PROGNOSTIC BENEFITS OF HISTOLOGICAL GRADING AND IMMUNOHISTOCHEMISTRY IN HUMAN PROSTATIC CARCINOMA

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DE PROGNOSTISCHE BETEKENIS VAN HISTOLOGISCHE GRADERING EN IMMUNOHISTOCHEMIE VOOR HET HUMANE PROSTAATCARCINOOM

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MARINUS PIETER WILHELMUS GALLEE

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Promotor:	Prof. Dr. R.O. van der Heul
Overige leden:	Prof. Dr. J.C. Birkenhäger
	Prof. Dr. F.H. Schröder
	Dr. F.H. de Jong
Co-promotor:	Dr. Ir. J. Trapman

The only wisdom we can hope to acquire is the wisdom of humility.

T.S. Eliot: East Coker (1940).

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CHAPTER I

GENERAL INTRODUCTION

1. Anatomy of the prostatic gland

The normal prostate surrounds the proximal part of the human male urethra, and is traversed by the ejaculatory ducts, which open into the prostatic urethra. These structures divide the gland into localised areas, known as the prostatic lobes-anterior, middle, posterior and two lateral. These lobes, however, are purely anatomical and have no functional implications.

Functionally, the prostate is divided into inner and outer gland groups. The inner gland group is made up of short periurethral glands and somewhat larger submucosal glands. The main ducts of the submucosal glands are lined by transitional epithelium. The inner group is separated from the main mass of the prostatic glands -the outer prostate- by an indefinite fibrous capsule.

Excretory duct and tubulo-alveolar glands (exocrine acini) arranged within lobuli form the functional entity of the prostatic gland. Acini of inner and outer glands are microscopically indistinguishable and are both lined by a double layer of epithelium: the inner cylindric cell-layer (with secretory activity) and the outer basal or myoepithelial cell-layer.

The inner glands are relatively inconspicuous in the young but become increasingly prominent with age (see I-3.4). Benign Prostatic Hyperplasia (BPH) develops from this inner gland group. A series of changes also occurs in the outer group of glands with age: atrophy of epithelium and stroma has been described in addition to hyperplasia and neoplasia (Franks et al., 1956).

2. Epidemiology of prostatic cancer

In those Western countries with a high socio-economic standard prostatic cancer (PC) is the second most common cancer, after lung cancer, occurring in males (Catalona, 1984). The incidence rate (the number of new cases per 100,000 males per year) within the United States between the years 1973 and 1977 was approximately 40. The incidence rate of clinically manifest PC in males above the age of 85 is even higher. Thus, PC is a disease more often found in the elderly male population. On the other hand in a representative group of males aged 47, the incidence rate counted was 0.001 (Cohen et al., 1985). Incidence- and mortality figures increase nearly logarithmically with age. Below the age of 40 PC is a rare tumor. However, PC is sporadically reported in male adolescents (Shimada et al., 1980). It is generally believed, that when prostatic carcinomas found on careful histological examination at autopsy are included, PC would be the most prevalent cancer in men.

In the Netherlands its incidence is still increasing and in 1982 the incidence rate was assessed at 25.1 (average annual age-standardized incidence rate per 100,000 population for PC; Zaridze et al., 1984). The increase in incidence is predominantly the result of a more frequent use of surgical

procedures in the treatment of BPH. Furthermore, complete submission of the entire specimen for histological examination has resulted into an increase of detection of stage A disease (see I-6) (Beckner et al., 1985; Dhom et al., 1983).

As is known for a long period, incidence-figures of PC show a remarkable variation of geographic distribution. The lowest incidence figures are found among the male population in Shanghai (0.84), whereas the highest incidence was recorded for the black population in Alameda County, California (1982: 100.2; Zaridze et al., 1984; 1987) and in Denver, Colorado (1977: 115.5; Beckner et al., 1985).

A similar geographic distribution was noted for the mortality with reference to PC. The age standardized mortality rate (recorded in 1978) ranged from 0.1 to 29.1 per 100,000 male population (0.1 for Thailand; 29.1 for St. Vincent and Grenadines; Zaridze et al., 1984; 1987). Northern European and North American countries have high rates, Israel and Latin and South America, and southern European countries have intermediate rates, and Oriental and eastern European countries have low rates. The mortality figure in Japan (1978) was assessed at 2.5; the mortality figure in the Netherlands was 15.5 (Zaridze et al., 1987). Interesting in this regard is the observation that the prevalence of BPH appears also low amongst Japanese compared with Caucasians (Wynder et al., 1971). In contrast with these overall figures regarding incidence and mortality, the incidence rate of stage A1 lesions (see I-6) does not show a geographic distribution, but is nearly equal within each country.

The difference between incidence- and mortality figures indicates the variation in biological behavior and success of intervening therapeutic regimens. Fortunately, only a fraction of patients suffering from PC will ultimately die from the sequels of this malignancy. However, increased life expectation and the introduction of sensitive diagnostic techniques have led to a relative growth of registrated mortality rate for PC within the last few years.

3. Etiology of prostatic cancer

Several different factors are assumed to play a role in the etiology of PC:

- genetic predisposition
- exposure to chemical carcinogens or chemical promoters of carcinogenesis
- endogenous hormonal influences
- venereal transmission of an infectious agent

3.1. Genetic factors in the oncogenesis of prostatic cancer

Evidence for genetic factors comes from several studies suggesting a high incidence of PC among relatives of PC patients (Schuman et al., 1977). In spite of this observation, a relationship between a specific HLA type and PC has not been reported as yet.

On the other hand difference in incidence rate among whites and non-whites in the United States provides evidence for some genetic factor in the ontogenesis of PC.

3.2 Environmental (socio-cultural) factors

The potential role of environmental factors in the etiology of PC is underlined by the observation that Japanese males migrating to Hawaii initially maintain a low mortality from PC for about one generation but finally account for a mortality closer to that of the American population (Winkelstein et al., 1979). A similar phenomenon for Asian immigrants in the United States was described by Ross et al. (1983). Furthermore, the importance of environmental factors is emphasized by the observation that Nigerian blacks show a much lower incidence of clinically manifest cancer than Nigerian blacks living in the United States.

Since a higher number of stage A2 lesions was detected in Japanese immigrants in Hawaii compared with Japanese in Japan (Akazaki et al., 1973), it is postulated that environmental factors may promote biological alterations within initially harmless stage A1 lesions resulting in an increase of tumor size or leading to a more aggressive variant (see I-6).

Among these environmental factors exhaust fumes or particular air-pollution can be considered (Rotkin, 1977). Also exposure or ingestion of cadmium and other heavy metal contacts have been introduced as potential promotors in carcinogenesis. Cadmium, a non-essential trace element, acts as a zinc antagonist in biological systems. Zinc is an essential trace element which is involved in the regulation of cell growth and its presence in large quantities is demonstrated in the prostatic gland (Ross et al., 1983). Compared with non-carcinomatous prostatic tissue the zinc concentration in PC seems to be lowered (Muentzing et al., 1974; Feustel et al., 1984).

Moreover, ingestion of a high fat diet may be associated with an increased risk for PC (Hill et al., 1981; Graham et al., 1983; Talamini et al., 1986). This is reflected in the strong positive correlation between body mass index and subsequent risk of developing PC. Changes in diet have been shown to affect endocrine function as measured by changes in androgen, estrogen and prolactin serum levels. Excess of body fat may influence the availability of steroid hormones (Wynder et al., 1971). Furthermore vitamin A is claimed to protect against PC (Osegbe et al., 1988; Hayes et al., 1988) and low daily intake of β-carotene and of vitamin A seems to correlate significantly with the subsequent development of PC (Oishi et al., 1988; Ohno et al., 1988).

Some reports deal with a potential sexually transmissible infectious etiology of PC similarly to the etiology of cervix uteri cancer in which it has been postulated that human papilloma virus (HPV 16/18) may play an etiologic role (Centifanto et al., 1973; Herbert et al., 1976; Jones et al., 1976; Baker et al., 1981; Campbell et al., 1985).

In addition there are numerous studies speculating about a

relationship to sexual practice (Mishina et al., 1985). Summarizing, these studies link sexual hyperactivity and promiscuity with an increased risk for PC (Catalona, 1984). On the other hand Ross et al. (1981) reported that Catholic priests had a similar and, in fact, slightly higher incidence of PC than controls.

3.3. Endogenous hormonal factors

The growth rate of the majority of PC is, at least in the initial phase, dependent on androgens. This may be explained by the functional presence of steroid receptors for androgens within prostatic tissue, especially within the nucleus of prostatic (epithelial) cells. Basic investigations of androgen action in prostatic tissue indicate the involvement of testosterone (T) uptake, conversion to dihydrotestosterone (DHT) by the enzyme 5α-reductase and transport of DHT to a nuclear localized androgen-binding receptor (AR; see also I-9.4) (Kliman et al., 1978). Since interstitial (Leydig) cells of the testis are the major source of T, castration (surgical or chemical) will benefit the clinical course of androgen-dependent PC. The interstitial cells are controlled by the interstitial cell stimulating- or luteinizing hormone (ICSH = LH) from the anterior pituitary, which in turn is influenced by gonadotrophin releasing hormones (LH-RH) from the hypothalamus. The pituitary gland also influences the prostatic gland by secreting prolactin which stimulates the metabolism of the prostate. Androgens are also produced by the adrenal cortex. The mechanisms involved have been thoroughly investigated in animal studies (Steenbrugge, van 1988a).

Further evidence of a potential etiologic role of the endocrine status of the host is provided by the observation that fertile human males have an increased risk of developing PC (Armenian et al., 1975). However, this is contradicted by other investigators (Wynder et al., 1971). In addition, PC in eunuchs is reported. Different alterations in levels of estrogens, adrenal steroids and androgens in urine and serum samples associated with prostatic malignancies have been reported (Hoisaeter et al., 1982; Levell et al., 1985; Nomura et al., 1988). This is partly attributed to the fact that T serum levels are age dependent. Moreover, the finding that the largest fraction of serum-T is bound to a carrier protein (sex hormone binding globulin = SHBG) might influence the accuracy of the data.

Contrary, experimental studies have demonstrated that long-term administration of T combined with estrogens resulted in the development of PC in Noble rats (Noble et al., 1977). Subcutaneous administration of T alone also produced prostatic adenocarcinomas in rats. T-induced synchronization of prostatic cells in a state of proliferation enhanced the carcinogenic effect of N-methyl-N-nitrosourea (Bosland, 1989).

Further evidence that changes in the endocrine status of the human host may be of influence on PC is provided by the finding that a lower prevalence of PC at autopsy was detected

amongst men suffering hepatic cirrhosis. Liver cirrhosis is associated with hormonal changes such as hyperestrogenism and alterations in the melanocyte stimulating hormone levels resulting in hyperpigmentation of the skin (Flanders, 1984).

3.4. Interrelationship of BPH with prostatic cancer

BPH has to be considered as a physiological process starting in the third decade of life and manifesting itself with complaints of prostatism in the male population at the age of 60. First pathological evidence of the disease appears in men between 40 and 50 years old (Berry et al., 1984). In the seventh decade the prevalence of BPH has increased to more than 70 per cent.

BPH results in an increase of the weight of the prostatic gland as a consequence of a relatively increased growth rate of the stromal and epithelial tissue. In the third decade of life the increase in weight is 1.6 g per year. In the subsequent decades a decline in growth rate is reported, leading to an increase of weight of only 0.4 g per year in the tenth decade (Berry et al., 1984).

It is generally accepted that prostatic hyperplasia occurs initially in the periurethral portion of the middle and lateral lobes and consists of fibromuscular hyperplasia with subsequent involvement of the stroma by proliferating glands (Mostofi et al., 1973). The nodular fibromyoadenomatous variant of BPH is most frequently encountered. The precise role of DHT in the pathogenesis of BPH has to be elucidated. Controversial reports are available concerning a possible relationship between the adenomatous (or glandular) variant of BPH and PC. Those authors who consider a possible causal link often do not realise that the frequently occurring coexistence of BPH with carcinoma does not exceed the incidence of BPH in an age-matched control group without PC (Catalona, 1984; Wynder et al., 1971).

According to Kastendieck (1980) PC may be preceded by the genesis of an area of disturbed architecture within a hyperplastic nodus (atypical prostatic hyperplasia; see I-4.1). On the other hand, the adenomatous variant of BPH is most frequently encountered in the periurethral prostatic region, whereas the majority of carcinomas originate in the peripheral (posterior) areas of the prostatic gland.

The description of rare types of hyperplasia (such as cribriform hyperplasia and basal cell hyperplasia = transitional cell metaplasia) and their possible relationship with PC is beyond the scope of this survey (see Dhom, 1985).

4. Histomorphology of prostatic cancer

Over 95% of prostate malignancies are adenocarcinomas (Utz et al., 1969; Flanders, 1984) and originate from the epithelial structures of the tubulo-alveolar prostatic glands. The morphologic diagnosis of carcinoma of the prostate is primarily based on the following criteria: cellular atypia (i.e. nuclear anaplasia, mitotic activity, and prominent

nucleoli), disorderly arrangement of glands and invasive growth (including perineural extension) (Kastendieck, 1980). Normal and hyperplastic glands are characterized by an epithelial lining consisting of two cell layers, whereas tumor glands are recognized by a simple often cuboid (or prismatic) epithelial lining of one layer lacking the outer myoepithelial cells. The inner surface of the malignant acinus is smooth and lacks the normal papillary projections. Since tumor glands are often densely packed, presumably caused by increased tortuosity of tubulo-alveolar units, interacinar stroma is found scarcely in tumor areas (dos à dos arrangement of tumor glands without interposition of stromal elements). Nuclei of epithelial tumor cells are enlarged (resulting into an increased nucleus-cytoplasm ratio) and appear to have prominent nucleoli. In general, mitotic figures are quite often not found in PC (see I-11). Nuclear anaplasia (variations from normal in size, shape, staining and chromatin distribution of tumor cell nuclei, sometimes variation in size and number of nucleoli are also included) varies widely and seems of potential prognostic value (see I-5.1).

For as yet it is believed that prostatic carcinomas (even stage A lesions; see I-6) have a multicentric origin (Parfitt et al., 1983ab).

4.1. Potential precancerous lesions within the prostate

Atypical, irregular epithelial proliferations in the prostate in conjunction with primarily benign lesions have been reported. The recognition of these lesions has been associated with the potential development of PC. However, the true existence of premalignant morphologic lesions that can easily be detected by pathologists is still a matter of controversy. Moreover, the nomenclature of these lesions is characterized by a total lack of uniformity among the different authors. In general, potential premalignant lesions in the prostatic gland have to be subdivided into lesions predominantly related with architectural and often with proliferative changes (dysplasia) and into those lesions with exclusive cytologic abnormalities (Kastendieck, 1980). The former lesions are often associated with BPH, whereas the latter are more frequently encountered in non-hyperplastic glands. However a simple dichotomy is not often possible since architectural disorders are nevertheless frequently associated with cellular atypia.

Lesions with predominant architectural abnormalities are characterized by an increase of small glands with a reduction of the amount of interacinar stroma. The boundaries of these proliferative foci with the surrounding tissue are usually well defined lacking obvious morphologic parameters of invasive growth. Moreover the majority of these glands are lined by a double layer of regularly arranged epithelium without any cytonuclear atypia. However, exceptions associated with nuclear anaplasia have been described. The lesions with predominant architectural disturbances are designated as adenosis (Brawn, 1982b) or described as atypical prostatic

hyperplasia (Kovi et al., 1988). Unlike the simple hyperplasia, a clearly raised 3H-thymidine index of these lesions is described (Helpap, 1980).

It can be very difficult to distinguish these potential precancerous dysplastic lesions from malignant tumors since the judgement of invasive growth within the prostatic gland is not always easy. In addition lack of precise definition hampers its general acceptance and recognition by pathologists. Mainly these lesions occur in combination with BPH; less often they are encountered in glands with signs of inflammation. For as yet the identification of such dysplastic lesions does not allow any conclusions referring its prospective potential. Interesting is the observation that the age-adjusted rate of dysplasia precedes that of latent (see I-6) PC. Out of the various forms of prostatic dysplasia the primary atypical hyperplasia shows the highest frequency in coincidence with manifest PC.

Apart from atypical proliferative lesions associated with BPH, potential pre-neoplastic lesions have also been recognized in non-hyperplastic prostatic ducts and acini (McNeal et al., 1986ab; McNeal, 1988b). The latter lesions are characterized by cytologic changes, whereas the architecture is normal or minimally distorted. These cytonuclear atypia includes nuclear enlargement (anisokaryosis), small prominent nucleoli, nuclear hyperchromasia, cell crowding with variable pseudostratification, and increased density of cytoplasmic staining.

4.2. Histologic typing of prostatic carcinoma

Unfortunately, an uniform and commonly endorsed histological classification of PC does not exist although the variety of divergent structural patterns of the common PC is rather limited (Melicow et al., 1976). The reason for this might be found in the variability of the histological proliferation patterns in a single case as is also demonstrated in this thesis (see Chapters II, V and VII). PC often shows a pluriform picture with simultaneous occurrence of several different growth patterns. Another reason might be the existence of differing concepts about the histogenesis with respect to the glandular system of the prostate. The Armed Forces Institute of Pathology (AFIP) proposed a classification into a few principal groups. In addition to adenocarcinomas that account for approximately 97 per cent of all malignant prostatic neoplasms (Utz et al., 1969), the AFIP distinguishes undifferentiated carcinomas, endometrioid carcinomas (of the prostatic utricle = periurethral gland carcinoma = papillary adenocarcinoma of the urethra, verumontanum; Bostwick et al., 1985; Walther et al., 1985; Epstein et al., 1986a), adenoid cystic carcinomas, transitional cell carcinomas (Nicolaisen et al., 1984), and squamous cell carcinomas (Mostofi, 1973). Furthermore, the existence of small cell carcinoma (sometimes combined with a "regular" prostatic adenocarcinoma; Wenk et al., 1977; Schron et al., 1984; Ro et al., 1987), true papillary adenocarcinoma (Kuhajda et al., 1984) and carcinoid

Table 1. Examples of histologic classification schemes designed for PC.

Mostofi and Price (1973)	Dhom (1977)
usual prostatic carcinoma:	carcinoma with uniform pattern:
glandular pattern (micro acinar, small, normal, large, mixed).	highly differentiated adenocarcinoma
papillary pattern	poorly differentiated adenocarcinoma
adenocystic pattern	cribriform carcinoma
cribriform pattern	anaplastic carcinoma
solid medullary pattern	urothelial carcinoma
	squamous cell carcinoma
	mucinous carcinoma
rare types of prostatic carcinoma:	carcinoma with pluriform pattern:
endometrioid carcinoma	highly and poorly differentiated adenocarcinoma
transitional cell carcinoma	cribriform and anaplastic carcinoma
squamous cell carcinoma	cribriform pattern in other types
adenoid-cystic carcinoma	

(Wasserstein et al., 1979) have been reported. Moreover, mucinous carcinomas and even signet ring cell carcinomas of the prostate have been described (Remmele et al., 1988; Ro et al., 1988). Some classification schemes proposed by different authors are summarized in Table 1. Finally, sarcomas can develop within the prostatic gland. Relatively frequently occurring sarcomas in the prostate are: rhabdomyosarcoma (most commonly found in children), leiomyosarcoma and fibrosarcoma.

One also has to realise the possibility of presence of metastases of an elsewhere localized primary tumor or the development of a carcinoma originating in the seminal vesicle. Also extension of bladder tumors and rectum cancers within the prostatic gland is regularly encountered.

In this thesis we distinguish well, moderately and poorly differentiated adenocarcinomas in addition to cribriform cancers, clear cell carcinomas (also known as the hypernephroid variant of PC), and undifferentiated carcinomas. Among the undifferentiated carcinomas we distinguish tumors composed of compact well-circumscribed areas of tumor cells (the so-called solid tumor areas or medullary cancers) and malignancies characterized by solitary cancer cells diffusely infiltrating the surrounding fibrous stroma (truely undifferentiated cancers). Many studies demonstrated that cribriform and solid patterns possess a higher malignant potential than purely glandular carcinomas. Some investigators support the idea that centrifugal extra acinous growth results in a

glandular pattern of PC, whereas a centripetal, intraglandular proliferation of tumor cells leads to a cribriform or medullary pattern.

4.3. Spread of prostatic carcinoma

4.3.1. Intra-prostatic spread

PC is considered to develop multifocally within the prostatic gland (Hayashi et al., 1987). For as yet it remains to be elucidated whether intra-prostatic metastases do occur in the natural course of PC. Lack of lymphatic vessels, and tissue clefts within the prostatic gland combined with the absence of intra-glandular extension makes a possible intra-organic spread less acceptable. However, recently the occurrence of ductal spread in nearly 50% of cases of PC was described by Kovi et al. (1988). Often tumor foci originate in peripheral parts of the gland. The areas of predilection are the dorsal and dorsolateral parts next to the capsule. Fortunately, at the dorsal aspect capsular invasion occurs relatively late as the fascia of Denonvillier seems to be an efficient barrier to tumor extension (Kastendieck, 1980). Additional tumor foci can sometimes be found in the ventral, pre-urethral region. Especially the stage A lesions (see I-6) seem to originate in the anterior portion of the prostatic gland (McNeal et al., 1988a). The stromal invasion results from destruction of the normal stromal-epithelial barrier. Invasive growth results from the splitting up of fibromuscular bundles.

The extension within the prostate first of all takes place into the outer area and intermediate part. The inner peri-urethral portion and the prostatic urethra itself will be infiltrated by tumor at the end of intraprostatic extensive spread.

Capsular involvement can be divided in: extension to the capsule; invasion without penetration of the capsule and penetration of the capsule by tumor. Extension in the peri-prostatic area may result into spread of tumor in the perineural clefts and angio-invasive growth. For as yet the perineural localization merely proves the spread into interstitial spaces and its precise predictive meaning for potentially wide spread (metastasized) disease is questioned (Kastendieck, 1980; Dhom, 1985).

According to Schroeder et al. (1975) invasion around the seminal vesicle did not influence survival or cancer death rates, when compared to histological stage C lesions (see I-6). However the latter is contradicted by other investigators (Myers et al., 1983). Progression of the disease after seminal vesicle involvement will lead to fixation of the prostatic gland to adjacent structures. A so-called frozen pelvis develops when the whole pelvis is filled with tumor.

Invasion of the rectum per continuitatem with mucosal ulceration is rare.

4.3.2. Extra-prostatic spread

PC may disseminate to regional lymph nodes. Regional lymph node centres encompass the common iliac, external iliac, hypogastric and obturator-fossa nodes. Furthermore the peri-aortic and sacral lymph nodes are also often involved. Invasion of the lymph nodes localized in the groin, supraclavicular region and hilar (tracheobronchial nodes) sites are usually very late events (Prout et al., 1973). Nodal metastases develop before skeletal metastases (Saltzstein et al., 1977).

Bone metastases most frequently develop within the vertebral column (especially the lumbo sacral spine) by means of extension within the venous vertebral plexus (Batson) and can be visualized by simple roentgenograms or by more sensitive techniques such as bone scanning after administration of a radio nuclide (Tc), CT- and NMR scan. Other frequent sites of bone metastases are the pelvis, the ribs and the upper parts of the femur (Mostofi et al., 1973). Metastatic lesions within the skeleton often induce bone formation resulting into the characteristic osteoplastic lesions associated with metastases of PC.

Visceral metastases are most frequently the result of hematogenous spread. The most common sites of visceral metastases are the lungs, liver, pleura, adrenals and kidneys, brain and spleen but almost every organ or structure may display involvement (Mostofi et al., 1973; Brawn et al., 1989).

As mentioned before, widespread disease is not the result of tumor extension by perineural invasion in the prostatic gland. Moreover, the tumor extension within the prostatic gland is unrelated to the capability of the tumor to metastasize.

Diagnostic punch biopsies seem not to accelerate the local and regional growth of PC (Kastendieck, 1980). On the other hand transurethral resection (TUR) must be avoided when PC is presumed. The influence of TUR upon the spread of PC was investigated by Hanks et al. in 1983. Their conclusion was that TUR disseminates cancer in patients with T3 and T4 lesions (see I-6) and has to be limited to relief of severe urinary obstruction within a palliative therapeutic regimen. However, one has to realise that shrinkage or reduction of the prostatic gland with relief of obstruction can also be achieved by immediate administration of estrogens or by radiotherapy. Moreover suprapubic urinary drainage can also be considered.

Histologically, the morphology of metastases agrees more often with the major component than with the worst differentiated areas of the primary tumor (Grayhack et al., 1983). However metastases of PC entirely composed of single malignant glands are rarely encountered. Therefore it is assumed that primary well-differentiated prostatic adenocarcinomas possess a lower metastatic capacity compared with less or undifferentiated primary tumors (Brawn et al., 1987). Since a differentiation arrest can occur within time, it is not permitted to consider primary tumors composed solely of

malignant single glands as totally harmless. Recently, it was also proven that arrest of differentiation may occur with time within the secondary tumor deposits (Brawn et al., 1989).

5. Grading in prostatic cancer: general comments

Efforts aimed at discrimination and determination of parameters with potential prognostic value extracted from cancerous tissue have resulted into the development of diverse grading systems. Using this wide definition biochemical, biological, immunological, morphologic (including morphometry, electron-microscopy, and immunohistochemistry) and tumor cell kinetic (DNA flow cytometry, DNA precursor uptake techniques, etc) data can be arranged in prognostic classes and regarded as grading systems.

Among all diverse grading techniques, histological grading is most intensively investigated in tumor pathology and applied by pathologists. Histological grading is based on the assumption that the degree of malignancy can be determined by the histological appearance (often including cytologic characteristics) of the original tumor. Broders reported in 1926 that the amount (percentage) of differentiation (i.e. keratinization) within squamous cell carcinomas of the lip was of utmost importance in the assessment of prognosis in the individual case. In the subsequent decades many grading systems have been introduced for cancers of diverse origin. In general, histological grading is predominantly based on considering the architecture and (or) cellular morphologic characteristics (including the presence and number of mitotic figures).

5.1. Histological grading systems in prostatic cancer

For PC numerous grading systems have been designed. The number of scores in each grading system varies from three (most grading systems) to 9 (grading system developed by Gleason). Though statistical analysis is often poor, all promise an optimal correlation of increasing grading score with progressive worse clinical outcome. A short (chronological) review of the most important grading systems for PC will be presented here (see also Chapter II and III). Histological grading systems that can be applied for all kinds of tumors including PC will not be discussed in this section (e.g. Broders system; 1926).

Contrary to all other grading systems designed for PC, Gleason (1966) has devised a grading method to solve the problem of histomorphologic heterogeneity within a single tumor. He observed that a majority of prostatic carcinomas had either a single pattern or at the most, two patterns of differentiation: a predominant pattern and a secondary pattern. Considering only tumor architecture within prostatic cancers, 5 different growth patterns could be distinguished with pattern 1 being the least malignant and pattern 5 the most malignant.

Pattern 1: Very well differentiated, small, closely packed, uniform glands in essentially circumscribed masses.

Pattern 2: Similar but with moderate variation in size and shape of glands and more atypia in the individual cells; cribriform pattern might be present; still essentially circumscribed, but more loosely arranged.

Pattern 3: Similar to Pattern 2 but marked irregularity in size and shape of glands, with tiny glands or individual cells invading stroma away from circumscribed masses, or solid cords and masses with easily identifiable glandular differentiation within most of them.

Pattern 4: Large clear cells growing in a diffuse pattern resembling hypernefroma; may also show gland formation.

Pattern 5: Very poorly differentiated tumors; usually solid masses or diffuse growth with little or no differentiation into glands (Gleason, 1966; Mellinger et al., 1967).

The Gleason score (ranging from 2 to 10) is determined by adding up the numerical values of the primary and secondary growth pattern. In those tumors in which only one growth pattern is present the numerical value of this single pattern is doubled to achieve the Gleason score. In the United States the Gleason system is often applied; despite of its complexity the interobserver agreement seems reasonable (see I-5.3; Fowler et al., 1985).

The grading system designed and utilized at the Mayo clinics (Utz et al., 1969) distinguishes four grades upon assessment of seven histological criteria (acinar structure; individual cellular structure; nuclear characteristics; presence of nucleoli; cytoplasmic characteristics; mitotic activity; and degree of invasiveness).

Combining differentiation with degree of nuclear anaplasia Mostofi (AFIP; WHO) has proposed the following grading system (Mostofi, 1975). Grade I: well differentiated glands with nuclei that show slight nuclear anaplasia. Grade II: gland formation but the nuclei show moderate nuclear anaplasia. Grade III: glands with marked nuclear anaplasia or tumors that are undifferentiated (do not form glands).

A grading system combining the histological features of glandular pattern and nuclear cytology was presented by Gaeta et al. (1980 and 1981; 4 grading scores) and has been recommended by the National Prostatic Cancer Project (NPCP).

Boecking et al. (1982) devised a grading system with 3 grades of malignancy combining tumor architecture and degree of nuclear anaplasia. In histologically heterogeneous tumors only the lowest grade of differentiation is considered. The score consists of the sum of the histological points and the points granted to the amount of nuclear anaplasia. Subsequently 3 grades of malignancy are deduced from the results. In their initial report an interobserver agreement of 91% was recorded. Moreover, they emphasize the phenomenon of changes of tumor grade in time as a result of dominant growth of a less differentiated tumor clone.

Brawn designed in 1982 the M.D. Anderson Hospital (MDAH) grading method based on the assumption that differentiated (gland forming) prostate carcinomas have a better prognosis than undifferentiated (non-gland forming) prostate carcinomas. Grade 1: 75-100% of the tumor forms glands; 0-25% does not

(grade 1 lesions do not include predominantly cribriform-papillary tumors). Grade 2: 50-75% of the tumor forms glands; 25-50% does not (includes tumors consisting of 50% or more of a cribriform-papillary pattern). Grade 3: 25-50% of the tumor forms glands; 50-75% does not. Grade 4: 0-25% forms glands; 75-100% of the tumor forms no glands.

Mostofi and Schroeder proposed a new grading system in 1985 based on a retrospective multivariate analysis on prostatectomy specimens with cancer of patients with a well-known long-term follow-up (Schroeder et al., 1985abc). Based on architectural features, mitotic activity and nuclear anaplasia patients could be arranged in 5 classes with statistically significant different prognostic outcome. Further studies have to be carried out on other patient groups to investigate the exact value of this new grading method. For as yet its relatively high interobserver agreement (this thesis) provides hopeful expectations (see Chapters II and III).

Other grading systems such as those designed by Evans et al. (1924), Young et al. (1927) and Shelley et al. (1958). These methods are excluded in this overview because they are at present of limited practical value.

5.2. Additional morphology-related prognostic factors in prostatic carcinoma

Apart from those features that are laid down in numerous grading systems especially designed for PC, still reports occur presenting additional morphologic and biometric characteristics within prostatic tumors with potential prognostic value.

The presence or absence of distinct or indistinct cell borders of tumor cells was noted to be of potential prognostic significance by Epstein et al. (1976). In their retrospective multi-variate analysis they also observed a prognostic significance of lymphocytic infiltration. The latter might suggest that cell-mediated immunity of the host plays a role in the natural course of PC.

Nuclear pleomorphism in PC is often used in grading systems and is known as nuclear anaplasia. However, according to others the latter term includes also variations in the nuclear distribution of chromatin. A subdivision regarding the degree of nuclear anaplasia (pleomorphism), which is sometimes used as solitary grading effort, was introduced by Epstein et al. (1976).

Class I — Nuclei are regular with little variation of size and shape.
Class II — Nuclei show a moderate variation of size and shape. The diameter of the largest nucleus is not more than twice the diameter of the smallest.
Class III — Nuclei vary markedly in size and shape.

Myers et al. (1982) introduced a method of grading nucleoli in neoplastic cells. The nucleolar grade (varying from 1 to 3) was attached as a superscript to the grade of tumor Gleason pattern (see also: Tannenbaum et al., 1982).

To objectivate and quantitate morphologic features and to diminish lack of reproducibility from examiner to examiner, morphometric and biometric studies of PC have been performed (Sharkey et al., 1984; Stamey et al., 1988). In general, tumor size correlates well with the biological behavior of PC and is regarded as a relatively reliable prognosticator (Fan et al., 1983). Evidence is available indicating a clear-cut relationship between histological grading scores and tumor size. This relationship has also been studied in the different clinical tumor stages of PC (see I-6). Stage A1 tumors are nearly always well differentiated adenocarcinomas. Guileyardo et al. (1982) stated that within stage A lesions (especially A2 neoplasms), tumors with Gleason pattern scores greater than 4, were significantly larger than those with Gleason scores 4 or less. Hence, for PC it is generally accepted that histological differentiation (reflected in grade) is related to size and clinical behavior of the tumor.

Apart from gross biometric parameters, which can be determined macroscopically or by low-magnification microscopy (Fan et al., 1983), now, computer assisted techniques are available to enable biometric studies of detailed histological and cytologic characteristics. The latter studies have been performed for many tumors including PC; many different parameters have been evaluated morphometrically and correlated with prognostic data. The major drawback of this technique is the subjective choice of the tumor area that will be submitted for morphometry.

Diamond et al. (1982ab) objectivated nuclear morphology (changes in size and shape of tumor nuclei) in clinically staged B2 prostatectomy specimens by means of computer assisted image analysis. It was determined that a nuclear roundness factor, the degree to which a nucleus deviates from a circle or sphere, could be used to distinguish those stage B-2 prostatic cancers with a high lethal potential from those B-2 lesions that are less aggressive (see I-6 for the explanation of the various clinical tumor stages). Recently Mohler et al. (1988ab) stated that the nuclear roundness factor correlated even better with prognosis than pathological grading by the Gleason method.

5.3. Controversy in histological grading of prostatic cancer

The large number of histological grading methods which have been developed during the last decennia already indicates the controversy that exists concerning the choice of the parameters and their distribution among scores to reach general acceptance. Grading methods differ widely as to which morphologic aspects of the cancer are most important for predicting the prognosis of PC. In some of them, large prognostic significance is attributed to architectural parameters (Gleason; Anderson), whereas others emphasize the prognostic value of cytological features (Mostofi, 1975). None of the systems reviewed in short previously is currently in use (Gardner, 1982). Reasons for this reluctancy of pathologists to use histological grading systems are:

- Lack of intra- and interobserver agreement when applied by others than the original designer of the grading method.
- Lack of sufficient statistical evidence of the prognostic value of each grading step (or score) within grading systems.
- Lack of precise definition of morphologic criteria such as for example nuclear anaplasia (Brooks et al., 1986).
- The morphologic heterogeneity of PC within a single patient, which complicates the use of those grading methods that offer only the possibility to judge one growth pattern (Dhom, 1985). Often the area of poorest differentiation (and highest cellular anaplasia; Boecking et al., 1982; McGowan et al., 1983) is selected for grading. In some grading methods the most dominant growth pattern of the cancer is examined.
- Lack of consensus concerning the type of the specimen on which histological grading has to be performed, because of lack of agreement between grade, defined in biopsy and radical prostatectomy specimens (needle biopsy specimen seems not representative enough; Catalona et al., 1982; McGowan et al., 1983; Lange et al., 1983; Babaian et al., 1985; Dhom, 1985; Mills et al., 1986).
- Lack of sufficient evidence that the prostatic tumor retains the same histomorphologic appearance throughout the life of the host and that the tumor is not subjected to morphologic changes (due to arrest of differentiation) during the natural history of the tumor (Murphy et al., 1979; Brawn, 1983b).

Looking at the numerous grading systems for PC introduced in literature, it is generally recognized that grading is arbitrary and subjective at best. Reasons why these grading methods are not generally applied are already mentioned in detail above. Here some criticism and comments are presented concerning the Gleason grading system, which is frequently used in the United States.

The grading system proposed by Gleason is criticized by some authors not to be reproducible and does not include all features associated with malignancy (Harada et al., 1977). Murphy et al. stated in 1979 that the margin of error of reproducibility applying the Gleason system from one institution to another could be as much as 50%. Contrary, a relatively high interobserver consensus of the Gleason system (74%) was found by Bain et al. (1982). Stage A PC (n = 143) was independently graded according to the Gleason system by two pathologists (Guileyardo et al., 1982). Perfect agreement (identical Gleason pattern score) was reached in 81% of cases.

Intra-observer concordance of the Gleason system seems also relatively poor. A considerable improvement can be achieved when Gleason scores are rearranged into three categories such as proposed by Babaian et al. (1985). Using his own grading system Gleason had an intra-observer reproducibility of 80% (Murphy et al., 1979). Svanholm and coworkers (1985) reached an intra-observer agreement of 65% and 42%. Criticism is also presented by McGowan et al. (1983) who found, in disagreement with Gleason, that the

qualitatively highest histological tumor grade (total lack of differentiation) is most important in respect of prognosis.

The resemblance or lack of resemblance of the primary tumor with secondary tumor deposits falls beyond the scope of this introduction.

Comparative studies concerning the prognostic accuracy and the reproducibility of grading are not frequently met in literature (Kate ten et al., 1986; Morenas et al., 1988; Gallee et al., 1989a). We have examined the interobserver consensus of 5 grading methods (Chapter II). In a subsequent study the prognostic accuracy of these methods was compared (Chapter III).

Referring the criticism about histological grading, therapeutic dicisions in the individual patient may not be made upon grading results alone (Murphy et al. 1979).

6. Staging in prostatic cancer

At the present time the individual prognosis and biological behavior of PC are predominantly based on (histological) grading and clinical staging. Histological grading of PC has been discussed in section I-5; results of histological grading studies are presented in chapters II and III. The clinical stage is assessed by clinical examination. In the case of PC these examinations encompass a physical survey (i.e. rectal examination), determinations of tumor-associated markers in body fluids (serum, urine, prostatic fluid etc) such as prostatic acid phosphatase (PAP) and prostate-specific antigen (PA), roentgenological assays (including computer tomography of chest, vertebral column, pelvis etc), bone scintigraphy studies, ascending (bi)pedal lymphangiography, ultrasonography (Fair et al., 1983; Jansen et al., 1989) and tissue biopsy information. Sometimes retroperitoneal lymphadenectomy (combined with frozen section analysis) is also part of the (clinical) staging procedure (Lieskovsky et al., 1980). Results of treatment are generally reported on the basis of clinical staging (Gibbons et al., 1983).

Whitmore introduced in 1956 the first classification of the clinically determined extension of PC. According to the extension of the tumor, PC was categorized into 4 stages. In 1975 Jewett presented a modified and more detailed description of the criteria of the 4 stages, which are briefly summarized in Table 2.

** Stage A PC's are clinically inapparent tumors. They are found incidentally by the pathologist either at autopsy (latent cancers) or during microscopic examination of surgical specimens without clinical suspicion of malignancy (TUR or simple suprapubic prostatectomy). They are nearly always localized anteromedially within the prostate and do not extend beyond the prostatic capsule (McNeal et al., 1988a). These tumors are also known as incidental or latent (unsuspected or quiescent) cancers. However, some authors confine the term "latent" to tumors that are

Table 2. The staging of prostate cancer (after Jewett, 1975)

A. Not clinically manifest
   1. Focal, low grade
   2. Diffuse and/or high grade

B. Localized
   1. One lobe only
   2. More than 1 lobe
   (3. Involvement of more than one lobe associated with deformity of the prostatic gland)

C. Local extension beyond the prostatic capsule but no metastases

D. Metastases

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encountered during postmortem examinations. Although latent cancers also develop in other organs, such as the thyroid gland, gastro-intestinal tract and lung, the prostate seems to be unsurpassed in its frequency of such tumor foci (Dhom, 1983; see also I-2). The incidence of latent cancers appears to increase when the step-section technique is employed and the whole specimen is submitted for histological examination (Scott et al., 1969; Moore et al., 1986). Using this technique Newman et al. (1982) found an occurence of incidental cancers in a large series of TUR specimens (n = 500) of 8 per cent. Bauer et al. (1960) and Parfitt et al. (1983ab) found in approximately 10 per cent of patients undergoing TUR or enucleation for presumed BPH an unsuspected PC. The incidence of latent cancers detected in necropsy specimens increases with age. An incidence rate of 6.4 per cent was recorded among men in the sixth decade of life, whereas above the age of 80 years 26 per cent of men appeared to have latent PC (Halpert et al., 1963). In general, the incidence of unsuspected PC varies widely ranging from 4 to 30 per cent (Golimbu et al., 1978; Bartsch et al., 1983a).

