# Negative control of epithelial cell proliferation by prostatic stroma

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# Negative control of epithelial cell proliferation by prostatic stroma.

De remmende invloed van het stroma op de groei van de epitheliale cellen van de prostaat.

**Proefschrift**

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam.
Op gezag van de Rector Magnificus Prof. Dr. P.W.C. Akkermans M.A.
en volgens het besluit van het college voor promoties.

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door

**Anko Kooistra**

geboren te Ermelo

## Promotiecommissie

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Voor mijn moeder
Aan Marianne en Kirsten
Ter nagedachtenis aan mijn vader

# Contents

# Contents

# Chapter 1

# General introduction

## 1.1. Prostatic disease

Prostatic diseases are rather frequently occurring disorders in the male population. Prostatic adenoma -better known as benign prostatic hyperplasia (BPH)- is the most common benign neoplasm in men. Prostate cancer is the most commonly diagnosed malignancy in men and second leading cause of male cancer death in Western countries[1]. In The Netherlands, the morbidity and mortality caused by this tumor are exceeded only by those originating in lung cancer; in 1991, 4,343 incident cases and 2,108 deaths from cancer of the prostate were recorded in a male population of 7.5 million[2]. The risk of developing prostate cancer increases dramatically with age, as does the occurrence of the proliferative disorder BPH[3]. Indeed, men over the age of 65 that do not present with symptoms of prostate cancer during life very frequently are found to contain microscopic malignant lesions upon autopsy[4]. Many patients with prostatic cancer are diagnosed with disseminated disease. The majority of these disseminated prostatic carcinomas are androgen dependent and respond to hormonal manipulation, i.e., medical or surgical castration[5]. The effectiveness of this therapy, however, is only temporary; after an initial response the tumor escapes from endocrine regulation to become insensitive to treatment based on the withdrawal or blockade of androgens. Such patients will eventually die of disseminated hormone-refractory prostate cancer. The etiology of prostate cancer is not clearly defined, it is thought to be multifactorial, involving genetic, hormonal, dietary and environmental causes. The mechanisms leading to progression of prostate tumors to autonomy are also largely unknown. Moreover, despite decades of experience with different treatment modalities, there are still controversies relating to patient selection criteria and choice of treatment[6]. The recent development of different (medical) treatments for BPH has given rise to similar discussions concerning benign enlargement of the gland[7].

## 1.2 Prostatic growth regulation

A better understanding of the mechanisms which regulate prostatic growth in the normal and neoplastic prostate may facilitate the eventual clinical manipulation of aberrant prostatic expansion. Prostatic growth and expression of differentiated function have been shown to be regulated through endocrine factors, extracellular matrix components, autocrine factors, and paracrine factors, all of which may be interdependent to various degrees.

### 1.2.1. Endocrine dependency

With respect to endocrine regulation, the dependence of stromal cells[8] and epithelial cells[9-11] in the prostate on androgens for their growth and development is well documented[12]. It is also known that in eunuchs and early castrates no (adult) prostate develops and no prostate cancer nor BPH is found later on in life[5]. The mechanisms of transition of prostate cancer to a hormone-unresponsive growth are not yet understood. Clonal selection and clonal overgrowth of hormone-insensitive cells has often been suggested to be the cause of autonomous growth. However, the basic events, either genetic or epigenetic, that induce cellular hormone independence remain to be identified.

### 1.2.2. Extracellular matrix

With regard to the extracellular matrix (ECM) there is considerable support for the concept that structural links between stroma and epithelium, formed by components of the extracellular matrix, mediate and transduce signals between both compartments. Such interactions may play a role in the control of prostatic growth and development[13,10].

The importance of ECM in stromal-epithelial interactions was demonstrated by the finding that extracts of ECM (from stromal cell cultures) can induce similar effects on epithelial cell behavior as intact stromal cells. Stromal tissues contain different types of macromolecules, including collagens, glycoproteins and proteoglycans. These molecules form extracellular matrix structures that contribute to tissue architecture by providing cell attachment sites. Adhesive interactions between cells and ECM structures play a crucial role in epithelial morphogenesis[14]. There is convincing evidence that ECM proteins also control differentiation and gene expression in adult organs[15-17]. Transmembrane receptors, for instance belonging to the family of integrins[18], are assumed to mediate the transmission of external signals to the inside of the cell upon binding to ECM molecules.

The recent observation that basic fibroblast growth factor (bFGF) binds with strong affinity to components of the extracellular matrix such as heparan sulfate proteoglycans and glycosaminoglycans, and that these bound forms of the bFGF can be mobilized in a stable form by proteolytic cleavage of the extracellular matrix[19,20], suggests another mechanism whereby the extracellular matrix may regulate prostatic growth and development.

