

Sex Hormone-Induced Prostatic Carcinogenesis in the Noble Rat: The Role of Insulin-Like Growth Factor-I (IGF-I) and Vascular Endothelial Growth Factor (VEGF) in the Development of Prostate Cancer

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BACKGROUND. Despite extensive effort, the mechanisms of prostate carcinogenesis are still unknown. We report on a modified method which enabled us to induce a high incidence of prostate carcinogenesis in the Noble rat and examined the role of insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) and their receptors during sex hormone-induced prostate carcinogenesis.

METHODS. Noble rats were implanted subcutaneously with a combination of testosterone and estradiol capsules for up to 12 months. Animals were sacrificed starting at 2 months after implantation, and the prostate gland was removed for histopathological and immunohistochemical studies.

RESULTS. The results showed that hyperplasia/dysplasia was detected as early as 2 months after treatment, while carcinoma in situ was induced in 4 months and adenocarcinoma in 7 months. Our data suggest that IGF-1, produced by stromal cells in hyperplasia, exerted its effects, through a paracrine mode, on epithelial cells which were IGF-1 receptor (IGF-1R)-positive. The production of IGF-1 appeared to switch to epithelial cells in adenocarcinoma, through which it regulated tumor cell growth via autocrine mode by binding to IGF-1R of carcinoma cells. On the other hand, VEGF was overexpressed in hyperplastic/dysplastic and carcinoma cells, while VEGF-R was detected in endothelial cells. The results suggest that overexpression of VEGF in deranged epithelia and arterial muscle cells may exert its influence on stromal angiogenesis and abnormal growth of prostate gland.

CONCLUSIONS. A modified Noble rat model with a high incidence of prostate carcinogenesis has been developed. Using this model, we have further established that IGF-1 and VEGF may be the critical regulators in mediating epithelial-stromal interactions in sex hormone-induced prostate carcinogenesis. *Prostate 35:165–177, 1998.* © 1998 Wiley-Liss, Inc.

KEY WORDS: sex-hormones; IGF-1; VEGF; receptors; prostate carcinogenesis

INTRODUCTION

Prostate cancer is one of the most common cancers in the Western world, but its etiology is not well understood. It is commonly believed that a change in ratio of testosterone and estrogens with advancing age is an important factor in initiation of benign prostatic hyperplasia (BPH) and prostate carcinogenesis [1–3]. Despite a wealth of epidemiological evidence implicating prolonged disturbance of hormonal status as a risk factor in prostate cancer [4–7], the mechanisms of

carcinogenesis in the prostate gland remain poorly understood. The slow progress in this area is, at least in part, due to a lack of a good experimental animal

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model which could subject the process of carcinogenesis to close scrutiny, and to the absence of early specific markers during the tumorigenic process. Attempts have been made to use testosterone alone [8,9] to induce prostate carcinogenesis in animals. However, this approach only achieved a low incidence of adenocarcinoma in Noble rats (18%) [8] and Lobund-Wistar rats (25%) [9]. The Noble rat is a strain which is particularly sensitive to hormonal manipulation and has been used for the study of sex hormone-induced prostate cancer [8,10,11]. Long-term implantation of a known ratio of testosterone and estradiol-17 β (T + E₂) pellets subcutaneously [10,11] has been shown to be able to induce carcinogenesis in the prostate gland, whereas short-term (16 weeks) treatment consistently induced a proliferative lesion, termed dysplasia, in dorsolateral prostate (DLP) of Noble rats [12–15]. In animals implanted with T or E₂ alone, no dysplastic lesion was observed [13,15]. However, the incidence of such hormone-induced carcinogenesis is low and renders it less accessible for detailed analysis of prostate carcinogenesis. In this study we used a modified method, involving a substantial increase in the amount of implanted testosterone and estradiol, and a very high incidence of prostate adenocarcinoma was achieved. This paper documents, in detail, the histopathology of sex hormone-induced prostate cancer.

On the other hand, investigations on the molecular and cellular mechanisms responsible for the initiation and progression of prostate cancer have produced evidence which suggests that the nature of mesenchymal/stromal and epithelial interactions are closely involved. It is well-accepted that epithelial-mesenchymal interactions play crucial roles in prostatic development. These interactions remain important in maintaining normal prostatic functional integrity in the adult. During prostatic carcinogenesis, the intricate balance of interactions is perturbed [16]. The importance of stromal-epithelial interactions in both normal and abnormal growth of the prostate is well-recognized [17,18], although the precise mechanism has yet to be worked out. It is widely recognized that a variety of growth factors have been implicated in epithelial-stromal interactions. Among these growth factors, insulin-like growth factor-1 (IGF-1) is a widely expressed polypeptide which plays a unique role in many tissues and has been considered a survival factor for cells, while VEGF is essential for angiogenesis accompanying the development of tumors. Here we present evidence that these two peptide factors and their receptors play an important role in mediating epithelial-stromal interactions during the process of prostate carcinogenesis.

MATERIALS AND METHODS

Animal Experimentation Model

Young adult male Noble (NBL) rats (n = 62, 3 months old) were surgically implanted subcutaneously with two 2-cm and one 1-cm Silastic capsules [no. 602-305 tubing; 1.54 mm inside diameter (i.d.) \times 3.18 mm outside diameter (o.d.), Dow-Corning Corporation, Midland, MI], tightly packed with 175 mg of testosterone propionate (T) and 25 mg of 17 β -estradiol benzoate (E₂) (Sigma Chemical Co., St. Louis, MO), respectively. Rats were treated for 2, 3, 4, 7, 10, and 12 months. Twenty-eight age-matched control animals were treated with empty capsules. All animals were housed in the Laboratory Animal Unit (LAU), University of Hong Kong, with food and drinking water ad libitum under controlled conditions (12 hr light, 12 hr dark, 20 \pm 2°C). The implanted hormone pellets were surgically removed and replaced with new pellets every 2 months.

Histopathological Examination

Animals were killed at various intervals starting at 2 months after the implantation of T + E₂, by injection of an excess dose (150–200 mg/kg body weight) of pentobarbitone, and the prostatic gland was carefully isolated. The three lobes, i.e., dorsal, lateral, and ventral, were dissected out and fixed separately in 10% buffered formalin, embedded in paraffin. Five-micron sections were stained with hematoxylin and eosin (H&E) to determine the histopathology of the gland. For this study, specimens from the lateral prostate (LP) were mainly used. However, appropriate specimens of dorsal prostate and ventral prostate were also used for comparison purposes.

