Biomed. Papers 147(1), 11–17 (2003)

THE SIGNIFICANCE OF KEY REGULATORS OF APOPTOSIS IN THE DEVELOPMENT AND PROGNOSIS OF PROSTATE CARCINOMA. II. PRODUCTS OF SUPPRESSOR GENES RB AND PTEN, CDKI, FAS

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Received: April 20, 2003; Accepted in revised form June 15, 2003

Key words: Prostate cancer / Apoptosis / Gene expression / Rb / PTEN / CDKI / Fas

The molecular basis for the transition of carcinoma of the prostate from androgen-dependent to androgen--independent growth is largely unknown. Currently for example, it is not clear whether the androgen-independent phenotype is a result of selection of a subgroup of genetically distinct prostate tumour cells which are already hormone-resistant or a genetic adaptation of prostate tumour cells to the hormone therapy itself. It has also been established that prostate tumour transformation is a result of homeostatic control defects, a line of thinking directed toward elucidating the apoptotic profile of prostate tumour cells that may be important in determining prognosis, response to therapy and illness progression. Main consideration in this part of rewiev is given to the role of tumour suppressor genes pRb and PTEN and also the natural inhibitors of cyclin dependent kinases – proteins $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. Attention is also given to the role of FAS-mediated pathways in apoptosis induction.

Rb

Retinoblastoma gene (Rb), a member of the tumour suppressor gene family, is localized on the short arm of chromosome 13. It codes the cell cycle regulating 110 kDa protein and it is expressed in the majority of cell types regardless of their proliferative status. Inactivation of both copies of the gene gives rise to ocular tumours (retinoblastomas) in the same way as other neoplasms[1]. Retinoblastoma protein (pRb) may be phosphorylated in various sites and its phosphorylation status changes during different phases of the cell cycle. Nonphosphorylated or hypophosphorylated forms of the protein block transition through the restriction point of the cell cycle. In this form, pRb and its related proteins p170 and p130 ("pocket proteins") bind transcription factors, mainly members of the E2F (E2F1-3) and DP families. These transcription factors are implicated in the regulation of the expression of genes necessary for progression to the S phase and binding by pRb blocks their activity. During progression through the G1 phase, pRb is phosphorylated. This facilitates the release and activation of E2F/DP transcription factors and permits progression to late G1 phase and completion of the cell cycle[2]. Key components of the cell cycle initiating pRb phosphorylation include D cyclins and associated kinases cdk4 and cdk6. Complex cyclin E/cdk2 is then responsible for pRb hyperphosphorylation. Cells with permanently overphosphorylated pRb, in other words those with overactive or insufficiently inhibited cdk4 (6)/cyclin D, and cells which are unable to dephosphorylate pRb or lack the ability to express pRb, do not control the restriction point and pass it without stopping. All these defects can lead to uncontrolled growth[1, 3]. As far as proliferation is concerned Hofman et al. demonstrated that a biphasic dose-dependent response of the prostatic cancer cell line LNCaP to androgens is closely reflected in pRb phosphorylation, E2F activity and $p27^{Kip1}$ protein expression[4].

Mutational changes in the Rb gene found in specific tumours are linked to aggressive behaviour and poor clinical prognosis[5, 6]. Molecular based studies of Rb alterations in prostate cancer are limited however and thus no firm conclusion can be drawn. Single stranded conformation polymorphism analysis (SSCP) of RNA showed that 16 % of human primary tumours had altered Rb[7, 8]. Other research groups evaluated genetic alterations in the region of the Rb gene on the basis of loss of heterozygosity (LOH). In 27–60 % primary prostate cancer cases LOH has been described. LOH in the Rb gene has also been found in benign prostatic hyperplasia (BPH) samples[8–10]. Debatable is whether inactivation of the Rb gene is a primary event in the pathogenesis of prostate cancer or a secondary event connected to the progression of the illness[11]. Bookstein et al. studied pRb protein expression in three different prostate cancer cell lines and found that the DU-145 cell line contained abnormally small protein translated from Rb mRNA transcript and lacking 105 nucleotides encoded on exon 21 (ref.[12]). The finding of short mRNA transcript in DU-145 cell line was confirmed in two further studies[13, 14]. In prostate cancer tissues however no similar

