THE RELATIONSHIP BETWEEN ANGIOGENESIS AND CYCLOOXYGENASE-2 EXPRESSION IN PROSTATE CANCER

Running Title: ANGIOGENESIS AND COX-2 EXPRESSION IN PROSTATE CANCER

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SUMMARY

Objective

To test the hypothesis that angiogenesis in prostate cancer is associated with tumour invasion and metastasis, and that this is mediated via increased COX-2 expression.

Materials and Methods

We studied angiogenesis in 105 patients with either prostate cancer (79) or BPH (26). We correlated this data with levels of COX-2 expression in the same dataset. Mean microvessel density was analysed, as a marker of angiogenesis, using the endothelial antigen CD34 stained by immunohistochemistry.

Results

There was no difference in MVD in progressive tumour stages compared to BPH. There was a negative correlation between MVD and COX-2 expression, however the effect of increased COX-2 expression on MVD was not marked.

Conclusion

This data would suggest that COX-2 drives tumour spread in prostate cancer by means other than the promotion of angiogenesis.

KEYWORDS prostate cancer, angiogenesis, COX-2

INTRODUCTION

Angiogenesis, the formation of new blood vessels, plays a vital role in the growth, progression and metastasis of many cancers [1]. Solid tumours induce the formation of new capillaries to avoid oxygen starvation and obtain the required nutrients to grow beyond 2-3mm in diameter [1]. The regulation of angiogenesis depends on a complex interplay between pro-angiogenic factors such as vascular endothelial growth factors (VEGFs) and inhibitory factors, known as the "angiogenic switch" [1,2]. Immunohistochemical (IHC) studies for endothelial antigens such as von Willebrands factor (Factor VIII), CD 31 and CD 34 are frequently used to quantify neovascularisation in tumours. Such studies have suggested that estimation of mean microvessel density (MVD) predicts local spread and recurrence in several cancers [1,3,4]. In addition, studies have suggested that angiogenesis contributes to the metastatic potential of prostate cancer [1,5] The administration of angiogenesis inhibitors has been shown to suppress the primary and metastatic growth of prostate tumours *in vivo* [1]. A number of retrospective studies have shown mean MVD to correlate with increasing Gleason score and disease progression (from extraprostatic extension to metastasis) in prostate cancer [4,5,5]. However, the clinical value of measuring angiogenesis remains controversial. Estimation of mean MVD has not always been shown to predict local recurrence, nor relate to Gleason score or stage [6]. In addition, some studies have been unable to confirm that MVD can act as an independent prognostic parameter for prostate cancer when subjected to multivariate analysis [1,7]

COX-2 is the inducible form of the enzyme cyclo-oxygenase, which is involved in the formation of prostaglandins such as PGE2 from arachidonic acid, which in turn regulates VEGF production, thereby promoting angiogenesis [8]. Aberrant or increased expression of COX-2 has been implicated in the pathogenesis of several human cancers including colorectal [9] , and breast [10,11]. A recent report revealed a correlation between COX-2 expression and tumour MVD as measured by CD31 in breast cancer [11]. This provides the rationale for the use of selective COX-2 inhibitors in the treatment of selected cancer patients, in order to reduce neovascularisation and therefore cell growth, which is currently under investigation [1].

Increased expression of COX-2 has already been shown in the prostate cancer cell lines LNCaP (androgen sensitive), and PC-3 (androgen insensitive) [12] [10]. Immunohistochemical studies, including our own, have confirmed high levels of COX-2 expression in human prostatic cancer tissue and high grade PIN [13-17]. Correlation between COX-2 staining intensity, Gleason score, and poor prognosis in prostate cancer has been shown in some studies [16-18]. but not others [13]. We have recently demonstrated higher levels of COX-2 expression in locally advanced prostate cancer [19]. Studies in hormone-resistant prostate cancer cell lines have demonstrated a link between COX-2, PGE2 production and the hypoxic upregulation of VEGF, which may be reversed by the addition of a selective COX-2 inhibitor [20]. Similarly, prostate

cancer studies in mice demonstrate that treatment with selective COX-2 inhibitors prevents the up-regulation of VEGF decreasing tumour MVD and tumour growth [21]. We tested the hypothesis that angiogenesis in prostate cancer is associated with tumour invasion and metastasis and mediated via increased COX-2 expression. The confirmation of angiogenesis and COX-2 as consistent prognostic markers in prostate cancer would support further clinical assessment of angiogenesis and COX-2 inhibitors, which have already shown promise in various trials [1,22].