Immunohistochemical Studies

Tissue sections (5 μ m) were cut and immersed in toluene to remove paraffin and were then hydrated in graded alcoholic solutions (100%, 95%, 80%, and 70%) and distilled water (DW). Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide (H₂O₂) in methanol (4 ml 30% w/v H₂O₂ in 200 ml of methanol) for 30 min, followed by washing in phosphate-buffered saline (PBS), pH 7.4. Normal goat serum (for polyclonal primary antibodies raised in rabbit) or normal horse serum (for monoclonal primary antibodies raised in mouse, and polyclonal primary antibody raised in chicken) were applied to the sections for 30 min to bind nonspecific antigens. The nor-

TABLE I. Summary of the Incidence of Hyperplasia, Dysplasia, and Prostate Cancer in Noble Rats After T + E₂ Treatment*

Month postimplantation	No. of rats with hyperplasia/total no. of rats (%)	No. of rats with dysplasia/total no. of rats (%)	No. of rats with cancer/total number of rats (%)
2	8/8 (100)	2/8 (25)	0/8 (0)
3	8/8 (100)	4/8 (50)	0/8 (0)
4	8/8 (100)	7/8 (87.5)	1/8 (12.5)
7	10/10 (100)	10/10 (100)	3/10 (30)
10	17/17 (100)	17/17 (100)	11/17 (64)
12	11/11 (100)	11/11 (100)	10/11 (91)

*The animals were implanted subcutaneously with a combination of testosterone propionate (T, 175 mg) and 17 β -estradiol benzoate (E₂, 25 mg) in Silastic tubings for up to 12 months, with replacement hormonal tubing implantation at 3-month intervals. Here is shown the very high incidence of prostatic carcinoma after 7 months of treatment; the incidence has reached 91% by 12 months. This shows the effectiveness of the T + E₂ regime in stimulating prostatic carcinogenesis.

mal serum-treated sections were incubated with optimally diluted primary antibodies overnight at 4°C.

In these experiments, the mouse anti-IGF-1 (catalogue no. 05-172) monoclonal antibody and the chicken anti-IGF-1 receptor (IGF-1R) (catalogue no. 06-429) polyclonal antibody were bought from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit anti-vascular endothelial growth factor (VEGF) (catalogue no. SC-507) polyclonal antibody and rabbit anti-Flk-1 (c-1158, VEGF-receptor) (catalogue no. SC-504) polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After incubation overnight at 4°C with appropriately diluted primary antibodies [i.e., IGF-1 (1:150), IGF-1R (1:200), VEGF (1:50), and VEGF-R (1:150)], the sections were washed in PBS (10 min \times 3), and then incubated for 30 min at room temperature (RT) in appropriate secondary antibodies. For polyclonal primary antibodies raised in rabbit, biotinylated goat anti-rabbit immunoglobulin (diluted with PBS at 1:200) was used. For monoclonal primary antibodies raised in mouse, biotinylated goat anti-mouse immunoglobulin (diluted with PBS at 1:200) was used. For polyclonal antibodies raised in chicken, biotinylated goat anti-chicken immunoglobulin (diluted with PBS at 1:200) was used. After incubation with secondary antibodies, the sections were washed again in PBS (10 min \times 3), and then incubated with avidin-biotin complex for 30 minutes at RT. After the last wash with PBS, the sections were developed for about 1–5 min using 3,3'-diaminobenzidine (DAB) in PBS and 0.03% H₂O₂. After checking under a microscope, the DAB reaction was stopped when adequate coloration had been reached. Sections were counterstained with hematoxylin, and dehydrated in alcohol. Control sections were parallel-processed with

the omission of primary antibodies. In addition, specific antisera preabsorbed with 100-fold concentration of the specific factors (i.e., IGF-1 and VEGF), and several nonspecific factors [i.e., basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF)], were also used to confirm the specificity of immunohistochemical reactivity.

RESULTS

Animal Model

Incidence of prostate cancer in Noble rats. In the experimental groups, the rats were sacrificed at time points of 2, 3, 4, 7, 10, and 12 months. A total of 24, out of the 62 prostates derived from these animals, contained tumors (Table I). All of these tumors were microscopic, with a high incidence observed in the group of rats sacrificed 7 months after the implantation of T + E₂. However, almost all the treated animals developed prostatic hyperplasia after 2 months of implantation, and 51 of the 62 prostates had prostatic dysplasia. In control groups, 28 animals were sacrificed at 2, 3, 4, 7, 10, and 12 months; there was no animal which showed any evidence of prostate cancer. Focal regions of hyperplasia were sometimes observed, but no dysplasia or carcinoma was observed.

Changes in weight of prostate. The growth rate of animals in the hormone-treated group was slower than in the normal group (Table II). However, there was a general increase in size and weight of prostate glands compared to the normal controls. By 12 months after hormonal implantation, the weight of prostate glands was 375.32 \pm 136.85 mg/100 g body weight (n = 6). This was significantly larger than in the age-

TABLE II. Profiles of Body Weight [mean \pm SD (n) in g]

Month postimplantation	Control	Experimental group	P
2	282.50 \pm 41.13 (4)	318.0 \pm 17.06 (8)	<0.05
3	423.64 \pm 52.11 (5)	336.57 \pm 32.80 (7)	<0.05
4	444.50 \pm 28.99 (5)	314.58 \pm 35.52 (8)	<0.05
7	490.50 \pm 19.51 (4)	326.55 \pm 23.71 (8)	<0.05
10	467.02 \pm 40.83 (6)	321.46 \pm 35.72 (6)	<0.05
12	455.87 \pm 8.16 (4)	339.73 \pm 64.18 (7)	<0.05

matched control group, which was 260.13 ± 43.69 mg/100 g body weight (n = 10).

Pathological Grading of the Development of Prostate Cancer

Normal prostate. Normal rat prostate was tubuloalveolar and consisted of epithelium-lined acini surrounded by a stromal matrix. It was an exocrine gland surrounding the urethra and was composed of several lobes, i.e., the dorsal, lateral, ventral, and anterior lobes (coagulating gland). The boundary between the dorsal prostate (DP) and lateral prostate (LP) was not distinctive, and difficult to separate easily even under a dissection microscope. The two were closely attached to one another so that they were often referred to as one lobe, i.e., the dorsolateral prostate (DLP).

The DLP consisted of a system of epithelium-lined tubules distributed within the loosely organized stromal tissue containing smooth muscle cells, fibroblasts, blood vessels, and lymphatics. The tubules were lined mainly by cuboidal cells that had centrally placed nuclei (Fig. 1) and a thin layer of smooth muscle. Secretion in the lumen of the tubules was intensely eosinophilic.