### 1.2.3. Autocrine growth modulating factors

Autocrine regulation of prostatic growth appears to involve a variety of growth factor polypeptides[21]. In this context, epidermal growth factor (EGF)-like and bFGF-like molecules account for a considerable amount of the growth factor activity in the prostate[21]. In addition,

acidic FGF[22], transforming growth factor-beta (TGF-ß)[23], and nerve growth factor (NGF)[24] have been identified in the prostate or prostatic tumor cell lines. Some of these growth factors may have a paracrine role as well.

### 1.2.4. Paracrine regulatory factors

Paracrine regulation of prostatic growth was first suggested by Franks *et al.*[25] after observing a lack of growth capacity of epithelia which had been separated from their stroma. Subsequently, Cunha *et al.* demonstrated that fetal mesenchyme (stroma) induced prostatic epithelial morphogenesis from urothelium[12]. The total prostatic growth correlated well with the amount of mesenchyme used[26]. Under normal conditions epithelial cells continue to express their characteristic morphological and functional properties also during adulthood. This stability of epithelial differentiation appeared to be based on instructive induction by the stroma rather than on irreversible commitment of the epithelial cells. Indeed, the proliferation of epithelial cells within the prostatic acini exhibits a regional heterogeneity, indicating that local control mechanisms, such as paracrine interactions with stroma, regulate differentiation and development[27,28]. However, even though several growth factors have been identified in the prostate from whole tissue homogenates and various cell lines, the specific growth factor interactions between epithelial cells and stromal cells, and therefore their identity as paracrine growth factors, remain to be investigated.

## 1.3. Prostatic size

### 1.3.1. Trophic influence of stroma

Upon maturation, accessory sexual organs reach a typical size and weight which is characteristic for that particular organ. Administration of hyperphysiological doses of androgen to intact male rats did not stimulate prostatic growth beyond normal adult size. Likewise, administration of testosterone to androgen-deprived rats restores the size of the prostate to its normal precastration level without inducing overgrowth, even after prolonged administration[12]. At that stage, epithelial cell proliferation is low and in balance with cell death[9]. Experiments performed by Chung and Cunha[26] showed that epithelial cell growth and, therefore, absolute prostatic size was regulated by the amount of mesenchymal (stromal) tissue present. During embryological development, androgen receptors are present only in the mesenchyme, suggesting that at this stage all androgen-induced events are mediated via the stroma[12,29]. Furthermore, it was shown that also in the adult rat prostate, the proliferative response to

androgens was mediated through the stroma[30].

Likewise, the *in vitro* growth of prostate epithelial cells was found to be stimulated by androgens in cocultures with prostate stromal cells[30,31], but not in isolated epithelial cultures[32,33]. Interestingly, prostate stromal cells were shown to express keratinocyte growth factor (KGF) in response to androgens while epithelial cells expressed the gene coding for a receptor which specifically binds KGF[31]. These observations strongly suggest that KGF is involved in the paracrine mechanism of androgen-regulated prostate epithelial growth.

### 1.3.2. Negative control via the stroma

At this point it is tempting to speculate about a regulatory role of the stroma also in castration-induced involution of the gland. Androgen-induced stromally derived inhibitors of epithelial cell growth could participate in keeping the delicate balance between cell proliferation and (programmed) cell death. Normal (non-transformed) murine and human prostatic epithelial cells grown *in vitro* were shown to proliferate in serum-free medium without the presence of androgens[32,34-36]. Androgen ablation *in vivo* (in the presence of connective tissue), however, induces a marked regression of the glandular epithelium[9]. These findings may be indicative of a role for the stroma in the negative control of epithelial cell proliferation. One of the well known inhibitors of epithelial cell growth, transforming growth factor-ß (TGF-ß), was shown to be present in prostate tissue[37]. Its expression appeared to be regulated by androgens in an inversely related manner[38]. However, both epithelial and stromal cells were found to be capable of expressing and secreting TGF-ß *in vitro*, and there is still doubt about the actual site of TGF-ß production in prostatic tissue.

## 1.4. Scope of the thesis

The aim of the present work was to study the stromal-epithelial interactions in the human prostate with special attention to exploring the hypothesis of a negative control of epithelial cell proliferation by prostatic stroma.

Chapter 2 of this thesis summarizes the available evidence demonstrating the profound influence of stroma on the epithelium.

Since the majority of the experiments published were performed *in vivo* using tissue recombinants such studies did not provide information about the exact nature of signal transducers between the two compartments as was mentioned in paragraph 1.2.4. For this reason we used an approach in which epithelial cells and stromal cells were physically

separated. To this end, human stromal cells were cultured from diseased prostates by means of tissue explant cultures; this method as well as the immunocytochemical characterization of such cells is described in chapter 3.

Using a double layer soft agar system separating stromal and epithelial cells, a negative control of epithelial cell proliferation by prostatic stromal cells was found, as presented in chapter 4.

