four weeks. The same paradigm was applied to non-castrated animals.

**Immunohistochemical analysis**

Thin (6 μm) sections were incubated with anti-vWF antibody (diluted 1:400; Dako), and anti-αSMA 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-PDGFR-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, Paris, France). 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 analysis of variance (ANOVA) or Fisher’s PLSD test (Statview 512; Brain Power Inc., Calabasas, CA, USA) was used for statistical analysis. Differences were considered significant at values of p < 0.05.
Results

Expression of AM, CLR, RAMP2, RAMP3 and NSE proteins in human CaP specimens

Serial sections of human CaP specimens (Gleason score >7; Fig. 1) were labelled with antibodies revealing NSE, AM, CLR, RAMP2, and RAMP3 proteins (Fig. 1). Overt NSE labelling 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 AM, CLR, RAMP2, and RAMP3 (Fig. 1). Dispersed among the stromal collagen septa, numerous clusters of labelled stromal cells for AM, CLR, RAMP2 and RAMP3 can be observed (Fig. 1). Illustrating the complexity of its localization, the AM protein was localized to either the nucleus and/or the cytoplasm in different biopsies (Fig. 1). The same localization for AM was recently reported in serial sections of lung cancer biopsies (24). Positive AM staining was completely abolished by preabsorption of the antibody with 50 μM synthetic AM peptide (Fig. 1) or CLR, RAMP2 and RAMP3 peptides (not shown). The other human CaP tissues (n=7) present the same staining paradigm for AM, CLR, RAMP2 and RAMP3 as shown in Figure 1. Furthermore, the expression of AM was analyzed with tissue microarray (TMA) in serial prostate sections from 72 patients with adenocarcinoma. More than 79% (57/72) of the biopsies were strongly positive for AM. Together, these data indicate that AM system is well expressed in CaP tissues and might be involved in tumor cell growth in vitro and in vivo.

Exogenous AM stimulates Du145 cells growth, cAMP activity and invasion in vitro

The effects of treatment with AM were studied in vitro on androgen independent Du145 cells which 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 73-76 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 73-76 kDa (supplementary Fig. S2B, lanes 3 and 5). These data suggest that Du145 cells could be sensitive to AM stimulus.
We next examined whether AM increases intracellular cAMP, the major second messenger of AM (25, 26) in cultured Du145 cells. AM increased the cAMP level in a dose-dependent manner with a peak at 5-20 minutes for the higher concentrations and then sudden decrease at 30 minutes, probably due to receptor desensitization (Fig. 2A). The cAMP accumulation by AM could be mediated by G protein, probably G\(\alpha_s\), which is involved in many systems of receptor-operated cAMP increase, or by other mechanism(s), such as facilitating the interaction between activated G\(\alpha_s\) and adenyl cyclase as demonstrated for other factors. The production of cAMP suggests that AM might be involved in the growth of Du145 cells. Accordingly, AM significantly stimulates the proliferation of Du145 cells in a dose-dependent manner by 8 days of treatment (Fig. 2B). To determine whether \(\alpha\)AM can inhibit cell growth \textit{in vitro}, Du145 cells were exposed to increasing concentrations of \(\alpha\)AM at 10, 30, and 70 \(\mu\)g/ml, and the effect on proliferation was assessed by MTT assay. \(\alpha\)AM 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 \(\mu\)g/ml of the control IgG of irrelevant specificity showed no inhibition of cell growth (Fig. 2B).

We next analyzed the effect of AM on Du145 cells invasion. The addition of AM (10\(^{-7}\) M, 10\(^{-8}\) M, and 10\(^{-9}\) M) to the bottom wells increased the number of invading cells in a dose-dependent manner after 12 h incubation, reaching 95 \(\pm\) 15\% (\(p < 0.001\)), 60 \(\pm\) 18\% (\(p < 0.001\)), and 10 \(\pm\) 3\% (\(p < 0.01\)), respectively (Fig. 2C). The induced effect of AM on invasion was inhibited when cells were pre-incubated with \(\alpha\)AM suggesting that the endogenous AM secreted by Du145 cells might be involved in the invasion process presumably by autocrine/paracrine manner (Fig. 2C). The effect of AM on invasion was similar to the one observed with bFGF (10\(^{-6}\) M) (Fig. 2C). Interestingly, a prostate hormone independent PC3 cells demonstrate the expression of AM and its receptors (supplementary Fig. S3A), inhibition of cell growth reaching 50\% upon incubation with \(\alpha\)AM (supplementary Fig. S3B), induction of migration (supplementary Fig. S3C) and invasion (supplementary Fig. S3C) by AM. Together, these data suggest that AM acts as an autocrine/paracrine growth factor to regulate many functions of Du145 and PC3 cells \textit{in vitro}.
AM mediates phosphorylation of MAPK