The ventral prostate (VP) was discrete and located on the ventral aspect of the urethra. The VP consisted of a series of ducts lined with mainly cuboidal epithelium that drained into the urethra. The epithelium lining the tubules/acini of VP was mostly columnar and/or cuboidal cells with basally located nuclei (Fig. 2). The acini were more tightly packed in the stroma of the VP. The secretion in the acini of the VP was lightly eosinophilic. The stromal tissue consisted of connective tissue, smooth muscle cells and fibroblasts, small nerves, and blood vessels.

Prostatic hyperplasia. Hyperplasia is the result of proliferation of epithelial cells and smooth muscle cells, fibroblasts, and other stromal components in variable proportions. Hyperplastic changes were observed in prostate glands as early as 2 months after the treatment with hormones. The tubules, of varying sizes, contained regions which were composed of more than one layer of epithelial cells (Figs. 3, 4). Numerous papillary projections were present in the tubules. In hormone-treated animals, the effects were seen not only in epithelium, but also in the stroma, which was more compact and with more smooth muscle cells, fibroblasts, blood vessels, and histocytes when compared with normal prostate. However, the morphology of the hyperplastic epithelial cells was generally normal. They did not show signs of derangement, such as loss of polarity and nuclear pleomorphism.

Prostatic dysplasia. Dysplasia is a pattern of epithelium that drained into the urethra. The epithelium lining the tubules/acini of VP was mostly columnar and/or cuboidal cells with basally located nuclei (Fig. 2). The acini were more tightly packed in the stroma of the VP. The secretion in the acini of the VP was lightly eosinophilic. The stromal tissue consisted of connective tissue, smooth muscle cells and fibroblasts, small nerves, and blood vessels.

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Fig. 1. Normal rat prostate (DLP). Note that tubules are lined with a single layer of cuboidal cells, with secretion in the lumen. Epithelial tubules, surrounded by a thin layer of smooth muscle cells, are loosely distributed within the loosely organized stromal tissue (H&E, $\times 240$).

Fig. 2. Normal VP. Note that acini are lined with mainly columnar epithelium and contain pale-stained secretions. The stroma is very loosely organized (H&E, $\times 240$).

Fig. 3. Hyperplasia in DLP. Note the increased epithelial thickness. More smooth muscle cells, fibroblasts, and blood vessels are present in stroma (H&E, $\times 240$).

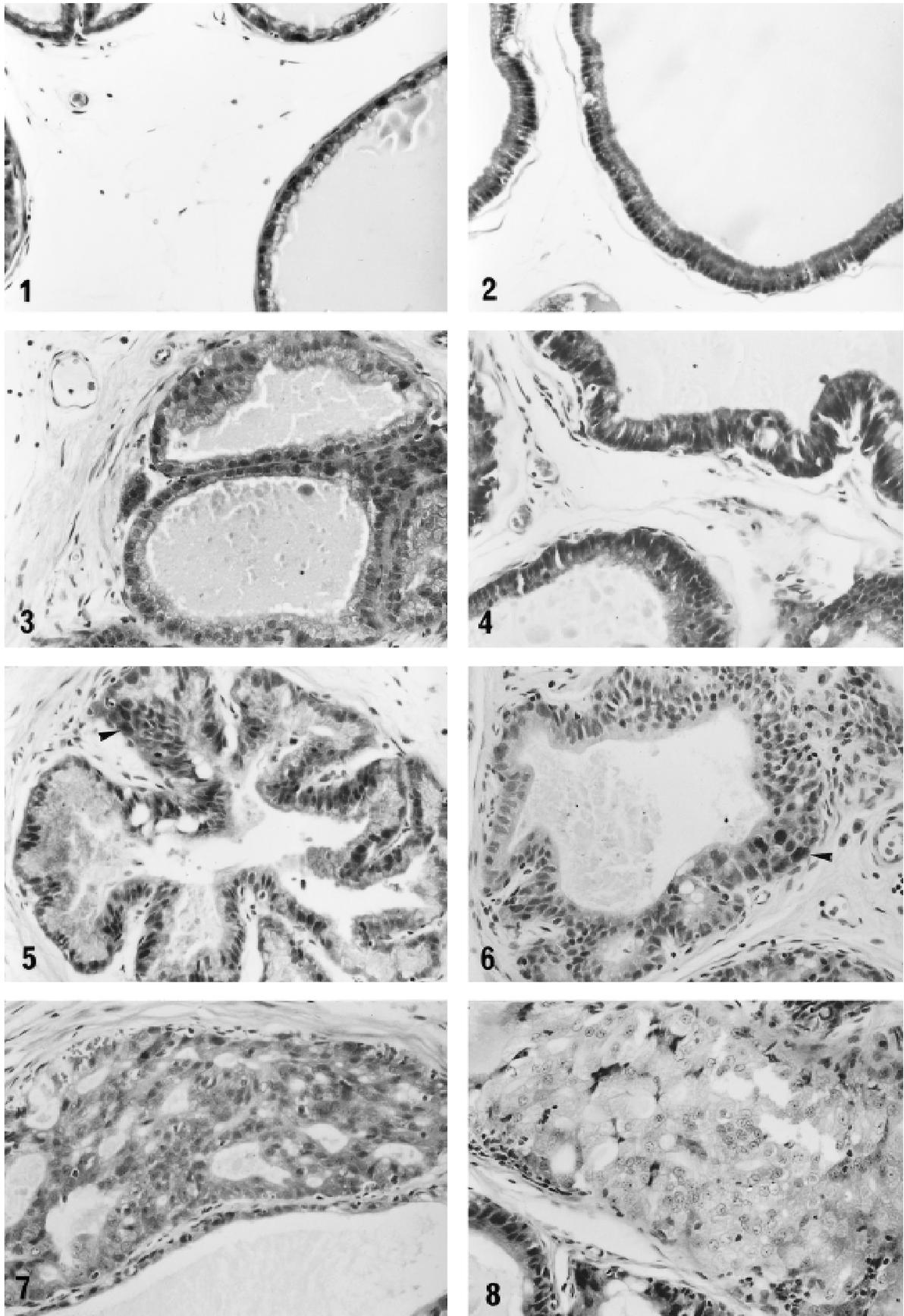
Fig. 4. Hyperplasia in VP. Many epithelial infoldings are seen projecting into the lumen. Note the crowdedness of epithelial cells. The stroma is more compact (H&E, $\times 240$).

Fig. 5. Dysplasia in DLP. The dysplastic epithelium is characterized by the presence of groups of deranged cells with hyperchromatic nuclei, and loss of cell polarity within an otherwise hyperplastic region. Foci of dysplastic cells are indicated by arrowheads (H&E, $\times 240$).