MATERIALS AND METHODS

Patient Recruitment and Tissue Retrieval

A database of 105 tumour biopsies was established with archival tissue specimens (formalin fixed, paraffin embedded). Detailed data on stage, presence of metastasis, Gleason grade and survival data if available was documented upon review of case notes. In order to develop this patient cohort, we obtained Multiple Research Ethical Committee approval and the support of the Scottish Urological Oncology Group and recruited patients throughout Scotland. Tissue specimens were then divided into 2 groups by stage (T1/2 and T3/4) with BPH specimens as control. Two further subgroups with non-metastatic and metastatic disease at presentation were also identified. COX-2 expression data was also available for each tumour in this cohort. [19].

Methods

Tumour angiogenesis was assessed by immunohistochemistry (IHC) using a monoclonal antibody to the endothelial cell surface marker CD34 (mouse IgG1, QBEnd/10, Novocastra, UK). Tissue sections (5μm) were dewaxed in xylene and rehydrated through graded alcohols. Antigen retrieval was achieved by incubating sections in 0.1% trypsin in 0.1% calcium chloride (w/v, pH4) for 25 minutes at 37^0C. Sections were then incubated with primary antibody at 1:50 dilution for 30 minutes at room temperature. Negative control sections were incubated with an isotype matched

control antibody. Bound antibody was visualised using a biotinylated secondary antibody, streptavidin-horseradish peroxidase complex (DAKO, UK) and 3,3'-diaminobenzidine (DAB) as chromogen (Vector). Tissue sections were counterstained with haematoxylin and dehydrated through graded alcohols and xylene. Subjective analysis of the tissue sections was used to identify 4 most vascular regions or "hot spots" (areas of maximal endothelial cell staining of microvessels) at low magnification (x 200). The number of vessels in each hot spot was then counted at higher magnification (x 400) in four fields of vision – the average of these counts was taken as the mean microvessel density (MVD). Every tenth slide was double-scored by an independent observer.

COX-2 expression was previously determined using a monoclonal antibody (mouse IgG1, Cat.No.160112, Cayman Chemical Co., USA) at a 1:80 concentration. This was quantified blindly by the same 2 observers using a weighted histoscore method, which is calculated from the sum of (1 x % weak staining) + (2 x % moderate staining) + (3 x % strong staining) and provides a semi-quantitative classification of staining intensity.

The inter-class correlation coefficients (ICCC) between each observer for each protein were greater than 0.7, which is classed as excellent.

Data Analysis

MVD scores are shown as mean +/- standard deviation. Statistical analysis was performed using the student's T-test to compare differences in scores between BPH

and individual tumour stages. Spearman ranks correlation coefficient was used to determine any correlation between COX-2 expression, angiogenesis (as determined by mean MVD), and Gleason score. Kaplan-Meier survival plots were used to correlate MVD scores with survival.

RESULTS

Angiogenesis

In total, 105 patients were retrospectively recruited into the study, 79 had prostate cancer (46 with stage T1/2 and 31 with stage T3/4, and 2 stage unknown) and 26 had BPH (see **Figure 1**). Tumour groups were also subdivided into metastatic (11 T1/2, 7 T3/4, 2 unknown) and non-metastatic (35 T1/2, 24 T3/4) at presentation. Median age, Gleason sum and mean survival for all patients in the database are shown in **Table 1**. There was no difference in angiogenesis, as measured by MVD, between tumour groups (all groups combined) compared to BPH (p=0.19), nor with T3/4 compared with T1/2 (p=0.95) (see **Table 2a**) when the data was subjected to the student's T-test. In addition, no significant differences were seen between patients with or without metastasis at diagnosis either in total (p=0.60) (see **Table 2a**), or when further sub-divided by individual tumour stage (p=0.39 for T1/2 and p=0.96 for T3/4) (see **Table 2b**). Patients with high MVD (above mean) did not show a significantly different survival time compared to patients with low MVD (below mean) (p=0.15).