ERK and Akt are known to regulate cell proliferation and this signaling pathway was reported to function downstream of the AM/cAMP pathway (27, 28). Therefore, we investigated the activation of different pathways and found that AM (10^{-7} M) increased CRAF phosphorylation as early as 5 min and declines to reach the control levels by 2 h treatment (Fig. 2D). To investigate MAPK activity, we measured the phosphorylation of MEK1/2, ERK1 (p44 MAPK) and ERK2 (p42 MAPK). The levels of pCRAF, pMEK1/2 and pERK1/2 in Du145 cells were increased as early as 5 min, respectively after AM (10^{-7} M) treatment (Fig. 2D). These effects sustained to be higher than control levels for up 1 h and decline to control levels by 2 h treatment (Fig. 2D). Inhibition of MEK, an immediate upstream activator of ERK1/2, with U0126 (10 \mu M, 30 min) prevented AM’s activation of ERK1/2 (Fig. 2D).

Pre-incubation of Du145 cells with \alpha AM or \alpha AMR inhibits the stimulus of AM on pERK1/2 suggesting that the effect of AM is specific and is one of the factors involved in the activation of MAPK pathway via CLR/RAMP2 and/or CLR/RAMP3 receptors (Fig. 2D). Interestingly, the effects of \alpha AM without additional AM demonstrate a decrease of pERK1/2 as early as 5 min to barely detectable levels by 2 h treatment as compared to control cells suggesting that AM is one of the Du145 cells-derived factors involved in the activation of MAPK pathway in autocrine/paracrine manner (Fig. 2D). Dose-response studies demonstrate that AM (10^{-6} to 10^{-9} M) induced a strong phosphorylation of CRAF, MEK1/2 and ERK1/2 by 10 min treatment (supplementary Fig. S4). These results suggest that AM-induced cell proliferation is mediated at least in part by the cAMP/CRAF/MEK/ERK pathway.

Expression of AM and its receptors in Du145 and LNCaP xenografts

To assess the steady-state levels of AM mRNA and AMR mRNAs, total RNA was prepared from s.c. and orthotopic Du145 tumors and LNCaP tumors from castrated and non castrated animals. Quantification of AM mRNA transcripts shows equivalent expression in s.c. and orthotopic Du145 xenografts (Fig. 3A). In LNCaP xenografts, the data show a clear increase of AM mRNA by approximately 5.5 \pm 0.3 fold (n=10; mean \pm sem) in castrated animals as compared to non castrated animals (p < 0.001; 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, the same finding was observed in LNCaP cells in vitro (22).

Radioimmunoassay demonstrates that castration increased immunoreactive-AM (ir-AM) levels by 3.5 ± 0.04 fold (n=10; mean ± sem) being 230 ± 15pg/μg protein in castrated animals and 65 ± 5pg/μg protein in non castrated animals (p < 0.01; Fig. 3B). The amount of ir-AM determined in both types of Du145 xenografts showed an average of 170pg/μg protein (Fig. 3B). Taken together, these data demonstrate that the AM system is expressed in both xenografts and that ir-AM, the target of αAM, is well expressed in Du145 and LNCaP xenografts in castrated animals.

