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Abstract

Prostate cancer is an enigmatic disease. Although prostatic-intraepithelial neoplasia appears as early as the third decade and as many as 80% of 80 year old men have epithelial cells in their prostate that fit the morphological criteria for cancer, only about 10% of men will ever have the clinical disease and less than 3% will die from it. There have been no significant proven interventions which have altered the natural history of the disease since hormone down regulation was introduced in the 1940s and new research has been poorly supported. There is however an urgent need to develop new criteria to distinguish those patients with localised disease who will benefit from intervention from those that do not require it or who will have occult extra prostatic metastases. Similarly, there is an urgent need to develop new treatments for those in whom the disease is extra-prostatic and therefore incurable by conventional treatments. This review covers the latest developments in epidemiology, cellular and molecular biology including new areas such as ion channels in the field of prostate cancer.

Prostate cancer – clinical perspectives

Prostate cancer (PC) is the commonest cancer in men and the second commonest cause of cancer death in the western world [1]. There were an estimated quarter of a million cases world-wide in 1985 [2] and the incidence appears to be increasing [3]. In the early 1990's, it was predicted that PC would account for 35,000 deaths annually in the European Community [4] and 38,000 in the USA [5] where it presents a lifetime risk of almost 10% [6]. More recently, it has been estimated that in 1996 there will be 317,000 newly diagnosed PC cases and 41,400 deaths in the USA compared to the 1995 figures of 244,000 and 40,400 respectively [7]. The difference between incidence rates and death rates is striking but the increase in the latter is at least in part because of demographic trends, with many more men surviving to an older age when PC is more frequent (1600:100000 in black American 80 year olds compared to less than 2:100000 in 40 year olds [3]). In contrast, the rise in incidence rates is almost certainly a consequence of the introduction of serum assays for prostate specific antigen (PSA) as well as more digital rectal examinations, transrectal ultrasound examinations and prostatic needle biopsies being performed in the clinic.

Whether PSA screening will enable the identification of cancers at a time when they will be curable is contentious [8–10]. This is probably because screening reveals many men with PCs that would never become clinically significant. Screening also finds men with PC which seem localised but with occult metastases which become apparent after local treatment (Table 1). Neither group would benefit from such treatment and it remains impossible, at the current time, to distinguish one group from another. The extent of the controversy can be demonstrated by one study which postulated that should all men in the UK be screened once and half the detected cancers treated with radical surgery, the probable operative mortality would exceed the an-

nual death rate from PC in the same population [11]. However, randomised trials of screening are underway, for example initiated by the National Cancer Institute [12] whose demanding protocol may cost US$89 million.

Aetiology of prostate cancer

There is evidence that differing incidences of PC are influenced by epigenetic factors. The increased incidence of clinical cancer in Japanese immigrants to the USA within 2 generations offers one such example [13]. Diet is believed to be relevant, with the predominantly vegetarian diet in Asia exerting a protective role [14]. Total fat consumption has been directly related to the risk of advanced PC, especially saturated fat as well as red meat and high caloric intake [15]. Other aetiological factors include vitamins A and D. Vitamin E is suggested to have a protective effect [16, 17]. Employment in agricultural occupations appears to have a positive association with PC [18, 19]. Even prenatal exposure to pregnancy hormones and growth factors may influence prostate carcinogenesis [20].

Management of prostate cancer

Whitmore has asked 'Is cure possible in those for whom it is necessary, and is cure necessary in those for whom it is possible?' [21]. There is no doubt that large numbers of patients require effective treatment for their disease, whether localised or extraprostatic. However, there have been no major changes in mortality and no major advances in treatment since the introduction of androgen down-regulation won the Nobel prize for Huggins in the early 1940s. There has however recently been a huge rise in the numbers of radical prostatectomies performed, in parallel with the current rise in diagnosis. The first description of radical surgery appeared over 90 years ago [22] and despite this, there is still no conclusive proof that it is of benefit [23]. Some commentators have actually described the use of radical prostatectomy as 'probably the longest running phase 1 cancer trial in the history of medicine' [24].

Clinically significant and latent cancer

Part of the problem in managing PC lies with the fact that there is a 'latent' form of the disease that is equally prevalent in communities throughout the world [25]. Latent cancer is generally an incidental finding, usually identified in post-mortem studies or in prostates removed surgically for apparently benign disease or more recently following PSA testing and imaging directed stereotactic biopsy. Latent PC is by definition small, generally well differentiated and the clinical behaviour is usually indolent. This cancer may be found as early as the third decade and has been reported in about 30% of fifty year olds and up to 80% of 80 year olds at post-mortem [26]. The incidence of latent cancer occurs equally across countries and racial groups. This is in sharp contrast to clinically important cancer which is rare in Asia (1:100000) but reaches epidemic proportions in the west, the highest rates being reported in Afro-Caribbean men in the USA (see above) [3].

New genotypic and phenotypic markers are therefore urgently needed to classify the disease accurately and guide management. The underlying molecular and cellular mechanisms governing initiation and promotion of the malignant and metastatic phenotypes are unknown in PC, although some clues may be gained from the study of other

Table 1. Clinical manifestations of prostate cancer

'Localised' prostate cancer	Extraprostatic prostate cancer
Latent (clinically unimportant)	Locally advanced without metastases
Clinical without local invasion or metastases (curable)	Locally advanced with metastases
Clinical with undetected local occult metastases (incurable)	Metastases but localised primary

cancers. Of particular relevance is the need to investigate factors governing hormone-regulation which may be specific to PC and the biology of the stromal proliferation in metastatic disease which governs the clinical manifestations. This may lead to rational strategies for cancer management rather than the currently unobtainable (and perhaps unnecessary) goal of cancer 'cure'/eradication.

Prostate cancer – biological aspects

Anatomical structure of the prostate gland

The prostate is a pear shaped glandular organ weighing up to 20 grams in the young adult male. It is enveloped by a capsule which is composed of an inner layer of smooth muscle and an outer fibrous capsule [27]. The prostate comprises three distinct zones: the peripheral (PZ), central (CZ) and transition zones (TZ) [28, 29]. The PZ is the largest zone (70% by volume) and is the commonest site for prostatic intraepithelial neoplasia (PIN) and adenocarcinomas which both arise from peripheral ducts and acini [30]. The CZ, which encircles the ejaculatory ducts, occupies approximately 25% of the volume of the prostate. The remaining 5% is formed by the TZ which is the primary site for nodular hyperplasia and the rare large duct carcinomas. Collectively the PZ and CZ are often referred to as the outer or cortical prostate, whereas TZ and the anterior fibromuscular stroma are known as the inner or periurethral prostate. In a normal prostate it is difficult to delineate the three zones histologically.

The glandular components (acini and ducts) of the prostate are composed predominantly of two types of epithelial cells: secretory and basal. These are interspersed with occasional neuroendocrine (NE) cells. These cell types can be phenotypically distinguished and are described below (Table 2).

Luminal (secretory) cells

Prostatic growth and differentiation is dependent on testicular derived androgenic hormones and on complex and as yet poorly understood epithelial-mesenchymal interactions. The glandular component of the prostate is composed of large (primary, major, excretory) and peripheral (secondary, minor) ducts. The acini and ducts contain secretory, basal and neuroendocrine cells. The luminal cells of the gland are secretory in nature and phenotypically express keratins 8 and 18, prostatic acid phosphatase (PAP), prostate specific antigen (PSA) and the androgen receptor (Table 2) [31–33]. They require androgens for maintenance of their differentiated function [34]. Adenocarcinomas of the prostate are considered to differentiate towards secretory cells as they express heterogeneously phenotypic markers typical of the secretory cell populations.

Basal cells

These are considered to be multi potential in nature giving rise to all epithelial lineages in normal, hyperplastic and neoplastic prostates [35]. They separate the secretory cells from the basement membrane and express high molecular weight cytokeratins reactive with the monoclonal anti-keratin antibody 34betaE12 (Table 2) [36]. This antibody is specific for keratins 1 (68 kDa), 5 (58 kDa), 10 (56.5 kDa) and 14 (50 kDa) which are characteristically found in complex epithelia [36]. It has been

Table 2. Phenotypic markers of glandular cell types of normal human prostate

Phenotypic markers	Secretory/luminal cell	Basal cell	Neuroendocrine cell
PSA	Yes	No	Yes
PAP	Yes	No	No
Androgen receptor	Yes	Focal	No
Cytokeratins	8, 18	5, 14 (H Mw Cks)	?
MUC1	Yes	No	No
Chromogranin (A/B)	No	No	Yes
Smooth muscle actin	No	No	No
S-100	No	No	No

used to help distinguish well differentiated adenocarcinomas (in which basal cells are absent) from benign conditions that simulate it such as adenosis, sclerosing adenosis, basal cell hyperplasia, atrophy, benign prostatic tissue with radiation atypia, and clear cell cribriform atypia [37–41]. Normal prostatic basal cells do not express PSA or PAP [42, 43]. They have been shown to express the androgen receptor focally [44, 45]. Neither basal nor secretory cells express S-100 protein or smooth muscle actin helping to distinguish them from neuronal or smooth muscle cells.

Neuroendocrine (NE) cells

These cells are present in all zones of the prostate although they are more abundant in the periurethral and ductal regions [46]. NE cells are found in the glandular component of the prostate among the secretory and basal cells [47]. They secrete a variety of biogenic amines (chromogranin A and B, secretogranin II, neurone specific enolase and serotonin) which are found in most prostatic NE cells (Table 2) [48, 49] or peptide hormones (somatostatin, calcitonin, bombesin) which are secreted by some of the cells [50]. They co-express PSA, suggesting an origin common with the secretory cells [48] but they do not express the androgen receptor (Table 2) [51]. For a recent review on NE cells readers are referred to articles by Noordzij and di Sant'Agnese [52, 53].

Prostatic stroma

This is a complex mixture of smooth muscle cells, fibroblasts, blood vessels, nerves and the intervening extra-cellular matrix. Normal prostatic development is critically dependent on complex and as yet poorly understood epithelial-mesenchymal interaction [54–58], and there is a highly complex dynamic interaction between tumour cells and the induced (desmoplastic) fibroblast population which may come to dominate some prostatic cancers.

Benign prostatic hyperplasia (BPH). Other conditions commonly co-exist with PC and are often found in prostate specimens. BPH is the most frequent benign condition found in the prostate and there are some similarities between PC and BPH (Table 3). Both require androgenic stimulation, show increased prevalence with age (although PC usually occurs 15–20 years later) may co-exist and may respond to androgen deprivation. Most PCs arise in prostates which already have BPH. In 10% of elective TURPs (transurethral resection of the prostate) for symptoms of bladder outflow obstruction clinically secondary to BPH, there is associated PC [59]. However, BPH is not a premalignant lesion nor is it a precursor of PC.