Recent knowledge of the biological charateristics has resulted into a subdivision within stage A PC of relatively indolent stage A1 and more aggressive stage A2 lesions. The former (focal or A1 tumors) have been defined variously as tumors involving less than 3 chips (Golimbu et al., 1981), no more than 3 microscopic foci (WHO), less than 5 chips and less than 5 per cent of the surgical specimen (TUR) (Cantrell et al., 1981; Newman et al., 1982; Bridges et al., 1983; Sonda et al., 1984), whereas the latter (A2 lesions) encompass more extensive lesions and reveal often a high histological grade (Wilson et al., 1983; Bartsch et al., 1983a). The A1 lesions are known in literature as incidental focal carcinomas. Though initially it was generally accepted that these lesions did not interfere with life expectancy and did not

need any therapy (Bridges et al., 1983), recently data have become available that in a subfraction of patients with stage A1 lesions (especially high grade lesions) relapses do occur after approximately 8 years (Epstein et al., 1986b; Blute et al., 1986; Lowe et al., 1988ab; Epstein et al., 1988). The A2 lesions are designated as incidental multi-focal or diffuse tumors. They are often considered as more aggressive (Lowe et al., 1988ab). When more than 5 per cent of the TUR tissue contained cancer, or the tumor histology showed a Gleason (sum) score of 8 to 10, 33 per cent of the patients had progressive disease within 4 years (Yatani et al., 1982; McNeal et al., 1988a). There is even evidence that some A2 lesions will behave more aggressively than many stage B tumors (Golimbu et al., 1978; Bartsch et al., 1983a; Parfitt, 1983ab).

The age distribution of unsuspected incidental PC indicates that the percentage of stage A1 tumors is relatively higher under the age of 60. Beckner et al (1985) found no poorly differentiated stage A2 lesions at younger ages. Conversely, more than two-thirds of the incidental tumors in men above the age of 85 were poorly differentiated and non-focal. In contrast with stage A2 lesions, the prevalence of small foci of latent PC (stage A1) does not show any geographical distribution (Flanders, 1984).

** Stage B cancers are those tumors that are clinically evident, apparently confined within the prostatic capsule and without evidence of regional or distant metastasis. This clinical stage has been further subclassified as B-1, a nodule or area of induration less than 1.5 cm confined to one lobe, or B-2, involving both lobes but still confined to the prostate (Gibbons et al., 1983). Finally, stage B-3 lesions are considered as intracapsular malignancies associated with deformation of the gland (Whitmore, 1980).

** Stage C lesions are clinically evident with apparent local extension beyond the prostatic capsule but without evidence of metastasis. Apart from capsular perforation involvement of seminal vesicles may be present. Eventually local tumor extension will lead to a prostate that is fixed to surrounding structures.

** Stage D tumors are clinically metastasized prostatic cancers. When a patient with clinical stage A, B, or C disease is found to have pelvic lymph node metastasis without bony spread or involvement of other organs, he is classified stage D-1. If a patient has distant bone metastasis, then he is staged D-2.

Apart from the Whitmore (1956) classification of PC the Veterans Administration Cooperative Urogenital Research Group (VACURG) suggested in 1974 a four-class staging system (Gleason et al., 1974). Moreover, detailed TNM clinical staging systems (T = the extent of the primary tumor; N = the

absence or presence and extent of regional lymphnode metastasis; M = the absence or presence of distant metastases) were developed for urogenital malignancies by the American Joint Committee for Cancer Staging and End Results Reporting (1978). The updated version of the clinical TNM system (1987) is summarized in Table 3. The most precise tumor stage, based on data obtained at microscopic examination, is represented by the pTNM system. Apart from these current staging systems, other rarely applied tumor extension classification systems have been developed (Hudson et al., 1972; VACURG, 1976; McLaughlin et al., 1976; Ray et al., 1976).

Table 3. TNM classification scheme for prostatic tumor extension (derived from "TNM classification of malignant tumors", International Union Against Cancer; 1987).

T1	Incidental
T1a	Less than 3 tumor-foci
T1b	More than 3 tumor-foci
T2	Clinically or grossly (macroscopically) limited to gland
T2a	Less than 1.5 cm
T2b	More than 1.5 cm or involvement of more than one lobe
T3	Invades prostatic apex/ beyond capsule/ bladder neck/ seminal vesicle/ not fixed.
T4	Fixed or invades other adjacent structures
N1	Single secondary tumor deposit less than 2 cm
N2	Single secondary tumor deposit larger than 2 cm and smaller than 5 cm, or multiple secondary tumor deposits smaller than 5 cm
N3	Secondary tumor deposits larger than 5 cm

Several studies have demonstrated that low stage cancers frequently show (histologically) low grade tumors (see I-5). However, highly specific tumor stage related histological characteristics have never been identified. In almost every stage of the disease, regardless of treatment modality, patients with well-differentiated tumors have a longer survival (Grayhack et al., 1983). On the contrary, high grade, high stage, and large tumor mass have an independent adverse effect on the probable patient course.

For all tumors including PC, a combined appraisal of histological grading and clinical staging raises the reliability of prognostic statements (Kastendieck, 1980; Mellinger et al., 1967; Gleason et al., 1974; Sogani et al., 1985).

6.1. Controversy in clinical staging of prostatic cancer

Underestimation of the exclusively clinically assessed tumor stage frequently occurs (Prout et al., 1973; Kramer et al., 1980; Bosch et al., 1986) and becomes evident when patients are submitted to surgical procedures i.e. retroperitoneal lymphadenectomy and prostatectomy and pathological staging can be compared with clinical staging (Lieskovsky et al., 1980). Various studies have suggested that up to 25 per cent of patients with clinically stage B lesions have regional metastases to the lymph nodes (stage D tumors). In patients with clinically staged C lesions this percentage is reported to be even higher (up to 60 per cent; Whitmore et al., 1973; Saltzstein et al., 1977). Grossly, approximately 20-50 per cent of tumors seems to be understaged. For this reason some authors stated that the stage of PC is of the least prognostic significance (Belt et al., 1972; Byar et al., 1981; Culp et al., 1973; Whitmore, 1973; Murphy et al., 1976).

On the other hand, local overstaging has also been reported. Bosch et al. (1986) found after thorough pathologic examination of the surgical specimen, that 23 per cent of patients with clinical stage T3 appeared to be overstaged.

Inaccurate staging can be considered as the result of limitations of the diagnostic armamentarium to judge precisely the extent of the disease. Concerning pathological examination it may also be the result of inaccurate or incomplete investigation of the surgical specimen. The necessity of microscopic evaluation of the entire specimen for detection of stage A cancer in clinically benign prostates is emphasized by many workers (Denton et al., 1965; Scott et al., 1969; Yatani et al., 1974; Battaglia et al., 1979; Newman et al., 1982; Vollmer, 1986; Yamabe et al., 1986). The necessity of repeated TUR's in accurate diagnosing stage A disease was stressed by McMillen et al. (1976), Bridges et al. (1983), Parfitt et al. (1983ab) and Sonda et al. (1984). The availability of advanced equipment such as ultrasonography, computer tomography and nuclear magnetic resonance scanning may help in more accurately determining pre-operatively the exact stage of the prostatic neoplasm.

7. Therapeutic management of prostatic cancer

To date, treatment of PC is primarily based on clinical stage. As there often is discrepancy between the assigned or clinical stage of tumor and its true extent, one can never be completely sure, which stage is being treated (I-6.1). This complicates a simple comparison of therapeutic modalities presented in literature. The choice of the most suited stage-related therapy is further hampered as in literature sufficient statistical analysis is frequently omitted or not comparable. Furthermore, follow-up is usually of too short duration to permit meaningful conclusions (Klein, 1979). Finally, it is difficult to define exactly objective treatment response criteria for PC (Schroeder et al., 1984a).

Because of the afore-mentioned reasons it is impossible to

state exactly which treatment is best for which tumor stage. In spite of these restrictions an attempt is made to present some major stage-associated therapeutic principles. In addition, the role of the major therapeutic approaches in the management of PC will be discussed briefly.

Stage A1 cancer needs no special therapy (Byar et al., 1981); an expectative regimen is justified. The simple prostatectomy or transurethral resection can be regarded as both diagnostic and curative at the same time. On the contrary, stage A2 cancer requires more than careful observation. Radiotherapy is applied with moderate success (Cobalt-60 teletherapy). Though radical excision of the prostate is not generally accepted in the treatment of stage A2 disease, it is quite often performed. These prostatectomy specimens obtained after previous transurethral resection contain in 50-80 per cent of cases residual tumor (Sonda et al., 1984). Hormonal manipulation has no proven efficacy in astage A2 disease.

Stage B cancer (see Table 2) has to be treated by radical prostatectomy (Myers et al., 1983). Equally successful results are described for external beam radiotherapy (Hanks, 1988). Hormonal treatment and orchiectomy have no particular role whatsoever in stage B PC.

Stage C cancer can be treated by external beam irradiation. Schroeder et al. (1975) and Bosch et al. (1987) propose a limited role for perineal prostatectomy in (early) stage C lesions performed for low grade (1 or 2) tumors (according the Mostofi grading system). However, in these cases surgical staging (consisting of pelvic lymphadenectomy, including immediate microscopic examination) has to precede radical surgery.

For **stage D** PC many therapeutic modalities have been installed. Among these hormonal manipulation and radiation therapy (sometimes combined with retroperitoneal lymphadenectomy in stage D-1 disease) have proven to achieve a maximum of palliation. Controversy exists concerning the precise timing of endocrine therapy; early or late treatment (when symptoms become manifest) (Aubel van et al., 1985b). Some authors report moderate success in patients treated with cytotoxic agents and specific or non-specific immunotherapy (Guinan et al., 1984; Deguchi et al., 1986).

7.1. Surgical intervention

Radical prostatic surgery for PC consists of perineal or retropubic prostatectomy. The latter approach has the advantage of simultaneous dissection of the retroperitoneal lymphnodes combined with the possibility of immediate (fresh-frozen) histological examination and is considered as the only treatment by which PC actually can be cured. Prostatectomy is nowadays an accepted treatment for localized limited PC (i.e.stage A2 and B lesions) (Paulson et al., 1986; Hanks, 1988). When radical prostatectomy is carried out for clinical stage C lesions, patients whose specimens contain tumor-positive margins of resection, can be treated successfully by

postoperative radiation (Paulson et al., 1986). Adjuvant therapy can be given either before or immediately after primary surgery. Some reports describe successful results following radical prostatectomy on patients with stage C lesions whose tumors were regressed during pre-operative endocrine therapy (Belt et al., 1972; Myers et al., 1983). To prevent complications such as impotence, urethral stricture, morbidity associated with anesthesia and incontinence the operation has to be carried out by surgeons with special experience in the field of urogenital oncology (Culp et al., 1973; Finkle et al., 1981). Post-operative follow-up of at least 15 years duration seems neccessary to determine whether a patient has been cured of this disease (Belt et al., 1972). The survival rates after prostatectomy are predominantly determined by the microscopic extent of the tumor within the prostatectomy specimen (Culp et al., 1973). When cancers were less than 1.0 cm in greatest dimension, 90 per cent of the patients lived at least 10 years post-operatively.

The precise role of TUR in the treatment of PC is still a matter of dispute. Some investigators believe that TUR may not be performed in the treatment (palliative or curative) of PC for the possible danger of wide spread dissemination (McGowan, 1980; Babaian et al., 1988). Others postulate that TUR might have a role in the palliative treatment of metastasized PC for relief of bladder outlet obstruction or as a curative treatment protocol for stage A and B cancers (Myers et al., 1983) and even stage C cancer (Bartsch et al., 1983b). Contrary to prostatectomy, TUR is regarded as a conservative treatment regimen.

7.2. Hormonal intervention

In choosing the optimal treatment regimen one must realize that approximately 80 per cent of patients respond initially to hormonal manipulation, suggesting that this should be the therapy of first choice. However, since hormonal intervention cannot be considered as a curative therapy, prostatectomy or radiotherapy has to be recommended for young patients with limited localized cancers, whereas an endocrine regimen has to be reserved for patients with high-staged prostatic tumors. Initially endocrine manipulation consists of administration of anti-androgens and estrogens or castration (orchiectomy). Treatment with stilboestrol was complicated by cardiovascular side effects (coronary artery disease and myocard infarct) especially when large doses were given. Later on, other estrogens at lower doses were applied showing considerably less cardiovascular side-effects. Among the alternatives of stilboestrol belong medroxyprogesterone acetate, diethylstilbestrol (DES), anti-androgens such as cyproterone acetate, Luteinising Hormone Releasing Hormone (LHRH) analogues, and the anti-fungal agent ketoconazole (Kirk, 1985; Schroeder et al., 1987). Nevertheless, endocrine manipulation is frequently accompanied by side effects such as painful gynecomastia and gastro-intestinal symptoms. Cardiovascular effects have already been mentioned. Ultimately, a large

number of prostatic cancers become insensitive for hormonal intervention. Recently, the role of modulation of the AR system of regulation of gene expression has been discussed in the development of androgen-independent growth of human PC (Coffey et al., 1987; see I-9.4).

7.3. Other therapeutic modalities

7.3.1. Radiation therapy

In a large patient group the effect of external beam radiotherapy was studied. Seventy-two per cent of patients with stage B cancers were alive at 5 years and 44 per cent at 10 years; a lower percentage was found in patients with extracapsular lesions (stage C): survival at 5 years 51 per cent and at 10 years 38 per cent (Ray et al., 1973). Application of X-ray treatment to truely localized carcinoma, such as stage A2, would be predicted to achieve a noteworthy success. Hanks did not find any difference in long-term outcome of external beam radiation therapy and radical prostatectomy for stage A2 and B cancer (Hanks, 1988).

Next to external beam radiation therapy, patients can be treated by implantation of radioactive seeds (interstitial irradiation) (Kirk, 1985; Fujino et al., 1986) or a combination of these two methods. The effect of radiotherapy (measured by ultrasonography) correlates with the histological grade of the tumor; poorly differentiated tumors react most favourable on radiotherapy (Fujino et al., 1986). Radiation therapy is often complicated by chronic proctitis, cystitis and impotence (Schroeder et al., 1976). Radiotherapy might also be of value in helping alleviate the pain of metastatic lesions.

7.3.2. Chemotherapy

For advanced stage endocrine-unresponsive PC (stage D lesions) the National Prostatic Cancer Project (NPCP) and the EORTC urological group have developed diverse multi-agent treatment regimens. These include various combinations of anti-cancer drugs (e.g. cyclophosphamide, 5-fluorouracil, estramustine phosphate (Estracyt), streptozotocin, vindesine, imidazole-carboxamide, dacarbazin, procarbazine, prednimustine, cis-platinum, methotrexate, vincristine, doxorubicin (adriamycin), mitomycin-C etc.). In some therapeutic regimens these agents are combined with hormonal therapy (Loening et al., 1983; Emrich et al.,1985). Torti et al. (1985) reported that the combination of doxorubicin and cisplatin showed no superiority to single-agent doxorubicin treatment in metastasized PC. These authors also state that the benefit of chemotherapy in PC is equivocal and that the response of diverse chemotherapeutic schemes can hardly be measured by objective parameters.

7.4. Therapy induced alterations in histo- and cytomorphology of prostatic carcinoma

Effects of hormonal therapy

The hormonally induced changes are mainly investigated in advanced stages of tumors since these more extended cancers are often treated by non-surgical regimens. The cytological changes observed in patients having a positive response to hormonal therapy include squamous metaplasia, hydropic degeneration, reticulation of the nuclear chromatin, and pyknosis and rupture of the nuclear membrane.

Estrogen-induced squamous metaplasia was first described by Bainborough in 1952. Most frequently it occurs in non-neoplastic glands (acini and ducts) and will sometimes lead to obliteration of glandular structures. However, Accetta et al. (1982) described the presence of large foci of squamous cell differentiation in the metastases of widespread prostatic adenocarcinoma and discussed the possible role of estrogen therapy in inducing these morphologic changes. Moreover, estrogen therapy causes also squamous metaplasia of the urethra. Experimental work in dogs by Leav et al. (1978) has shown that estrogens produce alterations in the two cell types. In the inner cylindric cells, features of both squamous and glandular differentiation were present with perinuclear tonofilaments and secretory vacuoles. In the basal cells of the glands, the cells were purely squamous, lacking any glandular differentiation.

Estrogen-induced squamous metaplasia is recently contradicted by the report of Voogt et al. (1987) who reported that, in prostates of young adults without cancer, treatment with a combination of estrogen and anti-androgen (for 8 weeks) resulted into stromal cell proliferation and atrofic glandular acini, lacking squamous metaplasia. However, the time relapse after initiation of therapy to the time point that these morphologic changes occured was presumably to short. Dhom (1985) postulated that the therapy-dependent morphologic phenomena become evident at least 6 months after therapy has started.

Apart from induction by estrogens, squamous metaplasia of prostatic ducts may also develop in the region of infarcts, after irradiation therapy, and after TUR (Utz et al., 1969). In metaplasia due to infarction, the acinar and ductal epithelium may also be replaced by transitional epithelium (Mostofi et al., 1973).

Effects of radiation therapy

The response of tumor tissue to radiation therapy, alone or in combination with endocrine therapy, has only recently become known. The regressive changes in tumor cells during hormonal therapy and radiation treatment (internal and external) are basically identical and are listed above. Hormonal- and radiotherapy induced cyto- and histological changes are similar and do not depend upon the histological differentiation of the carcinoma. However, radiation therapy

alone will not result in squamous metaplasia. Long-term radiotherapy will finally lead to a covert arrangement of tumor cells localized within a stroma that shows increased fibrosis. In cribriform or solid carcinomas radiation therapy seems to be superior to hormonal treatment, whereas endocrine or combined endocrine- and radiation therapy are more effective in the case of histologically better differentiated glandular carcinomas (Kastendieck, 1980; Dhom et al., 1982).

Benign prostatic glands in the vicinity of tumor areas may respond to radiation therapy by the development of moderate to severe cytologic atypia. Therefore a problem can rise by the differential diagnosis of radiation induced atypia and persistent adenocarcinoma when post-radiation biopsies are examined (Brawer et al., 1989).

The effects of therapy upon the expression of prostate-specific antigen and prostatic acid phosphatase will described elsewhere (see I-9.2).

8. Monitoring the clinical course of prostatic cancer

To monitor the natural course of PC or the potential effect of an intervening therapeutic regimen different approaches have been suggested. Histopathologic or cytologic follow-up (Dhom et al., 1982; Freiha et al., 1984; Bishop et al., 1985), repeated rectal examination, bone roentgenography or bone scintiscan have been more and more replaced by regular determinations of tumor related markers in the urine, in the prostatic fluid and above all in the blood. Nowadays, even periodic transrectal ultrasonometry has been proposed to monitor changes in size of the prostatic gland and intraprostatic alterations in acoustic impedance under endocrine management or radiation therapy (Carpentier et al., 1986; Fujino et al., 1986). However, the scarcity of truely objective criteria to measure the effect of treatment is emphasized by Schroeder et al (1984b).

Of those markers related to PC and that are shed in the blood circulation prostatic acid phosphatase (PSAP or PAP) and prostate-specific antigen (PSA or PA) are most commonly used for the purpose of monitoring patients with PC. They belong to the group of prostatic tissue-specific antigens (PTSA) with secretory activity. More detailed biochemical and immunological features of these antigens are presented in section I-9. In addition to PAP and PA, several less well-known secretory markers, detectable in the blood, prostatic fluid or urine and of potential use in monitoring PC patients, will be discussed briefly.

8.1. Markers present in the bloodcirculation

Prostate-specific acid phosphatase (PAP or PSAP)

In patients with advanced stage PC serum PAP levels can be raised (Maatman et al., 1984; Heller, 1987) and may fall after initiation of a successful therapeutic regimen. From

those patients with PC which have metastasized to the skeletal system (stage D PC), 75-90 per cent has elevated serum acid phosphatase levels. These high serum PAP levels may fall in response to treatment. Persistently elevated values after treatment indicate, in general, a poor prognosis. It is postulated that the large tumor load such as present in stage D cancer is responsible for the elevated PAP serum level, though the protein synthesis of individual tumor cells is lowered compared with non-neoplastic prostatic cells. Therefore, immunohistochemical data do not systematically correspond with serum levels of tumor markers. Moreover it has been postulated that as a result of tumor-associated abnormalities of the cytoskeleton (see I-9.3), the cellular translocation of the enzyme may be disrupted, altering the secretion kinetics. In patients with limited localized PC only a subpopulation will have raised serum PAP levels even when highly sensitive (immunological) methods are used (Bruce et al., 1981). After release into the circulation PAP is cleared rapidly with a half life of 0.5 to 2.5 hours (Wadstroem et al., 1985). Determination of the PAP serum level can be assessed by an enzymatic assay or by an immunological assay. Enzymatic assays for the assessment of serum PAP are hindered by fluctuations of the serum pH. Especially high pH (above 8) values and high temperature block the enzymatic activity of PAP. Therefore, depending on the specificity of the antibodies and the technique employed (EIA or RIA), in general, methods using immunological tools are preferable (Vihko et al., 1980, 1982 and 1985; De Vries et al., 1982).

False positive elevations of serum PAP levels not associated with PC, are encountered under several conditions. These conditions can be subdivided into prostatic and non-prostatic disorders. A transient elevation of the serum-PAP level has been associated with prostatic massage, after surgery in the prostatic area and in the case of prostatic infarction. Moreover, a rise in serum PAP level has been reported in a relatively high percentage of patients with BPH (up to 20 per cent; Oosterom et al., 1986; De Vries, 1987). Non-prostatic diseases associated with a rise of PAP serum level of different durations encompass multiple myeloma, osteogenic sarcoma, thromboembolic disease, thrombocytopenia (Prout, 1973). Furthermore, gastric carcinoma, lung and breast cancers, rectal carcinoid tumors, metastastic pancreatic islet cell cancer and several myeloproliferative disorders may be associated with elevated concentrations of PAP in enzymatic- and radio immuno-assays (Heller, 1987). For these reasons an elevation of serum PAP is considered as a semi-specific test for diagnosing PC. Since a rise is often absent in localized PC, measurement of PAP serum level cannot be used for mass screening purpose to detect early PC. However, in general, in patients with histologically proven PC, a rising PAP should be recognized as an objective criterion for progression.

Measurements of the PAP content in bone marrow aspirates (for early detection of bone metastases) falls beyond the scope of this survey (Huber et al., 1982).

Prostate-specific Antigen (PSA or PA)

Detailed biochemical and immunological characteristics of PA are presented in I-9.2.2. Even more specific for monitoring the biological course of PC is the determination of serum fluctuations of PA (or PSA). PA is released in the circulation of patients with advanced (stages B2-D) PC (Papsidero et al., 1980; Kuriyama et al., 1981; Wang et al., 1981; Pontes et al., 1982; Killian et al., 1985). However, similar to PAP, elevated PA levels were also observed in patients with BPH. Ercole and coworkers (1987) reported that 21 per cent of patients with BPH had increased PA levels before TUR. Stamey et al. reported in 1987 that the serum PA can be elevated in up to 83 per cent of patients with BPH. In high-staged PC (stages C and D) PA serum-levels usually exceed those in BPH. The PA level increases with advanced clinical stage and is proportional to the estimated volume of the tumor. Specificity of PA is considered to be higher compared with PAP. On the other hand, comparative studies concerning the sensitivity of both markers in their use as serum tumor markers evoke controversial results. Some agree with the opinion that the percentages of patients with high-staged PC with elevation of PA serum level without rise of PAP, and similarly staged patients showing the opposite serum tumor marker profile are almost equal. Other investigators found a somewhat higher sensitivity of PA and a better correlation with tumor size (Killian et al., 1985; Siddall et al., 1986; Stamey et al., 1987; Ercole et al., 1987; Schifman et al., 1987; Emtage et al., 1987; Buamah et al., 1988). An EIA for serum level measurements of PA has been described by several authors (Kuriyama et al., 1980; Siddall et al., 1986). In a review article Stamey and coworkers (1987) stated that repeated PA serum determinations in monitoring patients after radical surgery (total prostatectomy) have to be considered as extremely specific and regarded as superior compared with PAP values.

Summarizing, it is generally accepted that for as yet optimal monitoring of patients with PC has to be done by immunological measurements (EIA or RIA) of both prostatic markers at the same time and with regular time-intervals. However, PA might be prognostically somewhat more useful for patient follow-up (Killian et al., 1985). Both markers have insufficient diagnostic sensitivity (and specificity in the case of PAP) to be used in screening for cancer at an early stage.

Other markers shed in the blood circulation

Phosphohexoseisomerase (PHI) catalyzes the conversion of glucose-6-phosphate into fructose-6-phosphate and seems to be useful as a serum marker in monitoring the evolution of malignant breast- and prostate disease (Schwemmer et al., 1985). In prostatic malignant disease its serum level may provide a biochemical estimate of the extent of tumor mass.

The sensitivity for PC of the serum level of carcinoembryonic antigen is only limited (Schwemmer et al.,

1985).

Tissue polypeptide antigen (TPA) is a membrane bound polypeptide which can be present in the circulation in patients with cancer of different origin. However, the sensitivity of TPA for PC is low (Huber et al., 1983).

8.2. Markers present in the urine and prostatic fluid

Apart from fluctuations in the serum level of PAP and PA, the concentration of these markers in the urine and prostatic fluid have been described as potential parameters indicative for PC and presumably related to tumor extension (Tremblay et al., 1987). Tsai et al. (1984) found that the protein content of PAP in human prostatic fluid was extremely lowered in patients with PC.

The determination of the ratio LDH5/LDH1, complement components C3 and C4, transferrin and PAP in prostatic (seminal) fluid assists in identifying individuals with increased risk for PC (Grayhack et al., 1981). A comparative study of data obtained by two dimensional gel electrophoresis of prostatic fluid of individuals with PC and healthy volunteers revealed differences in the protein profiles between the two groups (Tsai et al., 1984).

As a secondary effect of PC growth, urine levels of fibronectin can be episodically elevated in patients with PC (Webb et al., 1980). Because of its transient character, sensitivity for the detection of PC can be increased when at least three successive analyses are performed.

9. Immunological aspects of prostatic cancer

In this short review only attention will be given to the immunophenotypic alterations of neoplastic cells of prostatic origin compared to their benign counterpart. Special emphasis is laid on the immunocytochemical identification of PC linked antigens. The immunological response of the human host (humoral or cellular mediated) induced by the presence of tumor and the mechanisms to modulate this response will not be discussed here (see for a review on this subject Guinan et al., 1984 and Wirth et al., 1985).

In general, neoplastic cells can display the following changes with reference to their immunophenotypic make-up:

- an increase of expression or modulation of normally present antigens (blood group antigens and tumor-associated antigens (TAA)).
- occurrence of new antigens associated with malignant transformation which are not expressed on normal cells. These antigens are known as tumor-specific antigens (TSA). Examples of this group are the activated ras oncogenes which are mutated in the amino acid sequence. In virus-associated malignancies viral antigens are also reckoned to this group.
- a decrease of normally present antigens (markers associated with cell differentiation).

Antibodies raised against the various antigenic determinants can be applied for detailed characterization of the tumor. Particularly in the case of PC one has to distinguish antigens that are exclusively present within the epithelial cells and antigens that are secreted in the seminal fluid and are sometimes also demonstrated in the urine (secretory antigens; SA). Since at least some of these latter antigens can be shed in the blood circulation in the case of invasive PC, they have been used for monitoring purpose. The antigens used for monitoring patients with PC and their attribution in tumor-staging are discussed in I-8. Among those secretory antigens that have been the subject of immunohistochemical studies and serological assays, are prostatic acid phosphatase (or prostate-specific acid phosphatase (PAP or PSAP); see I-9.2.1) and the more recently recognized prostate-specific antigen (PA or PSA; see I-9.2.2).

Many attempts have been made to isolate other prostate (tumor) markers. Cell lysates, cell membrane-enriched fractions of normal, BPH or cancerous prostatic tissue and prostatic tumor cell lines have been used to generate polyclonal and monoclonal antibodies (Bazinet et al., 1988). Efforts have also been made to generate syngeneic (human) monoclonal antibodies against human prostatic tumors (Lowe et al., 1984). In part of the studies the antigens detected by the antibodies were defined and characterized with reference to their tissue- (prostate) specificity and/or specificity for carcinoma. The intensive search for tumor-associated markers with high tissue-specificity is justified especially for their potential application as immunotarget agents carrying cytotoxic drugs or radioactive isotopes for therapy or highly specific immuno-imaging techniques (Goldenberg et al., 1983; Vihko et al., 1984; Deguchi et al., 1986; Vihko et al., 1987; Larson et al., 1988).

In addition to characterization of PC, immunohistochemical studies may reveal information concerning the functional state of the prostatic gland and may correlate particular marker profiles with diverse stages of development of the prostatic gland (Lehtinen, 1980; Wernert et al., 1987).

9.1. Tumor-associated - and Tumor-specific Antigens

Tumor-associated Antigens (TAA) are considered as antigens that are expressed on normal cells but are found at a higher level or in a modified form in malignant tissue (Carney, 1988). Tumor-specific Antigens (TSA) are, by definition, never found on any normal cell and their presence can be the result of either expression of mutated genes (the p21 product of the activated ras oncogenes) or the result of expression of viral antigens related with a presumed viral etiology of certain malignancies.

Tumor-specific and tumor-associated markers can be subdivided into markers related to malignant transformation in general (lacking tissue-specificity) and markers with even higher specificity i.e. associated exclusively with malignant transformation of a specific tissue type (in this survey

prostatic epithelium).

Many attemps have been made to characterize tumor-specific or tumor-associated antigens in order to generate antibodies against these molecules. Major characteristics of Tumor-associated - and Tumor-specific Antigens for PC are summarized in Table 4. Thus far sufficiently characterized prostatic tissue TSAs and TAAs have not been reported. TSAs identified for PC are cross-reactive with non prostatic malignant cells (and cell lines): the antibodies PrS5, PrE3, and PrD8 show cross-reactivity with breast cancer cell lines (Lindgren et al., 1986). The antibody D83.21 reacts also with bladder carcinoma cell lines (Starling et al., 1985). Some of the presumed TSAs have only been tested on several PC cell lines. Of special interest may be the recently described McAb's P25.48 and P25.91, which were not reactive with benign prostatic tissue, but reacted with a subset (especially the high grade lesions) of malignant prostatic tissues (Bazinet et al., 1988). The antibodies showed a very restricted pattern of reactivity in nonprostatic tissues. Some of the markers presented in Table 4 are secreted in the urine, the prostatic fluid or (in the case of in vitro studies) in the culture medium of PC cell lines.

We generated murine monoclonal antibodies against the human androgen-dependent PC cell line PC-82 (see I-10.2.1 and Chapter IV: Gallee et al., 1986). No antibodies were detected with exclusive binding for prostatic tumor cells. Many isolated antibodies displayed interesting cross-reactivity profiles with other malignant and non-malignant non-prostatic tissues. None of the antibodies produced against the PC-82 cell line was directed against a TAA or TSA. Antibodies against antigens exclusively present in prostatic tissue appeared to be reactive against PA and PAP (see I-9.2.1 and I-9.2.2).

In future, antibodies against TAA and TSA will enable (more or less) specific immuno-imaging and immunotherapy of malignant tumors. For immunotherapy and immuno-imaging cell membrane-related, tumor-specific antigens with relatively high tissue specificity, lacking antigenic modulation after antibody binding are preferable. A secretory characteristic of the antigen recognized by these antibodies might be a serious drawback. On the other hand secretory TSA and TAA might be of use in monitoring the course of PC.

Summarizing the literature with reference to prostatic tumor-specific markers, it becomes clear that sofar no antibody defined proteins are detected that are solely expressed in prostatic neoplastic epithelium. Of fundamental interest may be the McAb's recognizing PC cells which show cross reactivity with other malignant neoplasms or cell lines of different origin (see Table 4). Further research is needed to investigate whether these markers are just associated with less differentiated cells or whether they are linked to processes as malignant or premalignant transformation.

9.2. Prostatic-Tissue specific Antigens (PTSA)

The group of prostatic tissue-specific antigens encloses these markers which are exclusively expressed in prostatic tissue. An important use of these markers is the identification of the prostatic origin of metastatic lesions of PC by immunohistochemical techniques. Furthermore these markers are of fundamental interest to gain insight in the tissue-specific processes in the prostatic gland. In contrast to TSAs and TAAs, the biochemical properties of the tissue-specific markers are much more explored. The major representative markers within this group are secreted by the prostatic columnar epithelial cells and are under physiological conditions present in the prostatic fluid. Moreover under several pathological conditions they can be demonstrated in the blood circulation and are useful tools in the monitoring of patients with PC (see I-8). Among the PTSAs prostate-specific acid phosphatase (PAP or PSAP) and prostate-specific antigen (PA or PSA) have been most intensively investigated. Apart from these PTSAs with secretory capacity several less characterized tissue-specific markers for the prostatic gland have been developed which are largely not secreted and therefore are only demonstrated within the prostatic cells. A number of these have been isolated from PC cell lines (see also I-10).

In this brief review special attention is given to the immunohistochemical detection of these markers and the potential prognostic value related to tumor growth characteristics when fluctuations (i.e. lowering of the tissue concentration) occur during the process of malignant transformation.

9.2.1. Prostatic Acid Phosphatase (PAP or PSAP)

Enzymatic and biochemical features

Acid phosphatases (orthophosphoric monoester phosphohydrolase) designate, by definition, a group of enzymes that hydrolyze phosphate esters under acidic conditions to yield inorganic phosphate (Heller, 1987). Initially it was thought that multiple molecular forms with differing electrophoretic mobilities were the result of variations in sialic acid residues and carbohydrate content (Lad et al., 1984). However, presumably, the heterogeneity of acid phosphatases is even more complex than can be explained by differences in posttranslational modification (Lin et al., 1983). At least 7 isoenzymes (I, IIA, IIB, III, IV, VA, and VB) are distinguished.

The acid phosphatase secreted by the prostatic gland is considered as prostate-specific (PAP or PSAP; predominantly consisting of isoenzyme IIA, although isoenzyme IV might also be found) (Lam et al., 1979; Vihko, 1979; McTigue et al., 1982; Lad et al., 1984; Lin et al., 1983; Dang et al., 1986). Partial antigenic similarities of PAP exist with other acid phosphatases that originate from the pancreas, spleen, kidney,

Table 4. Major characteristics of antibodies against presumed TAA and TSA in prostate cancer

Immunization	McAb (immunoglobulin class)
Antibodies raised against single (DU-145) and mixtures of several prostatic cancer cell lines (DU-145; PC-3; 1013L).	Eight McAb's: PrK16 (IgG2α), PrN10 (IgM), PrL22 (IgG1), PrO11 (IgG1), PrHk (IgG2α), PrQ12 (IgG2α), PrM24 (IgG1), and PrP14 (IgG2α).
Extracts and viable cells of the PC-3 cell line were used.	PrS5 (IgG1); PrE3 (IgM); PrD8 (IgM).
DU-145 prostatic carcinoma.	D83.21 (IgM).
An equal mixture of PC-3, DU-145, and LNCaP.	α-Pro 13.
Fresh prostatic cancer cells.	P25.48 (IgG3); P25.91 (IgG2α)
PC-3 cell line (α-Pro 3, α-Pro 5).	α-Pro 3 α-Pro 5 α-Pro 1 α-Pro 2
Human lymphocytes from a regional draining lymph node from a patient with PC were fused with a murine myeloma cell line.	MHG-7 (IgM) (human McAb).

Characteristics	References
PrHk defined a 115 kD antigen; The ag's recognized by PrK16 and PrN10 are unknown. McAb PrN10 is reactive on fixed tissue; the antigen detected by this McAb is probably shed in the growth medium of PC-3 cultures. PrQ12 defined an antigen of 100kD; PrL22 and PrO11 were both reactive with a 160 kD antigen. The antigens defined by PrM24 and PrP14 are not fully characterized. Some of the antigens are shared with cell lines of other human cancer types.	Lindgren et al. (1985)
PrE3 and PrD8 are directed against the same ag (glycoprotein with MW 115 kD); cross reactivity with red blood cells. PrS5 directed against 90 kD ag. Cross reactivity with breast carcinoma cell lines.	Lindgren et al. (1985)
Reactivity limited to prostatic carcinoma and bladder carcinoma cell lines. The ag is a membrane glycoprotein consisting of 2 chains of of 180 and 110 kD. Cross reactivity was reported for CMV transformed fibroblasts.	Starling et al. (1982; 1985), Wright et al. (1983), Campbell et al. (1985)
Preferential binding to the ductal epithelium of prostatic tissue. MW under reducing conditions 40 kD. Lacking secretory activity. Most prevalent cross reactivity with blood vessel endothelium.	Webb et al. (1984)
No cross-reactivity with benign prostatic tissue. In high grade tumor lesions increased heterogenous immunoreactivity. Restricted pattern of reactivity in non prostatic tissue (no tissue-specificity). Antigen not preserved in formalin-fixed, paraffin-embedded tissues. Both McAb's directed against the same antigen. Antigen not detectable in PC-3; DU-145 and LNCaP.	Bazinet et al. (1988) Webb et al. (1983)
α-Pro 3 and α-Pro 5 recognize the same ag (MW 54 kD) but react with different epitopes. Cross reactivity with BPH, normal prostate and PC tissue. At least McAb α-Pro 3 and and α-Pro 5 are considered as TAA. α-Pro 3 is reactive with PC-3 cells but not with DU-145 cells and reacts more strongly with PC than with BPH.	Ware et al. (1982)
The ag is present on LNCaP and PC-3 cells; but is lacking in DU-145 cells. Cross reactivity with colon and lung carcinoma cell lines (TSA).	Lowe et al. (1984)

placenta, and neutrophil and eosinophil granulocytes (Vihko et al., 1988; Shaw et al., 1981; Yam et al., 1981a; Li et al., 1980; Choe et al., 1982; Bentz et al., 1982; Warhol et al., 1985). The existence of a natural substrate with an unique specificity for the PAP isoenzyme is still a matter of dispute (Serrano et al., 1976; Lee et al., 1982). Hence, the precise physiological role of PAP remains unclear.

PAP is a secretory glycoprotein with a molecular weight of 110 kD. It is composed of 2 (identical) subunits each of 48-54 kD. Recently cDNA coding for human PAP was isolated (Yeh et al., 1987; Vihko et al., 1988). The genetic information codes for a polypeptide of 354 amino acids.

There are still conflicting data about the hormonal regulation of the biosynthesis of PAP in the prostatic gland (Pontes et al., 1981; Bolton et al., 1981; Aumueller et al., 1983a; Dube et al., 1984; Warhol et al., 1985; Vihko et al., 1988). Alterations in tissue PAP concentrations under hormonal therapy have also been ascribed to a secondary response to a more general hormonal (growth and metabolism stimulating) effect on the cell. Steenbrugge van et al. (1983) reported that the PAP tissue concentration in the prostatic cancer cell line PC-82 (see I-10.2.1) was not controlled by androgens. However, changes in immunohistochemical staining activity for PAP have been described in patients following diethylstilbestrol therapy (Vernon et al., 1983ab; Grignon et al., 1985).

Initially, detection of PAP was only possible by enzymatic assays. Inconsistent results were often encountered and attributed to the different substrates used (Serrano et al., 1976; Vries de, 1987). Later on, more reliable enzymatic assays enabled to measure the presumed prostate-specific fraction based on the inhibitory effect of L(+) tartrate on the enzymatic activity of PAP (Jacobsson, 1960).

