Biochimica et Biophysica Acta 1831 (2013) 1579–1587

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Tumour epithelial expression levels of endocannabinoid markers modulate the value of endoglin-positive vascular density as a prognostic marker in prostate cancer ☆

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ARTICLE INFO

Article history:
Received 25 October 2012
Received in revised form 29 November 2012
Accepted 7 December 2012
Available online 20 December 2012

Keywords:
Cannabinoid CB_1 receptor
Fatty acid amide hydrolase
Angiogenesis
Endoglin
von Willebrand factor
Prostate cancer

ABSTRACT

Fatty acid amide hydrolase (FAAH) is responsible for the hydrolysis of the endogenous cannabinoid (CB) receptor ligand anandamide. Here we have investigated whether the expression levels of FAAH and CB_1 receptors influence the prognostic value of markers of angiogenesis in prostate cancer. Data from a cohort of 419 patients who were diagnosed with prostate cancer at transurethral resection for lower urinary tract symptoms, of whom approximately 2/3 had been followed by expectancy, were used. Scores for the angiogenesis markers endoglin and von Willebrand factor (vWf), the endocannabinoid markers fatty acid amide hydrolase (FAAH) and cannabinoid CB_1 receptors and the cell proliferation marker Ki-67 were available in the database. For the cases followed by expectancy, the prognostic value of endoglin was dependent upon the tumour epithelial FAAH immunoreactivity (FAAH-IR) and CB_1IR scores, and the non-malignant epithelial FAAH-IR scores, but not the non-malignant CB_1IR scores or the tumour blood vessel FAAH-IR scores. This dependency upon the tumour epithelial FAAH-IR or CB_1IR scores was less apparent for vWf, and was not seen for Ki-67. Using an endoglin cut-off value of 10 positively stained vessels per core and a median split of tumour FAAH-IR, four groups could be generated, with 15 year of disease-specific survival (%) of 68 ± 7 (low endoglin, low FAAH), 45 ± 11 (high endoglin, low FAAH), 77 ± 6 (low endoglin, high FAAH) and 21 ± 10 (high endoglin, high FAAH). Thus, the cases with high endoglin and high FAAH scores have the poorest rate of disease-specific survival. At diagnosis, the number of cases with tumour stages 1a–1b relative to stages 2–4 was sensitive to the endoglin score in a manner dependent upon the tumour FAAH-IR. It is concluded that the prognostic value of endoglin as a marker of neovascularisation in prostate cancer can be influenced by the expression level of markers of the endocannabinoid system. This article is part of a Special Issue entitled Lipid Metabolism in Cancer.

1. Introduction

The endocannabinoid system, comprising the endogenous ligands anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG), their target cannabinoid CB_1 and CB_2 receptors, and their synthetic and catabolic enzymes, are often perceived as being primarily involved in the regulation of brain function. However, the endocannabinoid system is found throughout the body, and plays important regulatory roles outside the brain in processes as diverse as fertility [1], energy balance [2], skin epidermal cell differentiation and proliferation [3] and the control of cancer cell proliferation and migration [3–8].

A case in point concerns the role of the endocannabinoid system in prostate cancer. AEA, its metabolically stable analogue *R*(+)-methananandamide, related *N*-aylethanolamines, 2-AG, 2-arachidonylglyceryl ether and synthetic and plant-derived cannabinoids affect the proliferation, migration or invasivity *in vitro* of prostate tumour cell lines by both CB receptor-dependent and -independent mechanisms, including the sustained production of ceramide and activation of ERK1/2 [4,9–18]. In an elegant series of experiments, Nithipatikom and colleagues [12] demonstrated that blockade of 2-AG synthesis increased the *in vitro* invasivity of the androgen-insensitive prostate cell lines PC3 and DU-145, whilst the blockade of the hydrolysis of this endocannabinoid reduced the invasivity. The primary enzyme involved in 2-AG metabolism is monoacylglycerol lipase, an enzyme which is overexpressed in

Abbreviations: bv, blood vessel; CB, cannabinoid; FAAH, fatty acid amide hydrolase; GS, Gleason score; IR, immunoreactivity; LT, tumour stage; †Pca, death due to prostate cancer (Pca); ROC, receiver-operated characteristic; vWf, von Willebrand factor

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http://dx.doi.org/10.1016/j.bbalip.2012.12.005

prostate cancer cell lines [19]. Genetic knockdown or pharmacological inhibition of monoacylglycerol lipase reduces the invasivity of PC3 cells *in vitro* [19,20], and decreases the rate of growth of these cells *in vivo* in a xenograft model [19]. The latter effect was partially prevented by treatment either with a CB_1 receptor antagonist or by a high fat diet, and completely prevented by a combination of the two, suggesting that the enzyme played a key role both as a regulator of antiproliferative 2-AG levels and as a generator of free fatty acids supporting tumour growth from the corresponding monoacylglycerols [19].