Prostatic intraepithelial neoplasia (PIN). This is considered the most likely precursor of invasive carcinoma, but studies have not determined conclusively whether PIN remains stable, regresses or progresses [59, 60]. It is characterised by proliferation of the luminal epithelium within pre-existing acini and ducts, with cytological atypia, including nuclear pleomorphism and nucleolar enlargement. By definition, there is no basement membrane invasion. Grading (I, II, III) depends on the severity of the following cytological and nuclear changes: (a) cellular crowding and stratification, (b) nuclear enlargement and pleomorphism, (c) chromatin pattern and nucleolar appearance. PIN is currently subdivided into two categories: low grade (PIN I) and high grade (PIN II and III) [60].

PIN has been reported to be present in greater than 85% of cases of PC [59]. Inter-observer variability for PIN grading is common, especially of low

Table 3. Similarities and differences between BPH and PC

	BPH	Prostate cancer
Common sites of occurrence	transition zone	peripheral zone and transition zone
Prevalence with age	increases	increases (lags by 15–20 years)
Androgens	required for growth	required for growth
Androgen deprivation	can decrease growth	can decrease growth
premalignant potential	none	

grade and this may account for the difference in reported associations [61]. In a recent study with patients that were re-biopsied following a diagnosis of PIN, 13% with low grade PIN and 48% with high-grade PIN had frank cancer in the subsequent biopsy [62]. Several studies suggest that PIN predates PC by some 10 years with low grade PIN first appearing in men in the third decade [63–65]. In prostates containing both PIN and PC there is a good degree of concordance in the DNA ploidy pattern of both lesions [66–68]. These observations suggest that high grade PIN may have a high predictive value as a marker for PC and some clinicians believe that patients with PIN should be closely monitored to include follow up biopsies. However, the identification of PIN should not influence or dictate therapeutic decisions [60] and there is a risk of inducing 'cancer-phobia' in such patients.

Molecular pathology

Advances made in molecular biology have had a major impact on the practice of surgical pathology [69–74] and the role of the academic pathologist. Many of the molecular biology techniques are being adapted for use on fixed paraffin embedded tissues. Microdissection has increased both specificity and sensitivity for the analysis of DNA, mRNA and protein in samples containing a heterogeneous population of cells. Fixed material is sometimes suboptimal for analysis and the availability of snap-frozen or specially fixed tissues is a major resource in academic pathology institutes.

Pathologists are increasingly being involved in the translation of techniques developed in pure research laboratories to routine use in diagnostic pathology. The pathologist is an integral member of multidisciplinary research teams interpreting gross and microscopical appearances in human diseases and animal models (e.g. transgenic and gene knockout mice). They are also important in assessing expression patterns at protein and mRNA level of specific genes and their alterations in differing disease states. The following sections illustrate the interface between basic sciences and clinical research in relation to PC.

Material

Human tissue

Most prostatic specimens result from trans-urethral resection of prostate (TURP) or radical prostatectomy (RP). RP tissue includes the whole prostate whereas TURP selectively samples the TZ, which is pathologically enlarged by the process of BPH, rather than the whole gland. The biology and clinical behaviour of cancer found in TURP specimens is therefore not necessarily the same as cancer found in RP specimens. It is usually of lower Gleason grade, with less frequent capsular penetration and lymph node metastasis [59]. There is also a major problem in obtaining samples of metastatic dis-

Table 4. Main distinguishing features of human PC cell lines

	LNCaP	DU-145	PC-3
Tumourigenicity in nude mice	+	+	++
Metastatic potential	–	±	+ [395]
Hormonal sensitivity	+	–	–
Biomarkers	PSA, PSM, prostatic acid phosphatase, prostatic inhibitory peptide, cytokeratins 8, 18 [396–399]	PSA and PSM negative [396–399]	Prostatic inhibitory peptide and prostatic acid phosphatase positive, PSA negative [396–399]
Origin	supraclavicular lymph node in a patient with hormonally sensitive cancer	brain metastasis	bone marrow metastasis in patient with hormone-independent PC

case. Some of these problems may be circumvented by PCR amplification of fine needle aspiration biopsy, where a thin needle is used to extract a few cells from a particular lesion, it will not be possible to evaluate the relative contribution of the malignant epithelial, entrapped benign, and stromal cells from any one sample. It is important to appreciate the differences in interpretations possible with results obtained from cells and tissues obtained through a wide range of surgical procedures.

Cell lines

Much of the understanding of the pathogenesis of PC arises from the study of cell lines. There are 3 principal human PC cell lines (Table 4) and, multiple rat cell lines derived from the original rat Dunning tumour as well as other less well known cell lines [75–78].

The LNCaP cell line was derived from a supraclavicular lymph node from a patient with hormone-refractory PC [79]. LNCaP cells express cytokeratins (8, 18) which are also expressed by prostatic luminal cells (see above). This cell line produces four biomarkers of the prostate: PSA, PAP, prostate-specific membrane antigen (PSM) and prostatic inhibitory peptide (PIP) [81, 82]. In addition, these cells express a high-affinity mutated (codon 868, Thr → Ala) androgen-receptor (AR), which is blocked by bicalutamide but behaves idiosyncratically with other antiandrogens (cyproterone acetate and flutamide). These latter act as agonists and stimulate proliferation [83, 84]. Although LNCaP tumours grow in nude mice and are hormonally responsive, they do not metastasise [85].

The PC-3 cell line, which was derived from a human bone marrow metastasis, does metastasise when injected subcutaneously into nude mice in about 30% of cases [86]. It expresses PSA as well as PIP and PAP, but not PSM [82, 87]. It does not produce an androgen receptor and is not hormonally responsive. These differences from the LNCaP cell line may reflect tumour dedifferentiation and resemble high grade and advanced stage human PC. A highly invasive subline of PC-3 cells, PC-3-ML has been developed which is considerably more invasive *in vivo* [88].

Microdissection

The application of molecular markers to pathological specimens is critically dependent on optimised procedures and the proportion of the sample with the feature of interest, e.g. a specific genetic mutation. Independently arising tumours vary in the combinations of genetic changes they contain and in the precise malignant phenotype they express.

PC is characterised by a remarkably low frequency of alterations in genes known to be associated with other malignancies such as *ras, myc, erbB-2, p53,* and *RB1* [89]. To date, no specific oncogenes or tumour suppressor genes (TSGs) have been conclusively correlated with PC initiation or progression. Given the heterogeneity of biopsies, and the infiltrative nature of tumours a low frequency of detection could stem from dilution of neoplastic DNA by stromal and/or entrapped non-neoplastic cellular elements. An important consideration in molecular analysis of tissues is the minimisation of the unavoidable contamination of tumour biopsies by such elements [90, 91]. Berthon and colleagues suggest that at least 20% of total DNA from a tumour sample needs to be mutated to score positively in PCR/SSCP analysis. They further suggest that when only one allele is affected greater than 40% of cells present within the specimen used for DNA extraction must be from the tumour [92]. Therefore the use of precise microdissection techniques is extremely important in analysing molecular markers from fresh and archival material.

Methods

Molecular genetic techniques have advanced over the last decade to a point where they can be used on routine clinical material. For those readers who may not be familiar with some of these techniques the following sections briefly describe methods with emphasis on those applicable to archival material where the greatest clinical reserve lies.

Detection of point mutations

There are at least three methods which can be used

for the detection of point mutations in surgical specimens.

Allele-specific oligonucleotide hybridisation. This method is based on the principle that a duplex of oligonucleotides with even one mismatch is unstable [93]. Two steps are involved: (a) extraction of DNA from tissue samples and its amplification by the polymerase chain reaction (PCR) and (b) screening of the amplified DNA on filters with radio-labelled synthetic oligomers whose sequences correspond to possible amino acid substitutions. Briefly, DNA is extracted from formalin-fixed paraffin-embedded tissue. Formylation of nucleic acids produces Schiff bases on tree amino groups of nucleotides, and exposure of nucleo-proteins to formaldehyde results in the formation of crosslinks between proteins and DNA [94]. These processes are reversible in aqueous solution, implying that DNA can be recovered from formalin-fixed tissue. DNA extracted from fixed tissues is not intact, but it is double stranded, cleavable with restriction endonucleases, can be hybridised with labelled probes and amplified by using PCR. If available, DNA extracted from fresh or frozen tissues is more suitable for a variety of standard molecular techniques [95, 96]. Following extraction and PCR amplification of the DNA it is immobilised to filters and hybridised with an oligonucleotide which spans the mutation of interest.

Direct sequencing of DNA amplified by PCR. In vitro amplification of DNA using PCR and subsequent direct sequencing of the product is an alternate approach to the detection of mutations [93].

Single strand conformational polymorphism (SSCP) analysis. In this method, the DNA sequences of interest are amplified and labelled by PCR using labelled primers or labelled nucleotide [97]. Most single base pair changes in fragments up to 200 bases can be detected as mobility shifts by SSCP analysis (e.g. *ras* point mutation) [98]. The limitation of this technique is that the position and exact nature of the mutation can not be determined. However, SSCP is a useful technique for screening point mutations as it is simple, fast and efficient. When gel shifts suggest mutations, other methods such as direct sequencing must be used to determine the base substitution.

Detection of gene amplification
Gene amplification can be detected in formalin-fixed paraffin embedded tissue by dot-blot analysis On fresh or frozen samples Southern blot analysis is preferable [95, 96]. Amplification is determined by comparing the signal intensity by densitometer with parallel samples hybridised with a reference single copy gene and the gene of interest.

Detection of loss of heterozygosity (LOH)
DNA restriction enzymes recognise specific sequences in DNA and by catalysing endonucleolytic cleavages, create fragments of certain lengths which are displayed by agarose gel electrophoresis. Individual variability in the lengths of a particular restriction fragment – restriction fragment length polymorphism (RFLP), result from a variety of genetic differences. Change in a single base pair could result in either loss or formation of cleavage sites. Insertion or deletion of DNA may also alter the size of the fragments. In either case the altered mobility of restriction fragments subjected to gel electrophoresis can readily be detected by Southern blotting and hybridisation with a labelled DNA probe which recognises an adjacent or linked DNA sequence. This method has been applied to genetic linkage analysis and also to unravel specific chromosomal deletions in PC identifying putative TSGs or PC genes and can be applied to extracted DNA from fresh or archival material. Using this technique it is possible to build up a picture of the genome and to identify which chromosomal regions are deleted or are rearranged in tumour cells of individual patients. The significance of the consistent loss of specific regions of genetic information from the genomes of tumour cells of particular histological type is now appreciated as such areas may contain as yet unrecognised TSGs. The characterisation of regions consistently lost forms the first step in localising such genes.