αAM inhibits growth of androgen-independent prostate cancer tumor xenografts

Based on our observations on the effects of AM on Du145 cell growth and invasion in vitro, we further wished to analyze the effect of AM on tumors developed in vivo in immunodeficient mice. We sought to determine if AM is just a classical growth factor involved in tumor cell proliferation in vivo, or if AM 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 s.c. xenograft tumors-bearing mice were treated with αAM and control IgG. In the first series of experiments, tumor cells were injected s.c. in the flanks of athymic mice (nu/nu). Once tumors reached a size of 600 ± 100 mm³, mice started to receive i.p. injection of 12 mg/kg of αAM or control IgG every 3 days for five 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 to control IgG treated-tumors (p < 0.001; Fig. 3C). At day 22, tumors in the αAM-treated mice reached a mean size of ~421 ± 179 mm³, whereas the tumors in the control group exhibited a mean size of ~2,612 ± 300 mm³. At this time of treatment, a group of animals was sacrificed; tumor weights taken and tumor tissues were saved to assess vascularity. The mean tumor weights in the control and in the αAM-treated animals were 2.5 g vs. 0.6 g, respectively.
Since the stromal environment can affect the tumor growth, we next tested the effect of αAM on tumor growth using the Du145 orthotopic model. Mice received i.p. 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 appeared sickly and cachectic, characterized by sluggishness, an unkempt appearance. At the same time point, antibody-treated mice appeared active and maintained normal grooming behavior. After 6 weeks treatment, animals were sacrificed and tumor burden was assessed. Nine of ten 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 ($p < 0.001$; Fig. 3C). Tumor weights were significantly lower in αAM-treated animals compared to control IgG-treated animals, further documenting tumor suppression ($p < 0.001$; Fig. 3C). Pathological 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.

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

Treatment of non-castrated animals with αAM showed no inhibition of tumor growth when compared to 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 AM expressed upon castration (Fig. 3A) might be involved in tumor growth, i.p. administration of αAM or control IgG was given to castrated and non-castrated animals. Interestingly, the growth of xenografts was significantly decreased by αAM treatment in castrated animals when compared to control IgG group suggesting that AM might play an important role in tumor regrowth process after androgen ablation (Fig. 3D).

**AM blockade depletes endothelial cells and pericytes in tumors**

Immunostaining of s.c. 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).
Co-staining with anti-vWF and anti-α-SMA 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 α-SMA stained pericytes demonstrates a clear decrease of both cell types in αAM-treated tumors when compared to control IgG-treated Du145 tumors in both xenografts models (p < 0.001; Fig. 4C and D). In contrast, control IgG-treated Du145 tumors showed a well-organized vascularization. Importantly, the vascularization in normal tissues was not disrupted by αAM treatment (Supplementary Fig. S5).

**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 to 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 to 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 lymphatic endothelial cells (Fig. 5A). The same finding is observed with α-SMA consistent with 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 pre-existing 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 to 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 AM secreted by LECs might be involved in the mural cells/pericytes recruitment. AM receptors are expressed in cultured primary HUVSMCs (not shown). This suggests that SMC could therefore respond to AM 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 AM (Fig. 5D). These data strongly suggest that AM must be one of the LEC-derived factor(s) responsible for SMC recruitment.

The reduction of lymphatic vessels upon αAM treatment indicates that AM 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 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 a ~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 CaP towards androgen-independent status is an oncological challenge. The mechanisms responsible for the tumor progression in androgen-independent manner are not well understood. Recently, we demonstrated that AM 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 AM system in androgen-independent tumor growth of CaP, we used in the current study the androgen-independent Du145 and PC3 cells. Our data showed that AM significantly increased Du145 cell proliferation, invasiveness, stimulation of cAMP, and the activation of the CRAF/MEK/ERK/MAPK pathway. We have also shown that AM is involved in the proliferation, migration and invasion of PC3 cells. These data indicate that hormone independent CaP cells are able to respond to AM in ways that would expect to further the aggressiveness of androgen-independent CaP. We demonstrated that αAM inhibited the basal levels of CaP androgen-independent cell proliferation and invasion in vitro; supporting the conclusion that AM can act in an autocrine manner in androgen-independent CaP. The presence of autocrine loop suggests that foci of AM-producing cells in a tumor could stimulate cells expressing AM receptors via autocrine/paracrine mechanisms.

The expression of AM and AM receptors by CaP cells and stromal cells suggests that AM system may play an important role in situ. The role of AM 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 AM system must be involved in neovascularization and/or vessel stabilization in hormone independent CaP. Since 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 physiological vascularization in normal tissues, which has a long acting doubling-
time (about 3 years) could not be disrupted by αAM treatment suggesting that AM system must be highly activated in tumor neoangiogenesis where far shorter doubling-time is observed (few days) (32).