AEA and other *N*-acylethanolamines are hydrolysed to their corresponding fatty acids by fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine-hydrolysing acid amidase, both of which are found in prostate cancer cells, albeit to different extents in different cell lines [21,22]. Overexpression of FAAH in PC3 cells, which normally express low levels of this enzyme, increases invasivity and migration of the cells *in vitro* [21], whilst inhibition of the enzyme increases the anti-proliferative potency of the *N*-acylethanolamine eicosapentaenoylethanolamide [16]. When all these data are taken together, a case can be made that the endocannabinoid system is an endogenous protective system to limit the growth and invasivity of prostate cancer cells, and that the tumour expression of endocannabinoid hydrolytic enzymes can affect the properties of the tumour cells. Additionally, it can be hypothesised that a dysfunctional endocannabinoid signalling may be involved in the pathogenesis of prostate cancer. Consistent with this hypothesis, FAAH expression is higher in epithelial tumour cells than in non-malignant luminal epithelial cells in formalin-fixed tissue from prostate cancer cases [21,23], and a high level of epithelial FAAH immunoreactivity (FAAH-IR) in tumour samples obtained at diagnosis is associated with a more severe disease phenotype [23] (as visualised by the Gleason score [24], a morphological assessment of disease severity). The FAAH-IR expression level is also associated with disease outcome (disease-specific survival) [23], although this effect is less robust than that seen for other potential disease biomarkers such as the cell proliferation marker Ki67 [25]. Tumour FAAH-IR is correlated with tumour CB_1 receptor immunoreactivity (CB_1IR), and a high CB_1IR is a very robust marker of poor outcome, with its prognostic value additive to that seen with the Gleason score [26].

Our previous studies [23,26] focussed upon the association of the endocannabinoid markers *per se* upon disease severity and outcome. However, it is possible that the level of these markers may have a more profound influence in prostate cancer by modifying the influence of processes essential for tumour growth. Thus, for example, the penetrance of a risk factor may be different when the tumour has a low endocannabinoid metabolic capability than when it has a high metabolic capability. One obvious process in this respect is tumour angiogenesis, given that the stable AEA analogue 2-methyl-arachidonyl-2′-fluoro-ethylamide inhibits angiogenesis in a thyroid tumour xenograft model [27], a property shared by the synthetic CB_2 receptor agonist JWH-133 in glioma and skin tumour xenograft models [28,29], and of the non-selective CB receptor agonist WIN55,212-2 in a prostate cancer xenograft model (published in abstract form [30]). Two markers of tumour vascular density, endoglin (CD105, a marker for immature blood vessels in prostate cancer tissue) and factor VIII-related antigen (von Willebrand factor, vWf; identifies both immature and mature blood vessels in prostate cancer tissue), have been investigated in the same tissue microarray as used for the FAAH and CB_1 receptor studies [23,25,26], allowing a unique opportunity to study the interaction between the endocannabinoid system and tumour vascular density in a well-characterised patient cohort. The present paper reports that the prognostic value of tumour microvessel endoglin as a measure of neovascularisation is tempered by the expression level of the endocannabinoid markers FAAH and CB_1 receptors.

2. Experimental

2.1. Patient material and immunohistochemistry

The present study has utilised data available in our database built up of clinical and immunochemical data for a large series of prostate cancer tissue samples. The tissue material was collected at the Regional Hospital, Västerås, Sweden, between 1975 and 1991 from patients undergoing transurethral resection for lower urinary tract problems and who were then diagnosed to be suffering from prostate cancer [31]. The patients were followed until 2003. Tissue microarrays were constructed and cores (usually 5, but between 1 and 8 for the tumour tissue) could be scored for the parameter in question. Non-malignant cores were also included in the tissue microarrays [31]. The data for the parameters used in the present study have been published, and are as follows:

- Tumour epithelial FAAH, blood vessel FAAH, non-malignant luminal (lu) and basal (ba) FAAH, scored on the basis of immunoreactive intensity (0, 1, 2 or 3) × distribution, to give a composite value between 0 and 3 [23]. The epithelial distribution components had seven tranches (0, 10, 25, 33, 50, 67 or 100% of the total distribution for each intensity), whereas the blood vessel distribution for a given intensity was either 0, 50 or 100%.
- Tumour and non-malignant endoglin and vWf, scored as the mean number of positively stained vessels per core [25].
- Tumour Ki67 index, scored as the % of tumour cells positive for Ki67 determined in eleven horizontal lines per tumour core [25].
- Tumour and non-malignant epithelial CB_1IR, scored on the basis of immunoreactive intensity × distribution, to give a value between 0 and 3 [26].

The research ethical committee at Umeå University hospital (Regional Ethical Review Board in Umeå, Sweden) approved of the studies.

2.2. Orthotopic injection of R3327 AT1 cells into the rat ventral prostate

Tissue obtained from a previous study [32] was used. Briefly, adult male Copenhagen rats were anaesthetised with pentobarbitol. After a small incision was made in the lower abdomen, 2000 Dunning AT-1 prostate cancer cells were injected into a lobe of the ventral prostate (for details, see [33]. On day 7 after injection, the animals were castrated by a scrotal incision. The animals were then treated daily by gavage with either vehicle (1% Tween 80) or the 50 mg/kg of angiogenesis inhibitor N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine (ZD6474). On day 10 after orthotopic injection of the cells, the animals were killed and the prostate glands were colected. Tumour-containing regions were collected and formalin-fixed paraffin-embedded samples were then prepared as described in [32]. FAAH-IR was determined using the same antibody (a rabbit anti-FAAH raised against the last 102 amino acids of rat FAAH [34], dilution 1/2000; antibody kindly provided by Dr. Ken Mackie, Indiana University, Bloomington, IN, USA), and the same immunochemical technique (Ventana system) as described in [23]. Ethical permission for the animal study was obtained from the local animal ethical committee.

2.3. Statistics

Two statistical programmes were used. Correlation coefficients, Kruskal–Wallis, Chi-squared and Fisher's exact tests, receiver operating characteristic (ROC) curves and Kaplan–Meier survival analyses were undertaken using the statistical package built into the GraphPad Prism 5 computer programme for the Macintosh (GraphPad Software Inc., San Diego, CA, USA). Cox proportional-hazards regression analyses were conducted using SPSS software (SPSS Inc., Chicago, IL, USA). In the survival analyses, an event was defined as death due to prostate cancer