In situ *hybridisation (ISH)*
In situ hybridisation (ISH) was first described by Gall and Parduc in 1969 [99]. It enables topographic

detection of nucleic acids (DNA or RNA) in cellular material (tissues and cell lines). One of the major advantages of ISH over solution filter hybridisation techniques (Southern/Northern blotting or gel electrophoresis) is the precise identification of specific cells within a heterogeneous population. Considerable information regarding the structure and function of cells within pathological lesions can be derived by this means. Combining ISH and immunocytochemistry provides the final link between transcription and translation.

The method employed for a given experiment critically depends on:
(a) the target nucleic acid (DNA or RNA)
(b) sensitivity and
(c) specificity desired.

DNA is relatively stable and is preserved in tissues even after routine fixation, unlike mRNA which is labile and highly susceptible to endogenous enzymatic degradation. Detection of mRNA requires different conditions (collection, storage, detection) to those of DNA. High sensitivity is necessary where sequences of low abundance are to be detected. Conversely, there are situations where only low sensitivity detection systems are desirable e.g. detection of specific repetitive sequences. The specificity of ISH is determined by the hybridisation and post hybridisation washes. The manipulation of these conditions provides flexibility and allows the detection of sequences related but not identical to the probe used. This is useful for example in determining viral subtypes. For a detailed description of DNA and RNA *in situ* hybridisation, readers may refer to specialist texts [100].

Fluorescent in situ *hybridisation (FISH)*

Cancers are characterised by genetic instability which may manifest as:
(a) abnormalities of chromosome number,
(b) gross deletions,
(c) translocations and
(d) gene amplifications.

Characteristic chromosome aberrations have been described in a wide range of tumours and some of these may serve as potential diagnostic or prognostic markers [101, 102]. Conventional cytogenetic analysis requires viable cells for the production of metaphase spreads followed by chromosome staining and visualisation for specific abnormalities. This is a slow and tedious process. Furthermore, many cancers do not grow in culture or are refractive to metaphase analysis rendering this approach inapplicable.

FISH provides a simple fast and reliable means for assessing genetic instability in cancer. It is possible to identify either entire chromosomes or regions smaller than a single gene. In addition, interphase cells can be used eliminating the need to culture cells and often touch preparations are adequate. FISH based analysis of chromosome aneusomy can be accomplished with interphase nuclei using fluorophore-labelled probes to highly repetitive centromeric regions. As the centromeric region of the chromosome remains condensed throughout the cell cycle, hybridisation to this region appears as a small spot within the interphase nucleus. Multiple chromosomal aberrations can be identified using multicolour-multitarget FISH [103]. As an example, one study from the Mayo Clinic examined paraffin embedded sections of PCs for chromosome aneusomy in two groups of patients: those who survived less than 3 years and those who survived greater than 5 years after initial diagnosis. All of the patients had either $T_2N_0M_0$ or $T_3N_0M_0$ stage cancer. A high correlation between chromosome 7 trisomy and poor survival was reported [104].

Comparative genomic hybridisation (CGH)

CGH provides a genomic fingerprint at a megabase level of resolution. Experimentally total DNA is extracted from normal tissue and from tissue with a neoplasm. Both DNA samples are labelled with a distinct fluorophore. The two labelled samples are mixed and hybridised to normal metaphase spreads. If the normal DNA was labelled with a green fluorochrome and the tumour DNA with a red fluorochrome, when they are present in a 1:1 ratio this results in a yellow signal. However, if there is a deletion in the tumour (no red label) there is green fluorescence. Gene amplification (e.g. androgen receptor) will result in multiple copies of red DNA and this region will therefore appear red. It is possible to computer map (utilising incremental colour ratios) areas of deletion and amplification.

Polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR)

The polymerase chain reaction was first described by Mullis and colleagues at Cetus corporation [105]. The subsequent inclusion of a thermally stable DNA polymerase [106] greatly facilitated its application and since its introduction, its uses and users have expanded significantly both in research and clinical applications. PCR allows specific *in vitro* enzymatic replication of nucleic acids directed by specific synthetic oligonucleotide primers. The PCR cycle consists of heat denaturation of the DNA followed by primer annealing and DNA synthesis. Primers are oriented to facilitate overlapping replication resulting in exponential increase in nucleic acids. Primers, which are in great excess, hybridise to opposite strands. The original target sequence and the synthesised PCR products serve as PCR substrates resulting in an exponential increase in the DNA sequence of interest. By using PCR it is possible to detect a single copy of a gene present in 100,000 cells compared to Southern blot which can detect one copy in 100 cells. This is a highly sensitive and specific technique. On average a PCR reaction may take 2–4 hrs depending on the sensitivity required, each cycle taking 2–5 minutes. There are on average 25–50 cycles [106]. PCR has at least two critical advantages: high sensitivity and short assay time. It can be readily applied to microdissected pathological specimens.

RNA is more readily degraded than DNA and its detection is more difficult. However, from optimally preserved samples, RNA can be extracted and PCR amplified after conversion to cDNA by the enzyme reverse transcriptase. This method is known as RT-PCR [106].

Molecular genetics of PC initiation, progression and metastasis

Studies on PC have been hampered by a number of factors such as restricted access to tissues, slow *in vivo* growth, difficulties in propagating tumour cells *in vitro*, limited availability of PC cell lines or immortalised cell lines for *in vitro* studies, lack of appropriate animal models and the complex epithelial-mesenchymal interactions. Cytogenetic studies (karyotypic analysis, FISH, CGH), loss of heterozygosity (LOH) and work on oncogenes and tumour suppressor genes are providing some insight into genetic factors contributing to the initiation, promotion and progression of PC *in vivo*.

Cytogenetics

Karyotypic analysis of many primary prostatic cancers has consistently revealed a normal male diploid karyotype (46XY) [107–109]. Cytogenetic changes can broadly be categorised as gains, losses, rearrangements and transversions. In PC, chromosomal rearrangements consisting mainly of deletions rather than translocations have been reported. Loss of chromosomes 1, 2, 5, 7, 14, and Y or gains of chromosomes 7, 14, 20, 22 have been reported. Also rearrangements of chromosome arms 2p, 7q and 10q are frequent. Aneusomy including trisomy of chromosome 7 and its association with higher tumour grade, advanced pathological stage, metastasis and early PC death have been reported by several groups utilising FISH [104, 110]. These groups suggest that genetic alterations of chromosome 7 play a significant role in the development of PC. To investigate this Takahashi and colleagues performed PCR analysis of 21 microsatellite loci on 54 paired samples. Chromosome 7 allelic imbalance was found in 30% of the cases of which 28% were losses and 2% gains. The commonest site of allelic loss was at 7q31.1 which correlated with increasing grade and metastasis [111].

LOH studies performed in search of consistent chromosomal losses can unearth putative TSGs. In 1990 Carter and colleagues examined 28 primary PCs most of which were localised stage B tumours measuring less than 2 cm in diameter. They found LOH in 61% of tumours on at least one of the chromosomes examined (3p, 7q, 9q, 10q, 11p, 13q, 16q, 17p and 18q). Most frequently deleted regions were on 10q and 16q [112]. In approximately 20% of the tumours there was LOH of either 13q, 17p and 18q or a combination [112]. These regions harbour the *Rb* (Retinoblastoma), *p53, DCC* (deleted in colorectal cancer) genes. Other studies have since suggested that chromosomes 8, 10, and 16 losses are

also common in PC [113, 114]. Both Bergerheim et al. and Bova et al. found high losses of alleles on chromosome 8p [114, 115]. Bova et al. also reported deletion of 8p22 in approximately 70% of their samples. These authors suggest the presence of a TSG on 8p [115]. Using different techniques (PCR, ISH) and a large number of microsatellites, other groups have also reported frequent loss of markers in this region [116, 117]. In a recent study Vocke and colleagues performed PCR based LOH studies utilising 25 microsatellite markers on the short arm of chromosome 8 on 99 microdissected PC. They found LOH on 8p in 86% of PC and the highest loss occurring in the 8p12-21 region which did not correlate with grade or stage [118]. The same group reported deletion at 8p12-21 in 63% of PIN [119] and they suggest that deletion of this region is an early event. Their findings are at variance with those of Trapman et al. who suggested that loss of 8p12-21 correlated with advanced disease [120]. Studies of chromosome 8 suggest early proximal involvement (8p12-21) and late distal involvement (8p22) suggesting that there may be two TSGs in this region. Bova et al. reported an increased copy number of sequences on the long arm of chromosome 8 in a subset of PCs suggesting the presence of an activated oncogene on the same chromosome [115].

To assess the effect of specific regional chromosomal losses Murakami et al. microcell transferred neomycin tagged normal human chromosome 10 into the PPC-1 (human PC) cell line [80]. This line does not contain normal copies of chromosome 10 but a complex translocation involving chromosomes 1, 3 and 10 [121]. Two of their 6 hybrid clones showed decreased tumourigenicity in athymic nude mice. Structural analysis of the fragment of chromosome 10 in each hybrid suggested a putative TSG in the distal region of 10p [122].

In a recent report by Dong et al., a potential marker for metastatic PC was localised to chromosome 11p12 and designated *KAI1* [91]. *KAI1* encodes a transmembrane protein of 267 amino acids with three potential glycosylation sites. The KAI1 protein is homologous to leukocyte adhesion molecules which function in cell-cell and cell-extracellular matrix interactions. Introduction of this gene into rat AT6.1 PC cell line suppressed their metastasis.

LOH involving 13q has been reported to occur in up to 30% of primary PC. Candidate TSG on 13q include *Rb1* and *BRCA2*. Cooney et al. examined LOH on 13q using 9 polymorphic markers spanning the entire chromosomal arm [239]. Thirty-seven of the 40 cases were also assessed for pRB, the protein product of *Rb1*, by immunohistochemistry. They report a lack of correlation of LOH at *Rb1* with absent pRB expression suggesting the existence of another TSG in the region of 13q14.

Assignment of a single specific chromosome or chromosomal aberration to the development and progression of PC is difficult to assess at the present time and requires further investigation.

In summary, chromosomes 2, 7, 8, 10 and 16 appear to have a greater incidence of involvement in the development and progression of PC.

Flow cytometric analysis

DNA flow cytometric analysis of solid tumours including PC has provided clinical information of questionable value. Most low grade PCs are diploid but may contain an extra one or two chromosomes without a distinct aneuploid peak being detectable by DNA flow cytometry [123]. Aneuploid and tetraploid tumours occur with worsening grade. It has been postulated that PCs progress from diploid to tetraploid to aneuploid to non-tetraploid aneuploid [124]. Stage A tumours were usually diploid whereas stage C tumours were non-tetraploid aneuploid and tumours may change ploidy with time [124].