alterations have been found to date[8]. Using immunohistochemical techniques, absence of pRb protein has been described in 17–78 % of cases. The significance of Rb alterations in prostate cancer, however, remains problematic[8, 9, 14]. There is insufficient correlation between LOH in Rb and missing pRb expression. In addition, evaluation of pRb expression may be influenced by fixation artefacts, variability of immunohistochemical reaction (sensitivity and specificity of antibody) and interpretation of staining results. Whether the reduction of pRb staining has any significance for predicting the malignancy potential of a given carcinoma lesion is a question requiring further study in a well characterised population of prostate cancer patients[8].

Rb activity is also connected to apoptosis induction in different cell environments. Day et al. have documented the functional role of Rb in signalling apoptosis in prostate tumour cell studies[15, 16]. Inactivation of the Rb apoptotic pathway is important in the case of metastatic progression of prostate tumours[6]. Kaltz-Wittmer et al. carried out a FISH analysis of gene aberration (myc, CCNDI, erbB-2, Rb and AR) in advanced prostate tumours before and after androgen therapy. Loss of the Rb gene was almost four times more frequent after therapy than before therapy. Changes in the number of gene copies before and after therapy demonstrate the possibility that these genes are connected to exit from androgen control[17]. Despite the fact that the predisposition of prostatic epithelial cells to cancerogenesis in the absence of Rb tumour suppressor gene has been shown in rats, the prognostic evaluation of Rb is not currently clear[18, 19].

PTEN/MMAC1/TEP1

Gene PTEN/MMAC1/TEP1 (PTEN) is localised on chromosome 10q23.3 and codes dual-specificity phosphatase. This has the ability to dephosphorylate both tyrosine phosphate and serine/threonine phosphate residues on proteins. The in vivo function of PTEN appears to be dephosphorylation of phosphatidylinositol 3,4,5-triphosphate[20–22]. PTEN possesses a PDZ [postsynaptic density protein (**P**DS-95)/Drosophila disc large tumour suppressor (**dl**g)/tight junction protein (**Z**O1)] motif represented by the amino acids Ile-Thr-Lys-Val. In addition to the site responsible for dual-phosphatase activity, the amino-terminal domain of PTEN protein contains a region which is homologous to cytoskeletal proteins tensin and auxillin[23]. PTEN may be also a component of the signalling complex associated with focal adhesion kinase (FAK)[24]. LOH in the region 10q23.3 is linked to a number of tumours including carcinomas of the prostate and mutations of PTEN were, contrary to other tumours, also found in prostate tumour cell lines, xenografts and tissue samples from hormone resistant prostate tumours[20, 25–27]. LOH in the PTEN locus was confirmed in 29–42 % tumours of prostate and screening for homozygous deletions of the gene showed a second mutational event in 43 % of prostate tumours. LOH of 10q was found in 20 % tumours localised in the prostate and in 46 % metastases[11, 28]. Suzuki et al. found 55 % LOH for PTEN in patients who had died of hormone-refractory prostate cancer with widespread metastases[29]. Wang et al. concluded that from 10 % to 15 % of stage T2 prostate carcinomas had homozygous deletion of the PTEN gene[30]. The aim of Rubin et al. was to find the frequency of LOH at 10q23.3 in the region of PTEN in men with and without lymph node positive prostate cancer. The findings of an elevated number of alterations in node-positive tumours of the prostate suggest that 10q23.3 is a marker for metastatic progression[27]. Mutations and genetic alterations (LOH) seem to be more frequent in higher stage cancers and prostatic tumour cell lines than in localised prostate tumours. In patients with an inherited predisposition to cancer of the prostate no germ line PTEN mutations were found[31, 32]. The majority of deletions involving PTEN eliminate the PDZ motif and thereby interrupt interactions with other proteins possessing PDZ domains[33]. These include Fas (CD95/APO-1) and adenomatous polyposis coli (APC) tumour suppressor protein containing the C-terminal PDZ motif. The majority of identified mutations block phosphatase activity or influence the stability of the enzyme. Hence phosphatase activity of PTEN is thought to be critical for tumour suppressor activity[34]. This suggests that PTEN may be an important tumour suppressor in a subset of prostate carcinomas. Inactivation of PTEN by contrast may be an important secondary genetic event contributing to progression of prostate cancer and giving prostatic tumour cells a selective advantage[11]. McMenamin et al. inquired into PTEN expression in a series of archival paraffin embedded samples of prostate cancer. PTEN expression was seen in secretory cells while a complete absence of PTEN expression correlated with Gleason score and with advanced pathological stage. Loss of PTEN protein thus correlated with the pathological markers of poor prognosis in prostate tumours[35].

