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Localization of Annexins I, II, IV and VII in whole prostate sections from radical prostatectomy patients

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Summary. Annexins (ANXs) represent a family of calcium and phospholipid binding proteins that are involved in several physiological processes e.g. signal transduction, cellular differentiation and proliferation. Since they are known to be dysregulated in a variety of cancers we investigated the immunolocalization of ANXs in whole prostate sections containing benign prostatic epithelium (BPE), benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and prostate cancer (PCa) in order to evaluate their possible role during tumorigenesis. Samples were obtained from 28 patients undergoing radical prostatectomy. Gross sections of whole prostates were examined immunohistochemically for the distribution of ANX I, II, IV and VII.

In BPE all ANXs were localized to the cell membranes and the cytoplasm of all gland cells. In BPH the immunoreactivity of ANX I and II was restricted to the basal cells of glands and expression pattern of ANX IV and VII was similar to BPE. In PIN only basal cells expressed ANX II. In PCa ANX II immunoreactivity was absent and weak ANX I and ANX IV immunoreactivity was restricted to the cytoplasm of tumor cells. ANX VII immunoreactivity was seen in some but not all tumor cells.

Since ANX IV and VII expression did not show significant changes in PCa compared to non-neoplastic tissue and PIN an essential role during prostate tumourigenesis seems unlikely. In contrast, as progression from PIN to PCa is characterized by a reduction of ANX I and II this suggests that downregulation of these proteins could represent an important event in prostate carcinogenesis.

Key words: Annexins, Prostate carcinoma, Immunohistochemistry, Prostatic intraepithelial neoplasia

Introduction

Annexins (ANXs) constitute a multigene family of Ca^{2+} and phospholipid binding proteins. They are characterized by a highly α -helical and tightly packed protein core domain building a conserved Ca^{2+} -regulated membrane binding module (Raynal and Pollard, 1994; Seaton and Dedman, 1998; Hawkins et al., 2000; Gerke et al., 2002). ANXs are involved in many cytological processes such as regulation of ion channel activities, endocytotic and exocytotic processes, signal transduction, cellular differentiation and proliferation (Gerke and Moss, 2002).

ANX I has been shown to participate in the regulation of inflammation by inhibiting phospholipase A_2 and to regulate the release of intracellular calcium (Errasfa et al., 1988; Kim et al., 2001). Moreover it is expressed during heat or oxidative stress (Rhee et al., 2000). ANX II has ion channel activity, acts as a possible anticoagulant (Waisman, 1995; Burger et al., 1996; Hawkins et al., 2000; Reutelingsperger, 2001) and is supposed as a RNA binding protein (Filipenko et al., 2004). ANX IV is characterized by binding of glycosaminoglycans and surfactant protein A in a calcium dependent manner. Furthermore, ANX IV is a substrate for proteinkinase C (Kaplan et al., 1988; Ishitsuka et al., 1998; Sohma et al., 1999). ANX VII has calcium channel activities (Gerke et al., 2002), catalyses the hydrolysis of GTP and is proposed as a tumour suppressor gene (Srivastava et al., 2001a,b).

In various carcinomas and malignant cell lines ANXs have been shown to be up- or downregulated. For instance, elevated levels of ANX I were demonstrated in malignant gastric (Sinha et al., 1998) and liver cell lines (de Coupade et al., 2000). In carcinomas of the breast (Pencil and Toth, 1998) and esophagus (Paweletz, 2000) ANX I was shown to be downregulated. For ANX II increased levels have been demonstrated in brain, breast, lung, liver, pancreatic, hematologic, gastric, and colorectal carcinoma (Frohlich et al., 1990; Cole et al., 1992; Kumble et al., 1993; Roseman et al., 1994; Chiang

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et al., 1996; Bastian, 1997; Menell et al., 1999; Emoto et al., 2001a,b). ANX IV seems to be involved in renal cell carcinoma (Zimmermann et al., 2004). Moreover immunohistochemical analysis of primary breast cancers has revealed a reduction of ANX VII (Srivastava et al., 2001a).

Previously, dysregulated ANX expression has also been observed in prostate cancer. For instance, ANX I and II have been shown either to be reduced or lost (Paweletz, 2000; Chetcuti et al., 2001; Kang et al., 2002; Banerjee et al., 2003) in PCa while other investigations noted no significant decrease of ANX I and II protein expression (Xin et al., 2003). Decreased ANX IV mRNA levels have been found in PCa (Xin et al., 2003) and for ANX VII a tumour stage dependent expression has been demonstrated in primary prostate tumours (Srivastava et al., 2001a,b).