Most investigators agree that in PC there is a correlation between DNA ploidy pattern and conventional histological grading [125]. DNA ploidy has been correlated with tumour volume and stage of disease. If a tumour has an abnormal DNA content, then the tumour volume is likely to be greater than 4cc [126]. However, the biological significance of ploidy in PC progression remains uncertain.

Oncogenes: introduction and definition

Over 100 oncogenes have been identified to date and the list is expanding. Proto-oncogenes partici-

pate in normal growth and proliferation, encoding a wide variety of proteins that may function as growth factors, growth factor receptors, regulators of DNA synthesis, regulators of RNA transcription, and modifiers of protein function by phosphorylation. The alteration that converts a proto-oncogene to an oncogene can take several forms. There may be a mutation (base substitution) in the gene sequence. The prototype of this mechanism is the *ras* oncogene. Single base substitutions (point mutations) at characteristic areas of the *ras* gene convert it into an oncogene. This gene has been widely studied in a variety of tumours including urological malignancies [127, 128]. A second mechanism occurs when there are extra copies of a normal DNA sequence gene. The prototype for this mechanism, called gene amplification include *N-myc* in neuroblastoma and *c-erbB-2* (also called *Her-2* or *Neu*) in breast cancer. Extra gene copy number correlates with adverse outcomes in both these cancers. A third mechanism for conversion occurs when the oncogene is moved (or translocated) to a different place on the same chromosome or to a different chromosome. In this scenario, the normal sequence of the gene comes under control of different promoters or regulators. The inappropriate or over-expression of such a gene may be oncogenic. Alternatively, a portion of the gene sequence is translocated and this portion may fuse with a different gene sequence at a new location to create a new hybrid gene, which is an oncogene. This is called translocational rearrangement. The prototype example of translocation/rearrangement is the *c-Abl* proto-oncogene in leukaemia.

Research regarding oncogenes in PC has been limited and to date no oncogene has been correlated with initiation or progression of PC [129]. Those that have been studied in PC include *ras*, *myc* and *c-erb-B-2*. Members of the *ras* gene family, e.g., Harvey (H)-, Kirsten (K)-, and Neuroblastoma (N)-*ras* have been most widely studied in PC.

Mutations in ras genes

C-H-ras, *c-Ki-ras* and *c-N-ras* proto-oncogenes comprise the ras family of genes. These closely related genes encode a 21 KD protein (p21). C-Ras genes are activated after a single base mutation or with deregulated expression of the wild type alleles. Ras p21 proteins accumulate in their GTP-bound state and initiate a cascade of events that lead to DNA synthesis through well characterised pathways in response to a mitogenic stimulus [130].

Mutations in *ras* genes have been reported frequently in human tumours [127, 128] Molecular analysis in primary and metastatic PC has demonstrated that none are commonly mutated in PC [131–137]. LNCaP cells transfected with non-mutated *c-ras* expressed high levels of p21 protein. High levels of p21 expression in LNCaP cells did not affect their growth properties. However, LNCaP cells transfected with a *mutated v-K-ras* became androgen independent and showed increased anchorage-independent colony formation [138]. Transfection of a mutated *v-H-ras* into a non-metastatic Dunning rat prostatic cell line resulted in acquisition of metastatic phenotype [139–141].

The frequency of *H-ras* point mutations in patients in the USA is estimated to be less than 5% [131, 134, 135, 142]. In contrast Konishi et al. found *K-ras* 12 mutations in approximately 25% of latent autopsy prostatic tumours in Japanese males [135]. The incidence of clinically manifest PC in Japanese men is approximately 5 fold less than that observed in the North American population. There may be a difference between latent and clinically evident cancers or there may be racial differences [135]. Anwar et al. reported a 24% mutation rate in clinical PCs from Japanese men. Approximately 70% of the mutations detected were restricted to codon 61 of *H-ras*. They also reported the detection of human papilloma virus (HPV) DNA in greater than 40% of PCs analysed [136]. The cumulative data on ras mutations suggests that the overall incidence in clinically manifest PC is less than 5% and that this is not a common event in either initiation or progression. Most of the mutations found in non progressive latent disease were those associated with *K-ras* (A to T transversion in codon 61) whereas clinically manifest ones are associated with *H-ras*.

Expression of p21 protein and mRNA. Several groups claimed enhanced ras p21 expression in primary prostatic carcinomas which correlated with increased Gleason grade [143, 144]. Antibodies utilised in these studies were later found to be non-spe-

cific and further studies have failed to show an association between p21 expression and tumour stage or grade [145]. Expression of p21 has also been assessed in primary and metastatic tumours from the same patient by Fan who found that most of the metastatic tumours expressed p21 whereas only a fifth of the primaries did. He postulated that the metastasis originated from the primary *ras* expressing tumour cells. Increases in *c-H-ras* m-RNA expression have been demonstrated in tumours which evolve from low grade androgen-dependent into high grade androgen-independent tumours [146, 147]. Expression of both *K-ras* and *N-ras* have been evaluated and there is no reported association between expression and tumour grade.

Myc genes in PC. Myc proteins are members of the helix-loop-helix-leucine zipper (HLH-Zip) family of transcription factors, which either homo- or hetero-dimerise with other members of the family. In most cases, dimerisation permits subsequent DNA binding, a function mediated by a highly basic region adjacent to the HLH-ZIP dimerisation motif. Myc proteins also contain distinct transcriptional activation domains that modulate the expression of genes to which the protein bind [148].

Genes regulated by *c-myc* are not known. Abnormalities (amplifications, chromosomal translocations or other genetic events) of *c-myc* have been detected in various types of human tumours. *c-myc* is amplified or rearranged in approximately 30% of breast tumours [149] and in differing rates in ovarian, colon, uterine and stomach tumours. There are conflicting reports in the literature regarding c-myc expression in prostatic carcinomas. Several studies have shown an increased *c-myc* m-RNA expression in PCs compared to benign prostatic hyperplasias and normal tissues and an association with tumour grade [150–154]. Other studies have failed to show such an association [155, 156]. The *c-myc* gene does not appear to be amplified in PC [132, 152]. These discrepancies are most likely to be due to the different methodologies employed. Collectively these studies would suggest that c-myc overexpression is not fundamental in PC.

In a mouse prostate model, *vH-ras* alone at high levels was found to induce dysplasia, while *c-myc* over-expression induced hyperplastic lesions [157]. Double *c-myc/ras* transgenics induced transformation and PC [158].

C-erbB-2 *in PC*

c-erbB-2 is on chromosome 17q21-q22 and encodes a transmembrane phosphoprotein which has considerable sequence homology to the epidermal growth factor (EGF) receptor. The most common mechanism of *c-erbB-2* activation is by gene amplification with a resultant over-expression of both the transcriptional and translational products. In addition to amplification, *c-erbB-2* activation in several tumours has been shown to occur by over expression of mRNA and protein without DNA amplification or just by protein over-expression [159]. This oncogene has been most widely studied in breast carcinomas in which amplification/over-expression has been correlated with poor prognosis [160–162]. *c-erbB-2* expression studies in PC have yielded conflicting results [163–170]. Overall it appears that a subset of prostatic adenocarcinomas express immuno-detectable *c-erbB-2* protein. The antibodies employed in the various studies (monoclonal and polyclonal), the detection methods, and whether the samples were fixed or frozen could all have contributed to the conflicting results.

Other oncogenes

The *c-sis* proto-oncogene has also been studied in PC. Its product is identical to the β chain of platelet derived growth factor (PDGF). C-sis may play a role in the growth of prostatic tissue by androgens and its suppression by corticosteroids [151, 171]. Dot blot RNA analysis showed an increase in c-sis expression in poorly differentiated prostatic adenocarcinomas [151].

c-fos oncogene encodes a protein FOS which forms heterodimers with JUN proteins that function as positive or negative transcription factors binding to specific DNA sequences via basic domains adjacent to the dimerizing helices. There has been little work in PC but some evidence that there may be a relationship between androgen receptor content and c-fos expression [151, 172, 173]. Androgen deprivation has been shown to reduce c-fos expression by 90% in some cell lines [172]. No gene amplification has been seen in PC (26).

Bcl-2 is a cell death 'suppressor' gene on chromosome 18q21 whose protein is normally undetectable in a majority of hormone dependent PCs. Hormone refractory PCs in contrast express high levels of bcl-2 protein [174]. Expression of bcl-2 protein in PCs appears to be associated with the transition to hormone (androgen) independence [175].

The steps involved in cellular transformation and the development of the metastatic phenotype are poorly characterised in PC. The formation and dissemination of a tumour could be due to an accumulation of specific, consecutive genetic alterations with time as in the case of colorectal cancer [176]. Results published by Carter et al. favour a multistep process, and this would explain the increasing frequency of PC with age [158]. Genes involved in this process are those involved in cell cyle control, apoptosis, proliferation, differentiation (oncogenes and tumour suppressor genes) and those involved in DNA repair, proteolysis, cell adhesion and motility

Tumour suppressor genes

Inactivation of tumour suppressor genes (TSG) results in loss of function. Usually both alleles of TSGs are lost, inactivated or damaged, resulting in either loss or attenuation of the gene product which was necessary in suppressing the neoplastic phenotype. Two well known TSGs, the retinoblastoma (*RBI*) [177, 178] and the *p53* genes [179, 181, 182], have been implicated in a number of neoplasms [183, 184]. Other recently identified TSGs are Wilm's tumour gene (*WT1*), *DCC* (deleted in colorectal cancer), *APC* gene (adenomatosis polyposis coli gene) type 1 neurofibromatosis gene (*NF1*), *MEN* (multiple endocrine neoplasia gene) and the *BRCA1* and *2* (breast cancer) genes [178, 179].