Later it was shown that the epithelial cells were not only inhibited in their clonal growth (in soft agar), but also in their anchorage-dependent growth (on culture plastics). Furthermore, the anti-proliferative activity was found to be present in the conditioned medium of the stromal cells. This enabled us to employ a more simple bioassay, the MTT-test, making it possible to test a larger number of samples at the same time and to manipulate the conditioned medium before testing its activity on the prostatic epithelial cells. As is described in chapter 5, the results from such tests suggested that the antiproliferative activity was specifically produced by prostatic stromal cells and that the reversible inhibition of (prostatic) epithelial cell growth was organ but not species specific.

The data presented in chapter 6 demonstrate that human prostate derived stromal cells cultured from neoplastic lesions produce a unique factor, tentatively called "prostate-derived epithelium inhibiting factor": p-EIF, which on the basis of its spectrum of biological activity as well as its physicochemical and immunoreactive properties, can be discriminated from previously described growth inhibitors.

Finally, in chapter 7 the importance of these findings as well as possible directions of future research are discussed.

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# Stromal-epithelial interactions in the prostate and its relation to steroid hormones

A. Kooistra

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## 2.1. Introduction

Since 1953, when Grobstein[1] published his study on the role of tissue interactions in the embryogenesis of the kidney, stromal-epithelial interactions have been shown to play a vital role in numerous organ functions. In a classic series of experiments on tissue recombinants, Cunha, Chung, and others have clearly demonstrated the importance of the embryonic mesenchyme in prostatic ductal morphogenesis, epithelial growth, secretory cytodifferentiation and function[2-7]. By the use of tissues derived from mice with the testicular feminization syndrome (containing non-functional androgen receptors) they were able to show that probably all androgen-induced events during fetal life are mediated by mesenchyme.

Tissue recombinants could be grown to maturity and striking similarities with the normal adult prostate were observed, especially during castration-induced atrophy as well as androgen-induced regeneration of the gland. These findings suggest that these models are applicable to the adult prostate too. Moreover, in systems containing undifferentiated stem cell populations, an orderly process of epithelial differentiation and functional maturation must be maintained as cells continually die and have to be replaced[8]. Thus, fundamentally similar processes seem to occur in both the embryo and the adult.

We have to evaluate these findings carefully to see whether they can provide us with any insight in the processes leading to neoplasia. This review will focus on those studies that show the importance of mesenchyme in the development of the gland as the mediator of androgenic effects upon the epithelium, and of the stroma in the homeostasis in the adult prostate. Further, studies on the stroma of neoplastic lesions are summarized and the leading theories about the etiology of benign prostatic hyperplasia (BPH) and prostatic cancer based on these observations are discussed. Special attention is paid to the action of androgens in the stromal-epithelial relationship in order to start unravelling the mechanisms that lead to hormone independent disease.

## 2.2. Mesenchymal regulation of epithelial morphogenesis in the male genital tract

The male genital tract develops from two embryonic structures: the Wolffian duct and the urogenital sinus. The epithelium of the Wolffian duct is of mesodermal origin and gives rise to the epididymis, ductus deferens, seminal vesicles and ejaculatory ducts. The urogenital sinus, of which the epithelium is endodermal in origin, gives rise to the prostate, bulbourethral glands, urethra, periurethral glands and contributes to the urinary bladder[2,9]. By analyzing the

development of heterotypic tissue recombinants, Cunha[10] showed that urogenital sinus mesenchyme cannot induce seminal vesicle epithelium to express prostatic differentiation, but it is able to support the expression of the characteristics of seminal vesicle development (Figure 1a). Similarly (Figure 1b), seminal vesicle mesenchyme permits the normal expression of prostatic differentiation in epithelium of the urogenital sinus, and does not elicit seminal vesicle development from urogenital sinus epithelium. Therefore, in these permissive inductions (as opposed to instructive inductions), the heterotypic mesenchyme promotes expression of the normal developmental fate of the epithelium. Epithelia of mesodermal origin may only be able to differentiate into mesodermal derivatives, whereas endodermal epithelia may only be able to form endodermal derivatives[3]. These findings were supported by analysis of tissue recombinants of bladder epithelium and mesenchyme from urogenital sinus[9]. Epithelium of the urinary bladder is endodermal in origin and in these experiments recombinants of urogenital sinus mesenchyme and bladder epithelium developed into prostate-like structures (Figure 1c). Prostatic differentiation could be induced whether the bladder epithelium is derived from either fetal or adult animals[11]. This represents an example of instructive induction because the mesenchyme reprograms the developmental fate of the epithelium[3].

In a series of similar experiments, prostatic ductal morphogenesis was induced by combining urogenital sinus mesenchyme and epithelium derived from urogenital sinus, fetal or adult bladder, postnatal vagina or adult prostate[9,10,12-14]. All these epithelia are derived from the primitive endodermal urogenital sinus. It should be noted that these results can only be obtained if the recombinant is grafted into an intact male host (where androgen levels are adequate) and if urogenital sinus mesenchyme is used. Extensive analysis of urogenital sinus mesenchyme and adult bladder epithelium tissue recombinants demonstrated that the epithelial ducts induced by urogenital sinus mesenchyme express histological, ultrastructural and functional features typical for prostate tissue, including androgen receptors, prostate-specific antigens, androgen- dependent DNA synthesis, characteristic protein patterns and histochemical profiles indicative of the prostatic phenotype[9,11,15,16].