The ability of PTEN to regulate apoptosis is the subject of active research. Davies *et al.* tested biological and biochemical effects of PTEN expression in LNCaP cell line which is devoid of functional gene products. Expression of PTEN in these cells was found to be related to inhibition of Akt/PKB activation, to apoptosis induction and growth inhibition. Overexpression of Bcl-2 blocked apoptosis induced by PTEN and p53, but not the growth-suppressive effects of PTEN suggesting that the growth-regulatory effects of PTEN involve multiple pathways[36]. Sharrard *et al.* investigated the influence of PTEN expression on prostate tumour cell lines. They showed that overexpression of PTEN in transfected cell lines led to shrinkage and rounding of cells but did not result in increased levels of classical apoptosis[37]. Huang et al. using prostate cancer cell lines showed that loss of PTEN leads to up-regulation of the bcl-2 gene, thus contributing to survival and chemoresistance of prostate cancer cells[38]. Studies of the mutual antagonism

between PTEN and androgen receptor (AR) was the subject of Li et al's work. On the one hand, PTEN repressed the transcriptional activity of AR, on the other hand, androgens protected prostate cancer cells from PTEN-induced apoptosis in an AR-dependent manner[39]. Loss of PTEN function may induce tumourigenesis through unopposed activity of AR as well as contribute to the resistance of prostate cancers to androgen ablation therapy. Also the combined tumour-suppressive activity of PTEN and $p27^{Kip1}$ through the control of the cell cycle progression shows the key significance of these proteins. Their co-operation in prostate cancer has been demonstrated in mouse model by Di Cristofano et al. PTEN activity leads to the induction of $p27^{Kip1}$ expression, which in turn can negatively regulate transition through the cell cycle. Thus, the inactivation of $p27^{Kip1}$ may be epistatic to PTEN in the cell cycle control[40].

$p21^{Waf1/Cip1/SdII}$

Protein $p21^{Waf1/Cip1/SdII}$ (p21) belongs to the KIP/CIP family of CDK inhibitors. Gene encoding for p21 is localised on chromosome 6p21. It has been shown that p21 inhibits activity of each member of the cyclin/CDK family and thus it is a universal inhibitor of cyclin dependent kinases. In normal cells p21 creates quaternary complexes together with CDK, cyclin and proliferative cell nuclear antigen (PCNA). It is interesting that according to stoichiometry of the cyclin/CDK/p21 complex it may function as a factor stimulating kinase activity or as an inhibitor[41]. Apart from inhibiting cyclin/CDK complexes, protein p21 also interacts with complex PCNA-DNA polymerase δ. It is, as well, an essential protein for DNA replication and repair[42]. In the case of DNA damage, p21 is a key protein for inducing G1 cell cycle arrest. Its expression is regulated by tumour suppressor gene p53[43]. In addition, in human cells p53 as well as p21 appear to be important for maintaining the G2 checkpoint[44]. This aside, p21 may be activated in a p53 independent way, mainly during development[45]. Protein p21 also exerts other cell functions such as differentiation, senescence or apoptosis. However, the role of p21 in apoptosis induction remains controversial[46].