Retinoblastoma gene (RB1)

This was the first identified TSG. It is located on chromosome 13q14.2 and its message size is approximately 4.7 Kb coding for a 105 kDa nuclear phosphoprotein termed $p105^{Rbl}$. It binds to double stranded DNA in a non-sequence specific manner and acts through a motif (the retinoblastoma control element, RCE). $p105^{Rbl}$ interacts with a large number of proteins many of which are transcription factors including cell cycle related proteins in particular the E2F family of transcriptional factors. E2F-$p105^{Rbl}$ association prevents transcriptional activation of a variety of genes, the products of which are central to the onset of DNA synthesis in the S phase of the cell cycle. Cell division is therefore prevented as cells are checked in the G1 phase. Mutations of the *RB1* gene have been described in a variety of human tumours in including osteosarcomas, soft tissue, bladder, breast, and lung tumours [185–190]. In 1990 Bookstein and colleagues infected the DU 145 human PC cell line (which produces a truncated isoform of Rb protein) with a recombinant retrovirus containing a normal *RB1* cDNA. Infected DU 145 cells expressing the normal *RB1* were non-tumourigenic when injected into nude mice compared to the parental cells. The authors concluded that *RB1* inactivation could play a role in the development of PC [191, 192]. Brooks et al. reported loss of a single *RB1* allele in 27% of PCs suggesting that inactivation of this gene may play a role in the development and progression of PC in a subset of tumours [193].

p53

The *p53* gene has been mapped to the short arm of chromosome 17 at 17q13.1. It encodes a 53 kDa nuclear phosphoprotein that is postulated to arrest cells in G_1 (for reviews see [194, 195]). For cellular growth to occur, cells must enter the S-phase and p53 cell growth arrest must be inactivated. Loss of the normal function of *p53* has been implicated in many different human tumours including urological tumours [182, 196–198]. Wild-type p53 protein is a sequence specific transcription factor which can activate genes possessing the *p53* consensus sites. Over expression of wild type *p53* can cause repression of many genes. p53 has been implicated as a key mediator of the cellular response to DNA damage. DNA damage induces accumulation of active wild-type *p53* which can arrest cells in G1 or induce apoptosis.

Analysis of p53 broadly falls into two categories: (a) Immunohistochemical and western blot analysis of accumulated p53 protein, (b) molecular analysis e.g. PCR followed by SSCP and direct sequencing. The frequency of *p53* mutations in primary PCs is low (10–20%) compared to the observed frequency

of mutations in other human tumours [199–202]. Several groups have found a higher frequency of *p53* mutations in PC bone metastasis [203–205]. Navone and colleagues found a higher rate of p53 accumulation in association with higher Gleason grade (30% of Gleason score 8–10 compared with none of the lower score tumours) and androgen independent tumours [203]. In summary, mutated p53 is found in less than 20% of PCs and is associated with increased rate of cellular proliferation, grade, stage and androgen independence. These studies suggest that p53 mutations are a late event in the development of PC.

Androgen receptor in PC

The human androgen receptor (AR) is a member of the ligand activated steroid thyroid hormone transcription factors [206]. The gene (> 90 Kb) is located on the X chromosome (Xq11-12) [207] of which the coding region is separated over eight exons [208]. Two AR mRNAs (11 Kb and 8.5 Kb) have been demonstrated in prostatic tissue which are generated by alternative splicing in the 3′ untranslated region [208]. The AR cDNA sequence possesses an open reading frame of 2730 bp encoding a protein of 910 amino acids with a molecular mass of 98.5 kDa. There are four functional domains: (a) a highly polymorphic, ~ 550 amino acid N-terminal portion which modulates the amplitude [209, 210] and probably the specificity of its target gene effects; (b) a central 67 amino acid DNA binding domain (DBD); (c) an 8 amino acid nuclear localisation signal [209, 210]; and (d) a C-terminal 250 amino acid hormone binding domain (HBD).

The N-terminal domain is characterised by a high abundance of acidic residues and by the presence of several homopolymeric residues of which expansion of one of the polyglutamine stretches results in decreased transaction. The AR's DBD has a 'D-box' on its C-terminal zinc finger for normal dimerisation [211]. Once bound, the AR's HBD has transcriptional regulatory properties and contributes to dimerisation and nuclear localisation [210, 212]. Phe-581 and Arg-614 are strictly conserved residues of the DBD with Phe-581 being a key component of the discriminatory N-terminal-helix of the DBD which is associated with the affinity and specificity of AR-androgen response element (ARE) binding.

Androgens are required for the development of both the normal prostate and PC. Although an initial subjective response to hormonal therapy is observed in 70–80% of patients with advanced disease, most tumours progress within 2 yrs to an androgen independent state. The proposed mechanism of progression to androgen independent growth includes the loss of AR expression [213], amplification of the AR gene [214] and structural changes in the AR protein [215].

Biochemical determination of AR levels have demonstrated their presence in both cytosol and nuclear fractions of benign and malignant prostatic cells [216, 217]. Nuclear AR (ARn) has been shown to be four fold less in metastases than in primary cancer [218]. Masai et al. demonstrated immunodetectable AR localisation in the nuclei of both benign and malignant cells [219]. In cancer tissue (n = 63 untreated) AR positive and AR negative cells were intermingled and the percent of strongly positive cells inversely correlated with Gleason grade [219]. In relapsed PC (n = 8) there was a significant decrease in AR positive cells compared to the untreated PC of a similar grade [219].

There are numerous reports of mutations associated with the coding region of AR but there appear to be conflicting data on the frequency of these mutations. Initial data suggested that in primary PC, mutation frequency was low [220, 221] and in a recent study Ruizeveld de Winter et al. found no mutations in exon 2–8 or major changes in exon 1 in 18 hormone refractory carcinomas [33]. However, Tilley et al. have presented data in which mutations occurred in 44% of the primary tumours (25 patients) analysed. These latter findings support the hypothesis that the rapid onset of androgen insensitive progression of PC may be due to selective outgrowth of cells with existing mutated AR rather than the acquisition of new mutations with the onset of treatment. A high proportion of mutations have been demonstrated in the ligand binding domain of the AR in hormone refractory PC [222] and in metastatic PC.

Overexpression of amplified genes is associated

with acquired resistance to cancer treatment. Visakorpi et al. demonstrated AR gene amplification in 30% of hormone refractory tumours or those analysed before androgen deprivation therapy [214]. The elevated copy number (up to 4 fold) is associated with enhanced AR transcription which may facilitate tumour cell growth in the presence of low serum androgen levels remaining after conventional endocrine treatment.

The transcription activation domain of the AR resides in the N-terminal region (encoded by exon 1) which contains the polymorphic trinucleotide repeats CAG [223] and GGC [224] coding for polyglutamine and polyglycine respectively. The average length of the CAG microsatellite in the population is 21 ± 2 (range 11–31 CAGs) [223]. Elimination of the polyglutamine tract resulted in elevated transcription activation (TA) and progressive increase of the CAG caused a linear decrease of TA [225]. It may be that shorter CAG alleles result in more active cell growth, due to greater AR transactivation activity, even at low levels of androgens during endocrine therapy. Irvine et al. demonstrated that short CAG alleles (< 22 repeat) were highest (75%) in African American males who also have the highest risk of PC and lowest (49%) in Asians who are at a low risk of PC [226]. In the 47 patients with < 22 CAG repeats 43% possessed long GGC alleles (> 16 repeats) [226]. Ruizeveld de Winter et al. found no expansion or deletions in the CAG repeat isolated from 18 hormone refractory and 9 normal prostate samples and in a study of an additional 40 PC specimens only one somatic CAG contraction (24 → 18 CAG's) was observed [227]. These data would suggest that somatic expansion or contraction of the CAG repeat is a rare event in the pathogenesis of PC.

Growth factors

In spite of maximal androgen deprivation therapy, many PCs become androgen independent, and autocrine and paracrine peptide growth factors assume greater importance [228].

Transforming growth factor-beta (TGF-beta)

The TGF-beta superfamily may be divided into subfamilies according to sequence homology. One group consists of TGF-beta 1, 2 and 3. The biologically active form of TGF-beta is a 25 kDa disulphide linked homodimer of mature segments although heterodimers have also been found. TGF-beta has been implicated in various biological processes affecting growth and differentiation with roles in angiogenesis, immunosuppression, as well as inhibition of cellular proliferation [229].

The mechanism of TGF-beta$_1$ action on prostatic cell growth is unclear. TGF-beta$_1$ binds the TGF-beta receptor II (TbetaR-II) which recruits the TGF-beta receptor I (TbetaR-I) inducing a signal transduction cascade. TGF-beta$_1$ prevents phosphorylation of the retinoblastoma gene product (RB) which would normally increase c-myc expression and accelerate proliferation. This would suggest that TGF-beta$_1$ should inhibit proliferation. Thus, one mechanism for escaping TGF-beta$_1$ control would be to down-regulate TbetaR-I or TbetaR-II. Proliferation in PC-3 and DU-145 but not LNCaP cell lines is inhibited by TGF-beta$_1$. LNCaP cells are insensitive because the gene for TbetaR-I is defective [230]. There is a variable response to TGF-beta$_1$ in the DU-145 cell lines, possibly due to a mutated RB product arising from clonal variation [231, 232].

Higher levels of immunoreactive TGF-beta have been reported in patients with PC compared to BPH. Increased expression of immunodetectable TGF-beta$_1$ was found in malignant epithelial cells compared to BPH samples [233]. Recent data suggest that elevated levels of plasma TGF-beta$_1$ discriminate between patients with invasive and non-invasive disease [234].

Reconciling the evidence from clinical data, *in vivo* and *in vitro* experiments is difficult. This may be because TGF-beta exhibits differing actions on the same cells at different concentrations. However, studies with Dunning rat MAT LyLu cells *in vitro* indicate that the effect of TGF-beta$_1$ depends also on experimental conditions. TGF-beta$_1$ does not inhibit MAT LyLu proliferation when cells are grown at high density, in the presence of serum, bFGF or

EGF (partial response only), or when grown on matrigel rather than plastic [235]. MAT LyLu cells transfected with TGF-beta$_1$ produce more and larger metastases *in vivo* than controls [236]. Growth *in vitro* can be inhibited in cells expressing TGF-beta$_1$ but could be restored to normal by TGF-beta$_1$ inhibiting antibodies. Evidence from human cell lines confirms the variable effect of TGF-beta$_1$. Growth in PC-3 and DU-145 cell lines is inhibited by exogenous TGF-beta$_1$ but only for 7 days after which the effect is lost [237]. PC-3 and DU-145 cells secrete TGF beta into the media *in vitro* allowing either for autocrine control of growth or potentially paracrine effects on stromal cells.

The difference in effect of TGF-beta$_1$ *in vitro* and *in vivo* probably relates to the effect TGF-beta$_1$ has on other factors affecting tumourigenicity including release activation of matrix metalloproteinases (MMPs) [238] reduced host immune response and angiogenesis. TGF-beta also has powerful effects on fibroblasts inducing growth, collagen synthesis and MMP secretion, which then modifies the extracellular environment which in turn affects tumour cell behaviour.

TGF-alpha, epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR)

EGF and TGF-alpha show sequence homology and both act through EGF membrane receptors (EGFR). EGFR is a receptor for several other ligands including heparin-binding EGF like factor, amphiregulin and vaccinia virus growth factor. Binding of EGF to its receptor increases receptor-receptor affinity, dimerisation of the cytoplasmic tyrosine kinase domain of EGFR and trans-phosphorylation. The receptor-ligand complex undergoes receptor mediated endocytosis. In some primary tumours EGFR is amplified by up to 50 fold including about 20% of bladder tumours. In $\sim$ 20% of primary breast cancers EGFR is amplified and strongly associated with early recurrence and death in lymph node positive patients. The EGFR gene is located on chromosome 7p13-12 and trisomy 7 has been reported in a significant number of PCs [110].