Correlation between COX-2 and angiogenesis

There was a negative correlation between COX-2 expression and angiogenesis, as measured by micro-vessel density, (p=0.02, $y = -0.027x + 18.58$) (see **Table 3** and **Figure 2**). In addition, Gleason score did not correlate with MVD nor COX-2 expression (p=0.75 and 0.40 respectively).

DISCUSSION

We have previously published an association between high COX-2 expression and increased tumour stage (T3/4), and increased COX-2 expression in prostate cancer compared with BPH [19]. This confirms previous studies in which COX-2 expression is associated with aggressive disease in prostate cancer [16-18] and led to the hypothesis that COX-2 drives increased neovascularisation. In support of this, a recent study in breast cancer demonstrated a positive relationship between COX-2 expression (as measured by weighted histoscore) and angiogenesis as measured by mean MVD using the CD31 antigen [11]. Interestingly our results demonstrated a significant negative correlation between angiogenesis, as measured by CD34 expression, and COX-2 expression. This would contradict the hypothesis that COX-2 acts as a pro-angiogenic stimulant at least in prostate cancer, and could suggest that COX-2 inhibits new blood vessel formation. However, the drop in MVD associated with a rise in COX-2 expression was lower than the observed variation in MVD at individual COX-2 expression levels (see **Figure.2**). For example, a rise in COX-2 expression from 100 to 300 histoscore units would result in a theoretical reduction in MVD from 15.88 to 10.48 microvessels/field (a fall of 5.4 units). However the actual mean MVD observed between COX-2 histoscores of 140-160, for example, is 17.76 +/- 9.9 (standard deviation) microvessels/field. At this point the variation in MVD scores is almost twice the maximum change predicted (since 90% of samples have a COX-2 histoscore between 100-300). This suggests that the variation in measurement of MVD would preclude its use as a prognostic or predictive factor. Furthermore, the high variation in

MVD scores at individual COX-2 scores would, in our view, undermine the biological significance of the observed negative correlation between MVD and COX-2 expression. *It is tempting to speculate that if a sub analysis could be performed comparing mean MVD in focal areas of high or low COX-2 expression alone, then less variation been may have been encountered. In practice, however, this would not be technically feasible due to the diffuse and heterogeneous nature of COX-2 staining within individual tissue sections. In addition there are inherent difficulties in accurately comparing protein expression within specific areas between different tissue sections, in the absence of a dual staining technique. The weighted histoscore technique for measuring COX-2 expression is therefore well established and has been used previously in comparison with mean MVD scores in breast tissue [11,16-19].*

Whilst our results therefore may reflect the methodological problems associated with MVD measurement, it may also imply that COX-2 drives tumour progression independently of neovascularisation. For example, COX-2 has well documented roles in the promotion of the inflammatory response, inhibition of apoptosis via the Akt/bcl-2 pathway, and is involved in the control of cellular proliferation via the IL-6 pathway [23]. Studies in breast cancer have revealed cellular proliferation, as measured by mitotic activity index (MAI), to have no association with angiogenesis as measured by MVD [3].

The importance of angiogenesis in tumour metastasis has been well established for over 30 years [1]. In theory inhibition of angiogenesis may provide a further