In the present study the expression of ANX I, II, IV and VII was investigated in gross sections of whole prostates containing BPE, BPH, PIN and PCa in order to resolve the conflicting reports regarding ANX expression and to gain insight into the possible role during tumorigenesis of PCa.

Materials and methods

The present study was based on 28 prostate carcinoma patients diagnosed and undergoing radical prostatectomy in the Department of Urology, University of Greifswald. The mean patient age was 61.5 ± 9.5 years. Tissue samples and patient data for this study were obtained and used after advice from the ethics committee of the University of Greifswald and in accordance to the declaration of Helsinki. Immediately after removal, specimens were fixed in buffered formalin (48 h, 4 °C) and subsequently embedded in paraffin. Follow up data were obtained from patient records. Histological diagnosis and Gleason grading on H&E stained sections was done by two experienced pathologists.

Immunohistochemistry

Paraffin sections were cut at 4 µm, mounted on glass-slides (Superfrost plus, Menzel, Braunschweig, Germany) and deparaffinized according to standard protocols. After blocking of endogenous peroxidase activity (Peroxydazed 1, Biocarta, Hamburg, Germany, 5 min), samples were cooked in a microwave oven for antigen retrieval (10 mM citrate buffer, pH 6.0, 20 min, 700 W). Slides were allowed to cool down, washed three times [first tap water, then two times distilled water and PBS-buffer (pH 7,3), 5 min each)], and incubated with blocking solution (Biocarta, 10 min). Slides were washed (PBS, 2x5 min) and incubated overnight (4 °C) with mouse monoclonal antibodies against ANX I (1:4000, Zymed, Berlin, Germany), ANX II (1:2000, Biotrend, Cologne, Germany), ANX IV (1:1000, Acris Antibodies, Hiddenhausen, Germany) and ANX VII (1:4000, Transduction Laboratories, Lexington, USA).

After washing (PBS, 2x5 min) antibody binding was achieved using the 4plusTM Universal Immunoperoxidase Detection System (Biocarta) by incubation with secondary antibody (10 min) and subsequent washing in PBS (2x5 min). The slides were applied to Steptavidin-HRP solution (Biocarta, 10 min), washed (PBS, 2x5 min), and antibody binding was visualized with 0,1% diamino-benzidine (Sigma, Munich, Germany) in PBS/0.01% H₂O₂ (5 min). Slides were counterstained with hemalum (1 min), dehydrated and mounted in Neo-mount (Merck, Darmstadt, Germany). Control reactions were done by i) incubation with PBS alone omitting all antibodies and detection steps except DAB-chromogen reaction, ii) omitting the primary antibody and iii) by substituting the primary antibody with mouse IgG at a concentration ranging from 1:1000 - 1:4000 dependent on primary antibody dilution.

Immunohistochemical scoring by visual assessment was 1 for cytoplasmic staining, 2 for membranous staining or 3 for both, cytoplasmic and membranous staining of cells. Photographs were taken on a BX50 microscope (Olympus, Hamburg, Germany) equipped with a DP 10 digital camera (Olympus, Hamburg, Germany).

Statistical analysis

Since it was assumed that immunolocalization could be related to Gleason, statistical analysis (chi square) was done to evaluate whether there was a correlation between membranous localization of ANXs and Gleason score and cytoplasmic staining and Gleason score. P<0.05 was regarded as significant.

Results

Routine histology and grading

All samples contained BPE, BPH, PIN and tumour tissue. Gleason score ranged from 3 to 8 and pT stage from 2a to 3c (Table 1). During the clinical survey (24 months) lymph node metastases were diagnosed in one patient.

BPE

ANX I staining was scored 3 in 27 samples whereas only in one sample immunoreactivity was restricted to the cytoplasm (score 1, Table 2). Staining for ANX II, IV and VII was cytoplasmic and membranous in all gland cells of all samples (score 3, Fig. 1A-D).