Fig. 6. Dysplasia in VP. Note the dysplastic cells (arrowhead) in this tubule from the VP. Multiple dysplastic sites are seen sometimes within the same tubule (H&E, $\times 240$).

Fig. 7. Carcinoma in situ in DLP. Tumor cells are seen extending into the lumen of tubules, with the formation of a few nested structures (H&E, $\times 240$).

Fig. 8. Carcinoma in situ in VP. Tumor cells are seen confined within a tubule (H&E, $\times 290$).



Figs. 1-8.

lial proliferation characterized by having more than one layer and, more importantly, demonstrating variable degrees of cytological atypia. In this study, the earliest sign was detected from animals sacrificed 2 months after T + E₂ implantation. The epithelial cells showed crowding of nuclei that were enlarged (Figs. 5, 6) and displaying a spectrum of pleomorphism. Multiple dysplastic sites were often seen within the same section. In large tubules, several dysplastic foci were sometimes seen within a single tubule. However, not all tubules were deranged in any given gland. In fact, many morphologically normal tubules were seen alongside tubules which showed different degrees of derangement and after a much longer period of treatment. On the other hand, many dysplastic sites were seen adjacent to hyperplastic foci. In still others, both dysplastic and hyperplastic features were observed in the same dysplastic regions. Dysplastic tubules were completely surrounded by fibromuscular tissue.

Prostatic carcinoma. Fully developed prostatic adenocarcinoma was first seen in animals 7 months after implantation of hormone pellets. However, smaller foci, often referred to as carcinoma in situ (Figs. 7, 8), were detected earlier in both the DLP and VP. Carcinoma in situ of rats is thought to correspond to latent prostate carcinoma in man. These pleomorphic tumor cells were often seen occupying one side of the tubule. With growth, tumors expanded to fill the tubule and increasingly involved interacinar connective tissue or invaded adjacent acini, resulting in the formation of tumor masses.

In fully developed carcinoma (adenocarcinoma) detected 7 months after treatment, different histopathological gradings were observed. They were classified as well, moderately, and poorly differentiated types by evaluating the differentiation (glandular structure) and the anaplasia (nuclear features) of the tumor. Well-differentiated tumors were composed of well-differentiated glands lined with slight nuclear anaplasia. Typical cribriform patterns were characteristic of these lesions, with cuboidal cells and a thin fibromuscular stroma (Fig. 9). Moderately well-differentiated tumors were composed of glandular structures which were sometimes not clearly delineated, and were often lined with cells with nuclear anaplasia. Cribriform glandular structures of variable sizes mingled with masses of less recognizable glandular patterns (Fig. 10). Poorly differentiated tumors were characterized by masses of tumor cells, which formed poorly recognizable glandular structures or no recognizable glandular structures. Cells were small and cuboidal, and with nuclear anaplasia (Fig. 11). The tumor cells sometimes formed cysts, or projected into the lumen of the tubules. Cribriform microglandular cystic pat-

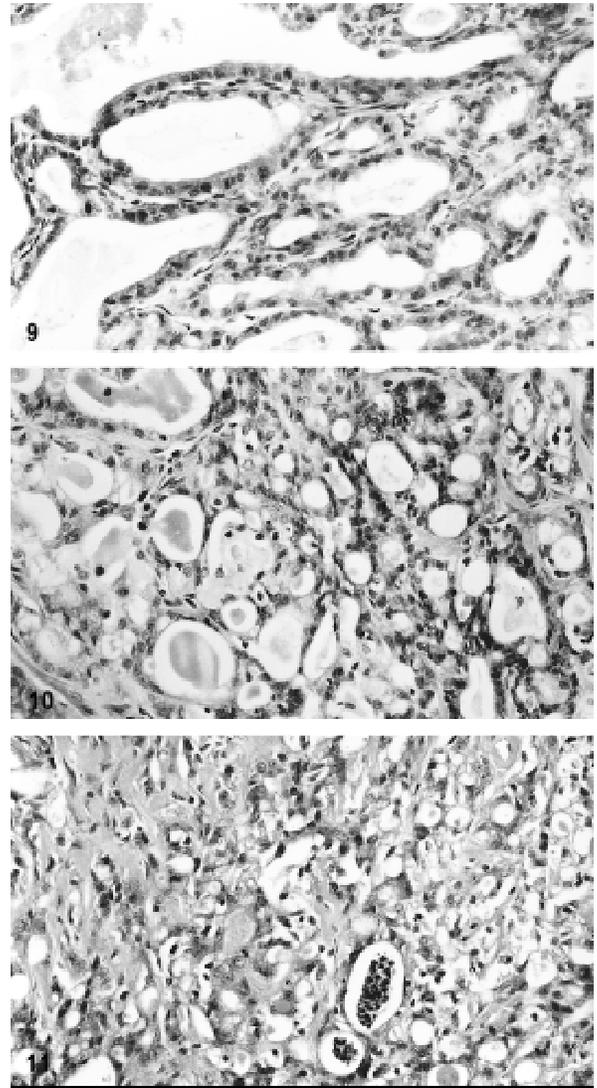


Fig. 9. Well-differentiated carcinoma in DLP. Note the easily recognizable glandular pattern of this tumor. Typical cribriform patterns were characteristic of this lesion, lined with cells with slight nuclear anaplasia. A thin fibromuscular connective tissue is seen permeating this tumor (H&E, $\times 240$).

Fig. 10. Moderately well-differentiated carcinoma in DLP. Note the multiple irregular acinar cell nests interspersed with a less recognizable glandular structure (H&E, $\times 240$).

Fig. 11. Poorly differentiated carcinoma in DLP. Small tumor cells with prominent nuclei and scanty cytoplasm and occasional glandular arrangements are apparent (H&E, $\times 240$).

terns were the predominant feature. The nuclei of cells were hyperchromatic and pleomorphic. The sizes of these cyst-like structures were variable, and loss of nuclear polarity was prominent.

Immunohistochemistry

VEGF and VEGF-R (Flk-1). No definite expression of VEGF was seen in the 28 cases of normal DLP (Fig.