We have reported that in castrated animals (absence of androgen in vivo), i.p. injection of AM stimulates the growth of LNCaP xenografts suggesting that AM 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 immunoreactive AM stimulated by androgen ablation must participate in CaP tumor growth. Interestingly, in intact animals or in the presence of androgen, αAM could not inhibit tumor growth suggesting that in non-castrated animals, LNCaP xenografts growth is AM independent in agreement with the barely detectable levels of AM in xenografts developed in non-castrated animals (22). Recently, AM was found to be overexpressed in AR-E231G prostates and shown to act as novel effector of AR-mediated prostate tumorigenesis by promoting cell proliferation and survival (33). Collectively, these results highlight the role of AM as a major factor that affects the tumor microenvironment to promote neoangiogenesis leading the nutrient and oxygen supply and hormone independent CaP 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 CaP 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 pre-existing 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 AM receptors by AM induces proliferation, migration, invasion, and survival of LECs.
(Supplementary Fig. S6 and S7) suggesting an important role of AM to build-up a functional lymphatic vessels during tumor growth. Interestingly, we demonstrate that AM secreted by LECs participate in SMCs recruitment in migration assay suggesting strongly that AM could be involved in SMC recruitment during collecting vessel formation in vivo. Together, our work provide evidence suggesting that the activation of AM/AM-receptors signalling pathway upon induction of AM 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).

AM has been postulated to possess lymphangiogenic properties (38, 39). Interestingly, 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 loss of AM signalling results in severely hypoplastic jugular lymph sacs (38). Systemic administration of AM 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 pre-existing lymphatic vessels in the tumor periphery or by eliciting lymphangiogenesis via growth factor production (40, 41). It is conceivable to propose that AM 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 vascular endothelial growth factor (VEGF) (44, 45) and platelet-derived growth factor-BB (PDGF-BB) (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 AM signalling and its pharmacological modulation via AM1 and AM2 receptors, might lead to novel therapeutic target to suppress angiogenesis and lymphangiogenesis as well as potentially tumor growth in hormonal independent manner in prostate cancer and dissemination. In fact, blocking tumor-induced angiogenesis and lymphangiogenesis is an increasing important strategy in the design of anti-tumor drugs. The present study and others identified AM system as a promising target for the development of a neutralizing mAb and/or the design of non-peptidic modulator that
could be useful for the treatment of cancer. Efforts are underway to develop mono-specific and/or bi-
specific mAbs targeting AM and/or AM receptors as well as the development of AM small molecule
antagonist (13, 16, 17, 24, 50).
Acknowledgments

This study was supported by grants from Institut National du Cancer (INCa) (grant CaPAM), Inserm, AP-HM, ARTC Sud and the Association pour la Recherche sur les Tumeurs de la Prostate (ARTP). We thank V. Gagna for her excellent secretarial assistance.
References


Figure Legends

Figure 1. Representative microscopic fields of AM, CLR, RAMP2, RAMP3 and NSE immunohistochemical analysis in CaP tissues. Section of CaP tissue is stained with H&E. Epithelial carcinoma cells as well as stromal cells showed NSE staining. Immunodetection of AM, CLR, RAMP2 and RAMP3 in serial sections of prostate cancer biopsies. AM, CLR and RAMP2 staining of cells dispersed among the stroma can be observed. In a second area of the biopsie, carcinomatous epithelium demonstrates AM staining localized to either nucleus and/or the cytoplasm (x400). AM immunoreactivity is completely canceled by the antibody preabsorbed with 50 μmol/l AM peptide.