Both instructive and permissive inductions by urogenital sinus mesenchyme appear to be mediated by similar, if not identical, signals in a variety of mammalian species. All heterospecific recombinants of urogenital sinus mesenchyme and urogenital sinus epithelium or bladder epithelium derived from mouse, rat, rabbit and human showed prostatic differentiation[17]. From these experiments it is evident that the mediators of these inductive interactions are highly conserved in different mammalian species.

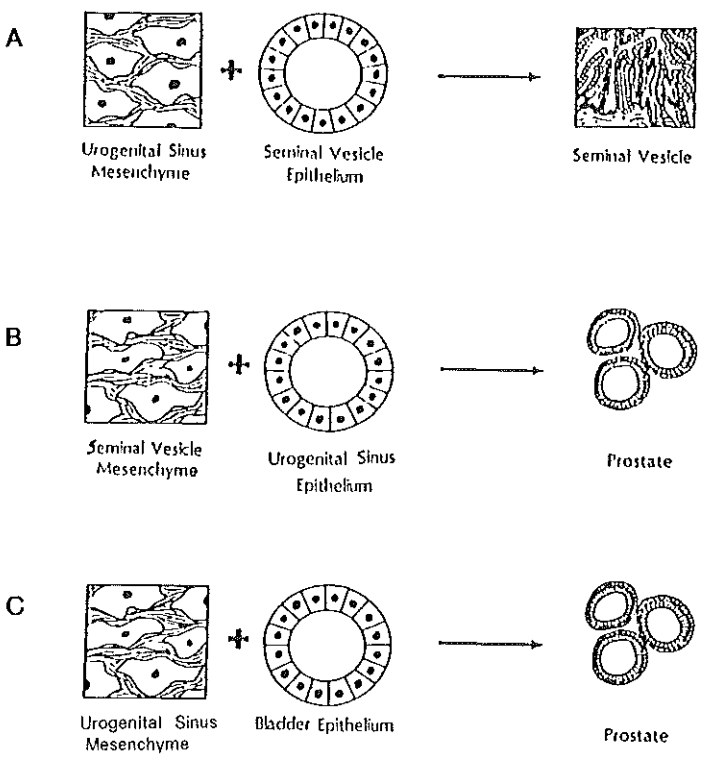


**Figure 1** Schematic representation of the experiments on recombinants constructed with embryonic mesenchyme (from reference 3, with permission).

## 2.3. Mesenchyme as a mediator of androgenic effects upon the epithelium

The development of the prostate as well as the regulation of its adult structure and function are dependent on androgens. These processes are thought to be mediated by androgen receptors. Study on the testicular feminization syndrome (Tfm) provides strong support for the essential role of androgen receptors in prostatic development. Despite adequate testosterone levels, prostatic tissue never develops because of a non-functional androgen receptor[3,11].

Now that the importance of androgens and its receptor has been shown, the question remains whether intraepithelial receptors play any role in this process, for the administration of androgens to castrated mice elicits growth of the prostatic epithelium. Although in the adult mouse androgen receptors are present in both epithelium and stroma the fortuitous association of androgen-induced epithelial growth with intraepithelial androgen receptors, does not in and of itself prove that androgen-induced prostatic epithelial growth is mediated via androgen receptors within the epithelial cells themselves[18]. Indeed, analysis of androgenic effects in Tfm/wild-type tissue recombinants provides strong evidence for the importance of mesenchymal, rather than epithelial, androgen receptors in prostatic development.

First, Kratochwil and coworkers established the idea that mammary mesenchyme is the actual target and mediator of androgenic effects upon the developing mammary gland whose epithelial component regresses in response to testosterone[19]. Later on, the relevance of this concept to the prostate was confirmed by similar experiments in which mesenchyme and epithelium from urogenital sinuses of Tfm and wild-type mouse embryos were combined [3,10,11]. The four possible types of tissue recombinants are indicated in Figure 2. All recombinants were exposed to physiological levels of androgens as they were implanted into intact male hosts. Prostatic morphogenesis occurred only in those recombinants constructed with wild-type mesenchyme. The genotype of the epithelium was irrelevant, as both wild-type and Tfm epithelia formed prostate in association with wild-type urogenital sinus mesenchyme. In contrast, prostatic differentiation did not occur in combinants constructed with Tfm mesenchyme even when wild-type epithelium was used. Thus, in the developing prostate and embryonic mouse mammary gland, the mesenchyme is the actual target and mediator of androgenic effects upon the epithelium, whereas the genotype of the epithelial element does not influence the developmental response. This concept is supported by the observation of nuclear [$^3$H]dihydrotestosterone (DHT) binding sites in mesenchymal cells of wild-type urogenital sinus and mammary gland using steroid autoradiography[20,21]. Tfm urogenital sinus mesenchyme lacks nuclear [$^3$H]DHT binding sites[18]. Surprisingly, the epithelial cells of the developing wild-type glands appear to be devoid of androgen receptors too. The only obvious difference in androgen receptor content between wild-type and Tfm urogenital sinus and mammary gland was observed in the mesenchyme: receptors were present in mesenchymal cells of wild-type embryos but were completely absent in Tfm embryos[18]. All the processes necessary for full prostatic development, i.e. ductal morphogenesis, epithelial proliferation and secretory cytodifferentiation, are expressed in the Tfm epithelium, which, in contrast to wild-type bladder epithelium[22], remains androgen receptor-negative even after prostatic induction[23]. This indicates that all these androgen-induced events must be regulated by the receptor positive stromal cells.