Recent knowledge of the immunogenic properties of PAP has resulted in the production of numerous antibodies (Choe et al., 1982). For preparation of antibodies PAP can be isolated from seminal plasma (Vries de et al., 1979) or extracted from prostatic cell lysates (BPH and/or PC) (Taga et al., 1983). The secretory acid phosphatase (PAP) is predominantly present in seminal plasma, whereas the cellular extract (prostatic homogenate) also contains lysosomal and microsomal acid phosphatases (Aumueller et al., 1983ab; Vries de, et al., 1986). Therefore, the source of the antigen and degree of purification determine the specificity of the prepared (polyclonal) antibodies. The antibodies have become an essential tool for detection and quantification of PAP in serum, in prostatic fluid, in tissue sections (immunocytochemistry) and in cell extracts. Compared with enzymatic assays the sensitivity of the immunological assays is considerably higher (Vihko et al., 1985). Both polyclonal and monoclonal antibodies recognizing PAP have become available (Joebsis et al., 1978; Joebsis et al., 1981; Nadji et al., 1982; Foti et al., 1975, Vihko et al., 1978; Vihko et al., 1980; Lee et al., 1978; Lee et al., 1980; Choe et al., 1978; Yam et al., 1981b; Sinha et al., 1988; Shevchuk et al., 1983; Dang et al., 1986; Shaw et al., 1981; Lam et al., 1979; Gallee et al., 1986; Teillac et

al., 1987; Kuciel et al., 1988). Prostatic tissue as well as prostatic fluid have been used for preparation of antibodies. In our attempt to generate tumor-specific antibodies against the prostatic cancer cell line PC-82, we isolated several antibodies directed against PAP. After further purification of PAP using the McAb 8F9 we prepared a monospecific polyclonal antiserum that could be applied on formalin-fixed tissue (Chapter V).

The first observation of an elevated serum acid phosphatase level in metastasized PC was described by Gutman et al. in 1936. For this reason PAP can be considered as the first tumor marker ever found. Details regarding the value of PAP for the purpose of monitoring patients with PC are outlined in I-8. The use of antibodies recognizing PAP in tissue sections (immunocytochemistry) will be discussed here in more detail.

Detection of PAP in tissue sections

Before antibodies against PAP were available, the enzymatic activity of PAP has been used for the detection of PAP in tissue-sections. However, since most of the enzymatic activity of PAP disappears during tissue processing (ethanol inhibits irreversibly the enzymatic activity; formaline seems not to interfere with enzymatic activity) assays were only successful on fresh-frozen or formalin-fixed cryostat sections (Gomori, 1950).

When antibodies became available immunohistochemical surveys could be performed for detection of PAP. Several antibodies were applicable on formalin-fixed and paraffin-embedded tissue sections and were even able to detect the antigen after previous decalcifying procedures (Joebsis et al., 1978; Henwood et al., 1982). Immunohistochemically, PAP has been demonstrated in normal prostatic glands, in BPH, in primary prostatic tumors and in metastases of PC (Lippert et al., 1982; Nadji et al., 1982 and 1983). Equivocal data are available regarding the presence of PAP in the epithelial lining of the seminal vesicle. This observation, and sporadically described cross-reactivity with other tissues, may be largely dependent on the (lack of) specificity of the antibodies used. In the non-neoplastic tissues PAP seems only present in the inner columnar (secretory) cells of normal and hyperplastic (BPH) prostatic glands (in the cytoplasm and on the cell surface) and is often detected within the glandular lumina. In fact, the majority of PAP is being located extracellularly (Bolton et al., 1981). PAP has not been demonstrated in the cytoplasm of the basal cells. Immuno electron microscopy studies revealed the presence of PAP in lysosomes and secretory vesicles (Warhol et al., 1985; Zondervan et al., 1986).

In cancerous tissue PAP can be identified within the cytoplasm of carcinoma cells. Demonstration of PTSAs such as PAP has become a valuable aid in the differentiation of primary transitional cell carcinoma from prostatic cancers and can be of great help in the elucidation of the initially unknown primary tumor in patients with metastasized malignant

disease. Most investigations indicate that the immunohistochemical staining intensity in carcinoma cells is weaker than in non-tumorous epithelium. This observation agrees with biochemical studies measuring the concentration of PAP (immunologically and enzymatically) in cancerous and non-cancerous prostatic tissue (Loor et al., 1981; Copland et al., 1983; Pretlow et al., 1985). However, the precise relationship between the amount of PAP detectable in PC and tumor differentiation status remains controversial (Joebsis et al., 1981; Ito et al., 1986). Pretlow et al. (1985) reported that the enzymatic activity of PAP extracted from primary cancerous tissue was reduced compared with BPH but that the decrease in enzymatic activity was not significantly related to Gleason grade. A similar absence of a relationship between Gleason tumor grade and PAP assessed by an immunohistochemical assay was recently described by Hammond et al. (1989). In contrast to these findings, Sinha et al. (1988) reported a progressive decline in the PAP staining in moderately and poorly differentiated tumors. Earlier, Bates et al.(1982) and Shevchuk et al.(1983) reached a similar conclusion. Further immunohistochemical surveys to gain more insight in the interrelationship between PAP expression and tumor differentiation have been carried out by Pontes et al. (1981), Bentz et al. (1982), Yam et al. (1983), and Gallee et al. (1989c). To circumvent problems associated with histological grading (see Chapter I-5.3 and Chapters II and III) we investigated the correlation between staining intensities of PAP and PA (see I-9.2.1 and I-9.2.2) and architectural growth patterns of PC within prostatectomy specimens. Using well characterized polyclonal antibodies against PAP we found no consistent relationship with growth pattern of PC. Though the immunohistochemically assessed concentration of PAP in PC was lowered compared to BPH areas, PAP was demonstrated more readily in neoplastic tissue than PA.

Apart from simple alterations in staining intensities in PC compared with non-malignant prostatic tissue, an increase in the variability of the staining intensity was detected in less differentiated tumors (Bates et al., 1982; Nadji et al., 1982). Irrespective the growth characteristics of PC, Hammond et al. (1989) found a significant correlation between survival and the immunohistochemical demonstration (subjective grading of intensity) of PAP.

Data concerning the sensitivity of antibodies against PAP for immunohistochemical detection of PC cells are conflicting (Lippert et al., 1982). In some cases well defined polyclonal antibodies are to prefere above monoclonals (Shevchuk et al., 1983). On the other hand, Choe et al. (1982) developed McAb's that were exclusively reactive with antigenic determinants of PAP that were not shared with determinants on lysosomal acid phosphatases. Tissue processing techniques may interfere with the detectability of PAP since antigenic sites might be destroyed by tissue handling.

Contrary to the afore mentioned subjects, consensus exists about the finding, that there is no consistent relationship between serum PAP levels and tissue concentration of PAP (Shevchuk et al., 1983).

PAP expression is retained in a number of different prostatic in vivo and in vitro cancer cell lines. These cell lines are therefore convincingly established as originating from prostatic tissue. Information refering the cell lines which have maintained PAP expression is presented in I-10 and Table 7.

9.2.2.Prostate-specific Antigen

Enzymatic and biochemical features.

In 1979 Wang and coworkers discovered a new highly specific prostatic antigen (PSA or PA). This protein was isolated from prostatic epithelium and is clearly distinct from PAP. It is a single chain prostate tissue-specific glycoprotein (Watt et al., 1986) with a molecular weight of 34 kD. The carbohydrate configuration and amino acid composition have been determined (Watt et al., 1986). Similar to PAP, PA is secreted into the prostatic fluid in high concentration (Ban et al., 1984) and can also be demonstrated in the urine (Tremblay et al., 1987). In addition, in advanced stages of PC, PA can be found in the serum of patients and it has been used intensively for monitoring tumor behavior and effects of therapeutic intervention (Papsidero et al., 1980; Stamey et al., 1987). For detailed information concerning this particular application of PA see I-8. Compared with PAP, specificity of PA for prostatic epithelium is even higher; it could not be isolated from any other organ nor was its presence reported in female serum (Chu et al., 1986). Under physiological conditions only small amounts of PA can be found in the male serum.

PA is a serine protease belonging to the family of kallikrein and kallikrein-like proteins (Akiyama et al., 1987). The homology with the human glandular kallikrein hGK-1 is 80% (Schedlich et al., 1987) and that with human pancreatic renal kallikrein 62% (Evans et al., 1988). The PA substrate is probably a high molecular weight seminal vesicle protein known as seminogelin, which is secreted by the seminal vesicles and is responsible for the gel-like consistency of the semen. Recently, the molecular cloning of human PA cDNAs has been described (Lundwall et al., 1987; Riegman et al., 1988; Schulz et al., 1988). The protein encoded by the cDNAs consists of 237 amino acids. It was proven that the PA gene and the human glandular kallikreine-1 (hGK-1) gene are closely linked and are both located on chromosome 19 (Riegman et al., 1989a). The human renal pancreatic kallikrein gene has also been mapped on chromosome 19 (Evans et al., 1988). Expression of the hGK-1 gene, like that of PA, seems also to be restricted to the prostate (Chapdelaine et al., 1988).

Various different antibodies against PA have been described (Frankel et al., 1982; Papsidero et al., 1983; Ford et al., 1985; Gallee et al., 1986; Chu et al., 1989; Gallee et al., 1989c). Major characteristics of the antibodies are summarized in Table 5. Using purified PA as immunogen we isolated McAb's directed against at least three different

Table 5. Major characteristics of antibodies reported in literature recognizing prostate-specific antigen (PA or PSA).

Immunization	Characteristics
benign hypertrophic prostatic tissue	1F3; 2G7; 1C5 (all of the IgG1 subclass) the three McAb's define two unique determinants on PA
prostatic tissue or seminal plasma	McAb F5 (IgG1; κ)*
prostatic tissue	IgG preparation of polyclonal rabbit antiserum; ag was demonstrated in LNCaP and PC-3 cell lines
purified PA	McAb's: 1A5, 2A4, 3F1, F5*, 3A12 (all IgG1 sub-class) directed against two distinct spatially related antigenic domains
human prostatic cancer cell line PC-82	McAb's ER-Pr 1 and ER-Pr 2 (both IgG1 subclass). Probably directed against the same antigenic domain
purified PA from BPH	McAb's: ER-Pr 8, ER-Pr 12, ER-Pr 27 (all IgG1 subclass). Directed against two antigenic domains distinct from ER-Pr 1, 2.; and rabbit polyclonal antiserum

* This is the same McAb; the F5 antibody was initially described by Papsidero in 1983.

epitopes of PA. All of these could be visualized on formalin-fixed prostatic tissue. Of potential interest was the observation of Chu et al. (1984) who found in patients with advanced stages of PC an elevated level of a serum PA-binding immunoglobulin. The precise role of this antibody as an auto-antibody or as an anti-tumor antibody as a result of patients' immune response is not understood.

Precise insight in the biosynthesis and mechanism of PA secretion are still lacking. Moreover, the exact role of androgens (T and DHT) in these processes remains to be elucidated. However, hormonal manipulation (DES therapy in patients with PC) seems to interfere with the immunohistochemical detectability of PA (Grignon et al., 1985). Similarly, in the LNCaP prostate cell line PA expression is androgen-regulated (Trapman et al., 1988b).

Detection of PA in tissue sections

The availability of antibodies enabled the immunohistochemical detection of PA in tissue sections. Many of the

Application of antibodies	References
radioimmunoassay immunocytochemistry (formalin fixation resistent)	Frankel et al. (1982)
immunocytohistochemistry	Papsidero et al. (1983)
immunocytochemistry; demonstration of PA in prostatic cell lines	Papsidero et al. (1981)
radio immunoassay and immunocytochemistry	Chu et al. (1989)
immunocytochemistry	Gallee et al. (1986)
immunocytochemistry and affinity chromatography	Gallee et al. (1989c)

antibodies generated can be applied on formalin-fixed and paraffin-embedded tissue samples (Sinha et al., 1986; Gallee et al., 1989c). At light-microscopic level PA is localized in the cytoplasm of the inner columnar cells and on the cell membrane, with slightly greater reactivity in the paranuclear area on the luminal sites of the cells. Immunoreactive PA is also found within the lumina of the normal and hyperplastic prostatic glands. There still remains doubt concerning the presence of PA in the cytoplasm of the basal cells. Immunoelectron microscopic studies provided data that PA is synthesized at the rough endoplasmic reticulum, stored in secretory vesicles and vacuoles and released into the glandular lumina by exocytosis (Warhol et al., 1985; Zondervan et al., 1986).

The majority of immunohistochemical studies provides evidence that the PA concentration is lowered in PC (Purnell et al., 1984). However, the precise relationship between PA expression and histological grade or tumor differentiation is still a controversial topic (Papsidero et al., 1983; Sinha et al., 1986). Controversy is partly caused by the absence of a

generally accepted histological grading system that is commonly used by pathologists and to which immunohistochemical data can be compared. Irrespective of the histological grade of the tumor Hammond et al.(1989) did not find a correlation between subjectively graded immunocytoreactive intensity of PA and survival. Contrary to this observation, it had been shown by several investigators that at least part of high grade prostatic cancers (poorly differentiated) do not stain for PA (Stein et al., 1982; Ellis et al., 1984; Feiner et al., 1986; Svanholm, 1986; Keillor et al., 1987; this thesis). These observations agree also with the finding of Epstein et al. (1984a) who reported that in stage A2 prostatic lesions an increase in immunostaining variability and a lowered immunodetectability for PA were significantly related with a subsequent progressive course of this initial incidental lesion.

In this thesis it was attempted to correlate the immunohistochemical expression of PA recognized by different antibodies with the histological growth pattern of PC. Results presented in Chapter V indicate a correlation of immunohistochemical detection of PA and architectural growth pattern of PC.

Within several established PC cell lines PA expression is retained (Trapman et al., 1988b; Riegman et al., 1988; this thesis). However, the concentration of PA detected in these cell lines may be lower than those found in prostatic specimens directly obtained from patients.

As was stated previously for PAP, no consensus has been reached concerning the existence of a relationship between serum PA levels and tissue PA concentration in patients with PC.

9.2.3. Comparison of immunohistochemical features of PAP and PA

Several studies that compare the immunohistochemical characteristics of PA and PAP have been published (Epstein et al., 1984a; Allhoff et al., 1983; Bentz et al., 1984; Shah et al., 1985; Svanholm, 1986; Keillor et al., 1987; this thesis). A simple comparison of those studies is hindered by the fact that different antibodies have been used. It is generally accepted that the specificity of PA is higher than that of PAP. However, concerning the question which of both markers displays the highest sensitivity, conflicting data have been presented (Shah et al., 1985; Keillor et al., 1987). In general the sensitivity of well-characterized polyclonal antibodies is higher than that of monoclonal antibodies. However, when several McAb's defining different predominant antigenic domains are mixed the sensitivity reached by McAb's may become the same as for (monospecific) polyclonal antisera. Stein et al. (1984) report in a small series a sensitivity of PAP to stain primary PC of 81% whereas that of PA was counted for 75%. Similar figures reported by Bentz et al. (1984) were 92% and maximally 89% respectively for PAP and PA. On the other hand Shah et al. (1985) and Svanholm et al. (1986 and 1988) reported a slightly higher sensitivity for PA (94%)

compared with PAP (90%) when commercial immunoperoxidase kits were used. Similar results were described by Ellis et al. (1984). Our results presented in Chapter V indicate a slightly larger sensitivity for PAP to label PC cells whereas PA expression in tumors is more related with differentiation status. Some of the monoclonal antibodies seem to be as good as a polyclonal antiserum.

Finally one has to realize that though no exact relationship of serum concentration of the two markers with histological differentiation of the tumor is known as yet and no generally accepted consensus exists concerning the exact sensitivity of these markers, the absence of PAP and PA in at least poorly differentiated cancerous tissue does not unequivocally eliminate the possibility of PC. The use of both markers in immunocytochemistry increases considerably the likelihood of correctly identifying PC.

9.2.4. Therapy-induced alterations in immunohistochemical expression of PA and PAP

Treatment induced effects on the immunohistochemical staining of PAP and PA in PC tissue has been sporadically described in literature. Hormonal treatment (androgen deprivation or estrogen therapy) reduces the intensity of immunostaining of PAP (Joebsis et al.,1983) and PSA (diethylstilbestrol; treatment interval 2-63 months; Grignon et al., 1985). Other investigators have disagreed with this observation and stated that especially the immunohistochemical detection of PAP, but to lesser extent also that of PA, were maintained even after different therapeutic regimens despite therapy-induced morphological changes (Vernon et al., 1983ab; Mahan et al., 1980). PAP staining remained present in areas of squamous differentiation in PC (Accetta et al., 1982). After radiotherapy Mahan et al. (1980) observed the immunohistochemical retention of PAP in prostate biopsy samples.

9.2.5. β-microseminoprotein (β-MSP)

β-microseminoprotein (β-MSP) is also known as β-inhibin and as PSP94 (Prostatic Secretory Protein of 94 amino acids; Seidah et al., 1984; Dube et al., 1987ab). The precise function of β-MSP is not known as yet. Together with PA and PAP, β-seminoprotein belongs to the group of three predominant proteins secreted by the normal prostatic gland (Tremblay et al., 1987; Lilja et al., 1988). Moreover they all are considered as PTSAs. When PC has developed, β-MSP can be demonstrated in the serum of patients and therefore it has also been used for monitoring. In addition, the excretion of β-MSP may be increased in the urine of men with PC (Tremblay et al., 1987).

The first report concerning the immunohistochemical tissue distribution of β-MSP in hyperplastic and cancerous prostatic tissue was presented by Okabe et al. (1983). Abrahamsson et al. (1988) reported an increase in the variability of the

immunohistochemical staining intensity for β-MSP in the moderately and poorly differentiated PC's. Though an uniform relationship with differentiation status could not be proven by these investigators, a decreased immunoreactivity parallel to the degree of differentiation was reported by others (Dube et al., 1987a; Doctor et al., 1986). For as yet no information is available refering a possible androgen-regulated expression and secretion of β-MSP.

9.2.6. Other prostatic tissue-specific markers

In addition to the most intensively investigated prostatic tissue-specific markers discussed above, several authors claimed to have generated antibodies directed against new PTSAs distinct from PAP, PA and β-MSP. A brief summary of these markers is outlined in Table 6. Most of these markers have initially been detected in prostatic cancer cell lines (PC-3; DU-145; LNCaP) and showed afterwards cross-reactivity with non-malignant prostatic tissue. Clarke et al. (1982), Frankel et al. (1982ab) and Starling et al. (1986) used BPH specimens to produce antibodies against new PTSAs. Some of these appeared later on also present in prostatic cancer cell lines. Of potential value are the markers isolated recently by Horoszewicz et al. (1987) out of the cell line LNCaP (see I-10.2). These markers could also be demonstrated in the serum of patients with PC. However, further characterization of all these new PTSAs is necessary to ascertain their prostatic tissue specificity and to compare their sensitivity with that of PA, PAP and β-MSP.

9.3. Markers related to cell differentiation status

Antigens related to cell differentiation status do not necessarily have to possess tissue (prostate) specificity. Antigens that belong to this group can be absent or structurally altered in tumor cells, whereas their expression is consistent in prostatic epithelial cells of the adult non-neoplastic gland. During the early development of the prostatic gland (embryogenesis) these antigens can be absent or present. Prostatic markers that have been associated with cell differentiation are:

- Prostate-specific antigen and prostatic acid phosphatase: Since the tissue concentration of PAP and PA is lowered in PC compared to benign prostatic tissue, PAP and PA are linked with prostatic epithelial cell differentiation. It has been demonstrated that immunoreactivity of secretory prostatic acid phosphatase (PAP) was not present in fetal and infantile glands, but developed progressively in puberal specimens with increasing age (Aumueller et al., 1983a). More details refering PAP and PA are presented in I-9.2.
- Blood group-related antigens (BGA; BgRAg): Posttranslational glycosylation of especially membrane and secreted proteins frequently occurs. The carbohydrate make up determines at

least partially the antigenicity of glycoproteins. Among the heavily glycosylated cell membrane-bound antigens are the blood group-related markers (BGA). Within this group the isoantigens of the ABO(H) blood group system have been most thoroughly investigated. Under non-pathological conditions the tissue-bound blood group and the blood group antigen recognized on red blood cells are identical. However, when malignant transformation occurs the tissue-bound blood group type may differ from the normal patients' blood group. These structural alterations of the BGAs are ascribed to changes of the processes involved in glycosylation linked to impaired differentiation of cancer cells (Hakomori, 1985; Coon et al., 1986). BGAs differences associated with malignant transformation have initially been described in transitional cell cancers of the urogenital tract (Cooper et al., 1982; Flanigan et al., 1983). In non-invasive bladder cancers conversion of tissue bound blood group A or B into blood group O(H) is considered to be associated with prospective agressiveness of the lesion and precedes invasive growth (Finan et al., 1982; Summers et al., 1983; Coon et al., 1985; Cuadrado et al., 1986). Availability of antisera and McAb's recognizing specific A, B, and O(H) carbohydrate configurations has facilitated the research in tumor-associated glycosylation abnormalities and has replaced the use of the specific red cell adherence test and the application of lectins for this particular purpose (Voak et al., 1980; Sacks et al., 1981; Stein et al., 1981; Cooper et al., 1982; Ghazizadeh et al., 1983; Ernst et al., 1984; Gooi et al., 1985). Several types of cancer have been studied for the immunocytochemical expression of BGAs (Vowden et al., 1986ab; Compton et al., 1987). Recently, interest was also focused on PC (Walker et al., 1984; Vowden et al., 1986c; Chastonay et al., 1986; Abel et al., 1987; Abel et al., 1989). Contrary to the patients' blood group, expression of BGA A and B was rarely found in cancerous tissue. Components of BGAs in prostatic tissue (epithelial cells) might be lipid-based or present as glycoproteins (Abel et al., 1989). Therefore, immunohistochemical studies related to this subject in prostatic tissue are recommended to be performed on fresh-frozen specimens to achieve reliable results (Abel et al., 1987). The precise meaning of this altered BGA expression in PC and especially its relationship with biological course warrants further research.

- Cytoskeleton proteins: In general, benign and malignant epithelial cells are characterized by the expression of cytoskeleton proteins that belong to the cytokeratin family (Cooper et al., 1985). The cytokeratin family is reckoned to one of the five classes of intermediate filaments. Apart from cytokeratins, vimentin, glial fibrillary acidic protein (GFAP), desmin and neurofilaments are also regarded as intermediate filament proteins or cytoskeleton proteins. Based on molecular weight and biochemical analyses at least 19 cytokeratins have been documented (Feitz et al., 1986). McAb's against nearly all these cytoskeleton proteins are available (Muijen et al., 1984). Combined expression of

Table 6. Major characteristics of Prostatic Tissue-Specific Antigens (PTSA) other than PAP and PA.

Immunization	McAb's (immunoglobulin class)
LNCaP cells were used as immunogen.	7E11-C5 (IgG1); 9H10-A4 (IgG1)
The PC-3 cell line was used as immunogen. The ag is secreted in the seminal pasma and urine from healthy individuals. It can also be demonstrated in the growth medium of PC-3 cultures. KR-P8 is reactive on formalin-fixed tissue.	KR-P8
Membrane preparations derived from TUR specimens of BPH and PC were used as immunogen. Reactive with both BPH and PC.	TURP-27 (IgG3)
Reactive with PC-3 cells; McAb 24 reacts more strongly to normal prostate than to PC. Membrane enriched fractions of human BPH were used as immunogen.	McAb 24 (IgM); McAb 35 (IgG2α)
The PC-3 cell line was used as an immunogen.	F77-129 McAb
Human BPH tissue extracts were used as immunogen.	several McAb's

clusters of these keratin filaments are specific for particular differentiation routes within epithelial cells (squamous-, transitional cell- (stratified epithelia), and adeno- (or glandular-)differentiation; Ramaekers et al., 1983). Hence, in the postpubertal prostatic acinus keratins from human stratum corneum, including cytokeratin 5, are exclusively demonstrable in the basal cells, whereas keratins 8 and 18 are only present in the inner secretory epithelium (Feitz et al., 1986; Wernert et al., 1987; Nagle et al., 1987). Keratins 7 (only focally present) and 19 are shared by both cell types (Wernert et al., 1987). The keratins 7, 8, 18 and 19 are regarded as typical keratins for the so-called simple epithelia. Differences in keratin profiles between basal and cylindrical cells of the prostatic acinus were also demonstrated by Molinolo et al.(1985), and Brawer et al.(1985). Of interest is the partial co-expression of keratins and vimentins in the secretory epithelium which was reported by Wernert et al. (1987) and was also found within the most malignant and highly metastatic subline of the Dunning rat prostatic tumor model system (Feitz et al., 1986; see also I-12). Hormonally induced metaplasia and malignant transformation

Characteristics	References
Antigen is detectable in the serum of patients with PC. No cross reactivity with PC-3 or DU-145 cells. The 9H10.A4 defined ag is exclusively present on LNCaP cells (TSA?).	Horoszewicz et al. (1987)
The ag (P8) is identified on the cell surface of 90% of PC-3 cells and 67% of DU-145 cells. Cross reactivity with benign protatic tissue. MW range of the ag 48-75 kD. The ag is heavily glycosylated.	Raynor et al. (1984; 1985; 1986)
The ag is not present in the cell lines DU-145, PC-3, and LNCaP.	Starling et al. (1986)
Antigens recognized by these antibodies are present on malignant and non-malignant prostatic tissue as well as on a limited number of other malignant tissues.	Frankel et al. (1982a,b)
	Carroll et al. (1984)
The ag is poorly characterized but is believed to be a product of differentiated prostatic acinar cells. The ag is suggested to be present in the prostatic fluid.	Clarke et al. (1982)

are associated with a different immunophenotypic keratin profile (Grignon et al., 1985). In PC diminished differentiation is accompanied with absence of expression of keratins from the stratum corneum. All other keratins are found in variable extension independent of the differentiation status of the tumor.

9.4. The Androgen Receptor (AR)

It is well established that prostate growth and differentiation are dependent on androgens (Cunha et al., 1987). In the initial phase most PC's also are androgen responsive. Therefore, a benificial effect of treatment with substances that block androgen products or inhibit further steps in androgen action is to be expected. Androgens evoke their effects by an interaction with the androgen receptor (AR). Interaction of androgens with the AR results into an activated AR complex that is presumed to be able to modulate specific expression of target genes. In vivo DHT has the highest affinity for the AR. Until recently it was only possible to detect the AR in tissue using a ligand binding assay (Bowman

et al., 1986). ^{3}H-Methyltrienolone (R1881) is mostly used for this purpose. Using this technique the AR was found to be within the nucleus of prostatic cells. Presumably its presence is largely restricted to the epithelial cells of the prostatic acinus (Bashirelahi et al., 1980; Peters et al., 1987). As the epithelium/stroma ratio is higher in the peripheral zones compared with the central zone of the prostatic gland, only a low AR concentration was found in the periurethral area of the non-diseased gland (Bowman et al., 1986).

Recently, the primary structure of the human AR as deduced from the cDNA has been elucidated (Trapman et al., 1988a; Tan et al., 1988; Chang et al., 1988; Lubahn et al., 1988ab; Faber et al., 1989). The primary structure of the human AR is closely related to that of other steroid receptors such as the progesterone receptor, the glucocorticoid receptor, the mineralocorticoid receptor, and the estrogen receptor (Faber et al., 1989). The amino acid composition of the human AR shows the highest homology with the human progesterone receptor. This is particularly true for that part of the receptor which is believed to interact directly with specific sequences in the promoter/enhancer region of target genes (DNA binding domain). Immunocytochemical staining using polyclonal antibodies localized the AR to epithelial cell nuclei in the rat ventral and human prostate (Tan et al., 1988; Lubahn et al., 1988b; Laar van et al., 1989 in press). Remarkably, staining seemed exclusively present in the nuclei of the inner cylindric epithelial cells (see Figure 1). Sporadically, a nucleus of the stromal cells showed immunoreactivity. Availability of series of antibodies should be effective in further analysis of structure-function relationship of specific domains of the AR.

Naturally occuring mutations resulting in defects of synthesis and/or properties of the human AR are presumably involved in human disease especially disturbed male sexual differentiation and development (androgen insensitivity syndrom; Wilson et al., 1985).

Detailed knowledge concerning the AR in PC is not available as yet. Increased, decreased and unaltered concentrations (compared with BPH or non-diseased prostatic tissue) of AR in PC have been described (Bowman et al., 1986; Aubel van et al., 1985a; Benson et al., 1985; Barrack et al., 1983). Using immunological tools and the ligand technique simultaneously it will be possible to correlate the presence of the AR with its androgen-binding capacity, while morphological features can be retained. The presence of a (functional) AR in PC might be associated with a favourable response of endocrine intervention. On the other hand modulation of the AR system may result in the androgen-independent growth of PC. Further studies have to be initiated to provide more evidence for these hypotheses.

9.5. Additional and functional markers in prostatic cancer

Apart from the markers associated with benign and

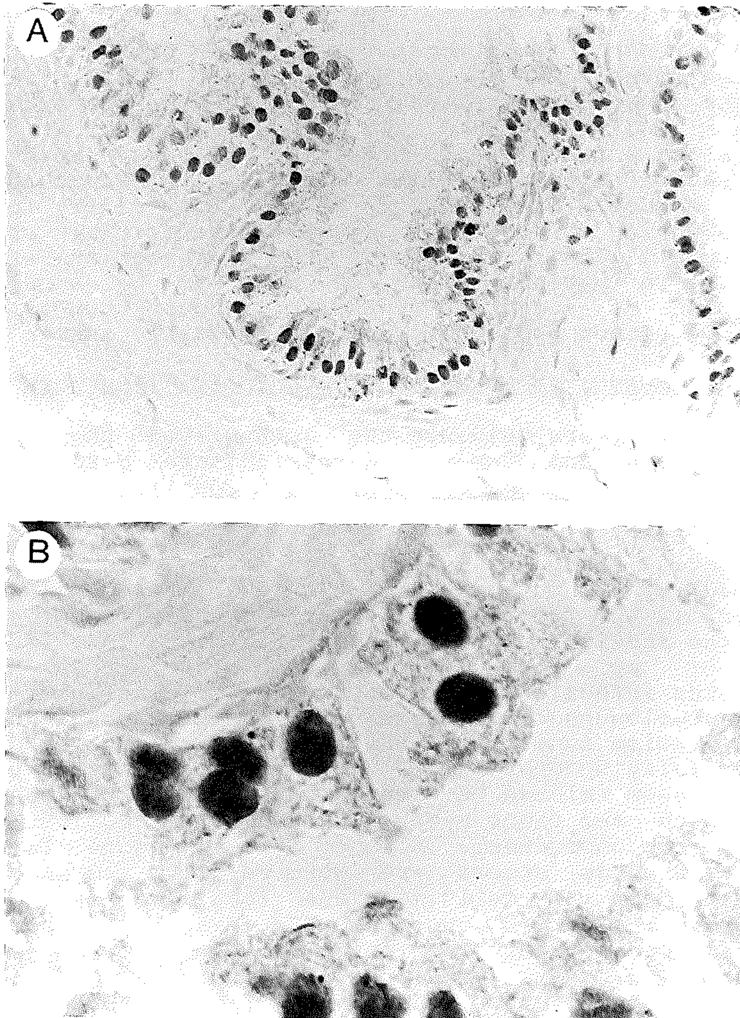

Figure 1:
Immunocytochemical localization of the androgen receptor in cryostat sections of glandular BPH. Notice the exclusive staining of the nuclei of the inner cylindric epithelial cell layer. (magn. 1A:150x; 1B:600x).

malignant prostatic tissue as discussed in the previous sections some other factors have been suggested to play a role in malignant transformation and are of potential interest in the management of PC. A limited review of the most important factors in this regard is presented here.

Estrogen Receptor (ER)

The presence of estrogen receptors in prostatic tissue is still controversial. Though Harper et al. (1986) failed to prove the immunohistochemical presence in the prostate, Seitz et al. (1987) described the localization of ER in normal prostate and a fraction of prostatic cancers. However, the concentration of the ER seems considerably lower than that of the AR. Moreover, the ER seems to be predominantly located in cells belonging to the fibromuscular interstitial stroma of the prostatic gland. Therefore, the therapeutical effects of estrogens in patients with PC have to be ascribed to suppression of androgen synthesis rather than to a direct inhibitory effect upon the growth of prostatic cancer cells.

Oncogenes, growth factors and growth factor receptors

In recent years evidence has accumulated that malignant transformation and tumor progression are associated with mutation and/or altered expression of specific genes. These genes are known as (proto-)oncogenes or cellular oncogenes. To date, over 30 oncogenes have been identified. The number of potential oncogenes is still increasing. Oncogenes may be activated by a mutation in the gene, resulting in a structurally and functionally altered protein (e.g. oncogenes of the ras family), by chromosomal translocation (e.g. myc, abl), or by gene amplification (myc). In non-human vertebrates infection with retroviruses containing mutated (viral) homologues of oncogenes can lead to tumor induction.

The precise function of the protein products of oncogenes is not fully understood. A number of the products of proto-oncogenes seem related to regulatory mechanisms controlling cell growth and trans membrane signal transduction (tyrosine kinases, ras oncogenes). Some of these encode for growth factors, whereas others are structurally and functionally closely related to cell membrane localized growth factor receptors. The erb-B oncogene, for example, encodes for a truncated variant of the epidermal growth factor (EGF) receptor. The c-fms gene product is related to the receptor for the macrophage colony stimulating factor (Slamon, 1987a). The sis oncogene encodes for platelet derived growth factor (PDGF). Most nuclear oncogenes probably are involved in regulation of gene expression (e.g. fos and myc). Recently, it has been shown that the fos oncogene exhibits its action by interaction with a second (onco)gene protein (jun). The expression of c-myc is rapidly induced prior to cellular proliferation; the fos gene is expressed within a few minutes after quiescent cells are stimulated to proliferate (Thompson et al., 1985). The function of the p21 protein of the ras oncogenes has been associated with signal transduction

involved in normal cell proliferation and differentiation. Ras proteins act relatively late in the G1 phase (as do the growth factors EGF and insulin).

In addition to DNA and RNA probes, antibodies recognizing mutated and normal oncogene products will enable the investigation of all kinds of tumors and to screen tumors for the presence of (activated) oncogenes. Apart from an increase in knowledge of the fundamental processes associated with malignant transformation, for some tumors (e.g. breast cancers) a direct correlation was suggested between proto-oncogene amplification (neu) and survival (Slamon et al., 1987b; Vijver van de et al., 1988).

Thusfar only a few of such studies have been performed on PC: Abnormalities referring the expression of the p21 protein or altered expression of ras genes at the mRNA level in prostatic (malignant) tissue were reported by Viola et al. (1986), Peehl et al. (1987), Fan (1988), Treiger et al. (1988). Recently, the role of ras oncogenes in the etiology of PC was emphasized by Bosland (1989). The latter investigator provided evidence for the occurence of a point-mutated c-Ki-ras proto-oncogene in chemically (N-methyl-N-nitrosurea) induced PC in the rat. Out of a small number (8) of human PC tissues only one activated Ki-ras oncogene could be detected in the 3T3 cell transformation assay (Peehl et al., 1987). Viola et al. (1986), using the McAb RAP 5 (that lacks the capacity to distinguish mutated and unaltered p21), provided data indicating that the levels of expression of the H-ras-oncogene in human PC correlate with the histological grade of the tumor. The p21 protein could not be detected in normal prostates and BPH samples. Recently, Fan (1988) presented results suggesting that p21 expression can be used to identify a subpopulation of a PC cell line with a potential metastatic capacity.

c-Myc mRNA levels can be low in normal human prostate and elevated in benign prostatic hyperplasia and prostate adenocarcinoma (Fleming et al., 1986; Buttyan et al., 1987). A correlation of an elevated c-myc expression and high tumor grade (according the Gleason system) was suggested by Buttyan et al. (1987). The c-myc gene is amplified in the PC-3 prostate cancer cell line (Trapman et al., 1988b). Fos expression is decreased in the PC-82 tumor after androgen depletion (Rijnders et al., 1985). It will be obvious from the fragmentary results summarized above that much work remains to be done in order to establish the role of (modified) oncogenes in prostate carcinogenesis.

Prostatic growth is influenced by prolactin that interacts with the prolactin receptor. Prolactin and prolactin receptors have been identified in benign and malignant prostatic tissue (Purnell etl al., 1982; Kadar et al., 1988a). In male mice the growth-stimulating effect of artificial hyperprolactinemia can by blocked by McAb's directed against the prolactin receptor (Sissom et al., 1988). In the Dunning rat prostate adenocarcinoma model membrane receptors for luteinizing hormone-releasing hormone (LH-RH), somatostatin, and prolactin were present (Kadar et al., 1988b). Moreover a

growth stimulatory effect on rat prostate epithelial cells in culture has been reported for EGF, TGF-α, glucocorticoid, insulin and cholera toxin (McKeehan et al., 1984). Similarly, EGF is able to stimulate the growth of the human prostate cell line LNCaP (Schuurmans et al., 1988a). Interestingly, so far transcription of TGF-β has only been found in androgen independent prostate cell lines and not in androgen dependent cells (Trapman et al., 1988b; Wilding et al., 1989). In PC specimens the EGF receptor concentration was significantly higher than in BPH (Eaton et al., 1988).

Other markers used in PC:

Independent of all markers discussed previously incidental reports announced additional markers with potential interest in characterization of PC. Among these are carcino embryonic antigen (CEA; Reynoso et al., 1972; Heyderman et al., 1977; Purnell et al., 1984); human choriogonadotrophin (Purnell et al., 1984) and the Leu 7 antibody with cross reactivity for prostatic epithelium (Wahab et al., 1985; Rusthoven et al., 1985; May et al., 1987).

10. Prostate tumor model systems

In the last few decades several PC model system have become available (Williams, 1980). Among these are spontaneously occuring carcinomas of the prostatic gland in laboratory animals. Some of these are well characterized and transplantable on recipient animals and possess metastatic capacity or can readily be propagated in tissue culture systems. In addition, hormonally or chemically induced tumors in rodents and domestic animals have been described. Finally several human model systems have been developed as transplantable xenografts on rodents or as in vitro models. A major disadvantage of human prostatic cancers (that is shared by other epithelial malignancies) is that these tumors are difficult to maintain and propagate in tissue culture systems (Okada et al., 1976; Merchant et al., 1983).

10.1. Non-human prostatic cancer models

In contrast to the human male population, prostatic carcinomas seldomly occur naturally in other mammalian species including laboratory animals. Among those rare tumors that have developed spontaneously belong the Dunning prostatic adenocarcinoma model (Dunning, 1963), the Pollard model (Pollard et al., 1973, 1975, 1980, 1984; Chang et al., 1977; Tazume et al., 1985), and the AXC rat model (Shain et al., 1975, 1977, 1981, 1984). Dunning reported in 1963 the development of a spontaneous PC in the Copenhagen rat. The Dunning R3327 rat prostate adenocarcinoma system consists of a series of in vivo transplantable tumors and in vitro growing cell lines which vary in their differentiation status, growth rate, androgen sensitivity and metastatic ability (Voigt et

al., 1974; Isaacs et al., 1978; Isaacs et al., 1981,b; Isaacs 1987). Numerous studies using the Dunning model have been carried out highlighting diverse aspects of PC (Isaacs et al., 1978; Vere White et al., 1983; English et al, 1986; Feitz et al., 1986; Zachary et al., 1986; Cooke et al., 1988ab; Treiger et al., 1988; see for a review Isaacs, 1987). Of special interest is the recent article of Treiger and coworkers (1988). These investigators reported a conversion of a tumorigenic nonmetastatic Dunning rat prostate adenocarcinoma cell line to a highly metastatic state after transfection with the v-H-ras oncogene. Using the same prostatic adenocarcinoma model Cooke et al. (1988ab) reported an increased expression of the c-H-ras gene at the mRNA level that seemed to coincide with a relatively high metastatic potential, aneuploidy, increased anaplasia and androgen-independent growth.