12a). In the majority of cases of prostatic hyperplasia (27/28) and dysplasia (25/27), the levels of VEGF expression in epithelial cells were very high (Fig. 12b). VEGF protein, however, was, strongly expressed by epithelial cells in areas of hyperplasia, with strong staining in the nuclei of some of the epithelial cells. However, no attempt was made to quantify the nuclear reactivity of these cells, as a spectrum of reactivity often existed, and it would be very difficult to obtain an accurate estimation of its number. Intensive staining was also seen in the muscular wall of arterioles. There was no detectable reaction in endothelial cells of the said arterioles and smaller vessels, i.e., venules and capillaries (Fig. 12c). The reactivity of VEGF remained high in prostate cancer (Fig. 12d), and the intensity of VEGF in most cases of carcinoma (15/19) was as strong as that in prostatic hyperplasia and dysplasia. Strong VEGF activity was frequently detected also in the nuclei of tumor cells (Fig. 12e). However, in 4 cases where the tumors were of well-differentiated type, the levels of VEGF expression were low, and staining was mainly located in the cytoplasm of tumor cells (Fig. 12d). In the cases where tumor cells were positive for VEGF, the main source of VEGF production may be the tumor cells themselves. Furthermore, the stromal vascular endothelial cells, where receptors of VEGF were located (Fig. 12f), were conspicuously negative to VEGF protein in the majority of cases of prostatic hyperplasia, dysplasia, and adenocarcinoma. The control sections with the omission of the primary antibody and primary antiserum preabsorbed with VEGF were all negative. The reactivity of anti-VEGF antiserum preabsorbed by other factors (i.e., bFGF and EGF) was not affected. This would suggest that VEGF from glandular cells and stromal arteriole muscle cells may act on endothelial cells to enhance proliferation of endothelial cells. It is strongly suggestive from this study that VEGF may be used as an indicator for differentiating abnormal growth from the normal prostate.

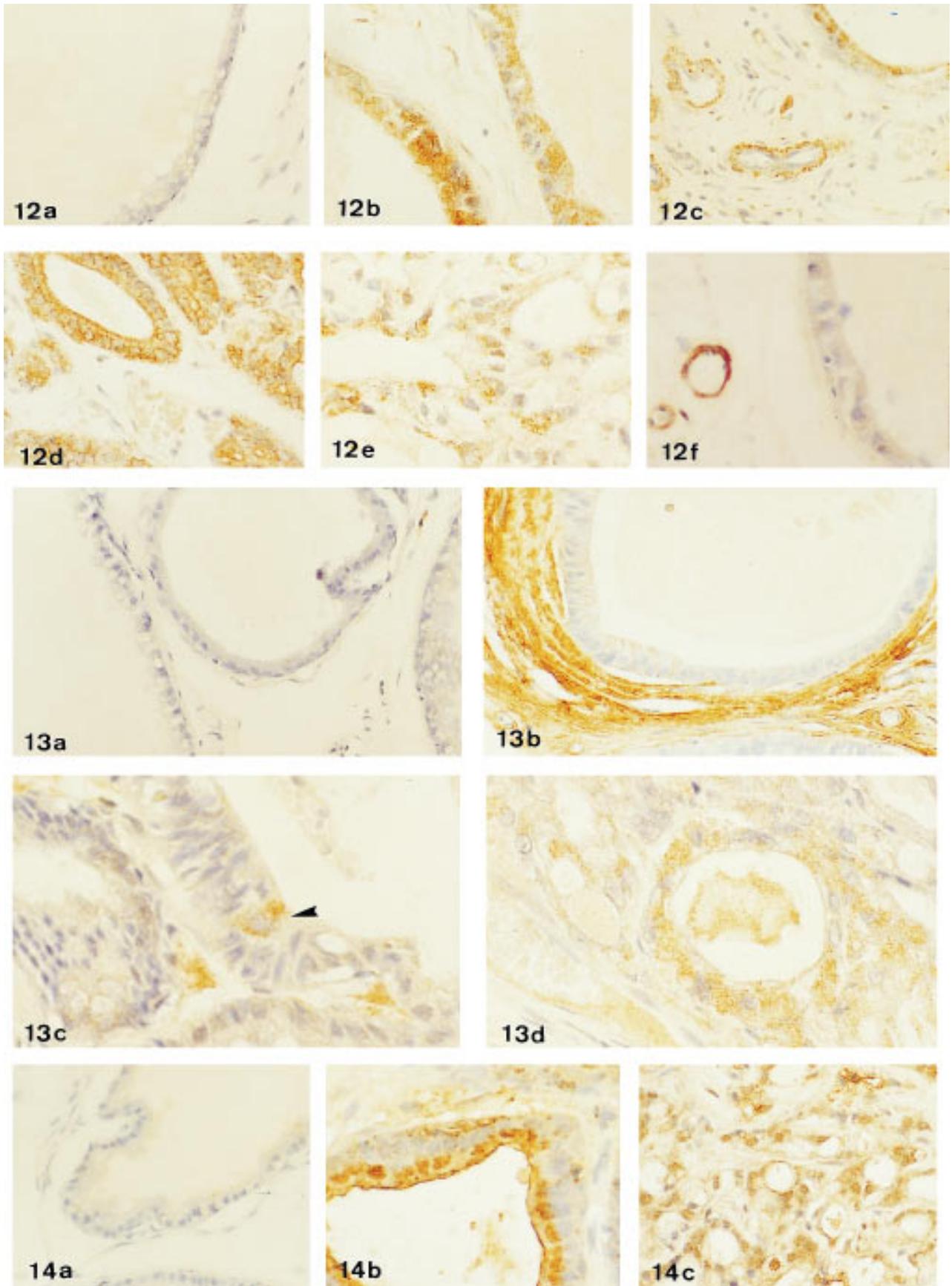
IGF-1. In 6 out of 12 normal rats, IGF-1 was undetectable in DLP (Fig. 13a), whereas in the other 6 cases, they were very weakly positive, if at all, for IGF-1, which was restricted to stroma, while epithelial cells were negative. In hyperplasia of DLP, 23 out of 24 rat prostates showed intense staining for IGF-1 in stroma, but all epithelial cells remained negative to IGF-1 (Fig. 13b). Within the stroma, reactivity appeared stronger in regions immediately adjacent to the tubules/acini than in regions away from them. The same pattern was also observed in ventral prostatic hyperplasia. In dysplasia, some dysplastic epithelial cells (malignant cells) in DLP showed positive reaction to IGF-1 (Fig. 13c), while the stroma still remained strongly positive

for IGF-1. In the majority of cases with carcinoma (17 out of 21 carcinomas) (Fig. 13d), the reactivity of IGF-1 was as strong as that in hyperplastic and dysplastic regions. The distribution of IGF-1 was mainly located in carcinoma cells instead of stromal components. However, in the remaining carcinoma cases, IGF-1 was weakly expressed or undetectable. The expression in prostate cancer was variable. Generally, the results showed that IGF-1 was undetectable in normal control tissue. Its level increased in the stroma in both non-hyperplastic and hyperplastic regions after hormonal treatment initially. The level was nevertheless higher in the stroma of hyperplastic regions. In dysplastic regions, however, IGF-1 was found not only in stroma but also in certain dysplastic epithelial cells. In prostate cancer, IGF-1 was detected mainly in carcinoma cells instead of stromal cells. The control sections (primary antiserum omission and preabsorption or neutralization by specific factor) were negative, while those preabsorbed with nonspecific antisera reacted positively. This showed the specificity of reactivity observed.