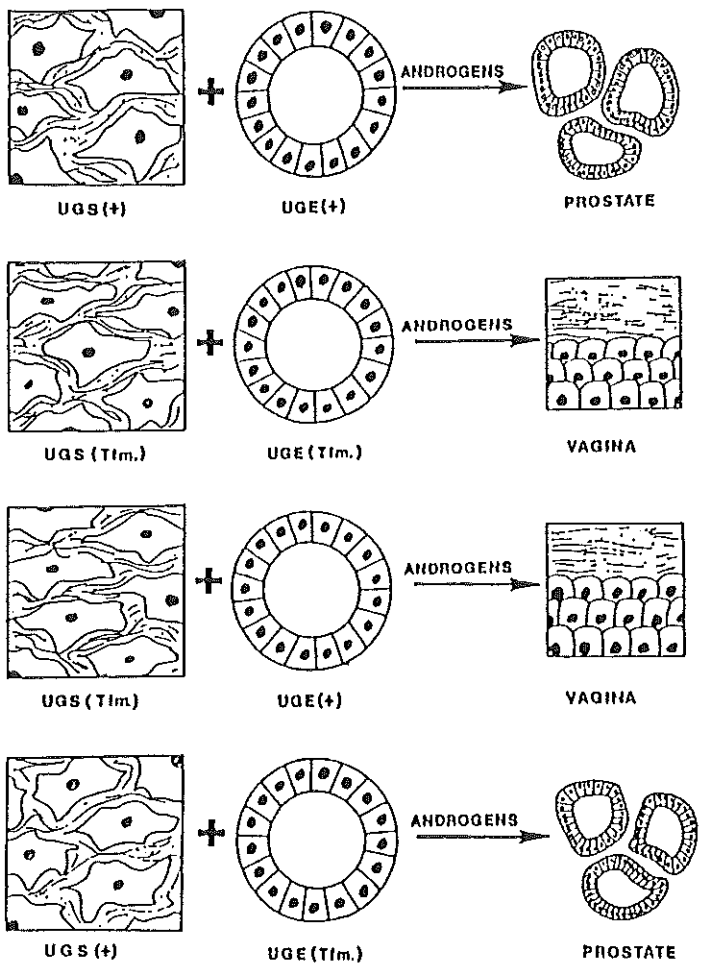


**Figure 2** Summary of Tfm / wild-type recombination experiments of prostatic induction in urogenital sinuses[11]. UGS: urogenital sinus mesenchyme; UGE: urogenital sinus epithelium; Tfm: Testicular feminization syndrome [non functional androgen receptor]; +: normal androgen receptor.

## 2.4. Stromal-epithelial interactions in adult organs

For all organs, epithelial morphology and functional activity must be maintained as cells continually undergo senescence, die and are replaced. In these systems, cellular proliferation must be regulated in order to maintain normal structure and function. Moreover, in systems containing undifferentiated stem cell populations, an orderly process of epithelial differentiation and functional maturation must be maintained[8]. Thus, fundamentally similar processes occur in both the embryo and the adult. In addition, for certain organ systems such as the female genital tract and the mammary gland, true morphogenetic processes occur in a cyclic fashion during menstrual cycles, pregnancy and lactation. These events closely resemble the primary developmental events that occur in the prostate during the perinatal period[3].