Incidental reports described the spontaneous occurrence of prostate malignancies in the dog, the hamster and the mastromy (Fortner et al., 1963; Snell et al., 1965).

Apart from the aforementioned PC models in laboratory animals hormones and chemical agents have been used to induce malignancies of the prostatic glands in rodents (Noble, 1977ab, 1980ab; Drago, 1984; Bosland, 1989). More recently, single oncogenes and combinations of oncogenes introduced by in vitro infection have been used to mimic in a mouse model system the process of prostate carcinogenesis (Thompson et al., 1989).

10.2. Human prostatic cancer models

Especially the gland forming (more differentiated) tumors are difficult to propagate in tissue culture. Those prostatic cancer cell lines that can be maintained in in vitro tissue culture are with only one exception (LNCaP) undifferentiated prostatic tumors. In addition to their lack of histomorphologic differentiation, the tumor cells have often lost their characteristic prostatic immunophenotype. The major characteristics of in vivo and in vitro human prostatic cancer cell lines are summarized in Table 7.

Among the human prostatic cancer cell lines that can be cultured in vitro and propagated in host animals are PC-3, LNCaP and DU-145. The PC-3 cell line is derived from a bone metastasis of PC (Kaighn et al., 1979). Growth in vitro resembles the histological appearance of a poorly differentiated carcinoma. Its growth is hormone-independent and the tumor lacks the biosynthesis and secretion of PAP. Controversial data exist concerning the retention of PA in this cell line (Papsidero et al., 1981; Trapman et al., 1988b). The PC-3 cell line is tumorigenic in nude mice. Xenografted onto nude mice the tumor can evolve into a spontaneously metastasizing malignancy (Ware et al., 1982b). The cell lines PC-133 and PC-135 can be maintained exclusively in vivo (Steenbrugge van et al., 1988a). They are derived from primary prostatic tumor deposits and are poorly differentiated. Both cell lines are characterized by the lack of androgen-dependent growth and absence of PAP and PA

Table 7. Major characteristics of the most well-known human prostatic cancer in vivo and in vitro model systems:

	PC-82	PC-3	PC-133	PC-135	DU-145	LNCaP	PC-EW	HONDA
PAP	+	-	-	-	+	+	+	+
PA	+	-	-	-	+/-	+	+	?
AS[a]	+	-	-	-	-	+	+	+
p/s[b]	p	s	s	p	s	s	s	s
vivo	+	+	+	+	+	+	+	+
vitro	-	+	-	-	+	+	-	-

[a]AS Androgen sensitivity/responsiveness/dependency
[b]p/s Derived from primary (p) or secondary (s) tumor deposit.

expression. The cell line LNCaP has been the subject of many studies (Hasenson et al., 1985; Berns et al., 1986; Schuurmans et al., 1988b). This cell line was derived from a lymph node metastasis of PC (Horoszewicz et al., 1980). In vitro the LNCaP shows an androgen responsive cell growth. AR's were demonstrated to be present in this cell line. The LNCaP model is the only in vitro PC cell line with hormone-responsive growth. However, growth in nude mice seems less dependent of the gender or hormonal status of the recipient animal (Horoszewicz et al., 1983). A variant cell line (LNCaP-r) has been established that seems to be hormone insensitive; the androgen receptor content is only 10% of the parental line (Hasenson et al., 1985). PAP and PA are still produced and secreted by LNCaP cells (Papsidero et al., 1981; Schulz et al., 1985). DU-145 is a hormone independent cell line orginating from a brain metastasis (Stone et al., 1978). Though the retention of PA in this cell line was doubted by Papsidero et al. (1981), Larson et al. (1988) performed successful immunoscintigraphy studies with antibodies against PA on mice bearing xenografts of the DU-145 cell line.

Among the human prostatic cancer cell lines that can be exclusively propagated as xenografts transplanted subcutaneously onto recipient animals, are the HONDA cell line, the PC-EW, the cell lines PC-133 and PC-135, and the PC-82 model. The HONDA cell line is derived from a human metastasis of PC localized in the testis (Ito et al., 1984). It is a hormone-dependent serially transplantable moderately differentiated adenocarcinoma. High levels of human PAP were detected in sera from tumor-bearing male mice. High affinity androgen receptors were also present (Ito et al., 1985). The PC-EW is a serially transplantable human prostatic carcinoma cell line, which has been developed through hetero-

transplantation of human tumor derived from a lymph node metastasis (Hoehn et al., 1984). Growth of this cell line is androgen-dependent. Histologically it appears a poor to moderately differentiated adenocarcinoma with cribriform areas. The serum PAP level of tumor bearing mice is elevated.

10.2.1. The PC-82 human prostatic cancer model

The PC-82 cell line was transplanted initially from a human primary prostatic tumor deposit and propagated subcutaneously into nude mice (Hoehn et al., 1980; Romijn et al., 1982; Steenbrugge van, 1988a). Tumor growth is androgen-dependent and therefore only successful in intact male mice, or female mice supplemented with T (Hoehn et al., 1982; Steenbrugge van et al., 1984). Radioactively labeled ligand studies have provided evidence for the presence of the AR within the nucleus. After numerous passages (approximately 40) the histology of the tumor still resembles the original PC (cribriform adenocarcinoma). PAP and PA can be demonstrated immunohistochemically in tissue sections (Hoehn et al., 1980; this thesis). Moreover, in tumor bearing animals PAP can be detected in the blood circulation of the recipient animals. The doubling time was determined as 18 days. Using this cell line McAb's have been prepared. Some of these recognized antigenic sites of PAP and PA (Gallee et al., 1986). Recently cDNA's coding for PA were isolated from a PC-82 cDNA library (Riegman et al., 1988). Several studies have been performed to gain insight into the effects of hormonal manipulation upon tumor growth. The latter was objectivated by biometry, AR content, PA and PAP expression (tissue concentration and serum levels), hormone levels in the serum and tumor cell kinetic data (Steenbrugge van et al., 1983 and 1984; Gallee et al., 1987).

11. Cell-kinetic data in benign and malignant prostatic tissue

It has been known for decades that mitotic figures, which reflect the growth activity in tissue sections, are often more numerous in malignancies associated with an unfavorable biological course.

Apart from counting of mitotic figures, cell kinetic information can be gained by several other established techniques. In viable systems, incorporation in recipient cells of DNA precursors is currently employed. Initially, autoradiographic studies were performed using ^{3}H-labeled thymidine. To date, the availability of antibodies against the synthetic DNA component bromodeoxyuridine (BrdU) enables a simple immunocytochemical procedure to determine S-phase fractions (Schutte et al., 1987). DNA flow cytometry is also applicable on (non-viable) fixed tissue. This technique enables to determine cell fractions in S-phase and M-phase (Tribukait, 1987). Different markers associated with cell division, cell growth, or cell proliferation have been

recognized. Out of these the antigen defined by the McAb Ki-67 has become a successful tool for cell biologists and pathologists (Gerdes et al., 1983, 1984a, 1985). This antigen with a still unknown function is retained in fresh-frozen tissue sections and exclusively present in the nuclei of cells in cell cycle. The antigen seems to be absent in the G0-phase (quiescent or resting cells) and in the early G1-phase. Growth fraction assessment by the Ki-67 method (Ki-67 score) reveals higher figures than those obtained with the previously mentioned techniques. The Ki-67 antibody has been used to determine the growth fraction in various malignancies (Burger et al., 1986; Gatter et al., 1986; Schrape et al., 1987; Landolt et al., 1987; Barnard et al., 1987; McGurrin et al., 1987; Walker et al., 1988; Brown et al., 1988; Gallee et al., 1987; 1989b).

In benign, hyperplastic and malignant prostatic tissue, mitotic figures are rarely found. Presumably for this reason these data are seldomly incorporated in the criteria of histological grading systems designed for PC. Nevertheless when present, mitotic figures are associated with a presumed unfavorable clinical course. In the grading system of Mostofi-Schroeder the presence of mitoses is considered as a bad prognostic sign (grade V tumor) (Schroeder et al., 1985abc). Data concerning cell kinetics in prostatic tissue are scarce in literature. In vitro exposure of surgical prostate specimens to ^{3}H-thymidine revealed extremely low S-phase fractions in normal and diseased prostatic tissue (Meyer et al., 1982; Helpap, 1985). The thymidine labeling index in normal prostate was estimated at 0.12%, in glandular BPH it was measured at 0.31%, whereas in PC an average value of 0.90% was determined (Meyer et al., 1982). Helpap (1985) found somewhat higher figures and a correlation with differentiation status. Well-differentiated adenocarcinomas showed a thymidine labeling index of 0.1-0.4%, whereas cribriform- and medullary cancers revealed an index of 2.3-5.6% (Helpap, 1985). In situ exposure of PC cells to BrdU revealed identical high S-phase fractions for PC (up to 6.29% for medullary carcinomas; Nemoto et al., 1989). Recently Raymond et al. (1988) determined growth fractions in human PC with the McAb Ki-67. An average Ki-67 score of 16.3% was obtained in PC, whereas in hyperplastic glandular epithelium a mean Ki-67 value of 4% was reached. A possible relationship between cell kinetics and scores of histological grading systems (i.e. the Gleason method and the Mostofi system) was suggested. We circumvented the use of histological grading methods and examined the correlation of Ki-67 score with generally accepted histological PC growth patterns (Gallee et al., 1989b). Ki-67 scores were generally lower when compared to those measured by Raymond et al. (1988). Cancers composed of solid undifferentiated sheets or cribriform patterns were characterized by relatively high Ki-67 scores. However, infiltrative, undifferentiated cancers showed growth fractions almost equal to those obtained in gland forming tumors.

Cell kinetic data are also available for the PC-82 cell line. DNA flow cytometry revealed that 85-90% of the cells are in G0/G1 phase, the fraction of cells in G2/M phase was

found to be less than 10%, whereas the fraction of S-phase cells was estimated not to exceed 5% of the total number of cells. In this thesis we describe growth fraction determinations in the PC-82 cell line using in vivo exposure of tumor cells to BrdU and the Ki-67 method (Gallee et al., 1987). In the intact model average values were assessed at: Ki-67 score 16% ; BrdU incorporation 9.3%. These figures are in agreement with those obtained with DNA flow cytometry. Approximately 15% of the cells in the PC-82 cell line are in cycle. In addition we have examined the influence of androgen withdrawal upon the Ki-67 score. Short-term androgen depletion resulted in a decline of the Ki-67 score, whereas repletion after 10-days withdrawal led to an increase and even a rebound effect of the growth fraction assessed by means of the BrdU uptake and the Ki-67 assay. Similar results indicating a relationship between cell proliferation and steroids, were obtained by other investigators (English et al., 1985; 1986; Shain et al., 1987). Others studied proliferative activity in the non-tumorous rat ventral prostate and in the Dunning androgen-dependent rat adenocarcinoma model using the ^{3}H-thymidine labeling technique (English et al., 1985; 1986). Special attention was given to changes in proliferative activity resulting from hormonal manipulation. Androgen depletion resulted in a slight reduction in the percentage of S-phase nuclei, whereas repletion initiated a significant increase of proliferative activity. A possible role of the induction of such an increase of cells in S-phase might be to potentiate cytotoxic chemotherapy for treatment of adenocarcinoma of the prostate. On the other hand, Bosland et al. (1989) have recently used this method of synchronization of cells in proliferation phase followed by intravenous administration of MNU to induce rat prostatic adenocarcinomas.

12. Aims and outlines of the study

This study was initiated in order to gain insight in the value of different parameters that are associated with the prognosis of PC. As it is generally accepted that morphological features are associated with clinical behavior, histological grading methods for all kinds of malignancies have been designed. These systems allow to score tumors based upon cyto-nuclear morphological characteristics and patterns of tumor architecture in such a manner that a relatively good correlation is found between these scores and the natural course of that particular cancer. In the case of PC numerous grading systems have been developed. The large number of grading systems available for PC leads to questions concerning which system is most suited to use in general practice and why the number of grading systems for this particular neoplasm is so extensive. The latter problem is presumably related to the high prevalence of PC and the subjectivity that is always associated with the performance of histological grading. Moreover, single prostatic tumors are often characterized by the presence of more than one histological growth pattern (histological heterogeneity). In an attempt to examine the

interobserver variation in histological grading of PC, we have studied the consensus of assigning histological scores to PC using a selection of 5 more commonly used grading systems. The general grading system described by Broders (1926), designed for histological typing of neoplasms irrespective of its origin was selected in addition to 4 systems especially designed to score prostate malignancies. These latter grading systems encompass the MDAH method (1982) and systems described by Mostofi (1975), Gleason (1966) and the more recently introduced method of Schroeder-Mostofi (1985abc). Out of these systems the Mostofi method is characterized by a large impact that is laid upon nuclear features, whereas the other methods also include architectural criteria of tumor growth. The Gleason system is commonly used in the United States. It is the only grading method for prostatic tumors that makes use of the possibility to score two histomorphological growth patterns within the same neoplasm. These 5 systems were utilized by 5 pathologists on tissue sections of 50 patients with a long follow-up period. Results of these studies are presented in Chapters II and III.

In addition, we have searched for other (more objective) parameters characteristic for PC. We attempted to generate PC specific antibodies using the well characterized human PC cell line PC-82. However, none of the antibodies displayed an exclusive specificity for PC. Interesting cross-reactivity profiles of some antibodies were observed. Some of the antibodies isolated were directed against epitopes of PA and PAP. Since some of the prepared antibodies were applicable on formalin-fixed tissue we correlated immunohistochemical expression of PA and PAP with generally accepted architectural growth patterns of PC. The use of grading scores was avoided. Results of these studies are presented in Chapters IV and V.

It is widely accepted that cell kinetic data are of prognostic value in many tumors. A high mitotic activity in malignant tumors has always been associated with a poor clinical outcome. In breast tumors the number of mitotic figures is regarded as an independent parameter with a high prognostic impact (Baak et al., 1985). As cell kinetic data available for human PC are scarce we investigated cell growth fractions in human PC samples within prostatectomy specimens and in the human prostatic cancer cell line PC-82. As data obtained with the Ki-67 method paralelled those measured by the BrdU uptake technique (Chapter VI), we could use the former method to investigate the correlation of growth fractions with architectural growth pattern of PC (Chapter VII). In Chapter VI an interesting correlation was noticed in the PC-82 tumor model between the endocrine status of the host animal and the Ki-67 defined growth fraction in the PC-82 tumor. Alterations of cell kinetics seem to precede light microscopic changes after short-term androgen depletion.

13. References

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CHAPTER II

CONTROVERSY IN GRADING OF PROSTATIC CARCINOMA: INTEROBSERVER REPRODUCIBILITY OF FIVE DIFFERENT GRADING SYSTEMS

F.J.W. ten Kate[1], M.P.W. Gallee[1],
P.I.M. Schmitz[2], A.C. Joebsis[3], R.O. van der Heul[1],
M.E.F. Prins[1], J.H.M. Blom[4]

Department of Pathololgy (1), Biostatistics (2) and Urology (4) of the Erasmus University Rotterdam, and the Department of Pathology (3) of the Bergweg Hospital, Rotterdam, The Netherlands

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SUMMARY

In order to investigate the reproducibility of grading systems for prostatic carcinoma currently in use, a comparative histological grading study was done. These studies were carried out on tissue sections from radical prostatectomy specimens (n = 50) stained with hematoxylin and eosin. Five pathologists with varying professional experience participated in the study, using five different grading systems: those of the Broders, Brawn, Gleason (for statistical compilation the modified version), Mostofi, and a modified Mostofi grading method recently described by Schroeder and Mostofi. Weighted kappa coefficients ranged from 0.21 to 0.52. None of the systems investigated demonstrated a high degree of reproducibility (k > 0.70). Reproducibility of the systems described by Broders and Brawn was reasonably good (k = 0.52 and 0.41, respectively). With the modified Gleason method (rearrangement of Gleason scores into 3 grades), a considerable difference was noted between the numerical agreement score (among at least 3 observers) and the measured kappa value (100% and 0.30, respectively). The methods described by Mostofi and Schroeder-Mostofi revealed only limited reproducibility (k = 0.21 and 0.34, respectively).

INTRODUCTION

Prostatic carcinoma is a major cause of death among the male population in most developed countries (8,36). Its prevalence and incidence is even higher, making this malignancy the most common neoplasm of the male urogenital tract (36). The discrepancy between mortality and morbidity rates reflects the wide scope of its clinical behavior, ranging from neoplasms found only incidentally at post-mortem examination (nearly 10%) to those that metastasize early and eventually lead to death (8,36).

In order to predict the clinical behavior and aggressiveness of prostatic carcinoma so that the most appropriate therapeutic regimen can be chosen and the prognosis determined, several grading systems have been developed by pathologists (10,11,13,15,19,21). Nevertheless, much controversy still exists as to the most reliable grading method (1,12,17,28).

Boecking et al. (5) have described the main objectives that a grading system should fulfil:

1. Each diagnostic criterion has to correlate with biological behavior and prognosis.
2. It must display sufficient reproducibility.
3. Grading done on random biopsies should, whenever possible, be representative of the tumor as a whole.

In some systems, grading is performed upon the least differentiated areas; in others, a selection of the most predominant growth patterns is made. Thus far, approximately 30 grading systems have been described. Some use low-power microscopy, taking into consideration only histological growth characteristics and their relationships to the surrounding

stroma (5,20,39). However, in most, prognostic significance has been attributed to cytological features (9,27,30,38). Combinations of both histological and cytological features form the basis of yet other systems. Also, in some studies, cytological features are examined by use of morphometry (9,12).

To investigate the degree of reproducibility, we have examined the interobserver variation of five grading systems in current use. Two methods, described by Broders (7) and Brawn (M.D. Anderson Hospital) (6), were chosen as their prognostic value is predominantly based upon histological criteria. The method described by Gleason (16,18,20,22,25,37) was included for its general reputation and the attention given to both growth characteristics and interaction of tumor with the surrounding stroma. To incorporate a system that utilizes cytological features as a prcgnostic indicator, the method of Mostofi was selected (27,28). Finally, a recently introduced method described by Schroeder and Mostofi was included (33,34,35). The latter system was based upon a retrospective multivariate analysis of a large number of histological and cytological criteria.

MATERIALS AND METHODS

Patients and Materials

Out of 464 patients of the original series of Belt and Schroeder (1930-1970), who had total perineal prostatectomy for limited prostatic cancer, 50 patients were randomly selected (4,32). Average age at the time of surgery was 66 years, ranging from 48 to 83 years. The mean period of follow-up lasted for 125 months, ranging from 8 to 317 months. The survival curves and curves of time interval until first recurrence for the 50 patients included in this grading study did not differ significantly from that of a much larger random selection in the series of Belt and Schroeder. None of the 50 patients of this study were lost to follow-up. In some of these cases the histology was not optimal. These 50 patients are a random selection of those previously graded by Mostofi and Schroeder (33,34,35), one of the few series to date with a follow-up of more than 10 years. The data recorded by Mostofi were also utilized for statistical analysis.

Prostatectomy specimens were fixed in a buffered 4% formaldehyde solution, and sectioned in a stepwise fashion. All sections were routinely stained with hematoxylin and eosin and an average of 4 slides per case was available for histological examination. The number of slides per prostatectomy specimen varied from 2 to 11. The number of slides in which tumor was present varied from 1 to 9 (mean: 3).

Grading systems

The 50 cases were evaluated by five pathologists using five grading systems in common use. The major criteria as well as the scoring systems are summarized briefly in Table 1 (6,7,18,25,27,33,34,35).

The final Gleason score was assessed by the total of the scores of the two quantitatively predominating growth patterns. In those tumors in which only one growth pattern was recognized, the value of the growth pattern selected was doubled. For statistical compilation as proposed in the literature (2,14), Gleason scores were rearranged in 3 main grades (or groups) in the following manner: specimens assigned as Gleason sum 2, 3, and 4 tumors were included in grade 1; tumors with Gleason scores 5, 6, and 7 in grade 2 and those with Gleason scores 8, 9, and 10 in grade 3. This is referred to as the modified Gleason grading method.

The grading method described by Schroeder and Mostofi was originally developed using 346 cases from the series of Belt and Schroeder (4,32). After collecting both cytological and histological features, they performed a retrospective multivariate analysis and were finally able to distinguish five categories of prognostic significance, predominantly based upon combinations of glandular differentiation, degree of nuclear anaplasia, and mitotic activity (33,34,35). The 50 patients included in the present study are a random selection of those previously graded by Mostofi and Schroeder. The data recorded by Mostofi were also utilized for statistical analysis.

Statistical analysis

Data was analyzed by calculating a measure of agreement among the five pathologists for each grading system. For most grading methods included in this study "standard" scores assigned by referee pathologists were not available. Therefore, weighted kappa coefficients were calculated as the best parameter of interobserver agreement. In order to incorporate the ordinal scales of the grading systems and to make the agreement of assessments for each system comparable, the so-called weighted kappa (k) coefficients were used together with quadratic disagreement weights (31).

RESULTS

Grading results obtained for all five methods are summarized in Tables 2 and 3. Table 2 presents the number of scores assigned by all pathologists to each grade for the five different methods evaluated in this study. A total of 250 scores per method were available (5 pathologists and 50 specimens). For the Mostofi and Mostofi-Schroeder methods, referee scores recorded by Mostofi himself were available and added to this table.

Table 3 presents data related to the measure of agreement and disagreement for each grading method.

Table 1. Major grading criteria of the five systems evaluated in this study

Broders (1926) (ref. 7)

Grade 1	100%-75% glandular differentiation; 0%-25% undifferentiated
Grade 2	75%-50% glandular differentiation; 25%-50% undifferentiated
Grade 3	50%-25% glandular differentiation; 50%-75% undifferentiated
Grade 4	0%-25% glandular differentiation; 100%-75% undifferentiated

Gleason (1966) (ref. 18,19,20)

Pattern 1	well-differentiated, small, closely packed, uniform glands in essentially circumscribed masses
Pattern 2	similar to pattern 1 with moderate variation in shape and size of glands and more atypia in the tumor cells; more loosely arranged though still circumscribed
Pattern 3	similar to pattern 2 with marked irregularity in size and shape of glands with small glands or individual cells invading the stroma
Pattern 4	raggedly infiltrating, fused glandular tumor, frequently with pale cells, may resemble hypernephroma of kidney
Pattern 5	anaplastic carcinoma with minimal glandular differentiation, diffusely infiltrating prostatic stroma

Mostofi (1975) (ref. 27)

Grade 1	glandular differentiation with slight nuclear anaplasia
Grade 2	glandular differentiation with moderate nuclear anaplasia
Grade 3	glandular differentiation with marked nuclear anaplasia or undifferentiated tumor

Brawn (M.D. Anderson, 1982) (ref. 6)

Grade 1	75%-100% glandular differentiation; 0%-25% of the tumor does not form glands. Excluded are cribriform-papillary tumors
Grade 2	50%- 75% glandular differentiation; 25%-50% of the tumor does not form glands. Included are tumors consisting of 50% or more of a cribriform-papillary pattern
Grade 3	25%- 50% glandular differentiation; 50%-75% of the tumor does not form glands.
Grade 4	0%- 25% glandular differentiation; the remainder is undifferentiated

Schroeder/Mostofi (1985) (ref. 33,34,35)

Class 1	glandular differentiation, absence of mitoses, slight nuclear anaplasia
Class 2	glandular differentiation, absence of mitoses, moderate nuclear anaplasia or: glandular differentiation, mitotic activity, slight or: undifferentiated tumor or cribriform growth variant, absence of mitoses and slight nuclear anaplasia
Class 3	glandular differentiation and mitotic activity combined with moderate or marked nuclear anaplasia or: glandular differentiation, absence of mitoses, marked nuclear anaplasia or: undifferentiated or cribriform growth variant, absence of mitoses, moderate anaplasia or: undifferentiated or cribriform growth variant, presence of mitoses, slight nuclear anaplasia
Class 4	undifferentiated tumor or cribriform growth variant, absence of mitoses, marked nuclear anaplasia or: undifferentiated or cribriform growth variant, presence of mitoses, moderate nuclear anaplasia
Class 5	undifferentiated tumor or cribriform growth variant, presence of mitoses, marked anaplasia

Broders grading method

Tumors in which glandular formation predominates (i.e. at least 50% consists of glandular formation) relatively outnumber tumors in which a minor part is differentiated. Of the 250 gradings performed, 207 (83%) were recorded as grade 1 or 2 (Table 2). Most agreement was also observed in the lower grades, i.e. 62% and 18% agreement for grades 1 and 2, respectively, by at least 3 observers (data not shown). The weighted kappa coefficient measured for the Broders method was 0.52 (Table 3). In 14 cases, the recorded scores differed by more than one grade. In 5 cases only 2 pathologists agreed with each other (10%). The latter demonstrates almost total disagreement among the participating pathologists.

M.D. Anderson method

Of the total of 250 scores, 210 were conferred upon tumors predominantly characterized by glandular or cribriform-papillary growth pattern (84%) (Table 2). The kappa coefficient calculated for the Anderson system was 0.41 (Table 3). Most agreement was found for grade 1 and 2. Agreement existed between at least 3 pathologists in 78% related to grade 1 and 2 tumors. In 16 cases (32%) more than one grade difference was observed. Concordance between only 2 pathologists was noticed in 7 cases (14%).

Table 2. Distribution of grades (1-5) and Gleason scores (1-10) tabulated separately for each grading system as assessed by the participating pathologists (A-E) and by Mostofi himself*

Grading systems	Grade										Total no. of scores
	1	2	3	4	5	6	7	8	9	10	
Broders	144 (58%)	63 (25%)	31 (12%)	12 (5%)	-	-	-	-	-	-	250
M.D. Anderson	102 (41%)	108 (43%)	26 (10%)	14 (6%)	-	-	-	-	-	-	250
Mostofi	22 (9%)	120 (48%)	108 (43%)	-	-	-	-	-	-	-	250
Mostofi*	1 (2%)	32 (64%)	17 (34%)	-	-	-	-	-	-	-	50
Gleason	-	0 (0%)	4 (2%)	24 (10%)	45 (18%)	117 (47%)	27 (11%)	21 (8%)	6 (2%)	6 (2%)	250
Modified Gleason	28 (11%)	189 (76%)	33 (13%)	-	-	-	-	-	-	-	250
Mostofi-Schroeder	5 (2%)	34 (14%)	91 (36%)	84 (34%)	36 (14%)	-	-	-	-	-	250
Mostofi-Schroeder*	1 (2%)	3 (6%)	21 (42%)	17 (34%)	8 (16%)	-	-	-	-	-	50

Table 3. Absolute and percentual measure of agreement and kappa values for each grading method tabulated separately

	Broders 4-grade system		Anderson 4-grade system		Mostofi 3-grade system		Mod. Gleason 3-grade system		Mostofi-Schroeder 5-grade system	
Agreement among:										
5 observers	13	(26%)	12	(24%)	6	(12%)	18	(36%)	1	(2%)
4 observers	14	(28%)	16	(32%)	19	(38%)	15	(30%)	8	(16%)
3 observers	18	(36%)	15	(30%)	20	(40%)	17	(34%)	29	(58%)
At least 3 observers	45	(90%)	43	(86%)	45	(90%)	50	(100%)	38	(76%)
A difference of more than 2 grades	14	(28%)	16	(32%)	12	(24%)	2	(4%)	29	(58%)
Total no. of specimens	50	(100%)	50	(100%)	50	(100%)	50	(100%)	50	(100%)
Kappa values	0.52		0.41		0.21		0.30		0.34	

Mostofi grading method

Glandular differentiation combined with slight nuclear anaplasia was only recorded in 22 scores out of a total of 250 (9%) (Table 2). The scores assessed earlier by Mostofi for the same material are also summarized in Table 2. The overall weighted kappa score was calculated at 0.21 (Table 3). Complete agreement among all pathologists was never found for grade 1 tumors (data not shown). More than one grade difference per specimen was observed in 12 cases (24%). Only in 5 cases was a total lack of concordance seen.

To obtain insight into the measure of agreement (or disagreement) of each participating pathologist with the grading results obtained by Mostofi himself, an individual kappa score was assessed. None of the 5 pathologists attained a weighted kappa coefficient of agreement above 0.40.

Gleason grading method

Grading results determined according the traditional and modified Gleason methods are shown in Table 2. None of the tumors received a traditional Gleason score of 2. In Table 3, the measure of agreement is presented only for the modified results of the rearranged Gleason scores (2,14). The weighted kappa score was calculated at 0.30. In all 50 cases (100%), at least 3 investigators agreed with each other concerning the chosen modified Gleason grade. As group/grade 2 was most frequently chosen, maximal agreement was found in this category. More than one grade disagreement of the modified system was found in only 2 cases.

Mostofi-Schroeder grading method

In Tables 2, 3 and 4, results using the Mostofi-Schroeder method are shown. Besides the distribution of scores obtained by the 5 participating pathologists, Table 2 also includes the results obtained by Mostofi. The weighted kappa coefficient as a measure of agreement among pathologists was calculated at 0.34. An overall percentage for all prognostic classes of 76% was a measure of agreement of at least 3 pathologists. In only 5 cases grade 1 was recorded. Regarding grade 5 tumors, agreement among 3 pathologists was only achieved in 2 cases. Disagreement reflected in the number of cases in which pathologists differed by more than one grade was recorded in 29 cases. Using the data recorded by Mostofi himself as a "standard", the individual weighted kappa coefficient as a measure of agreement for each pathologist was assessed (Table 4). With the Mostofi-Schroeder method, unlike the Mostofi method, 2 of the 5 pathologists scored a kappa value above 0.40.

Table 4. Measure of agreement of each participating pathologist (A-E) compared to the scores of Mostofi (M) using the Mostofi-Schroeder method (1-5)

Pathologist A

	1(M)	2(M)	3(M)	4(M)	5(M)	
1(A)	0	0	0	0	0	0
2(A)	1	2	2	2	1	8
3(A)	0	0	10	9	3	22
4(A)	0	0	9	5	4	18
5(A)	0	1	0	1	0	2
	1	3	21	17	8	50

Weighted κ coefficient A: $\kappa = 0.14$

Pathologist B

	1(M)	2(M)	3(M)	4(M)	5(M)	
1(B)	0	1	0	1	0	2
2(B)	0	1	4	1	1	7
3(B)	1	1	10	4	1	17
4(B)	0	0	6	8	5	19
5(B)	0	0	1	3	1	5
	1	3	21	17	8	50

Weighted κ coefficient B: $\kappa = 0.35$

Pathologist C

	1(M)	2(M)	3(M)	4(M)	5(M)	
1(C)	1	0	0	0	0	1
2(C)	0	1	2	0	0	3
3(C)	0	1	6	3	2	12
4(C)	0	1	10	9	2	22
5(C)	0	0	3	5	4	12
	1	3	21	17	8	50

Weighted κ coefficient C: $\kappa = 0.46$

Pathologist D

	1(M)	2(M)	3(M)	4(M)	5(M)	
1(D)	0	1	0	0	0	0
2(D)	1	0	0	1	0	2
3(D)	0	3	10	3	2	18
4(D)	0	0	8	7	2	17
5(D)	0	0	3	6	4	13
	1	3	21	17	8	50

Weighted κ coefficient D: $\kappa = 0.42$

Pathologist E

	1(M)	2(M)	3(M)	4(M)	5(M)	
1(E)	0	0	1	1	0	2
2(E)	1	2	7	3	1	14
3(E)	0	1	11	8	2	22
4(E)	0	0	1	4	3	8
5(E)	0	0	1	1	2	4
	1	3	21	17	8	50

Weighted κ coefficient E: $\kappa = 0.35$

DISCUSSION

Kappa values for each method separately indicate that none of the grading systems had a high degree of concordance ($k > 0.70$). Kappa values reflecting fair to reasonable reproducibility (kappa ranging from 0.40 to 0.70) were only found by the Broders and Anderson grading methods (Table 3). However, in contrast to these values, in both the Broders system and the Anderson grading method (in 14 and 16 cases, respectively), disagreement among observers of more than two grading steps was noted (Table 3). Such lack of agreement may be partly attributed to impaired tissue preservation and fixation. Furthermore, when the distribution of grades is considered for both methods, by far the most tumors were assessed as grade 1 or 2 (Table 2). Preponderance of such a relatively large number of well-differentiated tumors may be attributed to the inclusion of only prostatectomy specimens in this study.

In contrast to the low kappa score obtained by the Mostofi method, the percentual agreement was markedly better (Table 3). In fact, in 90% of the cases, at least 3 observers shared the same opinion about the assignment of tumor grade (Table 3). However, when this agreement per tumor grade was analyzed (data not shown), concordance was achieved most frequently for grade 2 tumors. Discrepancy of kappa values and percentual agreement score may be explained by the fact that observers are often inclined to classify tumors most frequently as grade 2 when a three-step grading method is used. In addition, inadequate preservation of tissue slides has undoubtedly influenced precise judgement of cytologic features such as degree of anaplasia. Furthermore, lack of sufficient experience and poor definability of slight, moderate and marked nuclear anaplasia may also contribute to this low kappa value.

Applying the modified Gleason method, a maximal numerical agreement score was achieved. In all specimens investigated, at least 3 observers agreed after rearrangement of results into a three-step grading system (100%). However, a low kappa value was a measure reflecting the low degree of accuracy. The latter may be explained by an unbalanced distribution, since grade 1 and 3 tumors are scarcely represented (Table 2). Although a kappa value was not determined for the traditional Gleason system, our results seem to be in contrast to those presented by others (16,22,24,26,29,37). Harada et al. (23) stated that, for the primary growth pattern, agreement was found in 64% of cases; for the secondary pattern, agreement was obtained in 44%. When the primary and secondary patterns were compared to those of Gleason, agreement was recorded in only 38% (23). Gleason (23) postulates that the margin of error of reproducibility from one institution to another could be as much as 50% and probably reflects the degree of experience of the particular observer. Bain et al. (3) stated in their study concerning the reproducibility of the Gleason method that agreement was reached among 7 pathologists in 74%-93% of the cases studied (n = 58; kappa ranged from 60.5% to 83.6%). However, grading in this study was performed predominantly upon transurethral resection specimens.

Although the percentual numerical scores of the Schroeder-Mostofi method demonstrate a nearly total lack of agreement (Table 3), the weighted kappa coefficient can be considered as relatively high (0.34). Again, lack of numerical concordance may be partly due to the fact that, in addition to histological criteria, cytological features, especially nuclear anaplasia and mitotic activity are incorporated in this grading system. Ordinal scale correction for this five-step grading method has undoubtedly influenced the kappa score favorably.

As the treatment decision for prostatic cancer is often influenced by the results of grading, accuracy and reproducibility of grading methods are of the utmost importance. However, even when sufficient tissue sections are available, accuracy of grading, as this study shows, is considered to be relatively low. Simple methods involving only histological features seem to have a better interobserver agreement than

those systems using cytological features. However, low agreement scores found for the Mostofi method and the recently described system introduced by Schroeder and Mostofi can be attributed to lack of optimal fixation and preservation of tissue sections, resulting in difficulty of evaluating nuclear anaplasia. In addition, our results may also indicate that, in practice, pathologists are more accustomed to dealing with well-defined (histological) criteria as compared to less circumscribed criteria such as degree of nuclear anaplasia.

Calculation of the weighted kappa scores may hamper precise interpretation of the value of the accuracy of grading scores, especially when the tumors selected are not equally divided among the diverse grades. Finally, reproducibility is largely dependent upon training and experience and is considerably facilitated by the elimination of ambiguity in the definition of predictive morphological criteria. As yet, it remains to be determined to what extent morphometrical techniques will enhance the accuracy and reproducibility of grading methods.

Since the final aim of grading is to predict the clinical behavior of neoplasms, grading results obtained so far will be correlated with survival and recurrence rates and the data will be presented in a second report.

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CHAPTER III

HISTOLOGICAL GRADING OF PROSTATIC CARCINOMA IN PROSTATECTOMY SPECIMENS: A COMPARATIVE STUDY OF THE PROGNOSTIC ACCURACY OF FIVE CURRENT GRADING SYSTEMS.

M.P.W. Gallee[1], F.J.W. ten Kate[1], P.G.H. Mulder[2], J.H.M. Blom[3], R.O. van der Heul[1].

Department of Pathology (1), Biostatistics (2) and Urology (3) of the Erasmus University Rotterdam, The Netherlands.

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ABSTRACT

The prognostic accuracy of 5 current histological grading systems (Broders, Anderson, Mostofi, Gleason, and Mostofi-Schroeder) was compared. The histological grading was performed by 5 pathologists upon 50 prostatectomy specimens. By averaging the grading results of the 5 pathologists the impact of the interobserver variation was eliminated. The Cox proportional hazards model was used for estimating the relationship between the average grading scores and time-to-recurrence (respectively, time-to-death by prostatic carcinoma). Age at surgery was adjusted for as a possible confounding factor. The prognostic impact of the 5 grading systems (related to both recurrence and death caused by prostatic carcinoma) was judged by the likelihood ratio (LR) test score. The LR test score (which is chi-square distributed with 1 df) for time-to-recurrence for the Mostofi-Schroeder score appeared to be 6.54, whereas a value of 1.79 was measured for the Gleason system. A stepwise procedure demonstrated that with the Mostofi-Schroeder - and Broders systems used together the best prognostic performance was reached (with Mostofi-Schroeder weighted 1.5 times larger than Broders). For time-to-recurrence also the median grading result was used giving results similar to the mean grading result. For time-to-death by prostatic carcinoma the LR test scores for all grading systems were relatively low. In this analysis the outcome of the Gleason system did show a minimum of prognostic ability, whereas the grading systems of Broders as well as the recently introduced system of Mostofi-Schroeder had a reasonable predictive power. Since the interobserver variation of the Schroeder-Mostofi system was large, the Broders system is to be preferred. Restrictions and implications of this study are discussed and a brief review of the prognostic importance of grading of prostatic carcinoma is presented.

INTRODUCTION

Carcinoma of the prostate is a frequently occurring malignancy. The incidence of prostatic carcinoma demonstrates a geographically and demographically related distribution ranging from 0.84 for Shanghai to 100.20 among the black population in Alameda County, California per 100,000 male population (Zaridze et al., 1984; Catalona, 1984; Ross et al., 1983; Wynder et al., 1971). The reason for these geographical and demographical differences in incidence and prevalence remains for as yet unknown. As latent or incidental carcinomas are erroneously omitted (Yamabe et al., 1986; Dhom, 1983), the actual figures concerning incidence and prevalence of prostatic carcinoma are often underestimated in the literature. Mortality resulting from disseminated prostatic tumours also shows a geographical and demographical variation ranging from 2.51 (Japan) to 29.1 (St. Vincent and Grenadines), per 100,000 male population (Zaridze et al., 1984; Catalona, 1984; Ross et al., 1983; Wynder et al., 1971). The large difference between incidence- and mortality rate

reflects a wide variation in clinical behaviour of prostatic carcinoma. At initial diagnosis prostatic carcinoma may present itself as incidental carcinoma which is considered harmless, or as an extended tumour with a tendency to metastasize, ultimately leading to death. As for histopathology nearly 97% of prostatic tumours shows acinar structures and these tumours are therefore designated as adenocarcinomas (Utz and Farrow, 1969).

Pathologists have searched for histological, cytological, immunological and morphometric parameters in an attempt to predict biological aggressiveness. Several histological grading systems have been developed to forecast the biological behaviour of the carcinomas (Murphy and Whitmore, 1979; Mostofi, 1976; Grayhack and Assimos, 1983).

The large number of grading systems presented in the literature already indicates the controversy concerning the choice of the most reliable grading system, due to large inter- and intra-observer variation as demonstrated in a previous report (Ten Kate et al., 1986). In this sequel report we will present these data again, now correlated with the clinical follow-up of patients incorporated in this survey.