The role of p21 expression in prostate cancer and its prognostic value is at the present little understood. Mutations of p21, in contrast to mutations of p53 and pRb, should be very rare in tumour cells[47]. However, SSCP analysis of primary tumours of the prostate, which lacked demonstrable p53 mutations, revealed that 18 % of the tumours carried p21 mutations[48]. LOH involving chromosome 6p, where p21 gene is also localised (6p21.2), has been found in prostate cancer. Loss of p21 activity may thus contribute to progression of prostate cancer[11, 49].

The relation between p21 and cell proliferation, apoptosis, expression of p53 and Bcl-2 is very variable and the clinical significance of p21 immunostaining is still unclear. In prostate cancer cell lines, p21 has an inhibitory influence on cell growth but in clinical studies of prostate cancer the role of p21 is almost untouched[50, 51]. Byrne et al. showed via immunohistochemical analysis of p21 protein in a series of 40 tumour samples no correlation between p21 immunoreactivity and tumour progression, grade or stage[52]. Further immunohistochemical analysis of p21 expression in 213 cases carried out by Aaltomaa et al. however demonstrated that p21 expression is significantly linked to high Gleason score, aneuploidy, high cell number in the S phase and also to expression of Ki-67, Bcl-2, cyclins A and D. p21 expression in this study significantly correlated with unfavourable prognosis and p21 was thus described as an independent predictor of survival in prostate cancer patients[51]. Other recent studies confirm these findings. Sarkar et al. showed the correlation between p21 expression, pathological stage and Gleason score. They also found differences in values of p21 as a prognostic marker of disease-free survival in Caucasians vs. African Americans and suggested that progression to prostate cancer may have different mechanisms in different ethnic groups[53]. A correlation between p21 and proliferation has also been described[54, 55]. In both studies positivity of p21 was linked to high proliferative index Ki-67. Overexpression of p21 before and after androgen deprivation therapy may characterise a subgroup of advanced carcinomas of the prostate with paradoxically high proliferative index and significantly poorer prognosis. In patients after radical prostatectomy, not treated by neoadjuvant therapy, a p21 positive phenotype occurred in connection with shortened relapse period[56]. According to Cheng et al. however, p21 positivity was a positive predictor of survival[57].

The relation between p53 and p21 expression is also debatable. A relation exists between these proteins for example in cases of breast cancer but not in tumours of the pancreas. Aaltoma et al. however found no correlation in prostate cancer samples[51]. The relation between p21 and p53 expression is complex and seems to be specific for single tumour types. Osman et al. found a significant relation between p21 and MDM2 expression. These authors assume that progression to prostate tumour involves inactivation of p53 due to overexpression of MDM2 and that transactivation of p21 is caused by an p53 independent signal pathway[55].

There are also interesting works focused on the relation between androgens, AR and p21 expression. Agus et al. studied factors connected to regression of prostate cancer after androgen deprivation. They found that in early phases after androgen withdrawal AR expression was decreased, followed by transient increase in p53 and p21 protein expression[58]. In an androgen-independent cell line derived from parental LNCaP cell line it has been shown that the use of antisense oligonucleotides against AR caused reduction of AR levels leading to increase in p21 expression and partial restoration of androgen-dependence[59]. In other work it has been demonstrated that androgens stimulated endogenous p21

gene expression at the transcription level. The androgen-responsive element (ARE), which mediates the response to androgens and increases transcription of p21, is located within 2.4 kb p21 gene promotor. Increase in p21 gene expression by androgens shows that p21 may have an antiapoptotic function in epithelial cells[60]. Modulation of p21 expression by androgens has been also confirmed immunohistochemically[61].