therapeutic option by targeting cancer growth and spread. In our own study however, angiogenesis, as measured by mean microvessel density in prostate cancer, was not seen to increase with increasing tumour stage or metastases in line with some studies [6,7]. However other studies have been able to demonstrate a relationship between mean MVD and advancing disease in prostate cancer [4,5,24,25]. There are similar inconsistencies with the use of MVD determination as a prognostic indicator in colorectal cancer, where MVD was lower in metastatic than primary tumours [26], and to a lesser extent in breast cancer [27]. These differences may be related to the use of different antibodies, as our MVD scores were lower than previously published results using Factor VIII and CD31 as an endothelial antigen [5,6,25]. This has also been reported in breast cancer when CD31 was compared to Factor VIII [6]. However disparities between MVD scores have also been reported between studies using the same antibody [4,6]. Furthermore, a recent report associating MVD with outcome after radical prostatectomy, quoted scores of a similar magnitude to ours when using CD34 as a antigen [28]. Similar scores to our study were also found when our protocol of MVD determination using CD34 was incorporated into a pilot study of breast cancer specimens within our laboratory (results unpublished). Other well-documented controversies do exist in angiogenesis determination, such as the presence of tumour heterogeneity [1,7]. Other aspects of methodology, such as the actual region selected for vessel counting may be as important [1,6]. If angiogenesis measurement alone is to be exploited for clinical use, current methods involving MVD analysis need to be simplified and standardised [1]. Further prospective studies are needed to explore its potential as a prognostic marker in prostate cancer. However, despite the possible

inverse correlation between mean MVD and COX-2 expression in our study, this study does not preclude the targeting of angiogenesis as a treatment modality. Angiogenesis is regulated by a complex series of molecular pathways, which, whilst they include the modulation of VEGF via PGE2 produced by COX-2, are subject to many other modulatory factors. We would therefore conclude that it is unrealistic to correlate COX-2 with a distant end-point such as angiogenesis. A more appropriate relationship may be found if VEGF expression itself was determined.

In summary, tumour angiogenesis, as measured by MVD determination using an antibody to CD34, does not increase with tumour stage. It also appears to have a weak negative relationship with the expression of the pro-angiogenic factor COX-2 in our study. This study raises the possibility that COX-2 may influence tumour progression in prostate cancer through mechanisms other than the promotion of angiogenesis. The use of selective COX-2 and angiogenesis inhibitors may still have a role in the targeted treatment of prostate cancer in the future and indeed this study might suggest these agents could be used in combination.

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References

1 Campbell SC. Advances in angiogenesis research: relevance to urological oncology. *J Urol* 1997: **158**(5):1663-1674.

2 Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996: **86**(3):353-364.

3 de Jong JS, van Diest PJ, Baak JP. Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. *Histopathology* 2000: **36**(4):306-312.

4 Bostwick DG, Wheeler TM, Blute M, Barrett DM, MacLennan GT, Sebo TJ et al. Optimized microvessel density analysis improves prediction of cancer stage from prostate needle biopsies. *Urology* 1996: **48**(1):47-57.

5 Brawer MK, Deering RE, Brown M, Preston SD, Bigler SA. Predictors of pathologic stage in prostatic carcinoma. The role of neovascularity. *Cancer* 1994: **73**(3):678-687.

6 Gettman MT, Bergstralh EJ, Blute M, Zincke H, Bostwick DG. Prediction of patient outcome in pathologic stage T2 adenocarcinoma of the prostate: Lack of significance for microvessel density analysis. *Urology* 1998: **51**(1):79-85.

7 van Moorselaar RJ, Voest EE. Angiogenesis in prostate cancer: its role in disease progression and possible therapeutic approaches. *Mol Cell Endocrinol* 2002: **197**(1-2):239-250.

8 Kirschenbaum A, Liu XH, Yao S, Levine AC. The role of cyclooxygenase-2 in prostate cancer. *Urology* 2001: **58**(2A):127-131.

9 Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K et al. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res* 1995: **55**(17):3785-3789.

10 Hwang D, Scollard D, Byrne J, Levine E. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. *J Natl Cancer Inst* 1998: **90**(6):455-460.

11 Davies G, Salter J, Hills M, Martin LA, Sacks N, Dowsett M. Correlation between cyclooxygenase-2 expression and angiogenesis in human breast cancer. *Clin Cancer Res* 2003: **9**(7):2651-2656.

12 Tjandrawinata RR, Dahiya R, HughesFulford M. Induction of cyclo-oxygenase-2 mRNA by prostaglandin E-2 in human prostatic carcinoma cells. *British Journal of Cancer* 1997: **75**(8):1111-1118.