MATERIALS AND METHODS

Patients and materials

From the original series of Belt and Schroeder of 464 patients, who underwent total perineal prostatectomy for localized limited prostatic carcinoma (Whitmore, 1956), 50 patients were randomly selected (Belt and Schroeder, 1972; Schroeder and Belt, 1975). In this group the average age at the time of surgery was 66 years with a range from 48 to 83 years. The mean period of follow-up lasted for 125 months, ranging from 8 to 317 months. The survival curves of time-to-death and time-to-first recurrence for these 50 patients did not differ from the curves of a much larger random selection of the series of Belt and Schroeder. The original series of Belt was recently used to devise a new grading method as described by Mostofi and Schroeder (Schroeder et al., 1985a; Schroeder et al., 1985b; Schroeder et al., 1985c).

We were able to obtain the follow-up of all the 50 patients. At the end of follow-up 11 patients were still alive without progression. For the analysis of time-to-first recurrence (19 patients), 31 patients were considered "censored"; and for time-to-death by prostatic carcinoma (16 patients), 34 patients were considered "censored" (Figure 1). None of the patients who showed progression is still alive. This series is one of the few to date with a mean follow-up period of more than 10 years. Recurrence after prostatectomy occurred in 19 patients. The time interval until first recurrence varied from 8 months to 133 months with a mean period of 51 months. Treatment modalities of these recurrences differed and were according to the current protocol at that time period. All of the patients who had a tumour which showed progression died at time intervals ranging

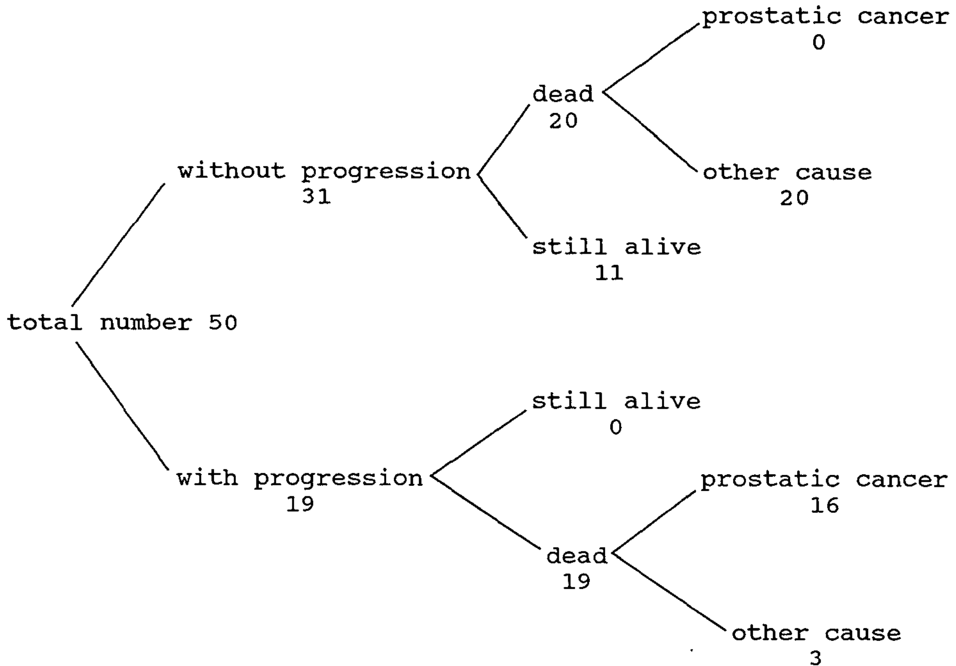

Figure 1:
Data concerning the follow-up of the 50 patients included in this study.

from 1 to 164 months with a mean period of 45 months. However, included are 3 patients who died from another cause. After excluding these patients time-to-death in the recurrence group ranged from 1 to 164 months with an average of 33 months.

Prostatectomy specimens were fixed routinely in 4% formaldehyde solution. Section was performed in a stepwise fashion and each specimen was submitted completely for histologic examination. Slides were stained routinely with Mayer's haematoxylin and eosin. The number of slides available for microscopic survey per prostatectomy specimen ranged from 2 to 11. The number of slides in which tumour was present varied from 1 to 9 (mean: 3).

Grading systems

The 50 cases were evaluated by 5 pathologists using the general grading system of Broders, the Anderson (hospital) system, the Mostofi system, the system described by Gleason and the Mostofi-Schroeder system (Broders, 1926; Brawn et al.,

1982; Mostofi, 1975; Gleason, 1966; Mellinger et al., 1967; Schroeder et al., 1985abc). The Gleason system was used as originally reported since, by definition, a crude linear transformation realised by rearrangement of Gleason scores in three main groups will not increase the prognostic impact of this system (Babaian and Grunow, 1985).

The percentual distribution of grading-scores for each grading system separately is listed in Table 1 (Ten Kate et al., 1986).

Table 1. Percentage of tumours attributed to the various grades of the Broders, Anderson, Mostofi, Gleason, and Mostofi-Schroeder grading systems assigned by all pathologists together.

	Broders	Anderson	Mostofi	Gleason	Mostofi-Schroeder
grade 1	58%	41%	9%	-	2%
grade 2	25%	43%	48%	0%	14%
grade 3	12%	10%	43%	2%	36%
grade 4	5%	6%	-	10%	34%
grade 5	-	-	-	18%	14%
grade 6	-	-	-	47%	-
grade 7	-	-	-	11%	-
grade 8	-	-	-	8%	-
grade 9	-	-	-	2%	-
grade 10	-	-	-	2%	-

Statistical analysis

Statistical analysis was performed using the Cox proportional hazards model (Cox, 1972; Cox, 1975). The following events ("endpoints") were considered:

Progression (N = 19) and

irrespective of the occurrence of progression:

Death caused by prostatic carcinoma (N = 16).

The BMDP-program 2 L was used (Dixon, 1983). The likelihood ratio (LR) test score was used for judging and comparing the predictive ability of each one of the 5 grading systems. The contribution of an explanatory variable to the likelihood function is supposed to represent its predictive ability (Green and Symons, 1983). This contribution is quantified by means of the likelihood ratio (LR) test score which has an asymptotical chi-square distribution with 1 degree of freedom. The contribution is significant (at the 5% level) if the LR test score exceeds the value 3.84. The five scores (of the 5 pathologists) of each grading system were summarized into one interval-scaled explanatory variable by taking the

average score. For the endpoint progression also the median score was considered and compared with the average score. For each grading system a separate analysis was done, with age at surgery always included as a confounding variable. For the endpoint progression also a stepwise analysis was performed, starting from a model including all 5 grading systems followed by stepwise elimination of insignificant grading methods. The purpose of this latter analysis was to investigate if two (or more) grading systems used simultaneously provide better prognostic information than one single system.

RESULTS

<u>Cox proportional hazard model and LR test scores for progression of prostatic carcinoma</u>

Patients' age (at time of surgery) and the average (respectively, the median) grading score were chosen as explanatory variables. The average (median) grading score was calculated for each patient and for each grading system as the arithmetic mean (as the median) of the scores of the 5 pathologists. Nineteen out of 50 patients developed progression (Figure 1). Time-to-progression was computed after date of surgery as zero-point. Progression was defined as local recurrence or metastastic disease irrespective of a possible lethal course. The estimated coefficients of the proportional hazard function and LR test scores are summarized in Table 2. Estimated coefficients for the average grading scores varied from 1.497 (Mostofi) to 0.327 (Gleason) and represent the estimated proportional increase in risk of progression to an increase of one score in the grading system applied. LR test scores ranged from 6.54 (Mostofi-Schroeder) to 1.79 (Gleason). As the latter values are used for judging the prognostic performance, our results indicate that the Mostofi-Schroeder system as well as the Broders system have the highest prognostic capability. The Gleason system reveals the poorest prognostic power.

An identical ordering in prognostic accuracy was obtained when median grading scores were used instead of the mean values (results are not tabulated).

The results of the stepwise procedure are presented in Table 3. It appeared that the Mostofi-Schroeder and the Broders systems (both mean- and median values) remained together in the model at the final step along with the forced presence of age at surgery. This result indicates that these two grading systems reflect different dimensions of grading prostatic carcinoma. From the estimated coefficients in Table 3 it can be concluded, that a weighted average of the Broders system and the Mostofi-Schroeder method might be the best prognostic tool for progression (with the Mostofi-Schroeder system weighted 1.5 times larger than the Broders method).

Table 2. Estimated proportional hazards model for progression (of prostatic carcinoma) and results of the chi-square likelihood ratio (LR) test per grading system (single regression analysis).

	coefficient	SE	LR test score
Mostofi-Schroeder:			
average grading-score over pathologists	1.145	0.474	6.54
age	0.058	0.031	
Broders:			
average grading-score over pathologists	0.822	0.309	6.32
age	0.038	0.032	
Anderson:			
average grading-score over pathologists	0.839	0.383	4.44
age	0.034	0.033	
Mostofi:			
average grading-score over pathologists	1.497	0.741	4.36
age	0.030	0.034	
Gleason:			
average grading-score over pathologists	0.327	0.240	1.79
age	0.054	0.032	

SE = standard error

Cox proportional hazard model and LR test scores for death from prostatic carcinoma.

Explanatory variables chosen for this statistical elaboration were identical to the ones described in the previous section. The endpoint considered here is time-to-death caused by prostatic carcinoma. Out of 50 patients, 16 patients died from the sequels of disseminated prostatic carcinoma (Figure 1). The estimated coefficients of the hazard function ranged from 0.999 (Mostofi) to 0.189 (Gleason). However, for all of the grading systems the prognostic performance of grading systems for death from prostatic carcinoma as reflected by the LR test score was poor and varied from 2.17 (Mostofi-Schroeder) to 0.59 (Gleason). Results of this analysis are shown in Table 4. Because of the similar results with the mean and median score obtained for the end-point progression, only the mean score was analyzed here.

Table 3. Final results of repeated inserting and withdrawing in the stepwise analysis for progression of prostatic cancer. Coefficients of the Cox proportional hazards model and the chi-square likelihood ratio (LR) test scores.

	coefficient	SE	LR test score
age	0.040	0.032	
Broders (median)	0.637	0.275	4.81
Mostofi-Schroeder (median)	1.157	0.400	9.19
age	0.041	0.032	
Broders (average)	0.649	0.318	3.82
Mostofi-Schroeder (average)	0.943	0.486	4.05

SE = standard error

Individual prognostic accuracy (including data of Mostofi) for progression of prostatic carcinoma.

For both the Mostofi system and the Mostofi-Schroeder system the scores assigned by Mostofi himself were available. In fact, the same slides of the 50 cases included in this study were evaluated by Mostofi. The individual prognostic accuracy for progression of prostatic carcinoma of 6 pathologists (Mostofi and pathologists A-E) was calculated for the Mostofi and Mostofi-Schroeder grading system (Table 5). Again, the LR-test was used for judgment of the prognostic performance.

In both grading systems a large inter-observer variation was noticed (Table 5). With reference to the Mostofi-Schroeder system 3 pathologists revealed statistical significance (Mostofi, pathologist A and pathologist B). LR test scores varied from 9.18 (pathologist B) to 0.18 (pathologist C). Using the Mostofi system statistical significance was only assessed for the grading data of Mostofi himself and pathologist B. The prognostic performance reflected by the LR test scores ranged from 6.26 (Mostofi) to 0.13 (pathologist D).

Table 4. Estimated proportional hazards model for death (caused by prostatic carcinoma) and results of the chi-square likelihood ratio (LR) test per grading system.

	coefficient	SE	LR test score
Broders:			
average grading-score over pathologists	0.436	0.294	2.01
age	0.051	0.036	
Anderson:			
average grading-score over pathologists	0.557	0.380	1.99
age	0.048	0.036	
Mostofi:			
average grading-score over pathologists	0.999	0.767	1.75
age	0.044	0.038	
Gleason:			
average grading-score over pathologists	0.189	0.245	0.59
age	0.061	0.035	
Mostofi-Schroeder:			
average grading-score over pathologists	0.672	0.465	2.17
age	0.059	0.034	

SE = standard error

DISCUSSION

In the present study the prognostic performance of 5 current grading systems was determined. Since an uniquely long period of follow-up after surgery of all patients was available, it was even possible to use time-to-death as a response.

In order to enable comparison of the prognostic accuracy of the grading systems the impact of the inter-observer variation on the various grading systems was eliminated by averaging the grading scores over the 5 pathologists. This might be debatable since a grading score is considered an ordinally-scaled covariate. Therefore, in the statistical analysis for progression of prostatic cancer also the median grading values (measured over 5 pathologists) were used. The dependency of the responses (recurrence or death by prostatic carcinoma) on these average (and median) grading scores was estimated by Cox proportional hazards model, while correcting

Table 5. Estimated proportional hazards model for progression and results of the chi-square likelihood ratio (LR) test for two grading systems (Mostofi and Mostofi-Schroeder) and 6 pathologists (including Mostofi).

	coefficient	SE	LR test score
Mostofi-Schroeder:			
grading-scores of Mostofi	0.668	0.310	4.89
age	0.087	0.034	
grading-scores of pathologist A	0.700	0.302	5.31
age	0.064	0.033	
grading-scores of pathologist B	0.847	0.288	9.18
age	0.057	0.030	
grading-scores of pathologist C	0.107	0.257	0.18
age	0.064	0.030	
grading-scores of pathologist D	0.395	0.316	1.58
age	0.052	0.031	
grading-scores of pathologist E	0.333	0.233	1.90
age	0.058	0.030	
Mostofi:			
grading-scores of Mostofi	1.147	0.456	6.26
age	0.066	0.031	
grading-scores of pathologist A	0.396	0.427	0.91
age	0.052	0.033	
grading-scores of pathologist B	0.924	0.457	4.37
age	0.049	0.030	
grading-scores of pathologist C	0.607	0.477	1.76
age	0.054	0.032	
grading-scores of pathologist D	0.219	0.617	0.13
age	0.060	0.033	
grading-scores of pathologist E	0.564	0.379	2.26
age	0.053	0.030	

SE = standard error

for age at surgery. This model allows one to relate multiplicatively the instantaneous risk of 'failure' (in this study progression or death by prostatic carcinoma) to a set of candidate covariates. Moreover, a stepwise analysis including initially six candidate covariates (age and 5 grading methods) was performed to study the influence of using two or more grading systems, simultaneously upon the prognostic performance. The antilog of the estimated coefficients represent the multiplicative increase in risk of progression or death related to one unit increase in the explanatory factor.

The chi-square likelihood ratio test score was used for judging and mutually comparing the prognostic performance of the grading systems.

In a previous report the interobserver variation in assignment of grading-scores to prostatic carcinomas of 50 patients was described (Ten Kate et al., 1986). As (dis)agreement score the kappa-value for each grading system was measured, but in none of the grading systems a high degree of consensus (kappa > 0.70) was reached as judged from the kappa-values (Ten Kate et al., 1986). However, the agreement among pathologists using the Broders and Anderson system was considered as fair to reasonable (kappa ranging from 0.40 to 0.70). The remaining grading systems showed a poor agreement among participating pathologists (kappa < 0.40).

When the results of this study presented in Table 2 and 4 are compared it becomes evident that histological grading can better predict the event of progression (Table 2) than the event of death by prostatic carcinoma (Table 4). This discrepancy could partly be attributed to the follow-up data of one patient who revealed progression 46 months after surgery, but lived afterwards for an extremely long period of 164 months before dying of the sequels of disseminated prostatic carcinoma. Therefore, we decided to use time-to-progression (Tables 2, 3) for the comparison of the prognostic performance of the different histological grading systems. From Cox regression it appeared that all but one grading systems were statistically significant; the Gleason system was the only one which was not significant (LR test score 1.79). The prognostic performance for the event of progression (Table 2) of the grading systems of Broders and Mostofi-Schroeder are superior to the other systems. The use of median values instead of average values did not result into a different ranking of grading systems referring their prognostic accuracy with respect to progression.

For the risk of death by prostatic carcinoma (Table 4), none of the grading systems reached statistical significance. Nevertheless, the trend reflected by the LR test scores for death is similar to that for progression (Table 2).

The stepwise analysis (performed for both average and median values) provided evidence that the Broders system and the Schroeder-Mostofi method used together, supplied additional prognostic information (Table 3), with the Mostofi-Schroeder method weighted 1.5 times larger than the Broders method. This might be explained by the fact that next to architectural features, that are exclusively used in the grading performance according to Broders, cytonuclear charac-

teristics such as encorporated in the Mostofi-Schroeder system, provide additional prognostic information.

Comparative studies on the prognostic impact of grading systems in prostatic carcinoma applied upon the same set of slides are scarcely found in literature. Comparison of these studies with ours is complicated by the use of different statistical techniques and diverse prognostic risk- or hazard functions. Mostofi (1976) reviewed grading systems for prostatic carcinoma introduced over a period of half a century (Broders 1926; Young 1926; Muir 1934; Kahler 1938; Shelley 1958; Evans 1942; Herbut 1952; Edwards 1953; Gleason 1966; Mobley 1968; Utz 1969; Hanash 1972; Vickery 1963; Corriere 1970; Rous 1972). He stated that according to the devisers they all promise to give the best prognostic value in the individual course of prostatic carcinoma. Nevertheless, reproducibility of all of these systems is poor.

In an extensive retrospective study in which multivariate analysis was used Schroeder et al. (1985abc) demonstrated that coexistence of several architectural patterns favours the prognosis as compared to tumours composed of only one prognostically bad histological pattern. In addition, nuclear and mitotic activity should also be implemented as these are important prognosticators to a grading system. Using these three parameters (gland formation; mitoses; and nuclear anaplasia) 5 different prognostic groups of patients can be identified.

Brawn introduced in 1982 (Brawn et al., 1982) a new grading system and compared the prognostic accuracy of his system (Anderson) with those described by Mostofi and Gleason in a group of 182 patients with locally advanced carcinoma (stage C) treated by radiotherapy. Follow-up of patients ranged from 3 to 7 years. Grading was performed upon transurethral specimens, needle biopsies or both. Survival-curves were obtained using the Kaplan-Meier statistical method. Statistical significance of survival curves by histologic grades was computed using the Wilcoxon method. Statistical significance was obtained for grade 1 and grade 4 lesions according the Anderson system.

The system of Gleason is probably most often applied and data about its prognostic potentials are frequently reported. Cantrell et al. (1981) studied the natural course of stage A (incidental) prostatic carcinoma in 117 patients followed for 2 to 15 years. Until relapse developed no therapeutic intervention had been performed. In 14 patients progression occurred. To obtain insight into factors that might predict clinically evident prostatic carcinoma at 4 years and/or predict a crude 4-year survival, a retrospective discriminant analysis using diverse variables was carried out. Of the variables chosen, grading scores of the Gleason system and extension of tumour at initial diagnosis revealed the highest prognostic impact. No patient with a low-grade lesion (Gleason score 2-4; n = 14) and only 2% of patients with less than 5% tumour-volume showed progression. Grading was performed on transurethral currettings or specimens obtained by simple enucleation.

Parfitt et al. (1983) studying stage A2 disease (incidentally found extensive carcinomas), reported that the Gleason grade had some predictive value. Of 16 patients with primary tumours with a Gleason score less than 4, none appeared to have lymphnode metastases on subsequent staging lymphadenectomy.

Kramer et al. (1980) also studied the predictive value of Gleason scores of primary tumours for nodal involvement at staging pelvic lymphadenectomy. In their group of 228 patients, irrespective of primary clinical stage, 93% of patients with Gleason sums more than 7 in initial biopsies showed evidence for lymphnode metastases. However, the sensitivity in this study was not as high since 31% of the patients (26 patients out of 84) with histologically proven lymphnode metastases revealed Gleason's sums ranging from 5 to 7. The latter might be attributed to a sample error resulting from the use of small tissue samples for grading. Especially when the Gleason system is applied on small tissue samples, it is possible that only one or two minor histological growth patterns are present, whereas a whole specimen contains often much more tumour patterns.

Results presented by Wilson et al. (1983) may also be confounded by grading inaccuracy resulting from sample error. They studied prospectively 115 fully staged (including pelvic lymphadenectomy) patients with localized prostatic carcinoma (stages A-C). Grading according to a modified Gleason system was carried out on needle-biopsies or transurethral specimens. Two of 6 low-grade (Gleason sum 2-4) lesions of 16 A2 tumours and 4 of 35 low-grade lesions of 53 B1 tumours revealed metastases during follow-up (ranging from 8 to 71 months). Wilson et al. (1983) postulate that low stage, low grade prostatic carcinoma can retain the potential for widespread metastatic disease. Even more interesting is their observation that 9 patients with high grade tumours and without evidence of metastases did not show any progression of their malignancy during follow up.

Guinan et al. (1983) compared the prognostic ability of 2 grading systems (Gleason and Broders) for the risk of recurrence in 111 patients with clinically localized carcinomas treated by radical surgery. Follow-up ranged from 6 months to 8 years. Statistical significance was computed by means of a chi-square test. Of the 2 systems studied the Gleason classification system was the least accurate.

Studies reviewed above do not give unequivocal information about which grading system reveals the highest prognostic power. Although most studies present relatively favourable results for the Gleason system, our data demonstrate a low interobserver accuracy (Ten Kate et al., 1986) and only a limited prognostic ability of the latter grading system (this study). The system of Mostofi also exhibits a large interobserver variation (Ten Kate et al., 1986). Nevertheless, compared with the Gleason system its prognostic accuracy of average (and median) grading scores for the occurrence of progression is relatively high. Our data indicate that less complicated systems as the ones described by Broders and Brawn (Anderson) reveal not only the highest interobserver reprodu-

cibility (Ten Kate et al., 1986), but also a high prognostic impact. Though inter-individual agreement was low, as reflected in the present study (Table 5), data obtained on the recently introduced system of Mostofi-Schroeder also seem promising. The finding of additional prognostic information provided by the use of more than one histological grading system upon the same tumour is of utmost interest and necessitates further investigation using larger series and other combinations of grading systems.

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CHAPTER IV

CHARACTERIZATION OF MONOCLONAL ANTIBODIES RAISED AGAINST THE PROSTATIC CANCER CELL LINE PC-82.

M.P.W. Gallee[1], C.C.J. van Vroonhoven[1],
J.A.G.M. van der Korput[1], Th.H. van der Kwast[1],
F.J.W. ten Kate[1], J.C. Romijn[2], J. Trapman[1].

Department of Pathology (1) and Urology (2) of the Erasmus University Rotterdam, The Netherlands.

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SUMMARY

For production of monoclonal antibodies (McAbs), hybrid cells were prepared by fusion of spleen cells of BALB/c mice immunized with the human prostatic cancer cell line PC-82 and the P_3-X6_3Ag8.653 murine myeloma cell line. Supernatants of approximately 500 hybrid clones were screened for prostate specific antibodies using an ELISA on PC-82 cells. A selection of antibodies was further tested for their specificity on a large series of different tissues. A broad cross reactivity pattern was obtained. Most cross reactivity was with pancreatic tissue, kidney, and bowel. One antibody turned out to react with prostate stromal cells. Two McAbs (ER-Pr 1 and ER-Pr 2) reacted solely with prostatic epithelium. Monoclonal antibody affinity chromatography combined with SDS-PAGE showed that both antibodies were directed against a 35-kD protein. Immunoblotting revealed that this protein is identical to prostatic antigen (PA). The epitope detected by ER-Pr 1 and ER-Pr 2 was largely preserved after formalin-fixation of prostatic tissues which renders these antibodies very suitable for routine examination of tissue sections.

INTRODUCTION

On the basis of the formation of gland-like structures, most tumors of the prostate are known as adenocarcinomas. Various grading systems, based on histological and cytological criteria, all trying to predict the biological behavior of the tumor, have been put forward (1-3). In addition to morphological data, the use of immunological methods can be of value for further characterization of human tissues of unknown origin and as differentiation markers (4-6).

Knowledge of prostatic tumors and their characteristics has increased by the isolation of several prostatic cancer cell lines derived from primary or secondary human tumor deposits (7-10). From these, the PC-82 cell line is one of the best characterized (8,11,12). It can be propagated in nude mice and its growth is androgen dependent (13,14). It has also been shown to contain prostatic acid phosphatase (PAP) (8,14). The solid tumor shows prostate carcinoma morphology, i.e. a predominantly cribriform growth pattern. The stromal component and vascularization are of murine origin (8,11). In this study we describe the generation and properties of monoclonal antibodies (McAbs) against this cell line.

MATERIALS AND METHODS

Cell line

In 1977 the PC-82 cell line was isolated from a primary tumor of the prostate and since then has been grown subcutaneously in homozygous male nude mice. Its characteristics have been well documented (8,11-14).

Immunization and cell fusion

Male BALB/c nu/+ mice were immunized subcutaneously with 2 to 5 x 10^6 PC-82 cells at three-week intervals. PC-82 cell suspensions were prepared from chopped solid tumor fragments that were incubated overnight in tissue culture medium supplemented with collagenase (200 U/ml). Prior to immunization, PC-82 cells were washed and resuspended in RPMI 1640 (Flow Labs, Irvine, UK) at a density of at least 8 x 10^6 cells/ml. Three days before removal of the spleen, a final combined intravenous/intraperitoneal booster was given.

For preparation of hybrid cells, spleen cells of immunized animals were fused to the P_3-$X6_3$Ag8.653 mouse myeloma cell line at a ratio of 5:1. The method used was essentially identical to that of Frankel et al. (15). Immediately after fusion, the cell suspension was seeded into 96-well tissue culture plates containing a macrophage feeder layer and grown in selective medium (HAT). Supernatants of large clones were scored for the presence of antibodies to PC-82 cells.

Enzyme linked immunosorbent assay

Terasaki plates were coated with PC-82 cells and human lymphocytes at a concentration of 5 x 10^4 cells/well. Cells were fixed by incubation in glutaraldehyde (0.1%) for 15 min. An indirect ELISA was performed utilizing a horseradish peroxidase conjugated rabbit anti-mouse serum (DAKO) as the second reagent. O-phenyl diaminedihydrochloride (Eastman KODAK, Rochester, NY) (0.2% in 0.1 M phosphate buffer, pH 6.0) activated by 0.03% H_2O_2 was used as a substrate for the peroxidase enzyme (16,17).

Immunodiffusion

Subclasses of the mouse antibodies were determined by standard agar gel diffusion(Ouchterlony). Specific antisera directed against the various subclasses were purchased from Nordic.

Human tissues

Prostatic tissue was obtained from various sources. Fresh tissue was collected from transurethral specimens obtained from clinically diagnosed benign prostatic hyperplasia (BPH) patients. In addition, samples of radical prostatectomy specimens were snap frozen and stored in liquid nitrogen. Tissues were used for preparation of BPH cell lysates and for immunohistochemical assays on cryostat sections. Paraffin-embedded tissues from prostatic carcinoma, BPH (transurethral, biopsy, and prostatectomy samples), and from a large series of different control tissues were used for the immunoperoxidase assay on formalin-fixed sections.

Immunohistochemial techniques

An indirect immunoperoxidase assay was carried out on acetone fixed 5 μ thick cryostat tissue sections using peroxidase conjugated rabbit anti-mouse antibodies (DAKO) as the second reagent. Diamino benzidine (0.05% in 0.2 M Tris-HCl, pH 7.0) was used as the enzyme substrate. Tissue sections were counterstained with hematoxylin. A similar technique was employed for formalin-fixed sections (8). In some experiments, slides were preincubated with pronase (0.1% in phosphate-buffered saline, PBS) for 7 min at room temperature (19,20). Rabbit polyclonal antibodies directed against PAP and PA were obtained from Immulok.

Preparation of cell lysate

For preparation of a lysate, BPH tissue (approximately 0.5 g) was minced into small pieces and suspended into 2 ml Tris-HCl buffer (0.01 M, pH 7.5) containing 0.15 M NaCl, 0.05% Triton X-100 and 1 mM phenylmethyl sulfonyl fluoride (PMSF). Cells were further disrupted by pottering and sonication (small probe, 30 sec, max speed, Branson B 12 sonifier). The lysate was clarified by centrifugation (15 min 12,000 g). Protein concentration of the supernatant was approximately 8 mg/ml as measured in the Biorad protein assay (BSA standardized).

Monoclonal antibody affinity chromatography

Protein in 1 ml of ascites of hybridoma bearing BALB/c mice was bound to 1 g CNBr-activated Sepharose 4B using conditions described by the supplier. A 3-ml column was poured and 0.5 ml of BPH lysate analyzed over this column. Elution of this affinity column was at 1.5 ml/h and 4°C in PBS containing 1 mM PMSF; 1-ml fractions were collected. After six fractions, the velocity was increased to 10 ml/h. After two hours, bound proteins were eluted by 3 M KSCN in phosphate buffer (pH 7.6, 1 mM PMSF). Protein containing fractions were pooled and dialyzed overnight against PBS at 4°C. The purified protein was concentrated 25-fold on an Amicon centricon-10 filter.

Electrophoresis

Fractions of a monoclonal antibody affinity column were further analyzed by SDS-polyacrylamide electrophoresis according to Laemmli (21). (Concentrated) column samples of 20 μl were mixed with 5 μl of sample buffer and boiled for 5 min, followed by electrophoresis on a 10% gel for 3½ hours at 17 mA. Gels were stained with Coomassie Blue using standard procedures.

Immunoblotting

Proteins were transferred from a gel to nitrocellulose sheets (22). After transfer, filters were washed and incubated with the appropriate mouse monoclonal antibody, polyclonal rabbit PA, or PAP antiserum (22,23). If mouse antibodies were used, filters were further incubated in a rabbit anti-mouse Ig antibody solution. Next, nitrocellulose sheets were incubated for 30 min at room temperature in alkaline phosphatase conjugated goat antirabbit IgG antibody solution (Tago). After washing, the filters were developed in a 0.2 M Tris-HCl buffer, pH 9.1, containing 10 mM $MgCl_2$ and supplemented with 4-amino-diphenylaminediazonium sulfate (0.3%, Sigma, St. Louis, MO) and Naphthol AS-MX phosphate disodium salt (0.1 g/100 ml, Sigma, St. Louis, MO). Incubation was for 5-10 min at room temperature.

RESULTS

Generation of monoclonal antibodies

Hybrid cells were obtained by fusion of spleen cells of BALB/c mice immunized with PC-82 cells with mouse myeloma cell line P_3-$X6_3$Ag8.653. Growth of hybrid cells was obtained in about 500 microtiter plate wells. Supernatants of the cultures were analyzed for their reactivity to PC-82 cells; human lymphocytes were used as control cells. In this initial screening, approximately 25% of the supernatants were found to contain antibodies positive to PC-82 cells and negative to the control cells. After rescreening by ELISA, supernatants of 54 stable cell lines were tested for their specificity using the immunoperoxidase assay on frozen tissue section of human prostate cancer, brain, and spleen. Thirty four samples which either reacted with all of these different tissues or did not react at all were discarded (10 of these preparations detected nuclear associated antigens). The remaining supernatants were further analyzed for reactivity with BPH, bowel, pancreas, liver, kidney, and skin, and were rescreened against prostate cancer, spleen, and brain tissue. The reaction pattern obtained is summarized in Table 1. Most cross reactivity in this final panel of McAbs was found with pancreas (this includes both ductal epithelial lining and exocrine acini), kidney (almost exclusively in tubuli), and bowel tissue. A positive reaction to liver was found predominantly in hepatocytes (diffuse or focal) or in the epithelial cells lining the bile ducts; positive reactions were also seen in sweat- and sebaceous glands, epidermis and in brain capillary walls.

McAbs ER-Pr 1, 2, 6, and 7 showed a very limited and/or highly specific cross reactivity pattern (Table 2). McAb ER-Pr 6 reacted with a fraction of stromal cells in the prostate and with smooth muscle cells in the large bowel (Fig. 1). This antibody also reacted with the stromal component of PC-82 tumor grown in nude mice, a surprise finding since the stroma is of murine origin. Some light

Table 1. Reactivity patterns of McAb's raised against PC-82 cells tested on frozen sections of different human tissues.

Tissue	No. tested	Percentage of positive
BPH	20	90
Prostate carcinoma	17	90
Pancreas	13	68
Kidney	15	60
Bowel	19	55
Skin	11	45
Liver	13	40
Brain	17	30
Spleen	17	25

Table 3. Reactivity of McAb Er-PR 1 against formalin-fixed and frozen sections of different human tissues.

	N	Positive (%)	Positive after pronase treatment (%)
BPH (fr)	11	11 (100)	ND
Prostate carc. (fr)	10	10 (100)	ND
BPH (ff)	16	13 (81)	13 (81)
Prostate carc.(ff)	18	11 (61)	14 (78)
Parotid gland (ff)	2	0 (0)	ND
Ureter (ff)	3	0 (0)	ND
Testis (ff)	1	0 (0)	ND
Seminal vesicle (ff)	2	0 (0)	ND
Stomach carc. (ff)	19	0 (0)	0 (0)
Colon carc. (ff)	11	0 (0)	0 (0)
Kidney carc. (ff)	12	0 (0)	0 (0)
Pancreas carc. (ff)	10	0 (0)	0 (0)
Lung carc. (ff)	10	0 (0)	0 (0)
Bladder carc. (ff)	8	0 (0)	0 (0)
Mammary carc. (ff)	19	0 (0)	0 (0)

fr = frozen sections; ff = formalin-fixed; ND = not done

Table 2. Specificity of four monoclonal antibodies, raised against PC-82 cells, determined by an immunoperoxidase assay on frozen tissue sections.

	Prostate BPH	Prostate carcinoma	Bowel	Pancreas	Kidney	Brain	Liver	Skin	Spleen
ER-Pr 1	+	+	-	-	-	-	-	-	-
ER-Pr 2	+	+	-	-	-	-	-	-	-
ER-Pr 6	+s	+s	+m	-	-	-	-	-	-
ER-Pr 7	+	+	+ga	+d,a	+gl	-	+p	+sg	-

s = stroma; m = muscularis; ga = ganglion cells; d = excretory ducts; a = exocrine acini; gl = glomeruli; p = parenchyma; sg = sebaceous glands

staining was also found in the PC-82 epithelial cells. In addition to the reaction to prostate, ER-Pr 7 was the only antibody of the entire series found to be positive to glomerular cells and large bowel ganglion cells (Fig. 2), indicating detection of an antigen shared by very diverse, but specific cell types.

McAbs ER-Pr 1 and ER-Pr 2 reacted with epithelial acinar cells of the prostate (Fig. 3). All other tissues tested, including salivary gland, bladder, ureter, and mammary gland, were negative. Because of their exclusive prostate specific reaction pattern, these antibodies were investigated in more detail. Experiments on the further characterization of ER-Pr 6 and 7 are in progress.

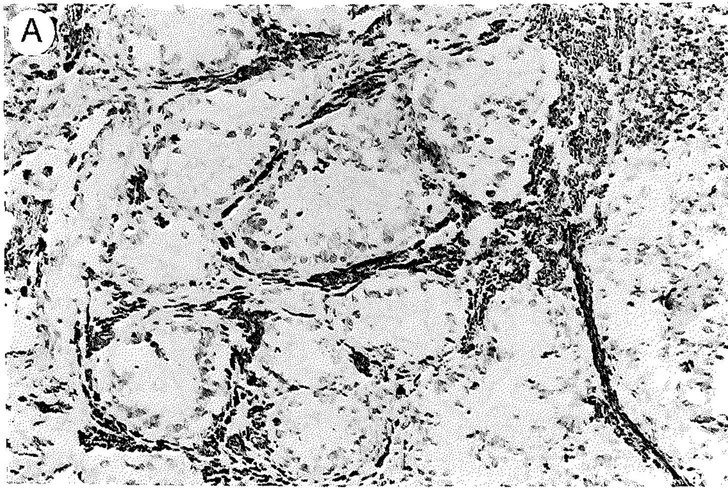
Characterization of ER-Pr 1 and ER-Pr 2

As measured in an Ouchterlony assay, both ER-Pr 1 and ER-Pr 2 were found to be IgG1 antibodies. The corresponding antigen was purified from a lysate of human BPH tissue by McAb affinity chromatography and further analyzed by SDS-PAGE. The results of this experiment are illustrated in Fig. 4. The electrophoresis pattern shows the presence of two major bands in the fraction eluted at high salt concentration. After reanalysis of this sample over an ER-Pr 1 column, most of the high molecular weight material was found in the flow through fraction, whereas the low molecular weight band (MW 35 kD) was retained in the high salt wash. In some experiments, an additional 25-kD component was observed in the high salt fraction. An essentially identical pattern, as shown for ER-Pr 1, was found for an ER-Pr 2 column.

For further characterization, the purified protein was analyzed by immunoblotting using ER-Pr 1, ER-Pr 2, and polyclonal PAP and PA antibodies. As demonstrated in Fig. 5, the 35-kD band is stained by ER-Pr 1 proving that this McAb is directed against this protein. Furthermore, incubation of filters with PAP (data not shown) and PA antibodies clearly demonstrated that the antigen against which ER-Pr 1 (and ER-Pr 2, data not shown) is directed is identical to PA.

Properties of ER-Pr 1 and ER-Pr 2 on frozen and formalin fixed tissue sections

To investigate in more detail the properties of ER-Pr 1 and 2, a series of 23 different prostate carcinomas and BPH tissue samples, all freshly frozen and obtained at autopsy or from surgery, were further tested by immunoperoxidase staining. Results are summarized in Table 3. All hyperplasia and carcinoma tissue sections investigated were stained by ER-Pr 1, ER-Pr 2, and polyclonal PA antibody. In contrast to the polyclonal antibody, ER-Pr 1 and ER-Pr 2 varied in intensity and in the number of epithelial acinar cells stained. These differences concern both the inner and outer cell layer of the acini. The most intense staining was in the cytoplasm, but positive reactions were also noted in the lumina of the acini.



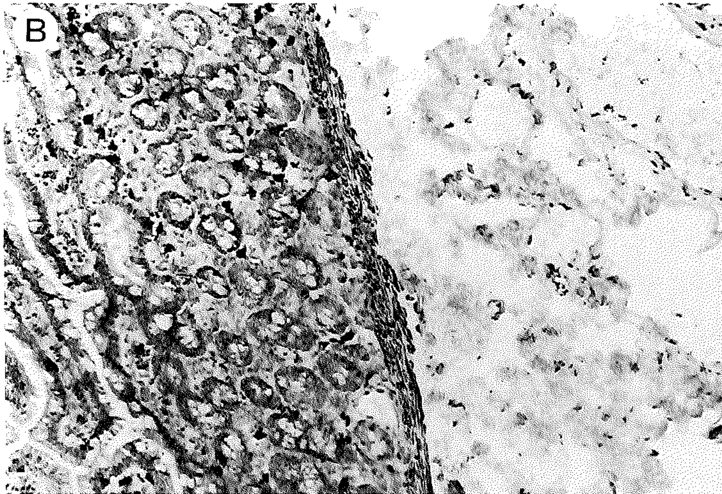
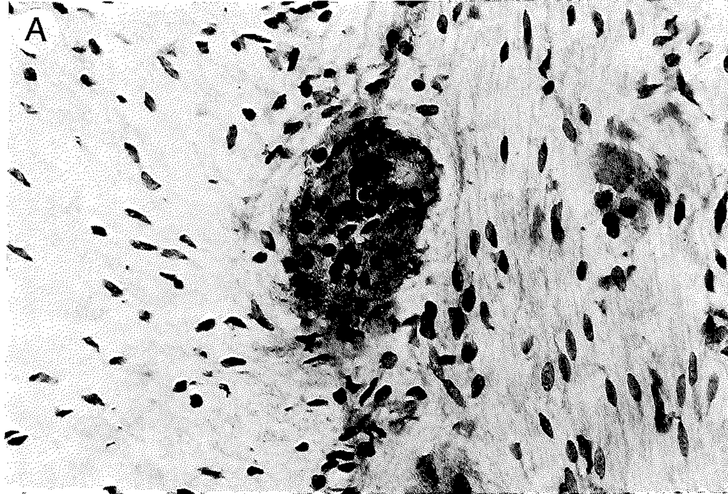


Figure 1:
(A) Immunoperoxidase staining of fibromuscular cells in the interacinar stroma of the prostate using McAb ER-Pr 6 (x150).
(B) Staining of the muscularis mucosae by McAb ER-Pr 6 (x60). The staining of eosinophilic granulocytes in the lamina propria is caused by endogenous peroxidase activity.