Increased immunodetectable TGF-alpha expression has been reported in samples of PC compared to benign prostatic tissue [240]. Ching et al. reported significantly increased mRNA expression of both pre-pro TGF alpha and pre-pro-EGF in PC compared to BPH and levels of EGF mRNA are higher in PC than BPH [241]. Increased EGFR mRNA has also been reported in PC where expression was found to correlate with grade [242]. However, classifying patients by EGFR status does not distinguish survival in patients with early versus late stage disease [243].

EGF stimulates proliferation of both DU-145 and LNCaP cells plated at low density [244, 245] and TGF-alpha has a similar effect on PC-3 cells [246]. Expression of EGFR can be modulated by exogenous androgens in LNCaP and ALVA 101 cell lines [247–249]. When the normally androgen-insensitive PC-3 cell was transfected with the human androgen receptor, levels of EGFR were increased and the proliferative response to exogenous EGF and dihydrotestosterone increased synergistically [250]. The role of the androgen response element in the transcriptional regulation of EGFR is unknown.

Transfection studies with a truncated EGFR into DU 145 cells have suggested a role for the TGF-alpha/EGF autocrine loop in invasion *in vitro* [251]. Stimulation of PC-3 cells with EGF increases urokinase-Plasminogen Activator (u-PA) levels on northern blot analysis and enhances their invasiveness *in vitro* [252, 253]. Similarly, EGF increases the migration of the TSUPr1 PC cell line through Boyden chambers.

Therapeutic strategies have been suggested based on EGF control of PC. EGFR expression is reduced in Dunning rat AT-1 cells and survival *in vivo* is prolonged after somatostatin [254] and LH-RH analogue therapy [255]. Suramin reduces binding of TGF-alpha to TGF-alpha receptors, inhibits growth and increases the fraction of PC 3 and DU-145 in the S phase [256]. Such therapies may eventually prove of use in clinical practice.

IGF

The insulin-like growth factor (IGF) axis is a multicomponent network of molecules involved in the regulation of cell growth. It includes two major li-

gands, (IGF-1 and IGF-2), cell surface receptors, (the type 1 IGF receptor family as well as the type 2 IGF receptor), a family of high affinity binding proteins which regulate IGF availability to the receptors (the IGFBPs) and a group of IGFBP proteases which cleave IGFBPs and modulate IGF action.

IGF-1 is a 70 amino acid polypeptide which is functionally similar to insulin [275]. Progression through G_1 to the S phase in the cell cycle is accelerated by IGF. IGF-1 and IGF-2 are similar and are usually bound to IGF-binding proteins.

PC-3 cells have been shown to produce IGF like factors [258]. IGF-1 induces proliferation of the DU-145 and PC-3 cell line, but LNCaP cell lines have fewer IGF-1 receptors and do so only in the presence of androgen [259]. There was no evidence in the latter study that the cell lines produced IGF themselves, but in another study using radioimmunoassay substantial quantities of IGF-1 were observed [260]. Another rat PC cell line exhibited increased growth when exposed to IGF and expressed predominantly IGF-1 receptors [261]. IGF-1 may act directly through the androgen receptor pathway [262].

FGF

There are at least 9 members of this family which bear 30–35% sequence homology with each other. Basic FGF (FGF-2) and to a lesser extent acidic FGF (FGF-1) are found in both benign and malignant prostates. FGF mediates its effect by tyrosine kinases and induces proliferation mediated by p21 ras. Keratinocyte growth factor (FGF-7) is produced by stromal cells and acts in a paracrine fashion on prostatic epithelial cells. As PC develops, the sensitivity of the FGF receptors for FGF-2 increases. FGF-2, FGF-5, and FGF-3 levels have been noted to be elevated in PC. Work from cell lines indicates a slightly different relationship. Human PC cell lines (DU-145, PC-3, LNCaP) do not appear sensitive to FGF-2 when added to culture medium [264, 265] although it can be found within the cells. More recently attention has focused on FGF8, also known as androgen inducible growth factor. This is expressed in prostatic epithelial cells and appears to correlate with AR expression (our unpublished observations).

Other growth factors

Endothelins

Endothelins (ET) are 24 amino acid polypeptides that are abnormally elevated in the plasma of men with metastatic PC [266]. Three endothelins (ET-1, ET-2, ET-3) have been identified of which ET-1 protein is detected in primary and metastatic sites of human PC [267]. There are two receptors for endothelins ET_A and ET_B. In BPH, ET_B receptors are found on prostate epithelial cells but in cancer ET_A receptors predominate [267, 268]. Loss of ET_B receptors may be of advantage because these mediate the production of nitric oxide (NO) [269, 270]. The role of NO in tumour biology is unclear, but it has been shown in other systems to induce apoptosis, be cytotoxic *in vitro*, inhibit metastases but paradoxically promote tumour growth *in vivo* [271, 272]. Exogenous ET-1 induces PC proliferation which can be prevented by selective block of ET_A receptors [267]. ET-1 also augments the proliferative response to IGF-1, IGF-2, PDGF, bFGF, and EGF in serum-free conditions. NO inhibits the release of ET-1 from endothelium and receptor binding of ET-1 to its receptors [273, 274]. The role of ET-1 and

Table 5. Integrins found in PC-3 cells

Integrin	ECM component	Ref
$\alpha 2 \beta 1$	collagen type I; laminin	[294, 400]
$\alpha 3 \beta 1$	entactin; laminin; decreased in invasive PC-3 sublines	[294, 297, 401]
$\alpha 6 \beta 1$	laminin; expressed less in invasive PC-3 subline	[294, 401, 402]
$\alpha 6 \beta 4$	laminin; increased in invasive PC-3 subline	[288, 294]
$\alpha v \beta 3$	vitronectin; fibronectin; osteopontin	[294, 400]

its activity through ET_A receptors and the loss of ET_B receptors in the actiology of PC remains to be fully established.

Nerve growth factor (NGF)

NGF is found in the prostatic stroma and NGF receptors are found in prostatic epithelial cells in BPH, cancer and normal tissue [275]. It is found in the conditioned media of TSUPr1, PA-III PC cells and human prostatic stromal cell lines and is important in paracrine interaction between stromal and epithelial cells *in vitro* [276, 277]. Cellular migration through Boyden chambers is enhanced by protein derived from stromal cells and reduced by addition of NGF antibodies [278]. Prostatic tumour cells growing near nerves show a lower apoptotic index than cells away from nerves and this may be due to NGF which is known to have proliferative effects on non-neuronal cells [279, 280].

Other growth factors

Spermine is an endogenous polyamine present in aqueous extracts from the prostate. It is growth inhibitory to PC-3 cells *in vitro* and AT-3.1 cells *in vivo* [281]. Levels of erythrocyte spermine may discriminate between patients with androgen sensitive and insensitive tumours and those likely to relapse following initial treatment [282]. Prolactin, which is probably necessary for citrate production by the prostate [283], appears to alter proliferation of the PC-3 cell line dose-dependently and increase it in the LNCaP cell line [284]. Early work suggests patients with PC treated by androgen deprivation showed lower serum prolactin levels with an improved response to treatment when bromocriptine was added [285].

Cell adhesion molecules (CAM)

A single cell uses multiple mechanisms to adhere to other cells and the extracellular matrix (ECM). CAM expression is relevant to understanding the genesis of the invasive and malignant phenotype, and also in defining prognostic categories and in the development of new therapeutic strategies.

Integrins

Integrins are a family of heterodimeric cell surface glycoproteins which connect the basolateral surface of the cell to the extracellular matrix (e.g. basement membrane) or partake in intercellular adhesion [286]. Multiple heterodimers of over 16 alpha and 8 beta subunits may be formed and are dependent on divalent cations (Ca^{++} or Mg^{++}). Patterns of integrin binding are complex. Some bind identical ECM components but transduce different signals from outside to inside the cell [287].

Normal human prostatic basal cells express alpha: 2, 3, 4, 5, 6, v and beta: 1, 4, 7 subunits [288–290]. In PIN, the hemidesmosomal proteins alpha6 beta4, alpha3 beta1, alpha6 beta1 integrins are preserved whereas in PC, alpha2, alpha4, alpha5, alphav and beta4, alpha6 beta4 integrins tend to be redistributed or lost [288–292]. In the DU-145 cell line, preservation of alpha 6 integrins increases invasiveness *in vivo* and motility on laminin *in vitro* [293]. Although some investigators have found reduced alpha3 levels in an invasive PC-3 subline [294], invasive carcinoma in patients is associated with preservation of alpha3 beta1 and alpha6 beta1 integrins which become expressed diffusely instead of basolaterally. This integrin may allow binding to the basal lamina in blood vessels, nerve and muscle [288–292]. A novel alpha3 subunit with the beta1 subunit has been identified that binds transferrin, which is implicated in PC cell proliferation in bone [295, 296]. Cytokine treatment of PC-3 and DU-145 cells reduces alpha3 expression and this suggests strategies for treatment [297].

A variety of integrins are expressed by PC-3 cell lines (Table 5). LNCaP cells express decreased levels of alpha3 compared to PC-3 or DU-145 cells and this has been suggested as one reason why this cell line is less invasive than the other two [297, 298]. When an invasive PC-3 subclone was generated, alpha3 beta1 integrins were expressed less than normal [294]. Interestingly, LNCaP cells grown on plastic express considerably less alpha3 than PC-3 cells which can be down-regulated by a variety of cytokines [297]. These results are difficult to reconcile, however these studies suggest ways that integrins may be important in the invasive process.

Cadherin

The cadherins are responsible for Ca^{++}-dependent adhesion. There are over 25 types of cadherins, each encoded by a separate gene, and named after the tissue in which they were first found. Cadherins are confined to epithelia and are a master molecule of differentiation inducing compaction in the blastomer, cellular polarity, and cell-cell adhesion. Cadherins act by a homophilic homotypic mechanism and play a pivotal role in cell-cell adhesion.

Most cadherins are 120 kDa single-pass transmembrane glycoproteins composed of 700 to 750 amino acid residues. The extra-cellular part consists of 5 domains, four of which are homologous and contain Ca^{2+} binding sites. Without Ca^{2+}, the extracellular domains collapse and the cells separate from each other.