IGF-1R. In all 10 DLP (Fig. 14a), IGF-1R was weakly expressed in glandular epithelial cells. This weak positivity was confined, in most cases, to the luminal border of epithelial cells. Meanwhile, intense IGF-1R reactivity was observed in ductal regions of normal LP, and the positive reaction was seen clearly on the epithelial cell membrane. The different expression between glandular and ductal regions may suggest the different susceptibility of the two regions to hormonal carcinogenesis. In all 20 cases with hyperplasia, 20 cases with dysplasia (Fig. 14b), and 14 cases with neoplasm (Fig. 14c), IGF-1R was strongly expressed in both epithelial/tumor cells.

DISCUSSION

The present work demonstrates that by using a protocol modified from that of Leav et al. [12,13], with an increased dosage of testosterone and estradiol, a very high incidence of prostate adenocarcinoma can be achieved. The exact difference in dosage from those used earlier [12–15,19] is difficult to work out. Although the number of capsules used is similar to those reported, the internal diameter of the Silastic tubing is slightly larger in the present study. Also, testosterone is packed tightly to ensure that the given amount (i.e., 175 mg for two 2-cm tubes) is reached. It has been estimated that our manual packing method for testosterone contained at least twice as much hormone as the conventional “pipette-suction” packing method. The estradiol level was only marginally higher, as we used a slightly larger Silastic tubing. The dosage



Figs. 12-14.

(25 mg) used represents an average amount which can be packed into 1-cm tubing by a "pipette-suction" packing method. In the present model, over 90% of animals treated with these sex hormones developed full-blown adenocarcinoma ranging from highly differentiated to poorly differentiated types, though metastasis was not observed. We have also demonstrated that in this system, prostate carcinogenesis follows a multistep pattern, with hyperplasia and dysplasia developing as early as 2 months after hormone implantation, which are followed by carcinoma in situ and fully developed adenocarcinoma. The earliest fully developed adenocarcinoma observed was 7 months after treatment, though carcinoma in situ was detected in animals 4 months after treatment. In fact, our latest results (data not included in this paper) indicate that adenocarcinoma occurs in animals 4 months after hormone implantation. This is much earlier than what was reported by Leav et al. [12,13,19]. In fact, they reported only dysplastic changes after 16 weeks, with no record of fully developed incidence of carcinoma in situ or fully developed adenocarcinoma. Our modified hormonal protocol has achieved, within a relatively short time period, what earlier models have failed to accomplish. The modified protocol, with a highly increased testosterone level, ensures a high incidence of prostate carcinogenesis in a relatively short time.

However, it must be pointed out that while the modified hormonal protocol has significantly increased the incidence of adenocarcinoma, no invasive adenocarcinomas or metastases were observed, although a number of animals had significantly enlarged prostate gland at laparotomy. In this sense, a clinically aggressive adenocarcinoma has not been obtained despite our observation of poorly differentiated prostatic carcinomas in a number of animals. It is therefore somewhat disappointing that despite the greatly increased incidence of carcinogenesis, our protocol has not produced a clinically relevant prostatic adenocarcinoma. However, this model has provided

us with an excellent opportunity to examine in detail the early changes of prostatic carcinomas.

We have also demonstrated the multistep nature of hormonal carcinogenesis with clearly identifiable stages from hyperplasia through dysplasia, and carcinoma in situ, to frank adenocarcinoma in the Noble rat. In time, this will prove to be a very useful model for detailed analysis of mechanisms underlying hormonal prostate carcinogenesis.

Also, the current model is effective in inducing carcinogenesis not only in DLP, which is believed to be more susceptible to hormonal carcinogenesis, but also in VP, though in a much lower frequency. Nevertheless, several cases of fully developed adenocarcinomas have also been achieved. Carcinogenesis in VP appears to follow a pattern similar to that of DLP with hyperplasia/dysplasia: carcinoma in situ and eventually adenocarcinoma have been observed.

It appears that, in the present system, the process of carcinogenesis is preceded by hyperplasia, as more hyperplastic sites or foci were observed in animals 2 months after treatment. Similar sites continued to be present in specimens with fully developed adenocarcinomas from animals treated with hormones for a much longer time. Very often, small dysplastic foci were present among hyperplastic cells. This leads us to believe that dysplastic cells may be derived from hyperplastic ones, and that hyperplasia/dysplasia is thus the earliest sign of prostate carcinogenesis. If this is the case, the present animal model might follow a slightly different pathway of carcinogenesis compared to that of human prostate cancer. It is generally accepted that human BPH is not precancerous, and carcinomas are believed to develop, as a rule, independently and not from BPH precursors. Human BPH is normally unable to develop further into carcinoma. In this sense, prostate carcinogenesis in our present model may be following a slightly different pathway from human prostate cancer. Despite this difference from human prostate cancer, with its clearly defined

Fig. 12. VEGF and VEGF-R. **a:** Normal LP. Note the absence of VEGF ($\times 240$). **b:** Hyperplasia in LP. Positive staining is observed in glandular epithelial cells. The intensity of staining for VEGF is strong in certain epithelial cells. The cells endowed with cribriform pattern are less intensely reacted with it ($\times 240$). **c:** Hyperplasia. This shows the strong reactivity of VEGF in the muscular layer of stromal arterioles. The endothelial cells appear negative in both the arterioles and venules as well as the capillaries ($\times 240$). **d:** Well-differentiated carcinoma. Note the presence of VEGF, mainly in adenocarcinoma cells ($\times 240$). **e:** Poorly differentiated carcinoma. VEGF is observed in most tumor cells ($\times 240$). **f:** Normal LP. VEGF-R reactivity is observed in the endothelial cells ($\times 240$).

Fig. 13. IGF-I. **a:** Normal LP. Note that there is no detectable IGF-I reactivity in normal LP ($\times 240$). **b:** Hyperplasia in LP. The reactivity of IGF-I becomes very strong in stroma of the hyperplastic region. However, the epithelium remains negative to it ($\times 240$). **c:** Dysplasia in LP. The reactivity of IGF-I is seen in both the stromal and some epithelial (dysplastic) cells (arrowhead). The majority of epithelial cells remain negative ($\times 450$). **d:** Carcinoma in LP. IGF-I reactivity is detected mainly in carcinoma cells. It must be pointed out that not all malignant cells are positive to IGF-I, even in the neoplastic state ($\times 360$).