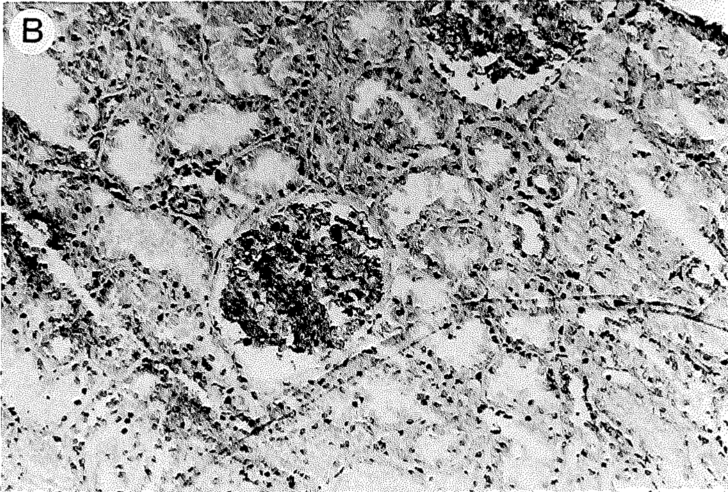


Figure 2:
(A) Immunoperoxidase staining of ganglion cells in the myenteric plexus by ER-Pr 7 (x380). (B) Staining of glomerular cells in the kidney by McAb ER-Pr 7 (x60).

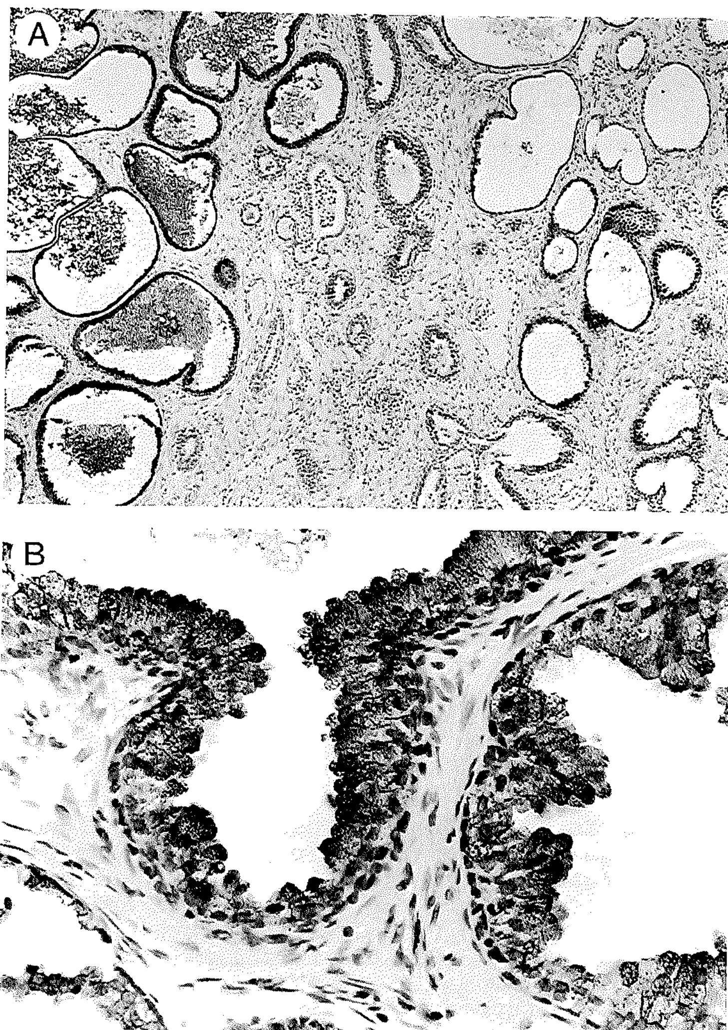


Figure 3:
(A) Immunoperoxidase staining by ER-Pr 1 of the prostate (x60). Notice variation in staining of cells lining prostatic acini. (B) Granular staining in cytoplasm of inner and outer cell layer of prostatic glands by ER-Pr 1 (x380).

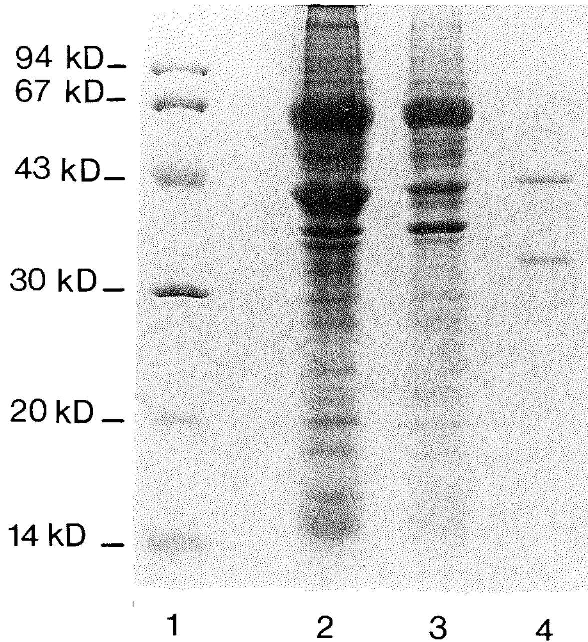


Figure 4:
SDS-PAGE analysis of a BPH cell lysate separated on an ER-PR 1 affinity column. Experimental details are described in Materials and Methods. Lane (1) marker proteins; (2) original lysate; (3) flow through fraction; (4) high salt wash fraction.

In addition to the frozen tissues, both antibodies were tested on a large series of formalin-fixed samples of prostate and control organs. Both normal and malignant counterparts were incubated (Table 3). The negative data obtained on non-prostate tissue confirm the results obtained on frozen tissues. In experiments with the formalin-fixed tissues, most of the BPH (81%) and 61% of carcinomas were stained. If tissue slides were pretreated by pronase for a short time period, the percentage of positive carcinomas increased to 78%. The data indicate that the epitope of PA detected by ER-Pr 1 is slightly sensitive to the fixative treatment. Moreover the results suggest that the PA concentration is higher in BPH than in carcinoma tissue, but additional experiments are necessary to substantiate this finding. Absence of positive reactions in a few biopsy samples could be explained by the absence of those acini with the antigenic determinant of ER-Pr 1 antibody, and was only observed in small biopsies with a limited number of tubular structures.

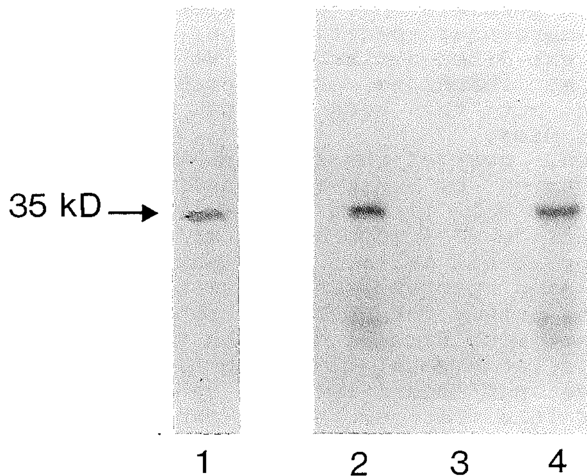


Figure 5:
Immunoblotting of BPH proteins separated by ER-Pr 1 antibody affinity chromatography. Details are described in Materials and Methods. Lane (1), high salt wash fraction incubated with ER-Pr 1; lanes 2, 3, and 4 are incubated with polyclonal PA antibody. Lane (2) high salt wash of ER-Pr 1 column; lane (3) flow through fraction; lane (4) original BPH cell lysate.

Data obtained so far show that ER-Pr 1 and ER-Pr 2 detect the same antigenic site of PA. Since ER-Pr 2 always shows some background staining to stromal components of the prostate, we believe that ER-Pr 1 and ER-Pr 2, although closely related, are not completely identical to each other.

DISCUSSION

In this study the properties of McAbs raised against the PC-82 prostatic carcinoma cell line are presented. The suitability of this cell line for generation of prostatic antibodies is based on certain of its features that are akin to differentiated human prostate carcinoma, including its histological appearance, androgen dependence for proliferation, and the presence of PAP. The isolation of antibodies against PA, as described herein, implies the presence of this prostate specific marker in PC-82 tumors.

In our panel of antibodies, we were unable to detect antibodies which were specific to carcinoma and which were less active or not active at all against BPH or normal prostate tissue. Several carcinoma specific antibodies have been described in sets of McAbs raised against the PC-3, Du-145 or

LNCaP prostatic cell lines (24-29). None of these antibodies was prostate specific. It still has to be seen whether these McAbs will be of value for characterization of patient tissue samples. It is possible that our series of antibodies was too small to find carcinoma specific McAbs, however, in additional experiments, not discussed herein, we also failed to isolate such antibodies. Therefore, it is possible that the PC-82 cell line is not the best source for raising carcinoma specific antibodies.

As expected, most of the antigens detected by the set of McAbs were shared by other cells originating from different malignant or normal tissues. Cellular components of the pancreas often showed immunologic similarity to the antigenic phenotype of prostatic cells. Four antibodies which showed striking staining patterns were described in greater detail. As found in this study for ER-Pr 6, two antibodies previously described react with stromal cells in the prostate and smooth muscle cells in large bowel tissue sections (30). Because the antibodies have not been further characterized, a direct comparison of the various McAbs cannot be made. Antibody ER-Pr 7 showed an unique cross reactivity pattern not previously described for an antibody raised against prostate tissue.

From all McAbs screened, two (ER-Pr 1 and ER-Pr 2) were selected for their complete prostatic specificity as demonstrated on frozen and formalin-fixed tissue sections. Characterization of the corresponding antigen provided evidence that the antibodies were directed against PA. In a recent experiment we also generated a McAb against PAP using PC-82 cells for immunization (data not shown). Prostatic antigen, which so far may be the only prostate specific marker besides PAP, was first described by Wang et al. (31). These authors also raised polyclonal and monoclonal antibodies against PA (15,32). Distinct from the previously described McAbs (15), ER-Pr 1 and ER-Pr 2 showed no cross reactivity to renal tubular epithelium nor to renal adenocarcinoma cells. Also, in contrast to previous findings (15), frozen sections of prostate tissue contained at least equal amounts of immunologically active PA reacting to ER-Pr 1 and ER-Pr 2 as compared to formalin-fixed tissue sections. ER-Pr 1 and ER-Pr 2 are also different from McAb F5, which is directed against PA and is prostate specific (33). This antibody recognizes over 95% of all acini. ER-Pr 1 and 2 react with 70% or less of the acini.

In comparison to the commercially available polyclonal antibodies against PA, the sensitivity of ER-Pr 1 and ER-Pr 2 was essentially equal. As mentioned above, differences in staining of the epithelium of adjacent acini were observed and therefore will be a minor disadvantage in utilizing McAbs in the immunoperoxidase assay on tissue sections. PA can be detected in sera from patients in more advanced stages of prostate cancer and can be used for monitoring (34,35). A panel of different McAbs, which probably will be available soon, could be useful for increasing the sensitivity of the assay.

ACKNOWLEDGMENTS

The authors are indebted to Dr. A.W.M. Rijnders for fruitful discussion and to Dr. G.J. van Steenbrugge for PC-82 tumor tissue. They also wish to thank Miss H.C.J.M. Geerts for technical assistance, Miss P.C. Delfos for skilful photographic assistance and Miss M. Hanegraaff for preparation of this manuscript.

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CHAPTER V

VARIATION OF PROSTATE-SPECIFIC ANTIGEN EXPRESSION IN DIFFERENT TUMOUR GROWTH PATTERNS PRESENT IN PROSTATECTOMY SPECIMENS.

M.P.W. Gallee[1], E. Visser-de Jong[1], J.A.G.M. van der Korput[1],
Th.H. van der Kwast[1], F.J.W. ten Kate[1],
F.H.Schroeder[2], J. Trapman[1]

Department of Pathology (1) and Urology (2) of the Erasmus University Rotterdam, The Netherlands.

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SUMMARY

A series of 55 randomly chosen radical prostatectomy specimens was analyzed for expression of prostate-specific antigen (PSA) by immunohistochemical techniques. Tissue sections were selected in such a manner that in addition to glandular benign prostatic hyperplasia (BPH), one or more different prostatic tumour growth patterns were present. Four monoclonal antibodies, directed against three different PSA epitopes, and one polyclonal anti-PSA antiserum were used. Expression of PSA was compared with that of prostate-specific acid phosphatase (PAP), recognized by two different polyclonal antisera. A critical dilution aimed at a maximum of staining intensity on BPH tissue sections was chosen for all antibodies. Anti-PSA and anti-PAP antisera stained essentially all BPH samples (over 90%). Irrespective of the nature of the antibodies used, PSA expression was found to be decreased in prostatic carcinoma. A clear cut relationship was found between immunoreactivity for PSA and the degree of differentiation of the tumour area. Under the experimental conditions used the PSA monoclonal antibodies stained only 1 out of 10 undifferentiated carcinomas, whereas 50% to 70% of the well- and moderately-differentiated carcinomas showed immunoreactivity. This correlation was less pronounced with the PAP staining pattern. If the PSA antibody titer was raised the percentage of clearly staining undifferentiated carcinomas could be considerably increased (up to 60%-100%), indicating that PSA expression is not absent, but lowered in most (if not all) undifferentiated carcinomas.

INTRODUCTION

Prostate-specific antigen (PSA) was first described by Wang and co-workers (21,28,29). It belongs to a family of kallikrein-like serine proteases and has a molecular weight of 33-35 kD (3, 30). Recently, the primary structure of the PSA protein obtained by direct sequencing or deduced from the cDNA sequence has been elucidated (18,24,30). PSA is exclusively secreted by the epithelial cells of the prostatic gland. Because of its tissue-specific expression pattern, PSA became of interest as being a prostate-specific (tumour) marker. Availability of specific polyclonal and monoclonal antibodies also made its immunohistochemical detection in neoplastic and non-neoplastic prostatic tissue possible (6,11,12,22). In addition, it turned out that the presence of PSA in sera of patients could be used for monitoring the natural course of prostatic cancer and to evaluate the success or failure of therapeutic (often endocrine) regimens (27).

Especially because of the general acceptance of PSA as being the most reliable and sensitive prostate (tumour) marker in serological assays, detailed analysis of PSA expression in prostatic tumours is of high importance. Several studies have been presented aiming to gain a better insight into the specificity and sensitivity of PSA expression in prostatic tumours (1,2,5,7,8,9,10,16,23,26). To extend our knowledge

further in this regard we investigated PSA expression in tissue sections derived from radical prostatectomy preparations. Prostatectomy was performed for localized prostatic carcinoma. Prostatic carcinomas are often composed of different growth patterns within a single tumour. This provided an ideal opportunity to compare in detail the immunohistochemical levels of PSA with prostatic tumour differentiation status. Since no consensus has been reached concerning the most suited histological grading system for prostatic cancer, immunohistochemical results were not correlated with grading scores. In addition to PSA expression we also studied prostate-specific acid phosphatase (PAP) expression in this material.

MATERIALS AND METHODS

Prostatic tissue

Prostatic tissues used in this study were obtained from prostatectomy specimens. Fifty-five patients (randomly selected from a much larger series) with clinically staged localized prostatic carcinoma (T1, T2 and T3) were treated by radical prostatectomy in the years 1980-1987 at the Department of Urology of Erasmus University Rotterdam. Patients did not receive any therapy prior to surgery.

All immunohistochemical assays were performed on surgical prostatectomy specimens. These specimens were routinely fixed in 10% buffered formalin, sectioned perpendicular to the prostatic urethra and submitted completely for histological examination. Tissues were routinely handled and stained with haematoxilin-azofloxin. The number of sections per specimen varied from 24 to 43. Of each specimen two slides were selected in which equally distributed glandular benign prostatic hyperplasia (BPH) and tumour areas were present. In all slides different subtypes of prostatic carcinoma based predominantly upon histological features could be distinguished. Apart from well-, moderately-, and poorly-differentiated adenocarcinomas, a cribriform growth pattern, and an undifferentiated (diffusely infiltrating variant without acinar formations) subtype were present in our material. In addition, a growth variant characterized by solid undifferentiated tumour areas (medullary growth pattern) and the so-called hypernephroid or clear cell type (showing resemblance with adenocarcinoma renis) were discerned (see Table 1). Tumour patterns with typical endometrioid features were not present in our material.

Primary Antibodies

PSA specific antibodies:

PSA purified from extracts of BPH tissue by affinity chromatography using the PSA-specific monoclonal antibody ER-Pr 1 (12) was applied to generate mouse monoclonal antibodies and a rabbit polyclonal antiserum (PSA(pc)) using standard procedures. Details will be published elsewhere. In short,

Table 1. Distribution of different histological patterns of prostatic carcinoma and benign prostatic hyperplasia in prostatectomy specimens obtained from 55 patients with localized carcinoma.

	N
well differentiated adenocarcinoma (WDA)	39
moderately differentiated adenocarcinoma (MDA)	43
poorly differentiated adenocarcinoma (PDA)	16
undifferentiated carcinoma (UC)	10
clear cell carcinoma (CCC)	12
solid tumour areas (STA)	8
cribriform carcinoma (CC)	19
benign prostatic hyperplasia (BPH)	55

specificity of the antibodies was checked by immuno-(Western-) blotting and immunohistochemistry using extracts and tissue sections of malignant and non-malignant human tissues from different origins. Three monoclonal antibodies (ER-Pr 8, 12 and 27) which showed the highest reactivity with formalin-fixed paraffin-embedded prostate tissue were analyzed in detail. All antibodies were reactive with a 33-35 kD protein, recognized as PSA. Monoclonal antibodies were of the IgG1 subclass. Ascites batches with high concentrations of monoclonal antibodies were used for further studies. The four monoclonal antibodies defined three unique antigenic determinants of PSA as established by competition ELISA assays. ER-Pr 8 and ER-Pr 27 were directed against the same PSA-epitope. Antibodies were titrated for optimalization of the most suitable dilution for immunohistochemistry. Dilutions of all antibodies were selected in such a manner that further lowering of the concentration resulted in a decrease of staining intensity of BPH control sections.

PAP specific antibodies:

A commercially available rabbit polyclonal antibody directed against PAP was obtained from Cambridge Research Laboratory, Cambridge, Massachusetts (Com-PAP(pc)). Additionally, a rabbit polyclonal antiserum (PAP(pc)) was prepared in our own laboratory. PAP was purified from BPH tissue by affinity chromatography using a PAP-specific McAb, which was able to bind PAP but did not recognize PAP in formalin-fixed tissue. Specificity of this polyclonal antiserum was assessed by means of the Western blot technique and immunohistochemistry. As cross-reactivity existed at immunohistochemical level, the rabbit anti-PAP serum was extensively adsorbed with acetone-dried human pancreatic and kidney tissue powder. The resulting purified antiserum (PAP(pc)) showed no reactivity with pancreatic island cells, kidney tubules, mast cells or granulocytes.

As described above for the PSA antibodies, PAP antibodies were used in such a dilution that further lowering of the concentration resulted into a decrease of staining intensity of BPH control tissue.

Immunohistochemistry:

Consecutive paraffin sections were cut at 5 μm thickness. After rehydration of the deparaffinized sections, the sections were treated for 20 min with absolute methyl alcohol, containing 0.3% H_2O_2 to block endogenous peroxidase activity. Subsequently, sections were rinsed in PBS. Primary antibodies were applied for 1 hr at 37°C. Subsequently, slides were incubated with peroxidase conjugated antisera (P-rabbit anti-mouse or P-conjugated swine anti-rabbit; Dakopatts Denmark) for 1 hr at room temperature. Peroxidase activity was visualized by incubating the sections in Tris-HCl buffer (0.05 M, pH 7.4) containing 0.0125% 3,3'-diamino benzidine tetrahydrochloride (Fluka AG, Switserland) activated by 0.03% H_2O_2 (10 min at room temperature). In all cases a brief nuclear counter stain with Mayer's haematoxilin was performed. Finally, sections were dehydrated, mounted in malinol and examined microscopically. Appropriate positive (BPH) and negative control slides were processed in an identical manner. Intra-epithelial staining intensities were judged subjectively as 0 (absent), 1 (faint), 2 (moderate), or 3 (strong). For purpose of presentation 0 and 1 were considered as negative, 2 and 3 as positive results. Since heterogeneity (variation in number of tumour cells that stain positively) even within the same histological subtype was often present, only areas with the highest staining intensity were judged in both BPH and prostatic carcinoma.

RESULTS

Histology of prostatectomy specimens

It is well established that prostate tumours are very heterogeneous. In the prostatectomy specimens investigated in this study, adjacent to prostatic carcinoma, areas of proliferative benign epithelial glandular structures (glandular-BPH) were present in all sections. In some areas groups of hyperplastic glands were arranged in nodular formations. In the 55 cases examined, tumour heterogeneity was reflected by the large number of different growth patterns present in each sample. Although the subdivision of growth characteristics of prostatic carcinoma such as represented in Table 1 is somewhat arbitrary, in our set of tumours the whole range of histological subtypes in nearly every combination was detectable and varied from only 1 histological carcinoma type in 4 cases to 5 different patterns in 3 specimens. Adenocarcinomas with gland formation, irrespective their degree of differentiation, were most frequently observed. Tumours consisting of glandular structures were subdivided into well-, moderately- and poorly-differentiated carcinomas based upon diameter of

tumour glands and their resemblance to normal prostatic glandular structures. A cribriform growth pattern was considered as a separate entity. A total lack of glandular formations in tumour tissue was only found in 5 specimens.

Expression of PSA in various prostate tumour growth patterns and BPH present in prostatectomy specimens.

The set of 55 prostatectomy preparations was analyzed for expression of PSA by immunohistochemical techniques using four different monoclonal antibodies directed against three different epitopes and a polyclonal antiserum (PSA(pc)) (see Materials and Methods). In BPH tissue sections some heterogeneity in immunostaining patterns was observed with all antibody preparations used. This heterogeneity was most prominent when monoclonal antibody ER-Pr 1 was applied. Heterogeneity was rarely detected in epithelial cells lining the same gland, but was observed as a variation of staining of different glands. The heterogeneity observed with monoclonal antibody ER-Pr 1 could partially be due to a less stable appearance of the PSA antigenic site recognized by this antibody. In spite of the presence of some unstained glands, essentially all (91%-98%) BPH specimens examined were clearly stained with three out of four monoclonal antibodies and the polyclonal antiserum against PSA. ER-Pr 1 gave somewhat lower figures (Table 2 and Figure 1).

We examined PSA expression in prostatic carcinoma in the same tissue slides as used for the study of PSA in BPH. Without subdividing the tumour regions into different growth patterns, the immunostaining for PSA was scored between 64% and 75% of the sections examined (Table 2). Similarly as noted in BPH tissue sections, ER-Pr 1 stained the smallest number of slides (35 out of 55), whereas the polyclonal antiserum (PSA(pc)) and the monoclonal antibody ER-Pr 27 showed the highest percentage of positives (75%). Figure 2 depicts four examples of lowered or even absent PSA staining in carcinoma areas as compared to the internal BPH control. If PSA expression was compared for the various tumour growth patterns as summarized in Table 1 an interesting distribution could be observed (Figure 1). Fifty to 70% of those tumour areas that consisted of glandular structures and designated as well- or moderately-differentiated adenocarcinomas stained positively for PSA. The variation of staining scored in our series of poorly-differentiated carcinoma is possibly due to some difficulties in determining the histological criteria for this subgroup. The same is true for the small group of solid undifferentiated tumours (Figure 1). In general, however, this growth pattern showed a low percentage of positives. The cribriform growing tumour areas also showed a low staining percentage (26 or less).

The difference between PSA expression in tumour tissue and BPH tissue was most striking in the sections containing undifferentiated carcinoma. In the latter group almost none of the tumour areas could be stained. As stated in the method section we used PSA antibodies at such a concentration that further dilution would cause a decrease in the staining

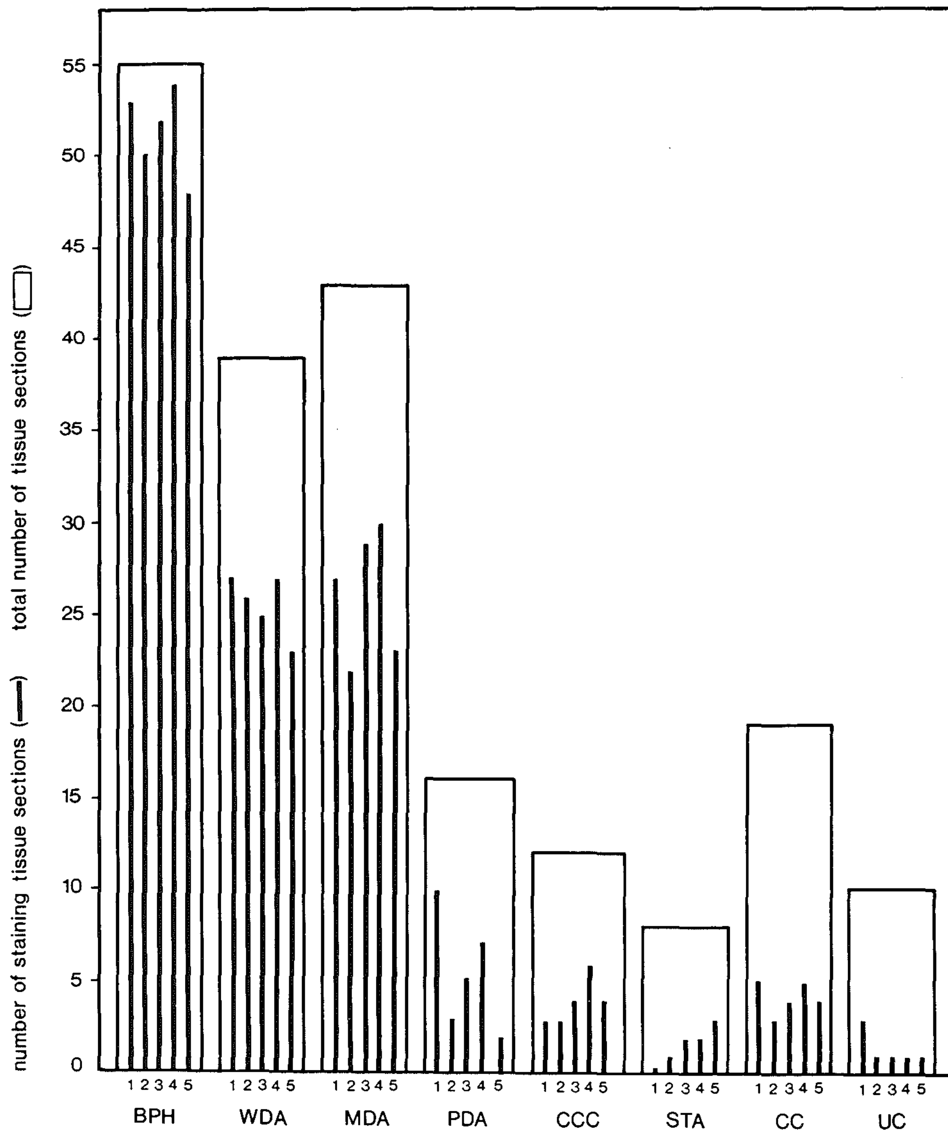
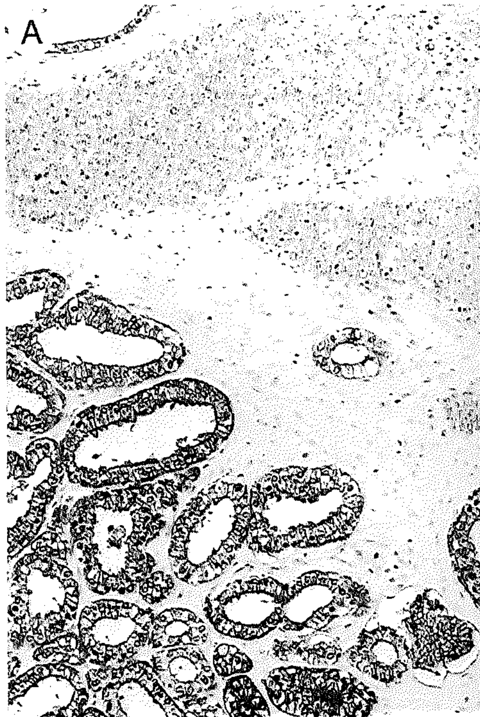


Figure 1:
Schematic presentation of PSA-immunoreactivity (scored 2 and 3) in BPH areas and various growth patterns of prostatic carcinoma, determined by polyclonal (PSA(pc)) - and McAbs (ER-Pr 1, 8, 12, and 27). 1: PSA(pc); 2: ER-Pr 8; 3: ER-Pr 12; 4: ER-Pr 27; 5: ER-Pr 1. Abbreviations of growth patterns are as described in Table 1.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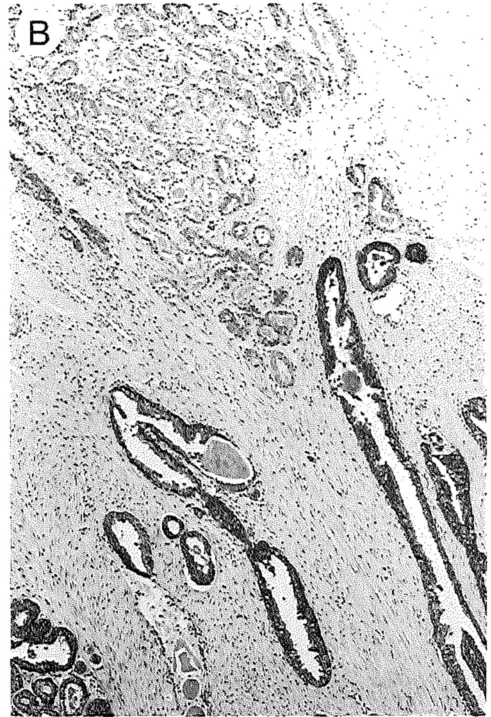
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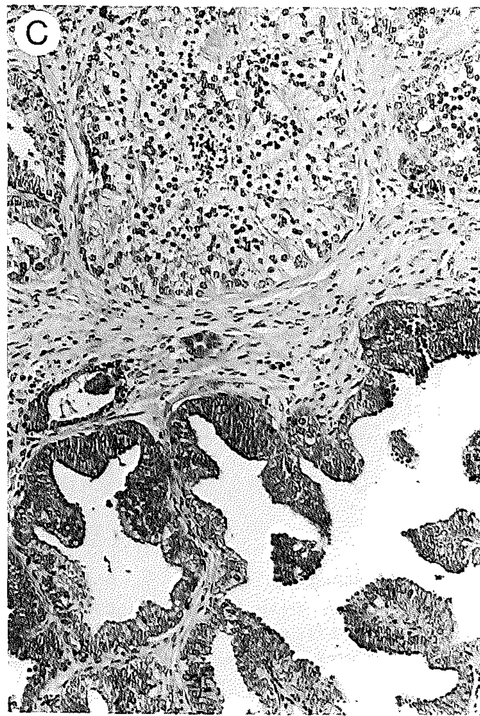
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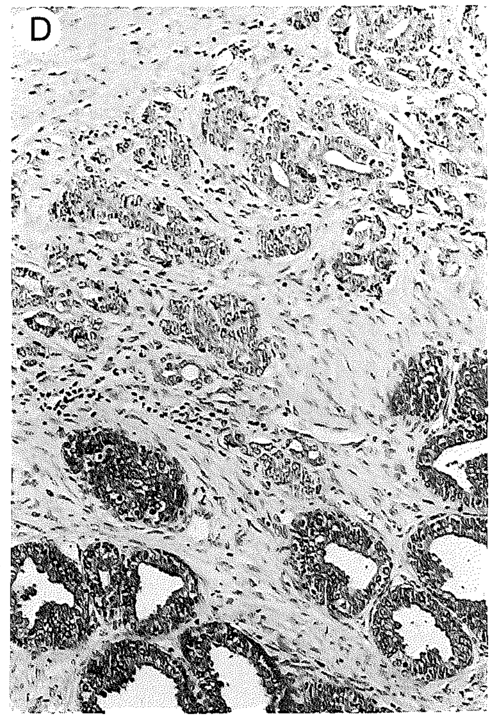
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intensity of BPH controls. Therefore, the results obtained reflect the maximal difference in PSA expression detectable with immunohistochemical techniques in the various tumour growth patterns and BPH.

To investigate the effect of antibody concentration on detection of PSA expression the 10 slides containing undifferentiated carcinoma areas were re-examined with a ten-fold higher PSA antibody concentration. Results are summarized in Table 3. All antibodies used showed essentially the same shift in staining intensity. At the low antibody titer staining could only be detected in a small number of the undifferentiated carcinomas, whereas application of high antibody titers resulted into staining of the majority of the undifferentiated prostate tumours. It is obvious from these results that differences in PSA expression level can only be clearly detected if carefully controlled experimental conditions are applied.

Comparison of PSA and PAP expression in prostatic tumour sections

Two different polyclonal antisera against PAP (Com-PAP (pc) and PAP (pc); see Materials and Methods section) were used for comparison of PAP expression with that of PSA in various prostate tumour growth patterns. Similarly as described for PSA antibodies, PAP antibodies were applied at such a concentration that further dilution would result in decreased staining of BPH tissue sections. Figure 3 shows an example of the immunostaining found with a PSA and a PAP antibody. In this particular tumour area PSA was absent using the initial antibody titer, whereas PAP could be readily demonstrated. Further results are presented in Figure 4. Identical to PSA, PAP expression assessed by either antiserum was found to be lower in tumour areas as compared to BPH. However, the differences observed were much less pronounced. Comparison of PSA and PAP expression in undifferentiated carcinoma and in cribriform carcinoma shows that the majority of sections examined were positively stained with PAP antibodies, whereas the PSA antibodies were in general negative (Figures 1 and 4). Both antisera against PAP gave

Figure 2:
Examples of diminished or absent (scored 1 or 0) PSA-immunoreactivity in growth patterns of prostatic carcinoma. At the lower half of every photograph an area of BPH is present. Original magnification 150x. Selected antibody titers were based on maximum staining in BPH areas.

A Solid tumour areas; section stained by the antibody ER-Pr 12
B moderately differentiated adenocarcinoma; section stained by the antibody ER-Pr 27.
C cribriform carcinoma; section stained by the antibody ER-Pr 27.
D moderately differentiated adenocarcinoma; section stained by the antibody ER-Pr 27.

Table 2. Number of prostatectomy specimens (%) with indicated staining for PSA (scored 2 or 3)/total no. specimens in benign prostatic hyperplasia (BPH) and prostatic carcinoma regardless its histological growth characteristics.

Antibodies	BPH		Carcinoma	
PSA (pc)	53/55	(96%)	41/55	(75%)
ER-Pr 1	48/55	(87%)	35/55	(64%)
ER-Pr 8	50/55	(91%)	40/55	(73%)
ER-Pr 12	52/55	(95%)	36/55	(65%)
ER-Pr 27	54/55	(98%)	41/55	(75%)

Table 3. Distribution of immunostaining intensity scores (scores 0 and 1 are combined) of PSA within undifferentiated carcinoma areas assessed by polyclonal (PSA(pc)) and monoclonal antibodies (ER-Pr 12; ER-Pr 8) using two different concentrations of the primary antibody.

		0,1	2	3			0,1	2	3
PSA (pc)	1:6400	7	3	0	PSA (pc)	1:640	0	8	2
ER-Pr 12	1:102.400	9	1	0	ER-Pr 12	1:10.240	3	4	3
ER-Pr 8	1:51.200	9	1	0	ER-Pr 8	1:5.120	4	4	2

essentially identical results. From these data we concluded that, in contrast to PSA, PAP expression is considerably less correlated with tumor-differentiation.

DISCUSSION

Although now accepted as a reliable marker for prostatic carcinoma both in serological and immunohistochemical assays the detailed possibilities of application of PSA expression as a tool for characterization of neoplastic cells of prostatic origin and its precise correlation with tumour differentiation are still a matter of dispute. This especially concerns fluctuation in PSA expression in various tumours and the sensitivity of PSA as compared to PAP. In this study PSA expression was investigated in various growth patterns of prostatic carcinoma present in prostatectomy specimens. Prostatectomy was performed without prior therapy. In general, several different growth patterns could be distinguished in each prostatectomy specimen and were often even present in one single tissue slide. As an internal control all tissue sections examined contained, in addition to tumour regions, areas of BPH. By this approach fluctuations in the data due to experimental artefacts or differences in

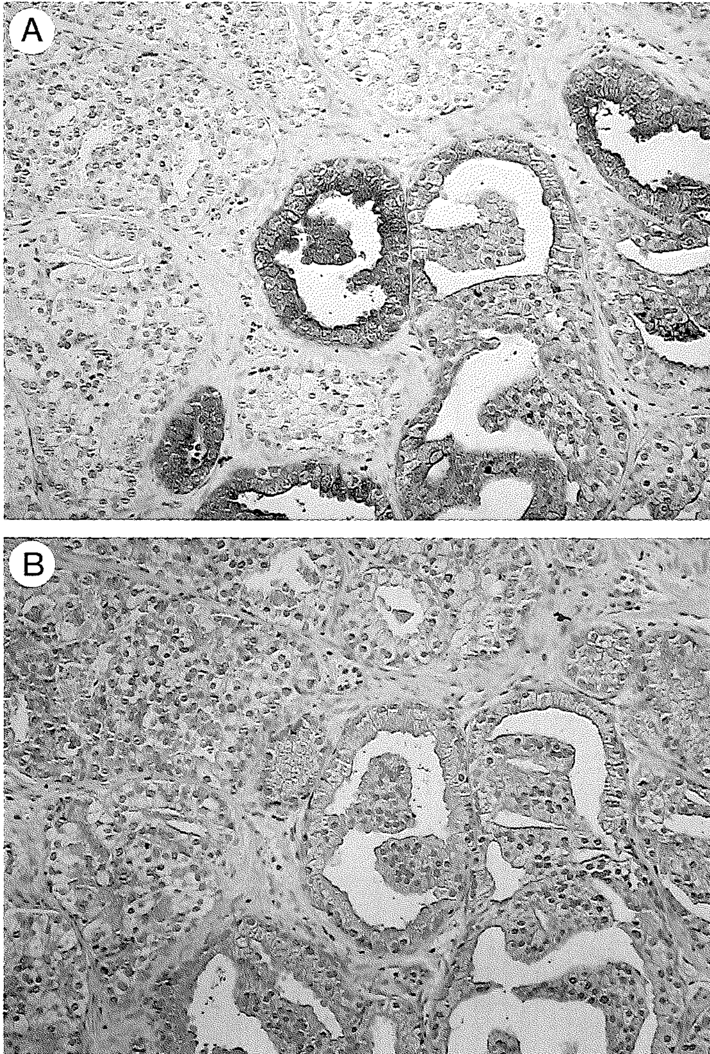

Figure 3:
Prostatectomy tissue section showing BPH and an area of moderately differentiated adenocarcinoma. Consecutive slides were stained by the anti-PSA antibody ER-Pr 12 (A) and the PAP (pc) antiserum (B). Chosen antibody titers were based on maximum staining in BPH areas. In the tumor area PSA could not be demonstrated, whereas PAP could be detected more readily. Original magnification 150x.