$p27^{Kip1}$

Protein $p27^{Kip1}$ (p27) belongs to the CIP/KIP family of CDK inhibitors. Gene coding for p27 is localised on chromosome 12p13 (ref.[62]). Expression of p27 is regulated by contact inhibition and specific growth factors (for example TGF-beta, IL-2, cAMP). p27 expression levels are higher in quiescent and differentiating cells. Protein p27 accumulates in the G1 phase of the cell cycle and causes its arrest. When the cell enters the cell cycle following mitogenic stimulation, p27 is proteolytically degraded and the p27 mediated repression of complexes cyclin E/cdk2 is released. Apart from its presumed role as a tumour supressor, many other functions are attributed to p27 such as regulation of drug resistence in solid tumours, as an auxiliary factor in apoptosis, etc.[63]

Elucidating the role of p27 as CDK inhibitor in normal and neoplastic cells has to date been the focus of major attention. It has been shown that loss of p27 protein expression may lead to tumour development and progression. Absence of p27 in tumours is connected to poor prognosis although surprisingly few p27 mutations have been described in tumours[47]. Lowered levels of p27 in tumours are caused chiefly at post-transcriptional levels owing to a higher degradation by the ubiquitin/proteasome pathway. Another important mechanism for p27 degradation seems to be phosphorylation. Among further mechanisms connected to regulation of p27 expression are methylation and regulation on the protein synthesis level (mRNA stability, localisation and translation)[64].

The actual diagnostic and prognostic significance of p27 expression in different tumours has been investigated more recently. Virtually all studies have shown low expression of p27 in more aggressive tumours and a number have confirmed the usefulness of p27 as an independent prognostic and/or diagnostic marker in a wide spectrum of human tumours including carcinoma of breast, tumour of large intestine, adenocarcinoma of prostate, esophagal adenocarcinoma, nonsmall cell lung cancer, malignant melanoma and endocrine tumours[63, 65]. Mutational inactivation of p27 in tumours of the prostate is infrequent[47]. Methylation CpG islets has also been described. However it is not frequent and does not seem to be the mechanism responsible for deregulation of p27 (ref.[66]). No further mutations have been identified[67].

A series of studies analysed p27 expression in prostatic adenocarcinomas. Guo et al. for example first showed the correlation between loss of protein expression, higher grade of tumour and proliferative status[68]. Their results were confirmed in further studies. In addition p27 expression indirectly correlated with higher Gleason score, with occurrence of metastasis to lymphatic glands, and anueploidy[69, 70]. It has also been shown that low p27 expression is an independent predictor of treatment failure and an independent prognostic factor for relapsing illness or shorter survival time[63, 71, 72]. Thomas et al. used p27 to preselect patients with high risk of relapse. In their group of patients p27 expression in preoperative samples significantly correlated with p27 expression in samples after radical prostatectomy[73]. p27 expression in benign lesions is not clear. Cordon-Cardo et al. found almost undetectable levels of protein p27 and p27 mRNA in BPH in epithelial cells and the stromal cells of BPH lesions. This finding would support the concept that BPH is not a precursor to prostate cancer[74]. By contrast, other work using immunohistochemical determination of p27 in normal and in benign prostatic epithelium, as well as studies of the possible existence of abnormalities in the progression of prostatic carcinomas, found that p27 expression is expressed constitutively in normal and benign prostate tissue[75]. Its expression is apparently lowered following neoplastic progression from preinvasive lesions to invasive carcinomas and metastasis. There is, in addition, an interesting study showing that cell cycle regulators are potential epigenic targets for the prevention of prostate cancer through suitable dietary supplements such as silymarin, genisten, and EGCG[76].

Fas (Apo-1/CD95)

Fas (Apo-1/CD95) is a death receptor belonging to the tumour necrosis factor receptor (TNFR) family. It is a cell-surface receptor protein which may initiate certain intracellular signalling pathways leading to apoptosis[77, 78]. It may be activated by its natural ligands (FasL/CD95L) or nonspecifically by antibodies against its internal domain. Fas and FasL play an important role in these types of physiological apoptosis where they exert control chiefly in the immune system. They may also contribute to cancer cell's escape from immune system control. Mutations of the genes coding for Fas or FasL may lead to lymphoproliferative and autoimmune diseases[79]. FasL is a homotrimeric molecule and each FasL binds three molecules of Fas. Trimerisation of Fas results in the recruitment of the cell death inducing signalling complex (DISC) which includes the adaptor protein FADD and procaspase 8 (ref.[78]). Activation of caspase 8 in turn leads to activation of the execution phase of the apoptotic programme. This appears to follow one of two pathways: (1) via direct cleavage and activation of caspase 3 or (2) by indirectly causing the

release of mitochondrial cytochrome c. This creates apoptosom with APAF-1 and procaspase 9 (ref.[80]).