Fig. 14. IGF-IR. **a:** Normal LP. Weak or undetectable reaction of IGF-IR in glandular epithelial cells is observed ($\times 240$). **b:** Dysplasia in LP. The reactivity of IGF-IR is very strong in cells of the dysplastic region ($\times 450$). **c:** Carcinoma. Most carcinoma cells are IGF-IR-positive ($\times 290$).

developmental stages, the present model may prove to be most useful and to offer even more advantages for detailed analysis of the various factors influencing prostate carcinogenesis, as it enables us to analyze the complete spectrum from hyperplasia/dysplasia to fully developed adenocarcinoma of the prostate gland.

As stated earlier, human prostate carcinogenesis has been viewed as a multistep process involving progression from prostatic dysplasia (precarcinoma) to carcinoma, from low histologic grade, small, latent carcinoma to large, higher-grade, metastasizing carcinoma. However, recent data suggest that a variety of pathogenic pathways may exist. The precise etiology and pathogenesis of human prostate cancer remain largely undefined. It is difficult to investigate various stages in human prostate cancer during development. Animal models, on the other hand, provide opportunities in this regard [20]. Our present model of obtaining a high incidence of prostate cancer in a relatively short time provides an excellent animal model for examining in detail the various factors and mechanisms involved in prostate carcinogenesis, especially early events. The fact that VP carcinogenesis can be induced by T + E₂ regime is significant, as it enables one to analyze the process of carcinogenesis in LP and VP, which are known to have different susceptibilities to hormonal carcinogenesis [12,13]. We have also shown that LP and VP have a very different TGF- α profile; the former is positive, while the latter is negative to this factor [21]. Carcinogenesis in LP involves overexpression of TGF- α , while in VP this factor remains negative throughout. In the present study, although the incidence of carcinoma in VP was low, hyperplasia and dysplasia were very common. It would appear that fewer dysplastic cases eventually succeed in developing into adenocarcinoma in VP. The reason for this difference in incidence is at present unknown. However, their drastic differences in levels of TGF- α in both normal and treated prostates, as reported [21], should be looked at closely.

In addition, this synergism between T and E₂ does markedly induce an increase in cell numbers and extracellular matrix in both epithelial and stromal components. As a result, the whole prostate-to-body weight ratio is significantly increased after hormonal treatment. An increase in prostatic wet weight may correlate with enhanced secretory activity, an increase in cell number and extracellular matrix, and an increase in DNA synthesis in the glands of treated rats. The hormone-induced prostatic carcinogenesis in the Noble rat model shows a clear multistep process which is similar to that of human prostatic carcinogenesis [13]. Thus, the modified animal model can be widely used for studies of prostate cancer, although

the precise role of hormones in carcinogenesis is unknown.

Staging of prostate cancer is a systematic classification of the extent of disease based on clinical and pathological criteria [22]. Knowledge of the histopathology of the normal and neoplastic prostate is essential [23], although the clinical course of prostate cancer is highly variable and cannot satisfactorily be predicted by histological criteria alone [24]. In the present study, we have reaffirmed, based on histopathology, that prostate carcinogenesis complies with this multistep process (i.e., normal, hyperplasia, dysplasia, carcinoma in situ, well-differentiated carcinoma, moderately well-differentiated carcinoma, and poorly differentiated carcinoma). The systematic studies based on the Noble rat model have provided an insight into the role of IGF-1 and VEGF during prostatic carcinogenesis.

It is well-known that VEGF is considered a crucial regulator of endothelial cell proliferation, migration, and permeability during embryonic vasculogenesis as well as in physiological and pathological angiogenesis. The importance of angiogenesis in the process of growth and progression in solid tumors has also been widely accepted. We believe that there should be a relationship between VEGF expression and prostatic carcinogenesis in our rat model system as well. To test this, we examined the expression of VEGF in normal prostate, hyperplasia, dysplasia, and carcinoma induced by sex hormones. The results show that no definite expression of VEGF is seen in the normal prostate. On the other hand, the levels of VEGF expression in epithelial cells of both prostatic hyperplasia and dysplasia are very high. Its reactivity in most cases of carcinoma remains as high as that in prostatic hyperplasia and dysplasia. In addition to epithelial cells, the stromal arteriole muscle cells are also strongly positive to VEGF protein in a majority of cases of prostatic hyperplasia, dysplasia, and carcinoma. In this connection it is of interest to state that no reactivities are detected in smaller vessels, venules, or capillaries where no muscle can be found. These observations are in agreement with Dvorak et al. [25], who found that VEGF mRNA is synthesized in tumor cells and accumulated in nearby tumor blood vessels. Taken together, our findings suggest that VEGF, a factor produced mainly by prostatic epithelial cells in hyperplasia, dysplasia, and carcinoma as well as by arterial muscle cells, may act on endothelial cells where receptors of VEGF are known to be located in stroma. Through this paracrine action VEGF exerts an important role in the angiogenesis which is associated with the development and staging of human prostatic carcinoma. Therefore, VEGF may play an important role in prostate carcinogenesis by stimulating angiogene-

sis. This is the first report on the relationship between VEGF and VEGF-R expression and the development of prostate cancer. Furthermore, this study strongly suggests that VEGF may be used as an indicator for differentiating abnormal growth (hyperplasia, dysplasia, and neoplasm) from the normal prostate. VEGF may serve not only as a biomarker for the development of prostate cancer, but also as a new therapeutic target in prostate cancer by anti-VEGF and antiangiogenesis.

The IGF system consists of two ligands, IGF-1 and IGF-2, which are potent mitogens for the growth of prostate cells, their receptors (IGF-Rs), and six IGF-binding proteins (IGFBPs) [26]. Thrasher et al. [26] further suggest that significant changes in the IGF system occur with malignant transformation in the prostate. We hypothesized that IGF-1 and IGF-1R play an important role in prostatic carcinogenesis, and that changes in their expression and/or distribution could be used as indicators for the development of prostate cancer. Thus, their expression and cellular location in normal rat prostate, hyperplasia, and prostate carcinoma were examined. The results showed that IGF-1 was undetectable in 50% of normal prostates, whereas in the other 50% of cases, they were very weakly positive, if at all, for IGF-1, which was restricted to stroma, while epithelial cells were negative. In hyperplasia, IGF-1 was intensely stained in stroma, but all epithelial cells remained negative to IGF-1. Meanwhile, the reactivity of IGF-1R was detected in epithelial cells of both normal and hyperplastic prostates, although it was weakly expressed in normal prostates but strongly expressed in hyperplasia. These results suggest that this growth factor, which is produced by stromal cells, may play a role in both normal and hyperplastic prostates by acting on epithelial cells through a paracrine mode. These observations are in agreement with results as reviewed by Culig et al. [27]. Earlier studies showed that neither IGF-1 nor IGF-1 transcripts or protein in primary culture of human prostatic epithelial cells derived from normal prostates and BPH could be detected [28]. On the other hand, Barni et al. [29] found that in prostatic hyperplasia, IGF-1 mRNA is localized exclusively in stromal cells, although IGF-1 is not detectable in these cells in culture [30]. The fact that IGF-1 could not be detected in conditioned medium from prostatic epithelial cells lends support to the belief that IGF-1 may be produced in the stroma, and acts on epithelium as a paracrine growth factor in the normal prostate. This pattern of IGF expression appears to be unchanged in BPH tissue [27].