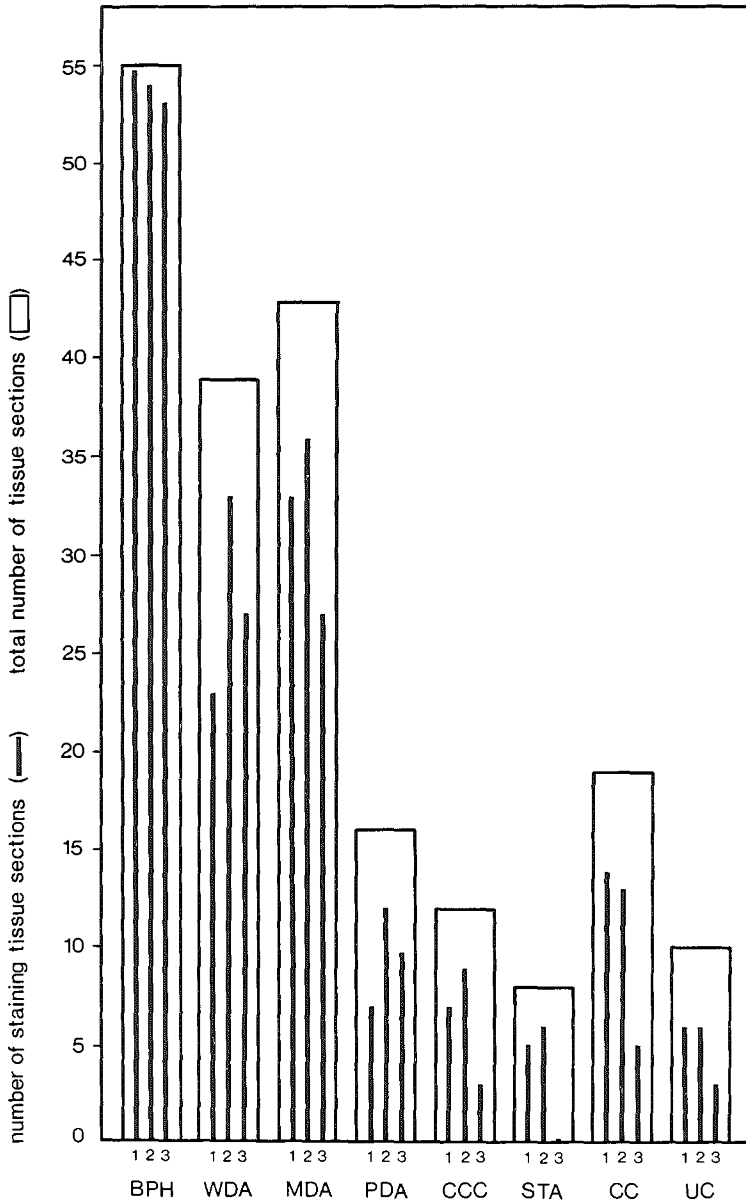

Figure 4:
Schematic presentation of PSA- and PAP immunoreactivity (scored 2 and 3) in BPH areas and various growth patterns of prostatic carcinoma, determined by polyclonal antibodies Com-PAP(pc)(1), PAP(pc) (2) and PSA(pc) (3). Abbreviations of growth patterns are as described in Table 1.

the quality of the tissue slides could be reduced to a minimum. PSA expression was correlated to the differentiation status of a tumour area. Since consensus is lacking referring the most appropriate grading method for prostatic carcinoma no attempts were made to compare immunohistochemical findings with scores of one of the various grading systems described (13,15,19,20).

The data obtained clearly show a difference in PSA expression in BPH and prostatic carcinoma irrespective of its histological features. Most pronounced differences were detected between undifferentiated tumours and the BPH control. Under well-defined conditions (low antibody concentration) the majority of undifferentiated tumour areas were negative when stained with five different PSA antibodies. In most studies described so far, a much higher percentage of PSA positive tumours has been reported (1,7,8,10,23), although in part of these less intense staining of less-differentiated tumours has been noticed. Two of the more recent studies mention the absence of PSA staining in (part of) undifferentiated tumours (9,16). Further experiments presented here clearly establish that the choice of the antibody titer for immunohistochemical detection of PSA in tumour material is of utmost importance. If higher concentrations of specific antibodies are used most of the undifferentiated tumour sections in prostatectomy specimens stain positively.

These data confirm and extend earlier observations that PSA is a very good marker for prostate carcinoma. Furthermore, the data presented here indicate that at least those monoclonal antibodies which are directed against dominant epitopes of PSA (in this study ER-Pr 8 and ER-Pr 27) are very useful in diagnostic pathology and do not differ in sensitivity from polyclonal antibodies. Our results may lend support to the assumption that a decrease of PSA concentration in the sera of patients with prostatic carcinoma not always has to reflect a successful therapeutic intervention, but may also be the result of dominant progressive growth of undifferentiated prostatic carcinoma. More extensive studies correlating immunohistochemical data with PSA serum levels have to be initiated to gain better insight in this phenomenon. Moreover, it is our opinion that during monitoring of endocrine therapy special care must be taken in the accurate interpretation of a decrease of serum PSA concentration, especially since there are indications that PSA expression is influenced by hormones (14).

In contrast to the results obtained on PSA a definite relationship between histopathological growth patterns and PAP expression was less apparent. Even at the experimental conditions chosen which were optimal for the visualization of differential expression, the differences in PAP expression level in the various tumour sections were small. From our data it can only be concluded that in tumour regions PAP expression is slightly reduced. Our data support those earlier observations showing that in undifferentiated prostatic tumours the presence of PAP can be more easily detected than PSA (4,5,9,14,16,17). However, our findings contradict the reports of others in which no difference or

even a better PSA staining was described in poorly- or undifferentiated prostatic carcinomas (1,2,7,8,31). Since the prostate specificity of PAP antibodies is sometimes questioned (25), we find it to be important to use both PAP and PSA as prostatic tumour markers. PAP expression might provide more information on the prostate origin of a tumour, whereas PSA expression (or its absence) seems to be better correlated with histological differentiation.

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CHAPTER VI

DETERMINATION OF THE PROLIFERATIVE FRACTION OF A TRANSPLANTABLE, HORMONE-DEPENDENT, HUMAN PROSTATIC CARCINOMA (PC-82) BY MONOCLONAL ANTIBODY KI-67: POTENTIAL APPLICATION FOR HORMONE THERAPY MONITORING.

M.P.W. Gallee[1], G.J. van Steenbrugge[2], F.J.W. ten Kate[1], F.H. Schroeder[2], Th.H. van der Kwast[1].

Department of Pathology (1) and Urology(2) of the Erasmus University Rotterdam, The Netherlands.

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ABSTRACT

The transplantable, hormone-dependent, human prostatic carcinoma PC-82 was used as an in vivo model for monitoring the proliferative fraction of tumor cells under the influence of androgen withdrawal and resubstitution. The number of cycling cells was assessed by means of an immunoperoxidase method and monoclonal antibody Ki-67. The number of Ki-67 positive tumor cells dropped from an average of 17% in androgen-supplemented, tumor-bearing female BALB/c mice to approximately 1.0% within 10 days after removal of the testosterone (T) implant. A similar effect was noted after castration of tumor-bearing male BALB/c mice. Androgen resubstitution after a 10-day period of T deprivation resulted in a rise in the tumor cell proliferation index to 20% within 4 days. The same pattern of response to androgen depletion and resubstitution was found when the number of cycling cells in S phase was assessed by the 5-bromo-2'deoxyuridine incorporation technique. Administration of supraphysiologic dosis of T in intact male mice did not lead to an increase in the number of Ki-67-stained nuclei. Androgen manipulation did not influence the immunohistochemically assessed expression of prostatic acid phosphatase and prostate-specific antigen. The rapid effect of hormone deprivation and resubstitution in the tumor cell proliferation fraction suggests that monoclonal antibody Ki-67 can be used for monitoring the short-term effects of hormonal treatment of prostatic cancer.

INTRODUCTION

Prostatic cancer is often treated by endocrine management, e.g. administration of estrogens, anti-androgens, luteinizing hormone releasing hormone agonists and antagonists, castration or other androgen-depriving procedures (1,2). In general, the therapeutic outcome can only be evaluated after a period of at least several weeks (1). In the majority of patients, the tumor becomes insensitive to further hormonal therapy as the disease progresses (1). To prevent unnecessary side effects due to hormonal treatment and to facilitate a timely change of the therapeutic regimen, early evaluation of the effects of hormonal manipulation of prostatic cancer could be of great value.

The human prostatic carcinoma PC-82 is an androgen-dependent cell line that can only be maintained by serial transplantation into intact male nude mice or into castrated male and intact female mice supplemented with androgen (3). Due to its androgen-dependent growth, its relatively slow growth rate in vivo, its moderately differentiated histological appearance (still resembling the original tumor) and the secretion of both PAP and PSA, the PC-82 tumor appears to be a suitable model for studying hormonal effects on human prostatic cancer growth (3-5).

Recently, a monoclonal antibody, Ki-67, was described (7) that identifies a nuclear DNA-related antigen associated with proliferation. The presence of this antigen is not only

observed in cells in mitosis but also in cells during the G_1, S, and G_2 phases (7,8). The antibody, used in an immunohistochemical technique, is a reliable tool for the quantification of the proliferative fraction of a human tumor.

In an attempt to detect an early effect of hormonal treatment on hormone-responsive prostatic carcinoma, we performed an androgen depletion-androgen repletion protocol to manipulate tumor cell kinetics in the human PC-82 prostate tumor model in nude mice.

MATERIALS AND METHODS

Tumor cell line

The PC-82 prostatic carcinoma was maintained by serial transplantation of tumor tissue fragments into intact male or into T-supplemented female or castrated male nude mice of the BALB/c strain, as described earlier (3). Routinely tumor grafts were inoculated subcutaneously into both flanks of the host animals. Single- and double-take tumor samples were distri-buted equally among the experiments described later in this paper. Administration of T in female mice was performed by using subcutaneous T-containing Silastic implants (10 mg T; average plasma T value, 25 nM/liter) (9). Castration was performed via the scrotal route under total anesthesia with the use of tribromoethanol (Janssen Pharmaceutica, Beerse, Belgium).

Experimental protocol

The effects of androgen withdrawal were determined by castration of PC-82-carrying intact male nude mice. Animals were sacrificed at 2, 7, and 10 days after bilateral orchidectomy.

An androgen depletion-androgen repletion protocol, used to manipulate tumor cell kinetics, was applied to PC-82-transplanted female mice that received at the same time a T implant (length, 1.0 cm). Androgen withdrawal (i.e., removal of the T implant) was performed when tumors were in the exponential phase of growth. The animals were sacrificed at different time points after androgen withdrawal or after reimplantation with T. This protocol was followed in 4 separate experiments with the use of different (overlapping) time frames. In each study, the duration of the androgen-free period was limited to 10 days. In experiment 4 tumor-bearing mice also were given ip injections of 10 mg BrdUrd/kg 1 hour before they were killed. This procedure was performed to attain simultaneous results with different techniques for measuring proliferation (10).

Some tumor-bearing, intact male mice were treated with a high dose (100 μg) of T for 5 days (resulting in high plasma levels of T) to examine the effect of supraphysiologic plasma T levels on the PC-82 tumor. After sacrifice of the animals, tumor nodules were excised, and a representative part of each tumor was immediately frozen in liquid nitrogen and stored at

-80°C until further use.

Immunohistochemistry

All immunohistochemical assays were performed on cryostat sections of 5 μm thick. Sections were fixed in acetone for 10 minutes. Rabbit antibodies directed against secretory PAP and PSA were purchased from Ortho Diagnostic Systems, Inc., Raritan,NJ. The antigen-antibody binding was visualized with an indirect peroxidase-antiperoxidase procedure.

The murine monoclonal antibody Ki-67, defining a human proliferation-associated nuclear antigen, was commercially obtained from DAKO, Immunoglobulins Ltd., Copenhagen, Denmark, and was applied in a dilution of 1:5 in PBS containing 0.01% gelatine and 0.1% sodium azide. To visualize incorporated BrdUrd, a murine monoclonal antibody (Becton Dickinson Monoclonal Center, Inc., Mountain View, CA), diluted 1:10 in PBS containing 0.01% gelatine and 0.1% sodium azide, was used. Pretreatment of slides before application of the monoclonal anti-BrdUrd antibody was performed in the same manner as described by Schutte et al. (10). Pronase treatment of frozen slides was, however, omitted. A peroxidase-conjugated polyclonal rabbit anti-mouse antibody (DAKO Immunoglobulins Ltd.) was used as second-step reagent in the indirect conjugated peroxidase assay for detection of Ki-67 and the monoclonal anti-BrdUrd antibody.

In both methods, 0.05% 3.3'-diaminobenzidine tetrahydrochloride, in 0.2 M Tris-HCl buffer (pH 7.2) and activated by 0.075% H_2O_2, was employed as a substrate for the peroxidase enzyme. After the immunoperoxidase staining reaction, slides were rinsed in absolute ethanol containing 5% acetic acid and 8% formaldehyde. Subsequently, the slides were rinsed in tap water and counterstained for 10 minutes in full-strength Mayer's hematoxilin. In each experiment, control PC-82 sections were incubated with PBS instead of the primary antibody.

Quantification of the number of cycling cells

To obtain objective data, in each section 500 epithelial tumor cell nuclei were counted in nonadjacent, randomly chosen nonperipherally localized, high power fields (objective 100x). The mean percentage of immunopositive nuclei per section slide was calculated. Counting of nuclei was facilitated using a grid of measured dimensions inserted in one of the ocular tubes of the microscope.

RESULTS

Staining properties of the Ki-67 antibody in PC-82 tumor

In all sections tested, only a fraction of the nuclei (<30% in all cases) showed immunoreactivity. The percentages of immunopositive nuclei in tumor tissue grown in intact male and T-supplemented female mice are listed in table 1. There

Table 1. Percentage of Ki-67-positive cells of PC-82 tumor tissue grown in intact male and in T-implanted female nude mice

Mouse	Ki-67 positive cells, %	Mean ± SD
	Males	
#1	12.2	
#2	18.8	16.1±4.0
#3	15.4	
#4	18.0	
	Females	
#5	14.6	
#6	8.8	
#7	20.0	17.0±4.1
#8	21.8	
#9	19.8	

was no statistically significant difference in the proliferation index between male and T-supplemented female host animals. In the immunopositive fraction, some variation in intensity of nuclear staining was observed. Irrespective of the degree of nuclear staining, staining of nucleoli was always marked (fig. 1), with only slight variation. Prominent nucleoli, without staining of the surrounding nuclear content, were only sporadically observed. The latter also were regarded as immunopositive nuclei. Independently of the gender of the host animal, most slides showed a slightly larger number of Ki-67-positive nuclei at the periphery of the tumor and in the perivascular region.

Inasmuch as the vascular and stromal compartments in the PC-82 tumor are both of murine origin and as the second antibody used was a rabbit antibody directed against murine proteins (see "Materials and Methods"), an inevitable but moderate background staining of these areas was observed. Since the monoclonal antibody Ki-67 was raised against a crude nuclear fraction of L428 cells (a Hodgkin's disease-derived human cell line), immunoreactivity with nuclei of the murine compartment was not found within the human PC-82 tumor.

Effect of hormonal manipulation on Ki-67 expression in PC-82 tissue grown in male mice.

Androgen deprivation by means of bilateral orchidectomy resulted in a decline in the percentage of Ki-67-positive nuclei (from an average of 16.1% for control mice (table 1) to 10.7% within 2 days (table 2)). This percentage decreased further to 1.5% at 7 days and to 0.2% at 10 days after castration. At this time point, changes in the tumor volumes or tumor weights were not yet demonstrable.

Table 3. Effects of androgen manipulation on proliferative activity in PC-82 tumor tissue assayed in 4 kinetic studies in female mice.

Mouse	Tumor nodule[a]	Endocrine state[b]	Ki-67-positive cells, % (BrdUrd labeling index, %)
		Experiment 1	
#1	R	T_0	14.6
	L	T_0	14.6
#2	R	T_0 10-	0.2
#3	L	T 10-/2+	14.3
#4	L	T 10-/10	17.9
		Experiment 2	
#1	R	T_0	3.9
#2	L	T_0 1-	2.7
#3	R	T 2-	2.3
	L	T 2-	0.2
#4	R	T 3-	1.5
#5	L	T 4-	0.2
#6	R	T 10-	0.2
#7	R	T 10-/1+	0.8
#8	L	T 10-/2+	0.0
		Experiment 3	
#1	R	T_0	21.8
	L	T_0	19.8
#2	R[c]	T_0 10-	1.4
	L	T 10-	1.9
#3	L	T 10-/1+	6.2
#4	R[c]	T 10-/2+	5.7
	L	T 10-/2+	2.8
#5	L	T 10-/4+	18.8
	R	T 10-/4+	21.1
#6	R[c]	T 10-/7+	30.9
		Experiment 4	
#1	L	T_0	16.3 (11.2)
	R	T_0	18.6 (7.4)
#2	L	T_0 10-	1.0 (0.4)
	R	T 10-	1.0 (0.4)
#3	L	T 10-/4+	19.3 (10.4)
	R	T 10-/4+	27.6 (16.0)

[a] R = right; L = Left
[b] T_0 = starting point (time 0); - = duration, days, of T withdrawal; + = duration, days, of T resubstitution
[c] Sections were photographed and are shown in fig. 2.

Table 2. Effect of castration on percentage of Ki-67 positive cells in PC-82 tumor grown in male mice

Mouse	Duration of castration, days	Ki-67-positive cells, %
#1	2	10.7
#2	7	1.5
#3	10	0.2

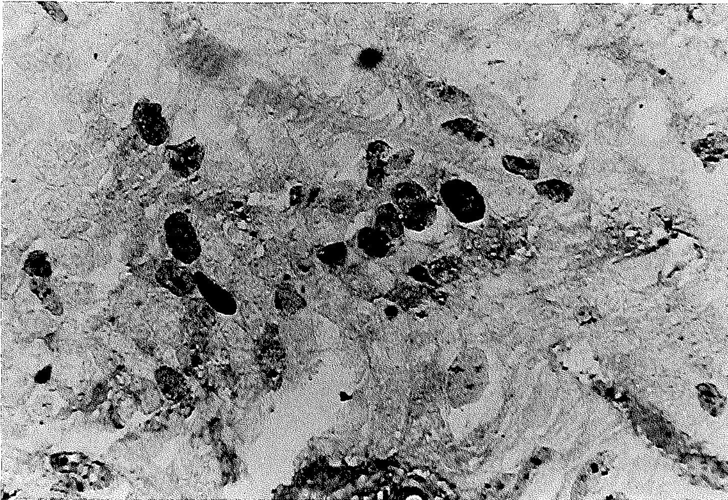
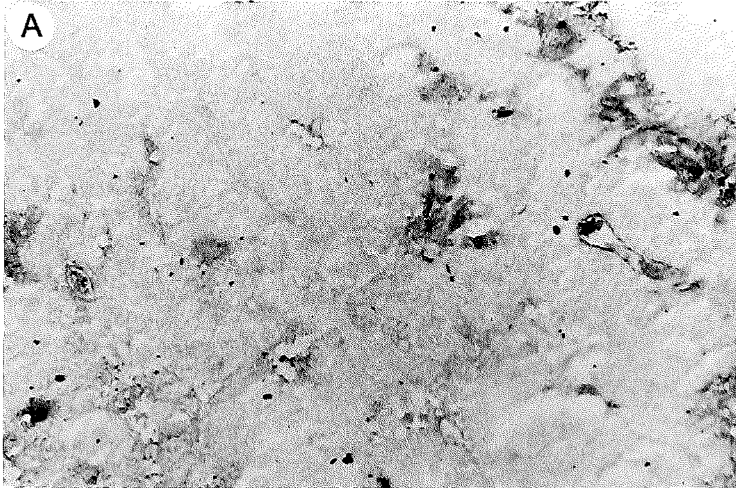


Figure 1:
Section of the PC-82 tumor, grown subcutaneously in intact male mice, and incubated by the monoclonal antibody Ki-67 (150x).



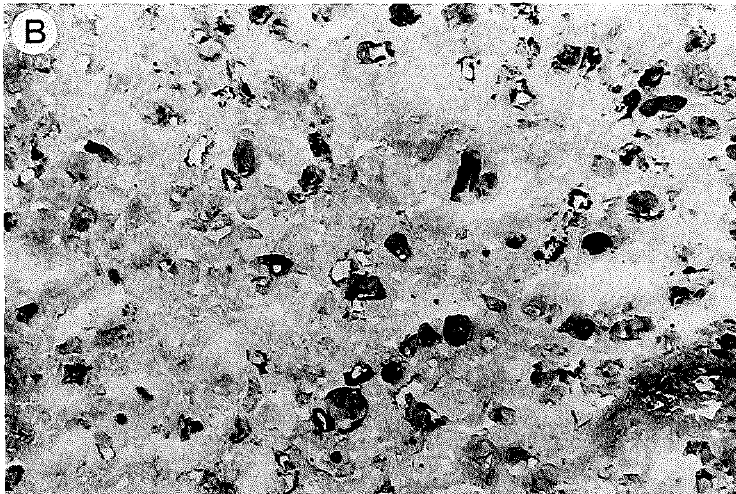
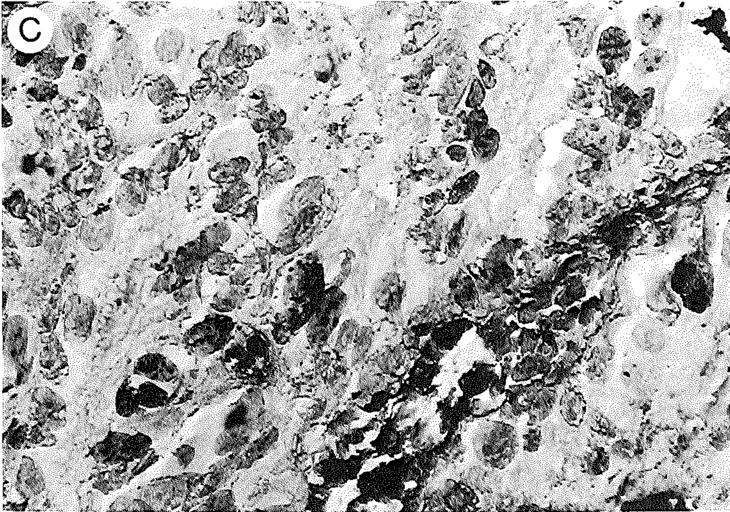


Figure 2:
PC-82 sections derived from tumors grown in female mice at three different time points during androgen manipulation (expt 3): at 10 days after androgen withdrawal (2A), and at 2 days (2B) and 7 days (2C) after reimplantation of T (150x).



Histologically, no areas of necrosis were found in either tumor in this series.

Administration of high doses of T (100 μg) for 5 days in intact male mice did not lead to a further rise in the percentage of Ki-67-positive nuclei when compared to the percentage for control mice.

As judged by the staining intensity in the immunohistochemical assay, androgen deprivation did not affect the expression of PAP and PSA.

Effect of androgen withdrawal and resubstitution on no. of cycling tumor cells in PC-82 tissue grown in female mice.

The results of 4 separate experiments, performed according to the depletion-repletion protocol (as described in "Materials and Methods"), are shown in table 3. Experiment 2 showed very low percentages of Ki-67 positive cells on day 1,2, and 4 after removal of the T implant, although an exceptionally low proliferation index (< 5% positive nuclei) was found in the control (T0) tumor at the start of the experiment (T_0). The other 3 experiments also indicated a drastic decline in the Ki-67-determined proliferative fraction on day 10 after removal of the T implant. A complete return of the number of Ki-67-positive tumor cells to the control value was attained in the first, third and fourth experiments 2-4 days after replacement of T (table 3; fig. 2) In the third experiment, prolonged resubstitution of T led to a further

increase in the proliferative fraction ($\leq$ 30% at 7 days). Results obtained by the BrdUrd assay for quantification of the number of cycling cells in the S phase (table 3, expt 4) were in agreement with those obtained by the Ki-67-determined proliferative fraction. At 10 days T withdrawal and 7 days resubstitution (time point T 10-/7+), the preferential perivascular localization of Ki-67-positive cells, noted at earlier time points was no longer detectable (fig. 3).

In all samples listed in table 3, no T-related variations in intensity of staining were noticed in the expression of PAP and PSA assessed in cryostat sections.

DISCUSSION

In this report we describe the early effect of hormonal manipulation on the proliferative activity of human prostatic tumor cells using the human hormone-dependent tumor model PC-82, which is serially transplantable in nude mice (3).

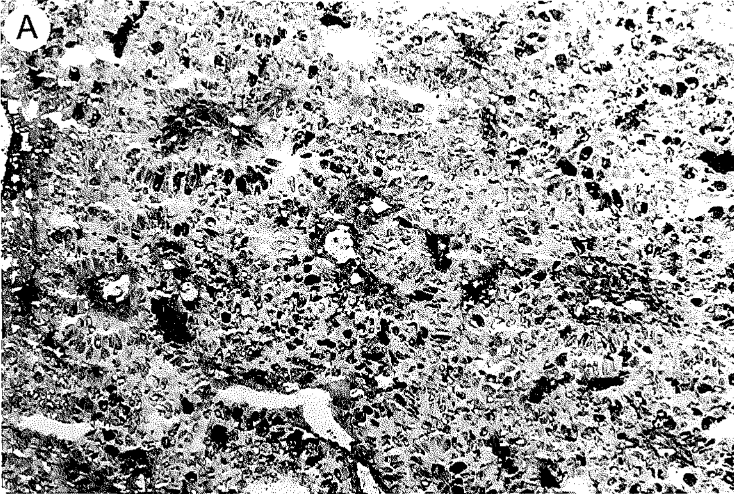
The sporadic occurrence of mitotic figures in human prostatic cancer (11), including the PC-82 tumor, precludes the use of this feature for the assessment of the tumor cell proliferative fraction. Compared to other malignant neoplasms, the low thymidine labeling indices for prostatic cancer, as reported by Meyer et al. (12) indicate that a large proportion of cells present are either quiescent with respect to cell cycle or are progressing through it very slowly with long intermitotic intervals.

Androgen withdrawal in PC-82 tumor-bearing mice leads to a complete inhibition of tumor growth but not to a drastic regression of tumor volume, whereas resubstitution of T (even after longer periods of time) results in a rapid onset of the growth rate (4). Therefore, we assumed that during androgen deprivation, the cells enter the G_0 phase of the cell cycle and remain in a quiescent state.

Although the precise nature of the human nuclear proliferative antigen, which is recognized by monoclonal antibody Ki-67, is still unknown, Gerdes et al. (8), demonstrated that it is expressed in the G_1, S, G_2, and M phases of all human continuously cycling cells but is absent in G_0 phase. Thus application of this antibody in the PC-82 model might discriminate between resting and cycling cells.

The present study shows that in PC-82 tumors, expression of the Ki-67-defined proliferation antigen declined within 4 days after removal of the T implant from PC-82 tumor-bearing female mice (table 3). A similar decline of cycling cells also was observed in castrated PC-82 tumor-bearing male mice (table 2).

In the PC-82 model, a rapid recovery of proliferation within 4 days (expts 3 and 4) after T-resubstitution was observed (table 3). The 30% of positively stained nuclei at 7 days after T-replacement (table 3, expt 3) may suggest a rebound effect of PC-82 cell proliferation. Further experiments covering the traject of 1-10 days after androgen replacement need to be performed to verify this phenomenon. Controversely, no rise in the percentage of cycling cells, as



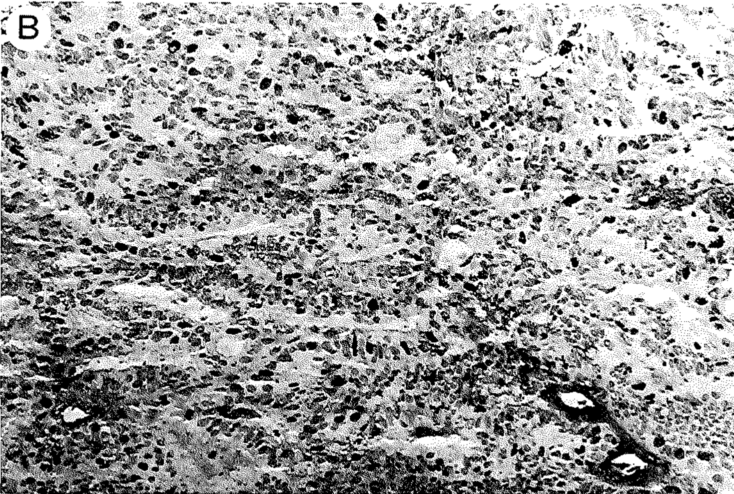


Figure 3:
Resubstitution effect as observed at time point T 10-/2+ (3A) in expt 3. Note the preferential localization of Ki-67 positive PC-82 tumor cell nuclei surrounding the medium-sized blood vessels (3A). This preferential localization was lost at time point T 10-/7+ (3B) (70x).

compared to control values, was achieved after administration of high doses of T administration in PC-82 tumor-bearing, intact, male nude mice. With the use of the BrdUrd incorporation technique, only cells in the S phase were detected (10), and as a consequence the percentages of BrdUrd-positive cells should be lower as compared to the Ki-67-determined proliferation indices. The effects of androgen withdrawal and repletion on the BrdUrd labeling indices (table 3, expt 4) provided further evidence for the assumption that the changes in Ki-67 labeling percentages reflected a decrease or an increase of proliferating cells rather than increases or decreases in numbers of tumor cells in the G_1 phase.

Rijnders et al. (13) recently showed that the expression of the oncogene *fos* in the PC-82 tumor is similarly regulated by androgen withdrawal and resubstitution as is the expression of the Ki-67 defined antigen. Analogous to this observation, a transient expression of *fos*-encoded proteins was induced in benign fibroblasts very early after growth stimulation by platelet-derived growth factor (14). Thus far, one might speculate that a deregulated constitutive expression of the *fos* oncogene can lead to unlimited cell proliferation and transformation (15).

Contrary to these findings, no T-related changes of the immunohistochemically determined expression of PAP and PSA were found in our study. Quantitative experiments related to the PAP concentration in PC-82 tumor samples, as reported earlier (16) are in agreement with these findings. A decline in PAP and PSA in the serum of patients under hormone treatment might thus reflect a decrease in tumor volume instead of a decline in secretory activity in the individual tumor cells.

Recently, comparable effects of hormone manipulation on proliferative activity were reported from similar experiments, in which the rat ventral prostate (17) and the Dunning rat prostate adenocarcinoma (18) were used. In the hormone-dependent Dunning R 3327H tumor it was shown that after a postcastration period of 12 days, androgen repletion resulted into a rapid increase (from 2 to 10%) of cells in S phase. A peak of reactivity exceeding the precastration value was reached at 72 hours after androgen administration, after which a decline to control levels was observed.

Further experiments, applying a fluorochrome-labeled Ki-67 antibody coupled to flow cytometric DNA analysis, may provide additional information on the distribution of PC-82 tumor cells over the G_0 and G_1 phases of the cell cycle at different hormonal stages of the host animal. Since both withdrawal and resubstitution of androgens have a profound effect on the Ki-67 determined proliferative activity of the PC-82 tumor, this technique might be useful for evaluation of the effects of hormonal therapy of patients with prostatic cancer.

Inasmuch as it is possible to perform the here-described assay on cytologic smears, the technique of sequential (fine-needle) aspiration biopsies of (PC-82) tumors might yield additional information on proliferative response during hormonal manipulation of individual tumors. The application of this technique to patients with prostatic cancer thus may contribute to the early identification of responders and

nonresponders to endocrine therapy.

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Chapter VII

MONOCLONAL ANTIBODY KI-67 DEFINED GROWTH FRACTION IN BENIGN PROSTATIC HYPERPLASIA AND PROSTATIC CANCER

M.P.W. Gallee[1], E. Visser-de Jong[1], F.J.W. ten Kate[1], F.H.Schroeder[2], Th.H.van der Kwast[1]

Department of Pathology(1) and Urology (2) of the Erasmus University Rotterdam, The Netherlands.

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SUMMARY

Growth fractions were assessed immunohistochemically in prostatic tissues with benign glandular hyperplasia (BPH) and in specimens of prostatic cancer using the monoclonal antibody Ki-67. This antibody is specific for a proliferation-associated nuclear antigen. In BPH tissues about 0.3% of nuclei of epithelial cells was reactive with Ki-67. The Ki-67 positive nuclei were distributed equally among the basal and luminal cells of the hyperplastic prostatic acini. In prostatic cancer the Ki-67 defined growth fraction ranged from 0.4% to 9.1% (mean value 2.9%). Cancers with a cribriform growth pattern and tumors composed of solid areas of undifferentiated cancer cells showed the highest growth fraction (average values 4.0%, respectively 7.6%). The investigated 4 tumors composed of undifferentiated solitary tumor cells with diffuse infiltration of the stroma demonstrated an unexpectedly low growth activity (average 1.2%). In cancers with a glandular growth pattern the Ki-67 defined growth fraction of tumor cells varied from 2.2% to 5%. Compared with other epithelial tumors these values are low, but they are in agreement with the earlier findings on prostatic cancer obtained with ^{3}H-thymidine labeling and bromodeoxyuridine incorporation. The observed variation in the level of Ki-67 defined growth activity partly related to the histological tumor pattern suggests that Ki-67 labeling may serve as a prognostic factor additional to the current histopathological grading criteria of prostatic cancer.

INTRODUCTION

The production and characterization of the murine monoclonal antibody Ki-67 directed against a human nuclear antigen associated with cell growth has enabled the immunocytochemical assessment of growth fractions in frozen sections (1-4) and on fine needle aspiration smears (5). The Ki-67 defined antigen is present throughout the cell-cycle except during G0 and early G1 (1,2). Recent studies demonstrated that the Ki-67 score correlates well both with mitotic index and with S-phase fractions determined either by DNA precursor incorporation techniques or by DNA-flowcytometry (6-12). Since only a minor fraction of growing cells is in mitosis or present in S-phase it can be expected that counting of mitotic figures and determination of S-phase fractions by tedious methods yield an incomplete reflection of the actual growth activity of a given tumor. The latter disadvantages are surpassed by the application of the Ki-67 antibody.

Prostatic cancers pursue a highly variable course. The predictive value of current histological grading systems for prostatic cancer is hampered by the lack of inter- and intra-observer reproducibility (13). In contrast with grading systems developed for other types of cancer (e.g. breast tumors; 14), most grading methods for prostatic cancer do not imply the mitotic rate in the criteria for grading. The use of this parameter as a prognostic criterium in prostate cancer

has probably been impeded due to the extremely low number of mitotic figures in these tumors. Meyer et al. (15) and Nemoto et al. (16) have confirmed this low proliferative activity in normal prostate, BPH and prostatic cancer by determination of S-phase fractions.

In this study we investigated the Ki-67 defined growth fractions in human prostatic cancer and BPH and we sought to establish a possible relationship between Ki-67 assessed growth rates and histopathologic characteristics of prostatic carcinoma.

MATERIALS AND METHODS

Prostatic tissue

Prostatic tissue was obtained from 33 patients with localized prostatic cancer, randomly selected from a much larger series. These patients underwent in the years 1983-1988 a suprapubic total prostatectomy. Prior to surgery no other therapy, especially no hormonal treatment was given. Immediately after surgery, prostatectomy specimens were sectioned in a step-wise fashion perpendicular to the prostatic urethra. Tumor areas were macroscopically localized and proven by means of histologic examination of cryostat sections routinely stained with eosin-Mayer's haematoxilin. Subsequently, samples of tumor areas were snap frozen in isopentane and stored in liquid nitrogen until further use. The remainder of the specimen was fixed in buffered 10% formaldehyde solution and submitted in toto for routine histopathologic examination. Similarly, 6 samples of transurethral specimens of patients with benign prostatic hyperplasia were snap frozen. Again, none of these patients received hormonal treatment prior to endoscopic resection.

Immunohistochemistry

The murine monoclonal antibody, Ki-67, was purchased from Dako Immunoglobulins Ltd., Copenhagen, Denmark. As second step reagent a peroxidase-conjugated polyclonal rabbit anti-mouse immunoglobulin serum was applied (Dakopatts, Denmark). In brief: 6 μm cryostat sections were cut from each sample, air dried and fixed for 10' in acetone. Afterwards, slides were rinsed in phosphate buffered saline (PBS; pH 7.4) and incubated with the Ki-67 monoclonal antibody for 60' at room temperature at a dilution of 1:5 in PBS containing 0.01% gelatine and 0.1% sodium azide. Subsequently, sections were rinsed in PBS to remove the excess of unbound antibody, followed by incubation with the second reagent. After a final thorough washing in PBS, antigen-antibody binding was visualized by incubating the sections in TRIS-HCl buffer (0.05 M, pH 7.4) containing 0.0125% 3,3'-diamino benzidine tetrahydrochloride (Fluka AG, Switserland) activated by 0.03% H_2O_2 (10' at room temperature). Sections were lightly counterstained with Mayer's haematoxilin for exactly 15 seconds to obtain a discrete nuclear staining pattern

without obscuring the Ki-67 reactivity. Replacement of the primary antibody by PBS served as a negative control whereas frozen sections of the prostatic cancer cell line PC-82 (grown in the presence of androgens) were considered as positive controls (17). Of all samples analyzed immunohistochemically with the Ki-67 antibody (human prostatic tumor; BPH and PC-82) consecutive sections were stained routinely with haematoxilin and eosin for optimal microscopic examination in order to evaluate the different histologic growth patterns. No attempt was made to determine the mitotic rate.

Assessment of the Ki-67 defined growth activity

Tissue sections stained with the antibody Ki-67 were examined by light-microscopy. Counting of negative and positive nuclei was performed at a magnification of 400x. For enumeration a number of high-power fields were randomly selected. To facilitate the counting procedure a grid of measured dimensions was inserted in one of the ocular tubes. The number of nuclei observed in every case ranged from 500 to 1000. Regardless of its intensity and distribution within the nucleus, cell nuclei were considered as positive if there was any nuclear staining present. As proposed by others (18) the Ki-67 growth fraction or Ki-67 score was determined by the quotient of Ki-67 positive cells and total number of cells. In those specimens showing more than one architectural tumor growth pattern counting was performed for each histologic growth pattern separately.

Statistical analysis

To investigate a possible relationship between the Ki-67 score and diverse histologic growth patterns of prostatic cancer the Mann-Whitney U test was used (19). One-tailed probabilities were determined.

RESULTS

Histology

The 6 cases of BPH investigated in this study were exclusively characterized by proliferation of the epithelial fraction resulting in hyperplastic glands showing intraluminal papillary folding of the lining epithelium. Apart from arrangement of hyperplastic glands in (often enlarged) lobular formations, a nodular appearance of proliferating glands was observed. No other types of prostatic hyperplasia such as fibromuscular proliferation or exclusive proliferation of the basal cell layer of prostatic acini were included in this survey. Nor was squamous metaplasia present in those sections used for assessment of the Ki-67 defined growth fraction.

Instead of grading, prostatic tumors were categorized into several growth characteristics according to generally accepted features. Apart from tumors consisting of glands and designated as well-, moderately, or poorly differentiated adenocarci-

nomas (respectively WDA, MDA, PDA), also undifferentiated cancers (UC), tumors with cribriform growth patterns (CF) and tumors composed of undifferentiated neoplastic cells arranged in well circumscribed islets (considered as solid tumor areas: STA) were discerned. The latter growth characteristic is by other investigators designated as a medullary pattern (16).

Heterogeneity of prostatic cancer was reflected by the fact that in the 33 cryostat sections derived from 33 different prostatectomy specimens a total of 40 growth patterns could be recognized (Table 1). Two growth patterns were present in 5 cases. In one case even 3 growth patterns could be detected. The human androgen-dependent prostatic cancer cell-line PC-82 shows an exclusive cribriform growth pattern.