E-cadherin is the best known of the cadherins and is concentrated in the adhesion belts of mature polarised epithelial cells between their apices and gap junctions. It is connected through the COOH terminal tail of E-cadherin to the cortical actin cytoskeleton via alpha-, beta-, and gamma-catenin, vinculin, alpha-actinin and plakoglobin. Gamma catenin is identical to plakogobin and alpha catenin shares homology at the protein level with vinculin which is located at the adherens junctions and focal contacts. This forms a transcellular network of proteins which can contract in the presence of myosin motor proteins.

Most cells express a combination of cadherins: basal cells in the prostate express both P- and E-cadherin whereas luminal cells express E-cadherin only [299]. They are of considerable interest because a significant proportion of prostatic tumours exhibit loss of heterozygosity at 16q, the region to which E-cadherin has been mapped (16q22), suggesting a putative invasive suppressor gene [300].

Several studies have shown strong relationships between the grade and stage of PC and E-cadherin [301]. Studies examining E-cadherin expression in formalin fixed paraffin embedded prostatectomy specimens show decreasing E-cadherin expression associated with increasing Gleason score with an accompanying decrease in E-cadherin expression in lymph node metastases compared to primary tissue [302–304]. Immunohistochemical expression patterns have also been associated significantly with survival after radical prostatectomy [305]. Whether E-cadherin expression is an independent predictor of survival when incorporated with models of Gleason score, PSA and stage has yet to be conclusively demonstrated.

In the Dunning model, there is an inverse relationship between E-cadherin expression and invasive phenotype [306, 307]. Following a single passage of the H cell line, a very aggressive anaplastic cell line was derived. This was metastatic immediately following administration and was named AT-6. It was shown that decreased E-cadherin expression occurred concomitantly with the acquisition of metastatic ability.

Aberrant hypermethylation of 5′ CpG islands within the proximal promoter region of E-cadherin has been shown in the PC cell lines TSUPr1 and DUPro [308] and may be one of the mechanisms leading to loss of functional E-cadherin expression. Demethylation can lead to re-expression of E-cadherin [309]. Functional E-cadherin can also be inactivated by phosphorylation of the associated catenins or aberrations in these proteins.

PC-3 cells express E-cadherin mRNA but are deficient in alpha-catenin because of a large homozygous deletion of the gene. Reintroduction of normal alpha-catenin DNA or microcell-mediated transfer of chromosome 5 restores E-cadherin function and the presence of tight junctions leading to the suppression of PC-3 growth *in vitro* and tumourigenicity *in vivo* [310, 311]. A relationship between the potential TSG product APC and beta-catenin exists in that the APC competes for binding with beta-catenin [312]. Binding of catenins to FGF receptor or to cadherin results in phosphorylation events. There is evidence from the LNCaP cell line that E-cadherin is regulated by androgens [313], which may be relevant in the sequence of events underlying the development of androgen independence and tumour progression.

C-CAM

Cell-CAM (C-CAM) is a member of the immuno-

globulin superfamily of proteins with sequences similar to the carcinoembryonic antigen (CEA) and whose human homologue is CD66a [314]. C-CAM binds by homophilic interactions with other cell adhesion molecules and is Ca^{++}-independent. C-CAM expression can be regulated by androgens [315] and is predominantly expressed continuously on the cell membrane of normal human tissue but becomes discontinuous in BPH and PIN and completely absent in PC [316]. The cytoplasmic domain of C-CAM 1, which is an isoform of C-CAM, contains several potential phosphorylation sites for cAMP-dependent kinase and for tyrosine kinase, but these are probably important in signal transduction rather than cell adhesion [317]. Single dose transfection of an isoform of C-CAM1 into PC-3 cells by an adenovirus results in stable expression of C-CAM1 and suppresses growth *in vitro* and tumourigenicity in nude mice [318, 319].

Intercellular-cell adhesion molecule-1 (ICAM-1)

ICAM-1 is an early marker of immune activation and response and it is the counter-receptor for LFA-1 (lymphocyte function-associated antigen) on T-cells. It is important in the conjugate formation between tumour infiltrating lymphocytes (TILs) and their target inducing both tumour cell death and natural killer cell-mediated cytolysis. Theoretically, up-regulation of ICAM-1 should enhance the host immune response against the tumour, but dissemination of tumour cells is enhanced because of tumour cell attachment to circulating lymphocytes. ICAM-1 levels are inducible by cytokines (IL-2, interferon-gamma, interferon-alpha, TNF-alpha) in PC-3 and DU-145 cells *in vitro*; LNCaP cells barely express ICAM-1 and it is not inducible by cytokine treatment [297]. It has yet to be shown that cytokine treatment reduces invasion and metastatic spread as a result of ICAM-1 expression. Circulating ICAM-1 levels are similar in controls, patients with PC and BPH, and are not useful as a staging tool [320].

Angiogenesis

Folkman and co-workers have provided new insights into possible mechanisms regarding tumour neovascularisation and how it affects clinical presentation [321]. Prostatic tumours are not usually angiogenic at the time of first development and avascular tumours stop growing after reaching 1–2 mm in diameter. Eventually some new capillaries are recruited from pre-existing blood vessels. The tumour grows not only because of the increased perfusion, but also because of the release of growth factors and extra-cellular matrix proteins from endothelial cells [322].

Angiogenesis is promoted by products released from tumour cells, infiltrating tumour-associated macrophages and the endothelium itself. Angiogenesis is of importance at two critical stages during the development of metastases: the time of shedding into the circulation and establishment at a new site [323]. Multiple factors can induce or inhibit an angiogenic response. Many of these factors may be produced or acted upon by the endothelial cells or prostatic tumour cells themselves.

In other tumour systems, the primary tumour releases both stimulatory and inhibitory agents which have markedly different half-lives in the circulation. The balance of these determines in part the clinical scenario [324]. Vascular Endothelial Growth Factor (VEGF) is produced in the human normal prostate [325] and its importance in the growth of the rat ventral prostate has also been demonstrated [326]. Expression is under the control of androgens and higher in prostatic adenocarcinoma specimens than normal or BPH tissues [326, 327]. In human tissue, VEGF has been found in tumour infiltrating lymphocytes (TILs) and tumour cells themselves [328]. Tumour associated macrophages have been shown in rats transplanted with Dunning MAT-Ly-Lu cell to be the major type of tumour infiltrating cell [329] which can secrete potent inducers of angiogenesis (e.g. Tumour Necrosis Factor alpha, bFGF, VEGF).

A novel angiogenesis inhibitor, angiostatin, was identified in a murine Lewis lung carcinoma and this inhibited the growth of metastases when released into the circulation by the primary tumour.

Recently, inoculation of immunodeficient mice with PC-3 cells inhibited neovascularisation of mouse corneas induced by bFGF. This suggests that PC-3 cells also release into the circulation inhibitors of angiogenesis. Whether this reduces metastatic spread is unproven [330].

Inhibitors of angiogenesis such as linomide have been shown to reduce the growth of the primary tumour and metastases of MAT-Ly-Lu cells *in vivo* [331, 332]. Linomide reduced the number of tumour infiltrating macrophages and the ability of these cells to produce TNF-alpha [329]. Microvessel density is reduced while apoptosis and necrosis are increased in the TSU and PC-3 tumours in SCID mice treated by linomide [333]. Such treatments may prove beneficial because they increase the death rate without affecting the proliferation rate which is low and partially explains the poor success with cytotoxic chemotherapy in PC.

Quantifying the extent of angiogenesis in human material has either been performed by analysis of microvessel density or quantifying levels of angiogenic factors in bodily fluids. Microvessel density is assessed by counting the number of vessels in randomly selected areas after staining for von Willebrand factor antigen [334]. Inter and intra-observer variability is minimised by computer image analysis. Microvessel density has been found to correlate with PSA levels and tumour grade in TURP specimens from patients with PC as well as with the response to external beam radiotherapy [335]. Microvessel density in prostatic tumours is greater than in surrounding benign tissue [334] and can distinguish between primary prostatic tumours from patients with metastases and those without [336]. Using computer image analysis, vessel density distinguished radical prostatectomy specimens with extraprostatic extension from those without [337]. Areas of PIN were distinguished from benign tissue by the number of vessels around the gland perimeter. Assessment of micro-vessel density may prove to be useful in devising selection criteria for those patients most likely to benefit from radical treatments.

As angiogenesis is crucial to the development of tumours, inhibiting it may be an effective treatment. Suramin, a polysulphonated naphthylurea, exerts angiostatic activity *in vivo* and inhibits the binding of PDGF, bFGF, IGF-1 and VEGF *in vitro*. It is variably effective in PC [338, 339] but with considerable toxicity. Further assessment of potential analogues may identify effective and less toxic substitutes [340]. Levels of bFGF or other angiogenic compounds in the serum or urine may eventually prove useful in the diagnosis or monitoring of treatment in PC [341].

Invasion

Invasive cells need to penetrate the basement membrane and interstitial stroma in order to metastasise. Attachment to the basement membrane is affected by integrins and expression of specific genes including MMPs is partially regulated through integrin-related signals [286]. Proteolysis is needed not only to break through the basement membrane but also for entry into the circulation, extravasation and successful settling at the site of metastasis. Increased motility identifies cells with raised metastatic potential in the Dunning model [342–344] and interference with adhesion can alter motility and slow tumor growth *in vivo* [345].

Matrix metalloproteinases (MMP)

The MMPs are a superfamily of homologous metalloendopeptidases that require an intrinsic Zn^{++} for catalytic activity and extrinsic Ca^{++} for enzyme stability. They include gelatinases and collagenases.

Multiple MMPs participate in an enzymatic cascade promoting dissolution of the ECM and tumour invasion. Their activity is controlled either by secretion as an inactive zymogen or by tissue inhibitors of metalloproteinases (TIMP).

MMPs have been shown to be important in invasion in human cell lines and primary tissue. A PC-3 cell line metastatic to bone (PC-3 ML) contained higher levels of MMP-2 compared to its relatively non metastatic precursor [346]. Secretion could be up-regulated by 'invasion stimulating factor' and conversely reduced by IL-10 with concomitant increases in TIMP-1 [347]. Levels of active MMP-2 were higher and there was almost no TIMP

in the conditioned media of primary cultures of human prostatic cancer tissue [348].

Similarly, matrilysin transfected DU-145 cells are more invasive than controls [349]. Transcripts for MMP-7 and collagenase type IV were found in the majority of prostatic adenocarcinomas, but collagenase type I and stromelysin I transcripts could not be detected on Northern blots [350]. Using paraffin sections, it was possible to detect MMP-7 and gelatinase A in prostatic epithelial tissue [351]. Levels of TIMP-1 and collagenase are higher in patients with PC than controls [352]. Patients with metastases had greater levels of collagenase than patients without. When combined with TIMP-1 levels, collagenase was as good a marker of disease state as PSA in identifying patients with metastases.