Figure 2. 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) Oppose effects of AM and αAM on the growth of Du145 cells (2 x 10^3) in vitro. Bars represent SEM of four independent experiments (*, p < 0.05; **, p < 0.01; *** p < 0.001). (C) AM stimulates Du145 cells invasion in vitro. The bottom wells of the all chambers were filled with RPMI 1640 containing 2% FBS in presence of control buffer (control), AM (10^{-7} M, 10^{-8} M, and 10^{-9} M), αAM (70 μg/ml) in presence of AM (10^{-7} M), or bFGF (10^{-6} M). Du145 cells (15x10^3) were placed in the upper chamber, then incubated for 12 hr at 37°C. The migrated cells were stained with DAPI and counted at 200X magnification using microscope. Bars represent the mean ± SEM of three independent experiments (**, p < 0.01; *** p < 0.001). (D) Intracellular signalling pathway induced by AM in Du145 cells. Du145 cells treated with AM (10^{-7} M) for the indicated times and immunoblotted for pCRAF, pMEK1/2, pERK1/2 and ERK1/2. MEK inhibitor (U0126) inhibits AM-induced phosphorylation of ERK (10 μM, 30 min). EGF was used as positive control known to stimulate phosphorylation of CRAF, MEK1/2, and ERK1/2. Pre-incubation of Du145 cells with αAM or αAMR for 30 min inhibits AM induced phosphorylation of ERK1/2. Pre-incubation of Du145 cells with αAM without additional AM decreases pERK1/2. β-actin was used as a loading control.
Figure 3. αAM inhibits growth of Du145 and LNCaP xenografts. (A) Expression of AM and its receptors (CLR, RAMP-2 and -3) in Du145 and LNCaP xenografts. Total RNA (1 μg) DNA-free from Du145 xenografts (s.c. and orthotopic (ortho)) and LNCaP xenografts from castrated (cast) and non castrated (non cast) animals were reverse transcribed into cDNA and subjected to real-time quantitative reverse transcription (RT)-PCR for the estimation of relative AM, CLR, RAMP2 and RAMP3 mRNAs to GAPDH mRNA ratio as described under Materials and Methods (***, p < 0.001). (B) Extracts prepared from Du145 xenografts (s.c. and ortho; n=5 each) and LNCaP xenografts (non cast and cast; n=5 each) were subjected to radioimmunoassay (RIA) to determine the ir-AM levels (**, p < 0.01). (C) Du145 cells were s.c. implanted in athymic nu/nu mice and mice bearing Du145 tumors were treated with αAM or control IgG of irrelevant specificity. The sizes of Du145 xenografts were determined by measuring the volume of the tumors. Values are means ± SEM. Asterisks indicate that the value is significantly different from the control IgG (***, p < 0.001). Orthotopic prostate tumors were established by injection of Du145 cells in the dorsal lobe of prostate. Tumor weight of αAM and control IgG-treated animals is shown and horizontal lines indicate the mean tumor weight in each treatment group (***, p < 0.001). (D) The LNCaP cells (8x10^6) in Matrigel (v/v) were s.c. implanted in nu/nu mice and 2 months later, when the primary tumors were 400 mm³ in size, animals were randomly divided into two groups. None castrated animals (n=10) were randomly divided into two groups (n=5) that received αAM or control IgG by i.p. administration. The castrated animals (n=10) were randomly divided into two groups and treated as above. The asterisks indicate that the tumor volume of αAM-treated xenografts is significantly different from control IgG-treated xenografts in castrated animals (**, p < 0.01; *** p < 0.001).

Figure 4. αAM-treated tumors are depleted of endothelial cells and pericytes. (A, B) Tumor sections from s.c. (A) or orthotopic (B) xenografts were evaluated by immunofluorescence for vWF (red) and αSMA (green). Tissue sections were counterstained with DAPI (blue). Scale bar = 50 μm. (C, D) Quantitative assessment of cells that stained positive for vWF in s.c. (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).

Figure 5. αAM disrupts tumor-associated lymphangiogenesis in orthotopic Du145 prostate tumor xenografts. Du145 cells (1x10⁶) 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, respectively and lymphatic endothelial cells in red. DAPI-stained nuclei are in blue. Pros: Prostate, T: Tumor tissue. (B, 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 means ± SEM; n = 8, ***, p < 0.001. (D) Migration of HUVSMC toward LEC-conditioned medium (CM) ± function-blocking antibody against AM. αAM and IgG-control were used at 70 μg/ml, AM (10⁻⁷ M), 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).

Figure 6. αAM induces LECs-associated tumors apoptosis. (A) Microphotographs of immunohistochemical-stained tumor sections for ssDNA using a Mab F7-26 antibody in control and αAM-treated tumors. (B) To determine cells undergoing apoptosis, tumor sections were evaluated by immunofluorescence for LYVE-1 (green) and ssDNA (red). DAPI-stained nuclei are blue. (C) Percentage of cells undergoing apoptosis was determined using MBF_Image J 1.43U software for analysis. Values are means ± SEM; n= 6; (***, p < 0.001).
Figure 1, Berenguer-Daizé et al.
Figure 3, Berson et al. clincancerres.aacrjournals.org on July 16, 2017. © 2013 American Association for Cancer Research.
Figure 4, Berenguer-Daizé et al.
Figure 5, Berenguer-Daizé et al.
Figure 6, Berenguer-Daizé et al.
Adrenomedullin blockade suppresses growth of human hormone independent prostate tumor xenograft in mice

Caroline BERENGER-DAIZE, Francoise BOUDOURESQUE, Cyrille Bastide, et al.

Clin Cancer Res  Published OnlineFirst October 7, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-0691

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2013/10/07/1078-0432.CCR-13-0691.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.