The importance of the Fas signalling cascade in prostate cells was shown in apoptosis induced by castration in normal rat prostate[81]. Fas is expressed in several prostatic carcinoma cell lines but its in vivo expression in normal prostate and in prostate cancer is poorly understood. Diaz et al. showed Fas expression in secretory cells in benign samples of prostatic tissue, expression in all locally growing tumours and significantly decreased expression in prostate carcinomas compared with benign prostate. The decrease was inversely related to the malignant grade of the tumours. Diminished expression of Fas according to this study seems to be an early molecular event in prostate cancer[82]. Mutations of the Fas gene might be involved in proliferative diseases of the prostate by prolongation of programmed cell death of prostatic epithelial cells. Takayama et al. detected Fas mutations exclusively in high-grade prostatic intraepithelial neoplasia (HGPIN) (14.3 %) and LOH occasionally found in HGPIN and prostate cancer. These results also show that genetic instability may occur during the early phase of prostate carcinogenesis[83]. The evidence from several studies shows that activation of the Fas/FasL pathway is connected with sensitisation of androgen-independent human prostatic cells during apoptotic response to different chemotherapeutic compounds[84, 85]. The pathways leading to Fas mediated apoptosis in prostate cancer cell lines are intact as suggested from apoptotic program which may be triggered either by Fas-ligation in the Fas-sensitive cell lines PC3 and ALVA31 or by rendering the Fas-resistant cell lines DU145 and JCA1 by Fas combined treatment with anti-Fas monoclonal antibody and cycloheximide[86]. Further works have shown that the mitochondrial pathway is implicated in Fas-mediated apoptosis in prostate cancer cell lines[87]. Two of the early events after Fas ligation are the release of cytochrome c from the mitochondria and activation of caspase 9. Processed is also protein Bid and this might activate the mitochondria-dependent apoptotic cascade. Induction of Fas-mediated apoptotis in prostate cancer cell lines using different external Fas agonists i.e., anti-Fas antibodies and membrane-bound FasL, does not seem to be very successful. Adenovirus-mediated intracellular expression of FasL seems to be more potent and thus potentially exploitable for gene therapy of prostate cancer[88, 89]. If prostatic epithelial cells harbour intact a Fas signalling pathway, sensitisation of androgen-independent tumours to Fas-induced apoptosis becomes an appealing therapeutic target, with potential clinical application in treating advanced prostate cancer[6].

CONCLUSION

Tumour progression can be ascribed to an imbalance between proliferation and programmed cell death, which is caused by alterations in the genes essential for regulation of cell growth, differentiation and apoptosis. Refractoriness of many types of cancer to available anticancer therapy is very often connected with the selection or acquisition of molecular mechanisms suppressing cell death induction. These mechanisms frequently involve an aberrant expression or function of cell death effectors and growth inhibitors, such as Fas, $p21^{Waf1/Cip1}$, $p27^{Kip1}$ or Rb and PTEN tumour suppressor genes. Recently, analysis of these gene products has yielded important prognostic information for many tumour types. Although a great deal of knowledge about the role of the tumour suppressors and CDK inhibitors in the cell cycle progression and tumour development has accumulated, there are still many unanswered questions. Loss of the function of these genes, potentially involving several different mechanisms, appears to play a role in prostate cancer tumourigenesis, particularly in cancer progression. These recent insights into the molecular basis of cancer and advances in our understanding of the integrated functions governing cell proliferation and apoptosis can permit development of novel therapeutic modalities and new strategies involving the restoration of cell death signalling pathways.

AKNOWLEDGEMENTS

This work was partly supported by IGA MH CR NC/6200-3 and NC/6779-3, and MSM 151100001. Our thanks go to Alexander Oulton for his kind help with translation.

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