Prostatic growth is stimulated by androgens while castration of adult males reduces prostatic DNA content to about 20% of the pre-castration level[2,24]. During this process about 35% of the ductal tips and branch points of the prostate completely degenerate from the distal (subcapsular) portion of the gland, while in proximal regions the epithelium merely undergoes simple atrophy, the ductal structures being maintained morphologically[25]. Administration of testosterone elicits a marked increase in DNA synthesis in both epithelial and stromal cells, primarily in the distal tips of the atrophied prostatic ducts[26,27]. This ultimately leads to complete regeneration of the previously lost distal architecture and restoration of the prostate to its normal size, weight and functional activity[24,25]. Androgens elicit these effects by acting primarily on the stromal cells which in turn induce epithelial proliferation and maturation. This conclusion is based on the experiments described in the previous section and is supported by another series on tissue recombinants[28,29]. In these experiments, tissue recombinants composed of wild-type urogenital sinus mesenchyme and Tfm bladder epithelium have been grown to maturity and compared with wild-type prostate or tissue recombinants of wild-type urogenital sinus mesenchyme and wild-type bladder epithelium. Both combinations gave rise to prostate tissues of which the cells expressed androgen receptors[16,22]. During this maturation the induced prostatic ducts became lined by differentiated secretory epithelial cells. The embryonic mesenchyme used to construct the recombinants differentiated into mature prostatic fibroblasts and smooth muscle cells. Adult rodent bladder epithelium also was shown to undergo prostatic differentiation including the appearance of androgen receptors, making this model relevant to mature prostatic epithelial and stromal cells. Two dimensional electrophoresis of the proteins produced by the recombinants of wild-type urogenital sinus mesenchyme and Tfm bladder epithelium and wild-type prostate showed striking similarities, although some minor differences were seen[14,28,29]. Moreover, prostatic epithelial cells of the recombinants containing wild-type urogenital sinus mesenchyme and Tfm bladder epithelium or wild-type bladder

epithelium underwent similar histological changes during regression in response to androgen deprivation. Androgen-induced DNA synthesis, as assessed by biochemical and autoradiographic procedures, was comparable in prostates that were either completely wild-type or were composed of wild-type urogenital sinus mesenchyme and Tfm bladder epithelium. This proliferation could be blocked by the anti-androgen cyproterone acetate. From these data it is evident that certain androgen effects (ductal morphogenesis, epithelial growth and secretory cyto-differentiation ) do not require the presence of intraepithelial androgen receptors[27]. So, several lines of evidence support the concept that in the adult prostate a multitude of epithelial features are regulated by androgens indirectly through androgen-dependent mediators of stromal origin.

The susceptibility of genuine adult prostatic epithelium to these embryonic inductors was shown by Chung and associates[30]. They implanted urogenital sinus mesenchyme or whole urogenital sinus in one lobe of the adult mouse ventral prostate and evaluated the growth (wet weight and DNA content) after different periods. As a result, after several months, massive growth was observed in the implanted lobe but not in the unimplanted, contralateral lobe. In these experiments they found that whole urogenital sinus induced 2.5-fold more growth than urogenital sinus mesenchyme while urogenital sinus epithelium was completely inactive. They explained this phenomenon by the possible modulation by urogenital sinus epithelium of the urogenital sinus mesenchyme gene expression that is responsible for inducing the proliferation of adult prostatic cells ( reciprocal mechanism ). However, since the urogenital sinus is the fetal counterpart of the adult prostate, given enough time and proper conditions, it will develop into an entire prostate gland. Indeed, Thompson and Chung showed that urogenital sinus, in contrast to urogenital sinus mesenchyme, grows to a considerable mass when inoculated under the renal capsule[31]. Therefore it was still unclear whether the massive overgrowth observed in the urogenital sinus-implanted ventral lobes was due to the stimulation of the adult prostate or to the normal growth and development of the implanted urogenital sinus. To answer this question, Chung and coworkers examined glucose phosphate isomerase isoenzyme patterns and found equal levels of donor and recipient tissue isomers, suggesting equal contributions by both tissues toward the new growth observed. Scatchard analysis of cytosolic androgen receptors, however, revealed a pattern resembling that of the host prostate[32]. Although these methods were described as a new mouse model for BPH, the question to what extent the enhanced effect was due to an increase in fetal urogenital sinus growth rather than to the growth of the host's ventral prostate could not be answered. Nevertheless, the growth caused by the urogenital sinus mesenchyme implants clearly demonstrated the capacity of mesenchyme to induce epithelial proliferation and differentiation in the adult prostate.

Further evidence for the importance of stroma in prostatic growth was provided from