Serine proteinases

Other degradative enzymes include serine proteases which have a highly reactive serine residue in their active site and can act independently of MMPs. Urokinase plasminogen activator (U-PA) is a serine protease which cleaves plasminogen activator releasing plasmin that acts on fibrin, fibronectin and laminin. It has been found to have prognostic significance in some tumour systems [353]. U-PA is associated with invasive potential *in vivo* [354] and is expressed more by PC-3 than LNCaP cells [355].

Other enzymes that degrade the ECM can also release sequestered angiogenic compound such as bFGF. Hyaluronic acid is broken down by hyaluronidase to fragments that promote angiogenesis *in vivo*. Levels of hyaluronidase are greater in PC tissue than BPH or normal prostate and are associated with the grade of the tumour suggesting that hyaluronidase activity may contribute to the invasive process [322].

Ion channels

Introduction

Ion channels are composed of glycosylated transmembrane proteins forming pores in the cell membrane that allow the passage of ions down their respective electrochemical gradients [356]. Ion channels are classified broadly by the principal ion they carry (e.g. Na^+, K^+, Ca^{++}, Cl^-) and by what 'gates' the channels open or close (including changes in membrane voltage, intracellular Ca^{++} or the binding of nucleotides). The unequal distribution of ions across the cell membrane results in the generation of the membrane potential, which is about 100 000 volts per centimetre. The positional conformation of charged molecules near the cell membrane will be affected by this potential [357]. More recently, ion channels have been implicated in the cell cycle [358–361] apoptosis [360, 362], cell adhesion [363–365], cell movement [363, 366, 367], exocytosis [368, 369], proliferation [358, 361, 370] and multidrug resistance [371, 372]; all of which have direct relevance to the neoplastic process.

Briefly, to identify ion channels either the membrane voltage or current flow are fixed and then deliberately changed whilst continuously monitoring the other. This allows the measurement of current flow across open channels. Using the patch clamp technique, for which Neher and Sakmann were awarded the Nobel Prize in 1991 [373], it became possible to study channels present in low density, in areas difficult to clamp or of low conductance and in real time.

Invasion

The first systematic examination of cell membrane ion channels with respect to known malignant phenotype was made in the Dunning model of PC. AT-2 cells which are invasive but not metastatic were compared with MAT-LyLu cells which are invasive and metastatic *in vivo* [374]. MAT-LyLu cells were found to have voltage-activated sodium channels (VASC) in about 50% of cells, which bore characteristics similar to neuronal type VASC. Voltage-activated K^+ channels (VAKC) were found in AT-2 cells, but K^+ current was twice as great as in MAT-LyLu cells. When VASCs were blocked by tetrodotoxin (TTX) (which is a specific blocker of VASCs), invasion was reduced significantly *in vitro*. Further work in human cell lines has supported a role for

VASC in tumour invasion. When LNCaP and PC-3 cells were evaluated, VASCs were found only in PC-3 cells and K^+ currents were considerably larger in LNCaP cells [375, 376]. Again the incorporation of TTX significantly reduced invasion *in vitro*. PC-3 cells were also more depolarised by a median of 5 mV (CI 0 to 11) compared to LNCaP cells. Similar observations were made when membrane potentials from cells of human infiltrating ductal breast carcinoma were compared to cells from non-malignant breast tissue [377]. Membrane potential has been shown to change dramatically through the cell cycle [359] and blocking K^+ channels can induce cell-cycle arrest [358, 370].

The importance of membrane ion channels may relate to the factors described above, but also to cell movement: transformed Madin-Darby Canine Kidney (MDCK-F) cells show directional migration depending on the position of K_{Ca} channels in the membrane [379] which may further mediate migration/invasion. In neurones, release of neurotransmitters contained within vesicles can occur upon membrane depolarisation and similar mechanisms could lead to the release of MMPs or serine proteinases in tumour cells.

Calcium, androgens and apoptosis

Programmed cell death (apoptosis) occurs following androgen withdrawal in androgen dependent PC cell lines. There is rise in Ca_i^{++} which activates Ca^{++}/Mg^{++}-dependent endonucleases present within the nucleus and fragments DNA initiating the programmed cell death sequence [380, 381]. Androgen-independent cells do not undergo an elevation in Ca^{++} after androgen withdrawal, but prevention of re-uptake of Ca^{++} into the sarcoplasmic reticulum by thapsigargin (TG) will induce apoptosis. This induces an early rise in intracellular Ca_i^{++} which probably originates from the endoplasmic reticulum and a prolonged further rise which most likely depends on the entry of Ca^{++} through Ca^{++} channels in the cell membrane [382]. In normal rat ventral prostate verapamil, a blocker of L-type voltage-activated Ca^{++} channels, prevents prostate tissue regression following androgen withdrawal, suggesting a putative role for voltage-activated Ca^{++} channels in the cell membrane [383]. The bcl-2 protein helps prevent cells from undergoing apoptosis and confers protection against elevations in Ca_i^{++} as recently demonstrated in PC-3 cells transfected with the Bcl-2 gene [384]. Androgen exposure raises Ca_i^{++} in LNCaP cells to similar levels as seen in the later stages of androgen-independent cells treated by TG [382, 385]. The rise in Ca^{++} can be blocked by 1 mM verapamil which also suggests that Ca^{++} channels are present [385]. These results suggest that the relationship between Ca^{++}, androgens and apoptosis is complex and merits further investigation.

Proliferation

It is known that progression through the cell cycle is Ca^{++} dependent in certain tissues [361] and there is evidence that Ca^{++} channels are important in the proliferation of prostatic cells. Maximum growth rates of DU-145 cells are dependent on extracellular Ca^{++} concentration [386]. Depolarisation of these cells by high external K^+ results in Ca^{++} entry which suggests the presence of voltage-activated Ca^{++} channels. Verapamil caused a reduction in cell growth with an IC_{50} of 55 mM but increased $^{45}Ca^{++}$ intake. Verapamil blocks K^+ channels with an IC_{50} in the range 50 to 150 mM in LNCaP and PC-3 cells when tested using the whole-cell configuration of the patch clamp technique, and the cells die within days when grown with verapamil at these concentrations (our unpublished observations). It is possible that DU-145 cells were depolarised under such conditions allowing the entry of Ca^{++} through voltage-activated Ca^{++} channels. Studies in other cell lines with a similar respone have suggested that extracellular Ca^{++} is not the origin of the rise in Ca_i^{++} and that there may be alternative mechanisms by which verapamil exerts its biological effects.

There is evidence in various tissues that growth factors elevate intracellular Ca^{++} via membrane channels, but such an effect has not yet been demonstrated in prostatic tissue. It is likely that such ionic responses are essential to the transduction of growth factor signals because similar responses have been found in cell lines from the embryonic zebra fish and

this suggests that these have been conserved through evolution [387]. Entry of Ca^{++} through voltage-activated channels results in the phosphorylation of the serum response factor (SRF) that activates the serum response element of the c-fos gene. The latter is important in growth factor induced transcription [388]. In Balb/c 3T3 cells, IGF-2 directly stimulates Ca^{++} influx in cells primed with EGF or PDGF independent of membrane voltage but not if cells are in G_0 [389]. A calcium channel accelerates progression through G_1 in the presence of IGF-2 such that priming with EGF or PDGF becomes less important [390]. K_{Ca} channels are induced following exposure to EGF and PDGF and this has been shown in murine fibroblasts to depend on p21 ras and raf kinase [391]. Such channels are present in PC-3 cell lines [376]. Furthermore, inhibition of K_{ATP} channels causes reversible cell cycle arrest in breast cancer cell lines. Such studies indicate that K^+ channels and Ca^{++} have important roles in translating extracellular messages to intracellular signals.

Problem areas and focal issues

It remains to be seen whether the hypothesis in the Dunning and human cell lines are borne out when more cell lines or indeed human tissue are examined. Specific studies focused on identifying Ca^{++} channels need to be carried out as these may well be pivotal in exerting local effects. Cloning of ion channels and transfection studies will indicate more clearly the importance of ion channels both *in vitro* and *in vivo*.

Concluding remarks

PC still poses many questions regarding its aetiology, pathology including definition, pathogenesis and clinical management. It is evident from this review that PC has been underinvestigated but as a direct consequence of recent public awareness there has been a stimulus to undertake fundamental research. (This covers early detection of PC so that treatments may be effective.) Perhaps more importantly, we need to identify those features which distinguish PC from other solid cancers. There is an apparent rush to repeat studies carried out on other solid tumours such as breast, colorectal or pancreatic – this may prove to be short sighted and unrewarding. The natural history of this enigmatic disease is rather different from most other solid tumours and therein may lie the key to unravelling its biologic mysteries. Some clues will be gained by studying the genes most frequently involved in PC, and the cytogenetic analyses are an important starting point to locate areas of gross genetic damage. Increased clinical effort in locating potential PC families would be a major help in identifying a PC gene and may well require international collaboration. In addition, the reasons for the differences in prevalence of PC among differing cultural groups may well reveal an important facet controlling the pathogenesis of PC.

The other areas which are distinctive to PC are the mechanisms underlying hormone regulation of cell growth and progression, and the development of novel anti androgenic therapies will be helpful. It is essential to understand the mechanisms underlying the phenomenon of resistance to androgen blockade in order to circumvent this problem; at the present time reduction of the androgenic drive is an effective management strategy but the effect is temporary. The other feature that characterises PC is the nature of the induced stroma. This is particularly relevant in metastatic disease where a fibrocollagenous response may dominate the histological picture. Factors produced by the tumour in bone metastasis cause bone matrix destruction and replacement by sclerotic stroma, resulting in debility and pain. If this response can be controlled, it may allow patients to live with their disease, prolonging quality and quantity in life. In PC, effective management and reduction of symptoms may be a more realistic and desirable aim than attempting to search for a cure for this disease.

Key unanswered questions

These are

1) What are the mechanisms underlying the development of resistance to androgen blockade?
2) What are the principal genes most frequently involved in PC?

3) Which genes govern the progression of PC from latent to aggressive and metastatic cancer?
4) What factors govern the development of the stromal response in PC?
5) How does the desmoplastic response modulate the growth of PC?
6) What are the factors underlying PC metastasis, particularly to bone?
7) Are there other serum markers which may be more discriminatory than PSA for distinguishing between latent and aggressive cancer?
8) What are the functions of PSA, PSM, PAP on prostatic epithelial cell growth and differentiation?
9) What factors govern successful propagation and maintenance of primary PC cell lines and immortalisation of benign prostatic epithelia?
10) Development of *in vitro* models more relevant to *in vivo* disease.

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