Table 1. Gleason score and tumour stage of PCa samples

	GLEASON SCORE			рТ	n
number of specimen	3-4 8	5-6 10	7-8 10	2a-2c 3a-3c 24 4	28

Hyperplastic glands were immunopositive for all

ANXs. ANX I was localized to the plasma membrane (score 2) in all cases. The immunoreactivity of ANX II was scored 2 in 25 samples and 3 in 3 samples. Scoring



Fig. 1. Immunohistochemical detection of ANX I (A), ANX II (B), ANX IV (C) and ANX VII (D) in BPE. x 20

for ANX IV was 1 (1 sample), 2 (20 samples) and 3 (7 samples). ANX VII immunoreactivity was 2 in 3 samples and 3 in 25 samples (Table 2).

PIN

ANX I score was 1 in 7 samples, 2 in 18 and 3 in 3 samples. ANX II was absent in 8 samples and 20 samples showed membranous staining in the basal cells only. ANX IV score was 1 in 10 and 3 in 18 cases. The cytoplasm of 16 samples was positive for ANX VII (score 1) and 12 samples had membrane staining as well as cytoplasmic staining (score 3, Fig. 2 A-D, Table 2).

Tumor tissue

ANX I was scored 1 in 26 samples while 2 samples were negative. ANX II staining was absent in 25 tumour samples while three cases showed a weak immunoreaction at the cytoplasm (score 1). ANX IV score was 1 in 23 samples and 3 in 5 samples. Staining for ANX VII was cytoplasmic in 21 samples (score 1) and membranous in one sample (score 2). 6 samples showed both cytoplasmic and membranous immunoresponse (score 3, Fig. 3 A-D, Table 2).

Statistical analysis revealed no correlation between ANX-immunolocalization and Gleason score (P>0.05).

In summary, the comparison of immunoreactivity scores between benign and malignant tissue revealed a loss of ANX II as well as a reduction of ANX I and IV in tumour tissue. ANX VII expression, however showed no differences when comparing BPE, BPH, PIN and PCa.

Discussion

The present immunohistochemical study of whole mounted prostate specimens has shown that ANXs I, II, IV and VII are expressed in non-neoplastic epithelial prostate cells and revealed i) a faint staining for ANX I in BPH, PIN and PCa, ii) a positive reaction for ANX II in the basal cells of BPH and PIN but no staining in PCa, iii) a faint immunostaining for ANX IV in BPH, PIN and PCa and iiii) no differences of ANX VII immunoreactivity when comparing BPH, PIN and PCa.

In recent studies ANX I was reduced in PCa as shown by both Western blot analysis of laser captured microdissected prostate tumour tissue and by

Table 2. Summary of patient data and protein expression.

PSA ANX IV SCORE ANX VII SCORE A G ANX I SCORE ANX II SCORE BPE BPH PIN PCa BPE BPH PIN PCa BPE BPH PIN PCa BPE BPH PIN PCa 2^b 8.40 3+4=7 2^b 8.40 3+4=72^b 7.50 3+4=72^b 13.70 2+3=52^b 2.12 2+3=5 З З З 0^b З З 7.64 2+3=52^b 10.50 2+3=5 З 2^b 6.50 3+4=72^b 3.83 3+5=8З 2^b З 5.27 3+4=7 2^b 6.00 2+2=46.38 2+2=4 0^b 6.38 2^b 2+2=4 2^{b} 5.91 3+4=72^b 6.05 3+4=72^b 3.81 3+3=6 2^b 13.8 2+3=52^b 4.05 1+2=30^b 7.3 2+3=5 2^b 7.95 2+3=5З 2^b З З З 0.071 3+5=8 0^b 12.1 1+2=31.13² 2^b 2+3=5З 0^b 29.3 3+4=70^b 3.77 1+2=33+4=70^b 7.52 0^{b} 7.01 2+2=4 5.63 2+2=42^b

A: age at diagnosis; PSA: prostate specific antigen (ng/ml), (1) preoperative antiandrogene therapy, (2) PSA-level was determined after transurethral resection of the prostate (TUR-P); G: Gleason score; BPE: benign prostate epithelium; BPH: benign prostate hyperplasia; PIN: prostatic intraepithelial neoplasia; PCa: prostate carcinoma. In PINs immunoreactivity was absent in all glandular epithelial cells and in basal cells(b) of 8 samples for ANX II.