In dysplasia, it is of interest to note that some dysplastic epithelial cells (malignant cells) begin to show positive reaction in cytoplasm while the stroma re-

mains strongly positive for IGF-1. Once again a strong expression of IGF-1R is observed in epithelial cells at this stage. Our results suggest that IGF-1 may be produced by both epithelial and stromal cells, and with epithelial/tumor cells as its target. Therefore, the action of IGF-1 in prostatic dysplasia may include both the paracrine and autocrine mode.

In most cases with carcinomas, IGF-1 is mainly located in epithelial cells instead of stromal cells, while IGF-1R is still detectable in epithelial/carcinoma cells. Our results indicate that IGF-1 may act as an autocrine growth factor in rat prostatic carcinoma. However, IGF-1 transcripts could not be detected in human prostatic cancer cell lines such as LNCaP, PC-3 and DU145 [31,32]. This may suggest a lack of autocrine mode of action in these cell lines. Such results support the hypothesis that IGFs continue to act as paracrine growth factors in advanced prostatic carcinoma. However, molecular and biochemical studies by other investigators have indicated that all these lines express IGF-1 mRNA and protein [33,34]. Pietrzkowski et al. [34] further reported that treatment of prostatic cancer cells with peptide analogs of IGF-1 that act as receptor antagonists slow down their growth. These results also suggest the existence of an IGF-1 autocrine mechanism in which the overexpressed peptide factor activates its receptor on the same cell, which is in agreement with our studies. Furthermore, Tennant et al. [35] reported that under *in vitro* conditions, the levels of IGF-1 and IGF-1R mRNA in prostatic intraepithelial neoplasms and prostatic cancer cells are significantly lower than those of benign prostatic epithelium. These results are in general agreement with our observations in this animal model, in which levels of IGF-1 and IGF-1R peaked at hyperplasia/dysplasia. Their levels became lower in carcinoma, though they were still significantly higher than in normal prostate epithelium. A direct comparison of the latter (*i.e.*, normal prostatic) epithelium of the rat with normal human prostatic epithelium is not possible, as Tennant et al. [35] included only benign prostatic epithelium and carcinoma cells in their study. However, the results from the present study agree with our preliminary observations of this ligand and its receptors in normal human prostatic tissue, *i.e.*, negative reactivity to IGF-1 and very low expression of IGF-1R immunohistochemically (Wong et al., unpublished observations). The present study presents for the first time the evidence available on IGF-1 expression and action in prostate cancer tissue.

Through our systematic immunohistochemical study of IGF-1 and IGF-1R protein in normal prostate, prostatic hyperplasia and dysplasia, and prostate cancer, several lines of thought may be advanced. First, there is a difference in IGF-1R reactivity between nor-

mal prostate and hyperplasia; the former is negative or undetectable, while the latter is intense. This suggests that IGF-1, which is produced by stromal cells, may be important for the development of prostatic hyperplasia. Second, IGF-1 activity is present not only in the stroma, but also in some epithelial cells of dysplastic regions which are believed to be at a precancerous stage. This indicates that certain dysplastic cells may begin to secrete IGF-1 on their own and become less dependent on ligands from stromal cells. Third, the reactivity of IGF-1 is mainly located in carcinoma rather than stromal cells in prostate carcinoma, which may suggest that these cancer cells can now secrete IGF-1. The changes in expression of IGF-1 during the progression of prostate cancer may be an important biomarker for the staging of hyperplasia, dysplasia, and early prostate cancer. Fourth, there is a significant difference in IGF-1R immunoreactivity between different regions of the normal prostate. The ductal epithelium, which is believed to be a high-risk region for prostate cancer, reacted intensely for IGF-1R, whereas the reaction of glandular cells was weak or undetectable. Based on these observations, we hypothesize that the differences of IGF-1R expression in the prostate gland may reflect the risk incidence for the development of prostate cancer. Fifth, IGF-1R is overexpressed under all abnormal prostatic conditions (i.e., hyperplasia, dysplasia, and prostate cancer) compared with normal prostate, which suggests that a high expression of IGF-1R may be associated with prostate diseases. Plymate et al. [36] reexpressed IGF-1R in malignant human prostate epithelial cells. This resulted in decreased tumor growth and anchorage-independent growth *in vivo*, while increasing the proliferative response to IGF *in vitro*. These findings suggest that the IGF system has a dual function, mediating both proliferation and differentiation. High levels of expression of IGF-1R may act as inhibitor of tumor growth, possibly by turning on a differentiation program. The implications of these *in vitro* results for our observations in this animal model require further investigation.

In short, both IGF-1 and VEGF are undetectable or very weak in normal prostate; however, IGF-1 is up-regulated in stroma of nonhyperplastic regions of the prostate after T + E₂ treatment. Therefore, IGF-1 can act via a paracrine mode on epithelial cells in which its receptors are located. In prostatic hyperplasia, IGF-1 appears to be secreted mainly by stromal cells which exerted its effects, through a paracrine mode, on the epithelial cells that were IGF-1R-positive. VEGF is detected in both epithelial and arterial muscle cells which exerted its effects, through a paracrine mode, on endothelial cells where VEGF receptors were located. In dysplasia, the expression of IGF-1 is not re-

stricted only to stroma, but is also found in certain dysplastic epithelial cells, where they gradually become less dependent on stromal factors. Hence IGF-1 exerts its effects on epithelial cells through both paracrine and autocrine modes. The cellular location of VEGF remains the same as in hyperplasia. Carcinoma cells might be the main producers for IGF-1 and VEGF. They exert their effects through both autocrine and paracrine modes. However, IGF acts on epithelial cells mainly via an autocrine mode, while VEGF exerts its effect on endothelial cells mainly via a paracrine mode. As carcinogenesis progresses, carcinoma cells gradually become more reliant on their own IGF-1 and VEGF. The present studies reaffirm our hypothesis that the interactions between stromal and epithelial cells during prostatic carcinogenesis are mediated at least partly by IGF-1 and VEGF.

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