studies that examined the factors which regulate prostatic size. For any accessory sexual organ, a typical size and weight can be determined which is characteristic of the mature organ[27]. Likewise, after a period of androgen deprivation, administration of testosterone restores the (rodent) prostate to its normal precastration size and no overgrowth is induced even after prolonged administration. Indeed, in intact adults androgen levels are high while prostatic epithelial cell proliferation is low and in balance with cell death[33]. To discern whether the regulation of prostatic growth is intrinsic or extrinsic, Chung and Cunha prepared homotypic recombinants with urogenital sinus mesenchyme and urogenital sinus epithelium in which the ratio of epithelium to mesenchyme was varied[34]. Their results strongly suggested that epithelial growth and, therefore, absolute prostatic size were regulated by the amount of mesenchymal (stromal) tissue present. From this perspective we have to evaluate the observations made on intact adult prostates and those after castration as reported by DeKlerk and Coffey[24]. They showed that in the intact male rat prostate the ratio of epithelial cells to stromal cells was 3.3:1, while after castration a disproportionate loss of epithelial versus stromal cells occurred resulting in an epithelial-stromal ratio 1:2. As mentioned above, Sugimura and associates reported that during prostatic regression the epithelial cells of the ductal tips completely degenerate and the stromal tissue, which could not be discerned in whole-mount preparations before regression, became visible[25,26]. This last observation raised the distinct possibility that the relative loss of epithelial compared to that of stromal cells after castration may be different in different regions of the gland[35]. This could, in turn, account for the heterogeneity of proliferative activity observed after androgen suppletion[26,27]. Furthermore, during prostatic development, a similar situation exists favoring the amount of stroma versus epithelium (prostatic buds). Since growth of the prostatic rudiment to the adult size is associated with a marked shift in the epithelial:stromal ratio, it may be that growth responsiveness of the adult prostate after androgen replacement therapy requires that the epithelial:stromal ratio favors the stromal element before growth can be induced. This would explain the inability of the prostate to be stimulated to grow beyond its normal size by exogenous sex steroids and the requirement of 4-7 days of androgen deprivation before DNA synthesis can be stimulated. This concept was tested by means of tissue recombinants prepared of a single epithelial ductal tip from adult prostate with mesenchyme from either the embryonic urogenital sinus or adult urinary bladder[14]. Recombinants were transplanted under the renal capsule and grown for one month in the intact male mouse. Individual ducts were maintained but did not grow by themselves or when combined with bladder mesenchyme or Tfm urogenital sinus mesenchyme. However, when combined with (wild-type) urogenital sinus mesenchyme, massive prostatic ductal growth was achieved, such that a single 300 $\mu$m ductal tip containing an extremely small number of epithelial cells gave rise to as much as 35 mg wet weight of prostatic tissue[14,27]. These ducts

were derived from intact donors and, therefore, the ducts did not require prior castration-induced atrophy or cell loss as a condition for growth response. Through analysis of similar recombinants with varying amounts of urogenital sinus mesenchyme, Neubauer and coworkers showed that new prostatic ductal tissue is formed in proportion to the amount of urogenital sinus mesenchyme used[36].

It has to be emphasized that the above-mentioned experiments were all carried out with fetal stromal tissue. Unfortunately, comparable experiments utilizing adult prostatic stroma have not as yet been performed due to difficulties in isolating prostatic epithelium and stroma in adults. Those experiments that were carried out with adult stromal cells did show either marginally inductive activity or complete absence of inductive activity when implanted in the rat's ventral prostate[31]. This inability could be due to the fact that these cells had to be cultured *in vitro* before they were used. However, in the mammary gland, stroma from both the fetus and adult are interchangeable in that they both induce the growth and branching morphogenesis of fetal or adult mammary epithelium[5,37].

In conclusion, it can be stated that short-range interactions between epithelium and stroma, which are of fundamental importance during morphogenesis in the embryo, are clearly involved in organ homeostasis in adulthood. For this reason, the development of cancer and other disease states is likely to involve a loss of coordination and alteration in the interactions between epithelial and stromal cells[38].

Several investigators have been searching for the molecular bases of this homeostatic constraint mechanism that curtails further increase in cell number once the prostate has reached its normal size. Different possible inhibitory mechanisms have been discussed by Bruchovsky and associates some years ago[39] and they considered the production of chalones[40] by the prostatic epithelial cell as an attractive possibility to explain the negative feedback on proliferative growth of the prostate. Experiments carried out by Müntzing and coworkers failed to show such an inhibitory effect[41]. They implanted atrophied ventral prostates from orchiectomized rats into the ventral prostate of other rats. Under the influence of endogenous or exogenous androgen the DNA content of the ventral prostates was increased above normal, probably due to the inductive properties of the stroma as discussed before. Studying the rat ventral prostate, Kyprianou and Isaacs more recently showed an 40-fold increase of TGF-ß mRNAs (a potent growth inhibitor of prostatic epithelial cells [42]), four days after castration. Androgen administration to four-day castrated rats led to a marked decrease in TGF-ß mRNA, to a level comparable to its constitutive expression obtained in intact control animals, showing that expression of TGF-ß is under negative androgenic regulation [43]. *In vitro* and *in vivo* experiments showed TGF-ß to induce programmed cell death of rat ventral prostate glandular cells [44]. Since these studies were performed on whole tissue homogenates, no conclusions

regarding the source of TGF-ß can be drawn from these experiments. Kabalin and colleagues focused their attention on the stroma and found a stimulatory response of the epithelial cells in coculture with prostatic fibroblasts using a serum-free clonal growth assay[45]. However, at higher cell densities they observed an inhibition of cell growth. Several other investigators have studied the stromal-epithelial interaction in the prostate *in vitro*, but their reports were mainly focused on the mitogenic influence of the stroma; all were using serum-free assays[46-49].