Ki-67 defined growth fractions in glandular BPH

The number of cells that showed nuclear immunoreactivity for the Ki-67 antibody in BPH tissue sections was low. Data are presented in Table 1 and Fig. 1. Immunopositive nuclei were equally distributed amongst basal cells and inner cylindric cells (Fig. 2A). In the 6 cases of BPH studied a mean value of 0.3% Ki-67 positive cells was found. In some hyperplastic glands cytoplasmic immunostaining was present in basal cells; nuclei in these cells lacked immuno-reactivity (Fig 2B). A similar cytoplasmic staining pattern was recognized by others in breast and cervical epithelium (12,20), but this reactivity seems not to be correlated with proliferative activity. Background staining was fully absent. Sporadically, nuclei of stromal cells showed Ki-67 reactivity.

Table 1. Ki-67 defined growth fractions (or Ki-67 scores) (%) in various histologic growth patterns of human prostatic carcinoma, in BPH and in the prostatic cancer cell line PC-82.

	N	range	mean value
benign prostatic hyperplasia	6	0.2-0.4	0.3
human prostatic cancer cell line (PC-82)	9	8.8-21.8	16.6*
well differentiated adenocarcinoma	4	0.7-4.0	2.9
moderately differentiated adenocarcinoma	15	0.4-4.8	2.5
poorly differentiated adenocarcinoma	8	0.6-7.1	2.5
undifferentiated carcinoma	4	0.6-2.5	1.2
cribriform carcinoma	7	1.7-7.5	4.0
solid tumor areas	2	6.2-9.1	7.6

* see reference 17

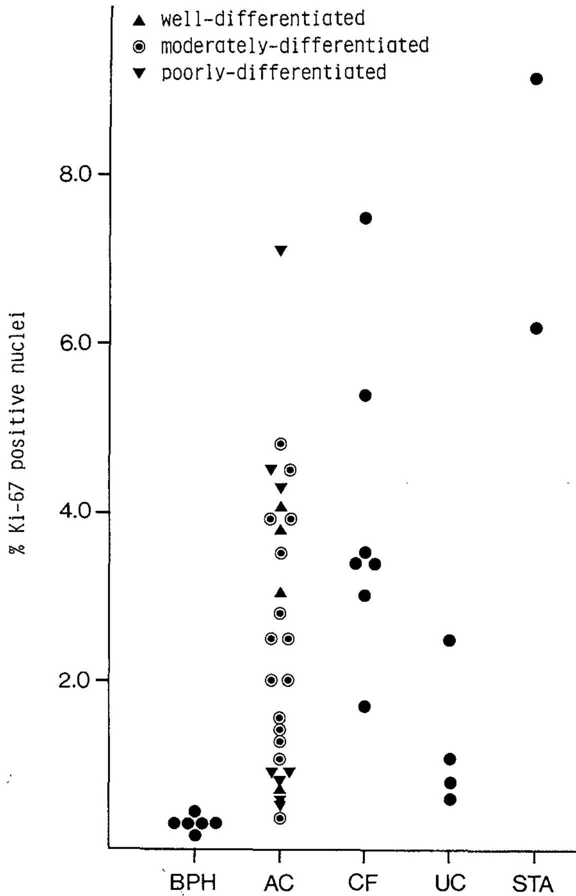

Figure 1:
Ki-67 defined growth fractions in glandular benign prostatic hyperplasia (BPH), and in the various growth patterns of prostatic cancer i.e. adenocarcinomas (AC): well differentiated adenocarcinoma; moderately differentiated adenocarcinoma; poorly differentiated adenocarcinoma (WDA: ▲, MDA: ◉, PDA: ▼), and cribriform cancer (CF), undifferentiated cancer (UC), and solid tumor areas (STA) (all latter tumor patterns including BPH are depicted in the figure as ●).

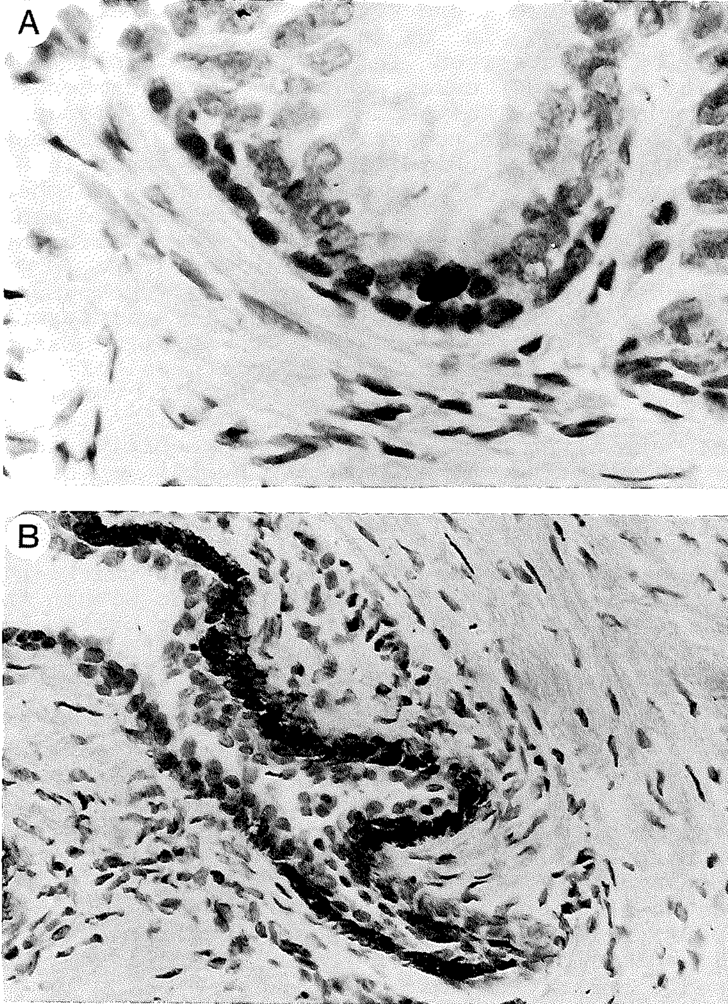

Figure 2:
Immunolabeling of glandular BPH with Ki-67. A: Immunopositive nucleus within the inner cylindric cell-layer of hyperplastic acinus (magnification 600x). B: Cytoplasmatic expression of Ki-67 in the basal cell layer of hyperplastic gland without nuclear immuno-reactivity (magnification 380x). Nuclear counterstaining with haematoxylin.

Ki-67 defined growth fractions in prostatic carcinoma

In general, staining was most prominent in the nucleoli of neoplastic cells. In some tumor cells only nucleoli were stained, whereas the remainder nuclear area was negative. Results of the Ki-67 defined growth fractions related to the different histopathologic growth patterns of prostatic cancer are summarized in Table 1 and Fig. 1. Comparatively high Ki-67 scores were obtained in CF and in STA tumors. The highest growth activity was observed in the PC-82 tissue sections (16.6%). In the 4 UC cases the Ki-67 values tended to be lower in comparison with those obtained in cancers with glandular differentiation regardless of their differentiation grade, but no statistical significance was reached ($p = 0.07$). The growth fractions of CF were significantly higher than those of UC cancers. Similarly, the CF cancers had higher Ki-67 values than cancers with glandular differentiation irrespespective of their differentiation grade ($p = 0.05$). When CF cancer combined with STA were compared with adenocarcinomas regardless their grade of differentiation statistical significance was obtained ($p = 0.01$). Examples of Ki-67 labeling in different growth patterns of prostatic cancer are presented in Fig. 3. In all tumor samples heterogeneity of the Ki-67 nuclear staining pattern was considerable and often clustering of immunopositive nuclei was observed. By counting at least 500 nuclei a bias in the selection of tumor areas was avoided as much as possible. Contrary to basal cells in BPH, tumor cells did not display cytoplasmic Ki-67 immunoreactivity. In morphologically heterogenous prostatic tumors it appeared that the Ki-67 score was identical in each of the growth patterns within the same tumor. For example, in the case characterized by the presence of WDA, MDA and CF tumor areas, the Ki-67 defined growth fraction was assessed at 3.8%, 3.5% and 3.5% respectively.

DISCUSSION

Since a close correlation between Ki-67 labeling on the one hand and mitotic rate or S-phase fraction on the other hand has been demonstrated in various tumor types, the immunocytochemical assessment of the Ki-67 defined antigen may be regarded as a reliable technique to measure cell-kinetics (8,10,11). A relative disadvantage is that the antibody is exclusively applicable on either fresh frozen tissue sections or fine needle aspiration smears (5,8).

In normal and diseased prostatic tissues mitotic figures are rarely encountered. The latter is reflected in this study by the very low Ki-67 defined growth fraction in glandular BPH. Our average value measured in BPH (0.3%; see Table 1 and Fig. 1) is consistent with the previously reported S-phase fraction (0.31%) assessed by in vitro uptake of ^{3}H-thymidine (15). The thymidine labeling index of stromal cells in prostatic hyperplasia was even considerably lower (15).

The low Ki-67 scores found in BPH hardly overlapped with the values obtained in prostatic cancer (Fig. 1). The

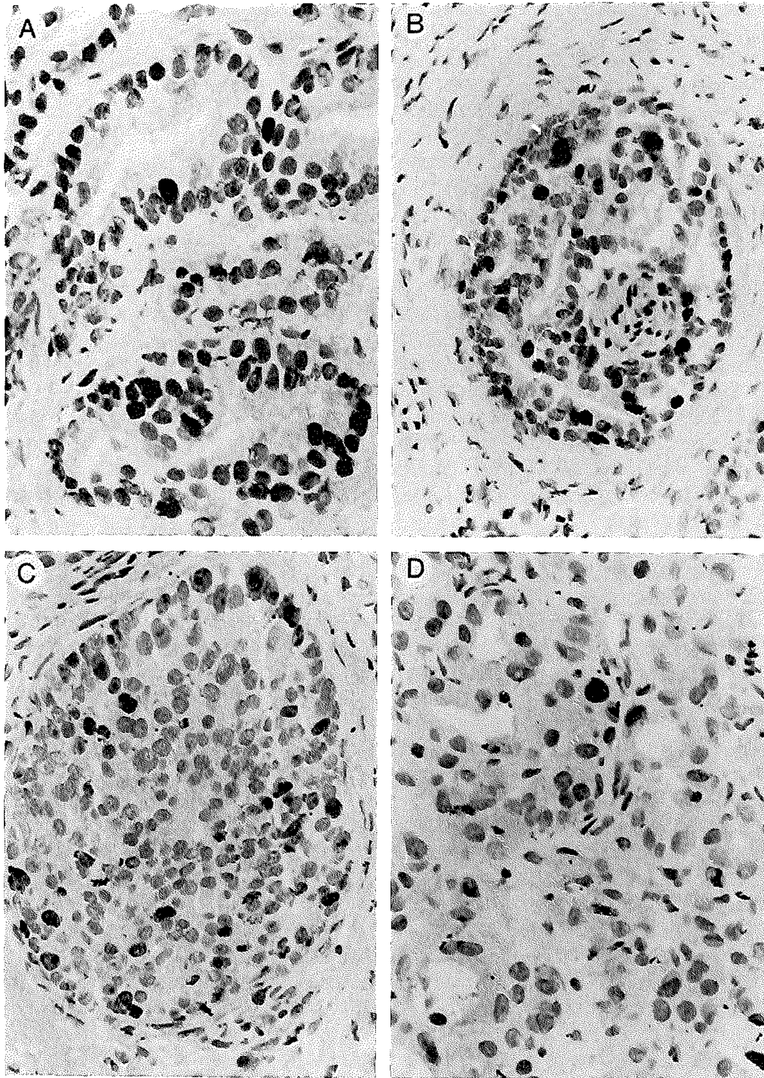

Figure 3:
Four examples of Ki-67 immunostaining reactions in different growth patterns of prostatic cancer. Magnification 380x.
A: Well differentiated adenocarcinoma
B: Perineural arrangement of a cribriform cancer
C: Solid tumor area
D: Poorly differentiated adenocarcinoma

observed distribution of Ki-67 immunoreactive nuclei among the inner cylindric and the outer basal cell layer of the hyperplastic prostatic glands supports the hypothesis that the basal cells do not solely account for the regenerative potential of glandular prostatic epithelium (22).

Occasionally, the Ki-67 antibody reacted with the cytoplasm of the outer basal cell layer (Fig. 2B). The nuclei of these cells remained unstained. Cytoplasmic immunoreactivity was not observed in the inner cylindric cell layer, nor in the cytoplasm of neoplastic epithelial cells or in the cytoplasm of stromal cells. To our knowledge cytoplasmic Ki-67 staining is not directly related with proliferative activity.

Prostatic carcinomas displayed a rather low growth fraction (mean value 2.9%); mitotic figures are scarcely found and the Ki-67 score is low especially when compared with epithelial benign and malignant lesions of other origin (see Table 2; ref. 5,7,8,10,12,17,20,21,23-28). With reference to other human epithelial tumors for as yet the highest Ki-67 scores are reported for high grade lung cancers (26), breast cancers (23), carcinomas of the cervix uteri (20) and high grade adenocarcinomas of the large bowel (27) (Table 2). In comparison with prostatic cancer these tumors display even at routine histological examination a much larger number of mitotic figures.

Similar observations concerning the relatively low growth rate in prostatic cancer were made by Meyer et al. (15). The average fraction of prostate tumor cells in S-phase assessed by in vitro ^{3}H-thymidine labeling measured 0.90% (15). After intravenous injection of BrdUrd in patients with prostatic cancer, Nemoto et al. recently found an average S-phase fraction of 2.86% (16). These latter authors selected well-labeled tumor areas for counting. In contrast to these low proliferation scores, Raymond and coworkers reported a mean Ki-67 score of 16.3% in human prostatic cancer samples (21). Their discrepant data might be explained by a different immunohistochemical method. We feel, however, that our results are more compatible with those obtained by the afore-mentioned authors who used in vitro or in vivo DNA-precursor incorporation techniques.

In human and rat prostatic cancer cell lines proliferative activity appeared to be considerably higher than in the patient material. Ki-67 defined growth fractions in the human prostatic carcinoma model PC-82 (cribriform growth pattern) ranged between 8.8% to 21.8% (17). In the androgen dependent Dunning R3327H rat model of prostatic adenocarcinoma flow-cytometry studies revealed 4.02% cells in S-phase (29). Synchronization of cell-kinetics by means of short-term castration subsequently followed by renewed administration of androgens resulted into a maximum increase in S-phase cells till 9.72% (30). In the human PC-82 model using Ki-67 as proliferation marker we found a similar rebound effect (17). In this study we have avoided to include patients who were treated by hormones prior to surgery.

The precise relationship of cell kinetic data and histologic growth patterns of prostatic cancer remains

Table 2. Comparison of Ki-67 defined growth fractions in benign and malignant epithelial lesions of various organs as reported in literature.

	percentual Ki-67 score mean values	Lit ref (no.)
<u>Benign Lesions</u>		
benign breast disease	3	23
mammary fibro-adenoma	1.0	24
mammary fibro-adenoma	1.1*	5
benign prostatic hyperplasia	0.3	this study
<u>Malignant Lesions</u>		
breast cancer	14.3	8
breast cancer	16.7	23
breast cancer	10.5*	5
breast cancer	22.0	10
breast cancer	16.0	12
breast cancer	20.7	7
breast cancer	16.2	25
cervix carcinoma	30.7	20
small cell cancer of lung	65	26
colorectal cancer	43.8	27
adenocarcinoma of lung	24.6	26
prostatic cancer	16.3	21
prostatic cancer	2.9	this study
prostatic cancer cell-line PC-82	16.6	17
metastases of breast carcinomas in brain	47.6	28

* Ki-67 rate was assessed in fine needle aspiration (FNA) smears. In stead of the mean values, the median figures are given

unclear. Our results indicate that CF (including STA) prostatic cancers strongly tend to have higher Ki-67 proliferative rates compared with cancers consisting exclusively of glandular formations ($p = 0.01$). In contrast, the 4 UC displayed an unexpectedly low proliferative activity as judged from Ki-67 labeling. These findings suggest that the level of Ki-67 labeling is partly dependent on the tumor growth pattern. On the other hand, we found that within the investigated 6 morphologically heterogenous tumors the same Ki-67 score was obtained for each of the composing growth patterns. The latter observations indicate that Ki-67 labeling is - to some extent - independent of growth pattern and seems to be a more characteristic feature for the

individual tumor. A possible trend of increasing S-phase fraction and Ki-67 defined growth fraction with increasing Gleason score was also reported by others (15,16,21). Unfortunately, these studies were performed largely on biopsy samples and the precise value of histopathological grading upon small tissue samples is often questioned (31,32). Moreover, the reproducibility of the rather complex Gleason grading system is still controversial (13,33). Of utmost interest was the observation that metastatic prostatic cancer tended to have a higher fraction of tumor cells in S-phase compared with primary tumor deposits (15).

Since a reliable, reproducible, and generally accepted grading system based on histopathologic features in prostatic cancer is not available as yet, the assessment of tumor cell kinetic data may provide additional information refering the prognosis of prostatic cancer. Of those methods currently in use (DNA precursor uptake and DNA flow cytometry) the Ki-67 technique can be considered as the most simple assay, which has proven its significance in many other tumors and is regarded as a reliable marker in the immunologic armamentarium of the routine pathologist. The precise elucidation of the prognostic value of Ki-67 labeling in prostatic cancer will, however, require extensive prospective studies, encompassing large numbers of patients. These studies should give insight whether Ki-67 labeling has to be regarded as a strongly independent prognostic factor or dependent on other variables such as histologic growth pattern.

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CHAPTER VIII

GENERAL DISCUSSION

In the papers presented in this thesis, we have searched for parameters of prognostic relevance for PC. In general our survey encompasses the study of three putative parameters linked with the biological behavior of PC.

Initially we have examined the reproducibility of five conventional histological grading systems currently in use for PC. Histological grading was performed by 5 pathologists with varying experience in both general pathology and pathological aspects of male urogenital oncology. All participants in the study received prior to histological grading the original papers in which the grading methods were explained. None of the grading methods displayed a good interobserver agreement such as measured by statistical evaluation. A reasonable interobserver consensus was reached in the methods based primarily on histological (architectural) criteria in simple grading methods as those designed by Broders and Brawn. Though inconsistent with several reports from the United States, the complex Gleason system showed only a poor consensus among the 5 observers.

The prognostic performance of the 5 grading methods was described in a subsequent paper. Extended data concerning time relapse to recurrence (progression) and time relapse to death were available. Results indicated that the methods of Broders and that described by Mostofi-Schroeder displayed the highest predictive value especially regarding the end point "recurrence". Of special interest was the observation that the application of more than one histological grading system on the same tumor may enhance the predictive value of grading. In our study this appeared to be the case with the afore mentioned two systems. Summarizing our data obtained with histological grading several conclusions and comments can be made. 1. Reproducibility of results derived from histological grading seems poor. 2. Experience of the observers will undoubtedly influence results positively. 3. Simple grading methods are preferable since some of these show at least a reasonable agreement.

A general drawback of histological grading is that the majority of grading methods designed for PC allow only the morphological judgment of one growth pattern. However, it is generally accepted that many tumors including PC are composed of several areas differing in morphological appearance (and also in immunophenotypical make up). On the other hand the only method investigated in our survey that permitted to grade two growth patterns (Gleason technique) showed even less interobserver agreement. The latter problems especially occur when large specimens are available for histological grading. Though theoretically the accuracy of grading has to increase when a large number of slides of each tumor is available, in practice the interobserver agreement will be hindered. For as yet there is, particularly in the case of PC, no consensus upon which type of specimen histological grading has to be performed.

Our survey regarding the prognostic accuracy of histological grading has to be evaluated with some restrictions. At first, the performance of a total prostatectomy has undoubtedly influenced the prognostic end

points studied. Secondly, our data set was derived from the same tissue slides that were previously used to set up the recently described new grading method by Schroeder and Mostofi. And thirdly, we have utilized for statistical calculations the average (or mean) values of grading data obtained from 5 observers. In spite of these restrictions, of interest might be our observation that the simultaneous use of more than one system on the same tumor may improve the predictive value of histological grading. As far as we know this has not been suggested previously for PC. One can imagine especially for PC, for which tumor diverse histological grading techniques have been developed, that one method supplies additional prognostic information to another. Preliminary results presented here have to be regarded as a challenge to combine other grading systems than the one described, not only for prostatic tumors but also for malignancies of other origin.

Subsequently, immunological aspects of PC have been studied. In the beginning we have attempted to generate TAAs or TSAs using the well-characterized human PC model PC-82. However, our attempts have been without success in this regard. Numerous McAb's produced by hybrid cell lines were generated. Though interesting cross-reactivity immunocytochemical profiles with human malignant and non-malignant tissues were encountered, none of the McAb's showed an exclusive binding for prostatic cancer cells. As was known already, the PC-82 cell line maintained PAP expression. Therefore, it was not surprising that some of the antibodies were directed against PAP. PA expression was also retained in the PC-82 cells as some of the antibodies were directed against antigenic sites of PA. McAb's prepared against PAP and PA enabled the further purification of both prostatic tissue-specific markers and resulted in the generation of sets of monoclonals and monospecific polyclonal rabbit antisera. At least three different epitopes of PA could be recognized by our set of monoclonal antibodies. As some of the monoclonal antibodies were applicable on formalin-fixed sections further immunocytochemical elucidation of especially PA expression was examined in human PC tissue sections and the relation was studied with histological tumor differentiation. Since the reliability of assignment of grading scores was questioned, no correlation was performed with data derived from grading systems. Our studies indicate that though the sensitivity of PAP is higher than that of PA as prostate-specific tumor marker, there are indications that especially PA expression is lost in undifferentiated prostatic tumors. Our results suggest that PA expression is correlated with tumor differentiation status. Therefore, PA expression interpreted quantitatively may be of help in forecasting the biological behavior of PC. The selection of antibodies and the concentration of the antibodies used seem of utmost importance and will have a large impact on the outcome. Since controversial data exist concerning the precise specificity of PAP, PA is to date the prostatic tissue-associated marker with the highest specificity. Our preliminary data are of further importance for the use of PA to monitor patients with PC. To date a fall in

serum PA level after a presumed curative prostatectomy for localized cancer is unequivocally linked with a total absence of remaining PC cells. However our data suggest that lack of detectable levels of serum PA may also be accompanied with the persistence of undifferentiated (PA-lacking) prostatic tumor cell clones. For these reasons further studies correlating serum PA levels in patients with histologically proven undifferentiated PC have to be carried out. Moreover one has to realize that in the course of human PC an initially differentiated tumor may become less differentiated or even undifferentiated. Time-related changes in the histomorphological appearance of PC are presumably associated with time related alterations in PA serum levels. Therefore, the precise interpretation of the serum PA level remains for as yet equivocal.

Not only several cDNAs for PA have been identified but also the structure of the PA gene has been elucidated. PA seems to belong to the human kallikreine-like gene family. These genes are located on chromosome 19. Of this group of genes the human glandular kallikreine 1 (hGK-1) gene appears also to be expressed exclusively in human prostatic tissue. This is of particular interest since the homology between the protein structures of the PA and the hGK-1 genes is high (79%). The latter implies that probably some of the antibodies described in literature (especially the polyclonal antisera) and presumed to be reactive against PA might be directed against antigenic sites of the hGK-1 protein. Recent experiments in our laboratory have demonstrated that at least the McAb ER-Pr 8 (and ER-Pr 27) is not reactive with hGK.

The tremendous interest during the recent years for PA has obscured the precise value of PAP as prostatic tumor marker. Both markers are shed in the blood circulation. Precise insight in the specificity of the numerous antibodies recognizing epitopes of PAP is difficult to obtain. The antigenic overlap of the so-called prostate-specific acid phosphatase with acid phosphatases originating from other sites remains for as yet unclear. All investigators in this field recommend the use of both markers for all studies of PC, whereas PA is generally accepted as the marker with the highest specificity.

The use of labeled antibodies against these PTSAs for in vivo immuno-imaging of primary and secondary prostatic tumor deposits has to be further investigated. Incidental articles report a limited success of visualizing retroperitoneal lymph node metastases in patients with widespread PC using Fab fragments of a polyclonal antiserum against PAP. The secretory features and the absence of PAP along the cell membrane are presumably the main reasons for the lack of success. Although PA is also a secretory protein, there is evidence available obtained by immuno-electronmicroscopy, that PA is somewhat more concentrated along the cell membrane of prostatic epithelial cells than PAP. Therefore immuno-imaging with PA antibodies might be more successfull.

Cell kinetic aspects are often associated with the malignant potential of neoplasms. However, PC is characterized by a relatively low growth rate. Mitotic

figures are rarely encountered. Other approaches than simply counting of mitotic figures to gain insight into the proliferative capacity of PC are DNA flowcytometry, DNA precursor uptake methodes and application of the Ki-67 antibody. Of these methods the DNA precursor uptake can only be performed in vivo. The availability of antibodies against BrdU has facilitated the visualization of the in vivo exposure of the tumor to BrdU. The monoclonal antibody Ki-67 is directed against a for as yet unknown antigen that is expressed in the nucleus of cycling cells. In the PC-82 model the Ki-67 defined growth rate responded quickly upon androgen depletion and repletion. As this technique is also applicable on fine needle aspiration smears of human prostatic cancers, the Ki-67 method might be of help in the judgment of the effects of hormonal treatment in patient with PC. A fall in Ki-67 defined growth fraction after initiation of treatment has to be interpreted as a success of therapy, whereas an absence of such a decline in Ki-67 defined PC growth fraction provides evidence for a lack of success of the endocrine therapeutic regimen. Further studies in patients have to be carried out for more precise elucidation of this relationship and for more accurate determination of the time interval after which these growth fraction changes occur. If the observations made by us can be repeated in patient material, the timely interruption of an unsuccessful endocrine therapy will circumvent the occurrence of unpleasant side effects associated with this therapy and will lead to a rapid transition to another hopefully more successfull therapeutic modality. The relationship of Ki-67 defined growth rate and tumor growth pattern is described in Chapter VII. Relatively high growth fractions were found in medullary cancers (solid tumors) and cribriform tumors. A correlation with histological grading scores was avoided for previously mentioned reasons. The precise prognostic value of the Ki-67 antibody has to be further investigated in large prospective studies with a long-term follow-up. Such studies will elucidate whether the Ki-67 defined growth fraction has to be considered as an independent (prognostic) parameter or is linked with histological grade.

Of the other functional markers which will become easily detectable in prostatic tissue, the AR (Androgen Receptor) has to be mentioned. The primary structure of the AR has been elucidated and the production of antibodies against the purified AR and synthetic oligopeptides of the AR has very recently been realized. Availability of a set of antibodies will enable structure-function analysis of the AR. In the case of PC the immunological visualization of the AR in tissue slides will enable detailed studies regarding the potential effects of hormonal treatment. Thusfar ligand binding studies were not able to demonstrate a relationship between AR concentration and responsiveness of the tumor to endocrine therapy. Immunocytochemistry will gain further insight into the variation of distribution of the AR in prostate malignant and non-malignant tissue. The prognostic value of the immunocytochemical demonstration of the AR can be investigated and related to architectural growth patterns or histological

grading scores of PC.

Of more fundamental interest is the research referring the specific (onco)genes which might be involved in the pathogenesis of PC. To date, it still is completely unknown which (onco)genes might play a role in PC and whatever mechanisms might be involved. Especially when amplification and/or overexpression of a particular oncogene can be proven, immunocytochemistry with antibodies against the oncogene product might help in recognizing PC in an early stage (more precise identification of for as yet doubtfull histological preneoplastic lesions) and expression of the gene product might be related with clinical behavior.

SUMMARY

Prostatic cancer is a highly frequent malignancy in the aging male population. When all clinical stages are considered it might even be the most frequent malignancy in men. As for most other malignancies the etiology of PC is not understood as yet. Epidemiological studies concerning PC have revealed interesting data. Whereas incidental (dormant) prostatic cancer figures are almost equal in each country, figures referring to clinically manifest tumors and mortality figures show a remarkable geographic distribution. Many studies have been performed to explain this phenomenon. Environmental factors, socio-cultural habits, endocrine status and genetic susceptibility have been investigated for their possible role in influencing the biological course of PC. However, the precise reasons for the large variation in clinical course remains unclear. Nevertheless one has attempted to predict the biological course by extracting parameters with presumed prognostic value of the malignant prostate tissue. Out of these parameters the morphological appearance of the tumor can be considered to be the oldest method to gain information concerning the clinical course. For PC numerous histological grading techniques have been developed. Five of these systems have been studied in Chapter II. The reproducibility of the grading data, when applied by several pathologists, was investigated. With exception of the methods using histological criteria, reproducibility was poor. In Chapter III a correlation of grading results and follow-up data was performed. The methods using predominantly histological criteria reached the largest prognostic impact. Of interest was the observation that two methods provided additional prognostic information when applied on the same tumor.

In Chapter IV an attempt was made to discover other parameters (antigens) linked to PC. The PC-82 model was used to generate monoclonal antibodies. None of the antibodies reacted with an exclusive PC-associated antigen. Among the antibodies prepared several recognized at least three different epitopes of PA. Moreover some antibodies identifying PAP were isolated. Those antibodies that were applicable on routinely handled tissue were used to examine the relationship between tumor differentiation status and PA and PAP expression respectively (Chapter V). Retention of PAP in PC was slightly higher than that of PA. Therefore the sensitivity of antibodies recognizing PAP in PC is higher. On the other hand, contrary to PAP, PA expression seems to be related to tumor differentiation. Diminished PA expression was noticed in undifferentiated prostatic cancers.

Cell kinetic studies related to PC are described in Chapters VI and VII. The monoclonal antibody Ki-67 was used to measure growth fractions in PC. In Chapter VI a relatively high Ki-67 defined growth fraction was assessed in the cribriform tumor model PC-82. Results obtained with the Ki-67 method paralelled those reached with the BrdU technique. Interesting was the observation that rapid fluctuations of proliferative acivity were found in androgen repletion and

depletion protocols. The use of this parameter in forecasting the effects of hormonal treatment are discussed. The relationship of cell kinetics and growth pattern of PC is described in Chapter VII. There seems to be a correlation between Ki-67 score and architectural growth pattern of PC. In less differentiated cancers higher proliferation scores were observed. The question whether the Ki-67 score has to be considered as an independent prognostic parameter for PC remains to be resolved.

SAMENVATTING

Prostaatcarcinoom is een zeer frequent voorkomende maligniteit. Wanneer de incidentiecijfers van alle klinische stadia van prostaatcarcinoom tesamen worden genomen, is het prostaatcarcinoom hoogst waarschijnlijk de meest frequente maligne aandoening bij de mens. Echter in tegenstelling tot deze hoge incidentie is de morbiditeit en mortaliteit veel lager. Zoals voor bijna elke maligne tumor is er nog maar weinig bekend over de oorzaak en ontstaanswijze van prostaatcarcinoom. Epidemiologisch onderzoek heeft interessante feiten aan het licht gebracht. Het voorkomen van incidenteel prostaatcarcinoom is voorzover onderzocht in elk land en iedere bevolkingsgroep gelijk. In tegenstelling hiermee is de waarneming dat het voorkomen van het klinisch manifeste prostaatcarcinoom grote verschillen toont van land tot land en ook van ras tot ras. Een goede verklaring hiervoor heeft men tot op de dag van vandaag niet kunnen vinden. Onderzoek is verricht naar tal van factoren gerelateerd aan socio-culturele status, genetische predispositie, hormonale status en factoren betreffende het milieu. Desalniettemin tracht men steeds opnieuw parameters te zoeken en toe te passen die het biologisch gedrag van prostaattumoren in een vroeg stadium kunnen voorspellen. Van al deze potentiele parameters moet de microscopisch morfologische bestudering van tumoren als een van de oudste worden beschouwd. Voor het prostaatcarcinoom zijn een groot aantal van histologische graderingssystemen beschreven. Steeds wordt aan een tumor op basis van histologische en of cytologische kenmerken een score toegekend die zou overeenstemmen met een bepaald te verwachten biologisch gedrag. In hoofdstuk II en III wordt een aantal van deze graderingssystemen voor prostaatcarcinoom nader bestudeerd. In hoofdstuk II wordt de reproduceerbaarheid van een 5-tal methoden vergeleken na toepassing op een serie van 50 tumoren door 5 beoordelaars. De reproduceerbaarheid van de systemen gebaseerd op hoofdzakelijk histologische criteria bleek redelijk, terwijl de methoden waarin ook gebruik werd gemaakt van vaak slecht gedefinieerde cytologische kenmerken een slechte reproduceerbaarheid toonden. In hoofdstuk III werd de prognostische waarde van de histologische graderingssystemen onderzocht. Ook hier bezaten de systemen gebaseerd op histologische kenmerken de hoogste prognostische relevantie. Opvallend en voorzover bekend nooit eerder beschreven was de waarneming dat twee graderingssystemen indien toegepast op eenzelfde tumor ten opzichte van elkaar additioneel prognostische waarde bleken te bezitten.

In hoofdstuk IV wordt een overzicht gegeven van de productie van antistoffen gebruik makend van de humane prostaatcarcinoom cellijn PC-82. Het bleek niet mogelijk antistoffen te isoleren met een exclusieve binding voor humaan prostaatcarcinoom weefsel. Wel werden antistoffen geisoleerd die gericht bleken tegen PA en PAP. Tenminste 3 verschillende antigene determinanten van PA konden worden herkend. In hoofdstuk V wordt een immunohistochemische studie beschreven waarbij de relatie PA en PAP expressie met tumordifferentiatie wordt onderzocht. Zowel monoclonale als polyclonale

antistoffen werden hiervoor toegepast op prostaatcarcinoomweefsel in prostatectomiepreparaten. PAP bleek aanwezig in iets meer tumoren dan PA. Echter in tegenstelling tot PAP, bleek de PA expressie gerelateerd aan de differentiatie-status van het carcinoom. De PA expressie was aanzienlijk verlaagd in ongedifferentieerde tumoren.

Celkinetische aspecten van prostaattumoren worden beschreven in de hoofdstukken VI en VII. De monoclonale antistof Ki-67 werd gebruikt voor het bepalen van de groeifracties in prostaatcarcinoom. In de humane prostaatcarcinoom cellijn PC-82 met een cribriforme groeiwijze werd een relatief hoge groeifractie bepaald. De resultaten verkregen met de Ki-67 methode liepen parallel met die bepaald met de BrdU incorporatie-techniek. In het algemeen liggen de waarden bereikt met de Ki-67 techniek hoger. In het PC-82 model bleek dat de Ki-67 bepaalde groeifractie snel reageerde op depletie van androgeen en hernieuwde toediening van androgenen na 10 dagen onttrekking. Het potentiele gebruik van de Ki-67 techniek voor het beoordelen van het effect van hormonale therapie bij patienten met PC werd besproken. De relatie Ki-67 score en histologisch groeipatroon van prostaatcarcinoom wordt besproken in hoofdstuk VII. Deze correlatie bleek aanwezig. In het algemeen geldt dat medullaire carcinomen (solide ongedifferentieerde carcinomen) een hogere groeifractie tonen dan buisvormende carcinomen. Vooralsnog zal moeten blijken in hoeverre de Ki-67 score rechtstreeks gerelateerd is aan groeipatroon dan wel beschouwd moet worden als onafhankelijke parameter met (potentieel) prognostische waarde.

POST SCRIPTUM

Zoals bijna elk proefschrift, is ook dit boekje beslist niet alleen het resultaat van de inspanning van de promovendus, maar tot stand gekomen dankij de hulp en inzet van velen. De beschikbare ruimte maakt het mij slechts mogelijk enkele van hen her met name te noemen. Allereerst wil ik mijn promotor, Prof.Dr. R.O. van der Heul, danken voor de mij geboden gelegenheid het hier beschreven onderzoek gedurende mijn opleidingsjaren binnen ijn instituut uit te voeren. Ook wil ik hem danken voor de bereidwilligheid het manuscript van kritische kanttekeningen te voorzien en zijn bijdrage aan een aantal artikelen.

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CURRICULUM VITAE

Schrijver dezes werd geboren in 1957. De gymnasium-β opleiding werd gevolgd aan het St. Ignatius College te Amsterdam en afgerond in 1975. In aansluiting hierop werd aangevangen met de studie geneeskunde aan de Vrije Universiteit eveneens te Amsterdam. Na het behalen van het doctoraal examen in februari 1981 werd de studie onderbroken voor een assistentschap aan het pathologisch instituut der Vrije Universiteit (hoofd destijds Prof.Dr. R. Donner). Het artsexamen werd behaald in januari 1983. Vanaf februari 1983 tot mei 1989 was de schrijver verbonden aan de afdeling Pathologische Anatomie I van de Erasmus Universiteit te Rotterdam (hoofd Prof.Dr. R.O. van der Heul) alwaar het hier beschreven onderzoek werd verricht en de opleiding tot patholoog-anatoom werd gevolgd. Sedert 1 juni 1989 is de schrijver weer terug in de hoofdstad en verbonden als patholoog-anatoom aan het Nederlands Kanker Instituut (Antoni van Leeuwenhoek Ziekenhuis).

ABBREVIATIONS

AC	Adenocarcinoma
AFIP	Armed Forces Institute of Pathology (Washington)
Ag	Antigen
AP	Alkaline Phosphatase
AR	Androgen Receptor
BGA	Blood Group Antigens
BGrAg	Blood Group-Related Antigens
BPH	Benign Prostatic Hyperplasia
BrdU	BromodesoxyUridine
BrdUrd	BromodesoxyUridine
CC	Cribriform Carcinoma (=CF)
CCC	Clear Cell Carcinoma (hypernephroid carcinoma)
CEA	Carcino-Embryonic Antigen
CF	Cribriform carcinoma (=CC)
DES	Diethylstilbestrol
DHT	DiHydroTestosterone
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EGF-R	Epidermal Growth Factor Receptor
EIA	Enzyme ImmunoAssay
ELISA	Enzyme-Linked Immunosorbent Assay
EORTC	European Organization on Research and Treatment of Cancer
HLA	Human Leukocyte Antigen
Ig	Immunoglobulin
β-Inhibin	β-MSP
LH-RH	Luteinizing Hormone Releasing Hormone
LNCaP	Prostatic cancer cell-line; Lymph Node Carcinoma of Prostatic origin
MAGIC	Mixed AGgregation Immunocytochemical technique
McAb	Monoclonal Antibody
MDA	Moderately Differentiated Adenocarcinoma
MDAH	M.D. Anderson Hospital grading system designed by Brawn (1982)
MNU	N-methyl-N-nitrosurea
MSP	β-microseminoprotein = β-inhibin
NPCP	National Prostatic Cancer Project
PA	Prostatic Antigen (= PSA)
PAP	Prostatic Acid Phosphatase (= PSAP); secretory subtype (iso-enzyme) of acid phosphatases in the prostatic gland
PBS	Phosphate Buffered Saline (pH 7.2)
PC	Prostatic Cancer/ Prostatic Carcinoma
PC-82	(human) Prostatic Carcinoma cell-line 82
PDA	Poorly Differentiated Adenocarcinoma
PDGF	Platelet-Derived Growth Factor.
PHI	Phosphohexose Isomerase
PSA	Prostate-Specific Antigen (= PA)
PSAP	Prostate-Specific Acid Phosphatase (=PAP)
PSP94	Prostatic Secretory Protein consisting of 94 amino acids = β-MSP
PTSA	Prostatic Tissue-Specific Antigen
RIA	Radio Immuno-Assay
SA	Secretory Antigen

SHBG	Sex Hormone-Binding Globulin
STA	Solid Tumor Areas (medullary growth pattern)
T	Testosterone
TAA	Tumor Associated Antigen
Tc	Technetium (radio-nucleide)
TGF	Transforming Growth Factor
TNM	Tumor, Node, Metastasis; classification of malignant tumours
TPA	Tissue Polypeptide Antigen
TSA	Tumor Specific Antigen
TUR	Transurethral Resection
UC	Undifferentiated Carcinoma
UEA-1	Ulex Europaeus Agglutin 1 (lectin)
VACURG	Veterans Administration Cooperative Urologenital Research Group
WDA	Well Differentiated Adenocarcinoma

OMNIA VANITAS EST

Ontwerp omslag:
Paula Delfos
Elly Visser-de Jong