Fig. 2. Immunohistochemical detection of ANXs in PIN. Cytoplasmic and membranous staining (representing score 3) for ANX I (A) and ANX IV (C) in neoplastic cells (score 3). ANX II immune response is limited to basal cells only (B). Staining for ANX VII (D) is cytoplasmic in neoplastic cells (score 1). x 20



Fig. 3. Immunohistochemical detection of ANXs in PCa. ANX I is predominantly localized in the cytoplasm of tumor cells (A). ANX II is absent in tumor cells but note the strong staining of nerve fibres (B, asterisks). ANX IV is present in the cytoplasm of tumor cells (C) and ANX VII is found cytoplasmatically and membranous (D). x 20

immunohistochemistry (Paweletz, 2000). This is in accordance with our study but in contrast to Xin (2003) which has not found significant differences between ANX I immunoreactivity in benign and neoplastic tissue (Xin et al., 2003). However, a downregulation of ANX I mRNA-levels has been demonstrated by quantitative PCR (Kang et al., 2002). These results were confirmed by cDNA microarray analysis (Xin et al., 2003), but high levels of ANX I protein suggest an accumulation of ANX I by tumour cells (Xin et al., 2003).

The differentiated expression pattern of ANX II among BPH, PIN and PCa as shown in the present and recent studies (Chetcuti et al., 2001; Banerjee et al., 2003) indicates that the loss of ANX II is involved in prostate cancer progression. Although ANX II protein was shown to be absent, low levels of ANX II mRNA were still detectable in PCa (Chetcuti et al., 2001). In contrast to our findings a previous study reported ANX II immunoreactivity in PCa as well as in non-neoplastic tissue (Xin et al., 2003). Due to the fact that ANX II is associated with proliferating and invasive cancer it has been proposed to be a marker for malignancy (Reeves et al., 1992). Consistently it is upregulated in a variety of other tumours including pancreatic (Vishwanatha et al., 1993) and astrocytic brain tumours (Roseman et al., 1994). Moreover, in gastric and colorectal carcinoma, ANX II overexpression correlates with poor prognosis (Emoto et al., 2001b). So far, loss of ANX II seems to be unique for PCa.

Since ANX II is absent in glandular epithelial cells of PIN, but present in basal cells PIN can be unequivocally differentiated from BPE (all cells immunopositive) as well as from PCa (all cells immunonegative). Currently 34BE12 represents the standard by highlighting the absence of basal cells in infiltrating prostate cancer. However, 34BE12 is not suitable for differentiation of PIN and non-neoplastic tissue, since basal cells are present in both. Therefore, since ANX II is present exclusively in basal cells of PIN but is lacking in glandular cells it seems to be a suitable marker (Chetcuti et al., 2001). The functional role of ANX II during cancer is yet poorly understood but there is evidence for an involvement in cell proliferation and differentiation under the control of receptor tyrosine kinases and DNA replication (Niki et al., 1996; Rothhut, 1997; Chiang et al., 1999). For instance, ANX II inhibits cell migration and nuclear ANX II reduces cell proliferation as shown by in vitro studies on malignant prostate cell lines (Liu et al., 2003a,b). In addition ANX II affects cellular DNA synthesis and cell proliferation of other human cancer cells (Chiang et al., 1999).

Current data of ANX IV expression in PCa indicate a decrease of mRNA levels whereas similar protein levels were seen in non-neoplastic and tumour tissue (Xin et al., 2003). In contrast our study revealed a weaker immunoreactivity in PCa when compared to non-neoplastic tissue. Obviously, from these findings, ANX IV does not necessarily seem to be related to the pathogenesis of PCA.

Previously a significant reduction of ANX VII

protein was detected in a stage-dependent manner in PCa (Srivastava et al., 2001b). Thus, a complete loss of the protein has been observed in a high proportion of metastasis and in local recurrences of hormone refractory PCa. However, in primary tumours (T2-T4), PIN and BPE the ANX VII protein remains high (Srivastava et al., 2001b) which is consistent with Xin et al. (2003) reporting no significant discrepancy of ANX VII protein as well as mRNA levels between BPE and localized PCa (Xin et al., 2003) and our immunohistochemical findings. Although ANX VII reduces cell proliferation and colony formation in LNCaP cells and thus could act as tumour suppressor it seems unlikely that ANX VII expression is involved in the progression from PIN to PCa. On the other hand there is increasing evidence that ANX VII exhibits tumor suppressor activities since it is lost during progression from primary PCa to metastasis (Srivastava et al., 2001b).

Taken together, our data strongly indicate that ANX I and II might be involved in prostate tumorigenesis rather than ANX IV and VII.

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