13 Kirschenbaum A, Klausner AP, Lee R, Unger P, Yao S, Liu XH et al. Expression of cyclooxygenase-1 and cyclooxygenase-2 in the human prostate. *Urology* 2000: **56**(4):671-676.

14 Uotila P, Valve E, Martikainen P, Nevalainen M, Nurmi M, Harkonen P. Increased expression of cyclooxygenase-2 and nitric: oxide synthase-2 in human prostate cancer. *Urological Research* 2002: **29**(1):25-28.

15 Gupta S, Srivastava M, Ahmad N, Bostwick DG, Mukhtar H. Over-expression of cyclooxygenase-2 in Human prostate adenocarcinoma. *Prostate* 2000: **42**(1):73-78.

16 Madaan S, Abel PD, Chaudhary KS, Hewitt R, Stott MA, Stamp GWH et al. Cytoplasmic induction and over-expression of cyclooxygenase-2 in human prostate cancer: implications for prevention and treatment. *BJU International* 2000: **86**(6):736-741.

17 Yoshimura R, Sano H, Masuda C, Kawamura M, Tsubouchi Y, Chargui J et al. Expression of cyclooxygenase-2 in prostate carcinoma. *Cancer* 2000: **89**(3):589-596.

18 Lee LM, Pan CC, Cheng CJ, Chi CW, Liu TY. Expression of cyclooxygenase-2 in prostate adenocarcinoma and benign prostatic hyperplasia. *Anticancer Research* 2001: **21**(2B):1291-1294.

19 Edwards J, Mukherjee R, Munro AF, Wells AC, Almushatat A, Bartlett JMS. HER2 and COX-2 expression in human prostate cancer. *European Journal of Cancer* 2004: **40**(1):50-55.

20 Liu XH, Kirschenbaum A, Yao S, Stearns ME, Holland JF, Claffey K et al. Upregulation of vascular endothelial growth factor by cobalt chloride-simulated hypoxia is mediated by persistent induction of cyclooxygenase-2 in a metastatic human prostate cancer cell line. *Clinical & Experimental Metastasis* 1999: **17**(8):687-694.

21 Liu XH, Kirschenbaum A, Yao S, Lee R, Holland JF, Levine AC. Inhibition of cyclooxygenase-2 suppresses angiogenesis and the growth of prostate cancer in vivo. *Journal of Urology* 2000: **164**(3):820-825.

22 Fujita J, Mestre JR, Zeldis JB, Subbaramaiah K, Dannenberg AJ. Thalidomide and its analogues inhibit lipopolysaccharide-mediated Iinduction of cyclooxygenase-2. *Clin Cancer Res* 2001: **7**(11):3349-3355.

23 Hussain T, Gupta S, Mukhtar H. Cyclooxygenase-2 and prostate carcinogenesis. *Cancer Letters* 2003: **191**(2):125-135.

24 Hall MC, Troncoso P, Pollack A, Zhau HY, Zagars GK, Chung LW et al. Significance of tumor angiogenesis in clinically localized prostate carcinoma treated with external beam radiotherapy. *Urology* 1994: **44**(6):869-875.

25 Huss WJ, Hanrahan CF, Barrios RJ, Simons JW, Greenberg NM. Angiogenesis and prostate cancer: Identification of a molecular progression switch. *Cancer Res* 2001: **61**(6):2736-2743.

26 Mooteri S, Rubin D, Leurgans S, Jakate S, Drab E, Saclarides T. Tumor angiogenesis in primary and metastatic colorectal cancers. *Dis Colon Rectum* 1996: **39**(10):1073-1080.

27 Axelsson K, Ljung BM, Moore DH, Thor AD, Chew KL, Edgerton SM et al. Tumor angiogenesis as a prognostic assay for invasive ductal breast carcinoma. *J Natl Cancer Inst* 1995: **87**(13):997-1008.

28 Bono AV, Celato N, Cova V, Salvadore M, Chinetti S, Novario R. Microvessel density in prostate carcinoma. *Prostate cancer and Prostatic Diseases* 2002: **5**(2):123-127.