## 2.5. Role of epithelial-stromal interactions in prostatic disease

### 2.5.1. Benign prostatic hyperplasia

Benign prostatic hyperplasia can be viewed as a new organ developing from the internal or periurethral portions of the prostatic gland between the bladder neck and the verumontanum as well as the adjacent transition zone[50]. McNeal identified two cell types and sites of origin for small lesions less than 3 mm[51]. First, those found in the periurethral smooth muscle were purely stromal in character or showed only a few small glands penetrating from the periphery. He noticed that the stroma was distinctively reminiscent of embryonic mesenchyme, with an abundance of pale ground substance and only a few collagen fibers. Secondly, lesions found in the transition zone were of epithelial origin. Characteristic features of glandular nodules were the abrupt increase in closeness of acinar packing and hypertrophy of individual epithelial cells. Furthermore, ducts adjacent to the nodule were found to send out branches exclusively on the nodule site growing into the nodular mass, but no branches were found on the opposite wall. These small lesions can be found occasionally in men in their fourth decade. Their number increases linearly with age, while the subsequent growth of each newly formed nodule is generally slow. Around the seventh decade there is a second phase of evolution, characterized by a rather abrupt and marked increase in the number of prostates having nodules of large mass. This latter increase in size is distinctively caused by glandular expansion and consequently in most men is limited to the transition zone. Nevertheless, in the fully developed state, the ratio of muscle tissue or fibromuscular stroma to epithelium is significantly greater than in the normal gland[52].

As far as the etiology of BPH is concerned, a primary endocrine imbalance is not admissible, for the early lesions are observed during a period in life in which no abnormalities in sex steroid levels are apparent be observed. Furthermore, nodule genesis, by definition, is sharply focal while an endocrine aberration would probably be expressed diffusely, at least throughout a given region. These observations have lead to the hypothesis that the initial abnormality in nodule genesis is a spontaneous reversion of a clone of stromal cells to the

embryonic state and that growth regulation in BPH may be under stromal regulation, as proposed by McNeal[50]. Moreover, the putative embryonic (diffusible) mediators could be responsible for the observed eccentric growth of epithelial ducts at the border of a nodule towards its center.

Indeed, from data presented in previous sections it is clear that (fetal) stroma is capable of inducing epithelial hyperplasia in adult prostates. Thus, an apparent similarity and parallelism can be shown between BPH stroma and embryonic mesenchyme in that both tissues express inductive activities that elicit morphogenesis and growth of prostatic epithelium.

This concept does not rule out the possibility that hormones play a role in pathogenesis. The observation that BPH does not develop in men castrated prior to puberty[53-55] indicates that testicular factors are involved in the etiology of BPH. Furthermore, androgens are needed for the activation of the embryonic developmental program of the prostate and are necessary for the expression of reawakened embryonic capacities in adults. Interesting in this context is the observation made by Schweikert and associates that BPH fibroblasts metabolized testosterone to a much higher extent than fibroblasts grown from prostatic carcinoma, foreskin and non-genital skin[56]. This was caused by the higher levels of 5$\alpha$-reductase and 3$\alpha$-hydroxy steroid dehydrogenase activity in these cells. Whether this must be appreciated as a causative feature or merely as a result of earlier abnormalities remains unclear. However, androgenic stimulation can elicit the formation of new gland architecture only in tissues which already have that intrinsic capacity which is probably the result of a dysbalance in stromal to epithelial cell number as seen for instance in an androgen deprived (regressed) prostate, whether or not containing BPH nodules, or a ventral lobe of the rat's prostate implanted with urogenital sinus mesenchyme (Chung's model for BPH[32]). Androgen repletion restores the prostatic size to its precastration level but no growth beyond this threshold value will occur despite of prolonged administration of hormone. These findings suggest a permissive role for androgens in nodule genesis. Walsh and Wilson[57] were the first to demonstrate, however, that enlargement of the prostate, comparable to that seen in spontaneously occurring canine prostatic hyperplasia, could be produced experimentally by treating castrated dogs with 3$\alpha$-diol in combination with estradiol. These lesions were induced at a time when dogs normally have no indications of starting hypertrophy demonstrating that certain endocrine changes 'at puberty' could create a particular hormonal or biochemical environment which initiates the early cellular or molecular dysfunction that leads to hypertrophy[58]. However, it still remained unclear whether the androgen or the estrogen was the main causative factor.

On the other hand there are indications that another type of endocrine influence may be involved in the marked enlargement of nodules which is seen in the second stage of BPH evolution. The acceleration of growth of the glandular component appears to affect multiple

nodules nearly at the same time. This pattern suggests the possibility that some marked alteration in endocrine status occurs in most men in their seventh decade. It is of special interest that this condition becomes clinically manifest at a period in life when testicular function is declining. Despite a number of conflicting data on the plasma hormone levels that have been published in recent years[59] it is now generally believed that in older men the ratio free estradiol to free testosterone is increased in plasma[60]. Taking into account the observed accumulation of estrogens in BPH tissue, especially in stromal nuclei[61], it is attractive to speculate about a stimulatory role for estrogens in BPH.

**Estrogens and stromal-epithelial relationship**

The male's estrogen production occurs mostly by extratesticular aromatization of androstenedione to estrone and of testosterone to estradiol. This conversion takes place in the peripheral tissues (e.g. adipose or muscle tissue). Some investigators found that this takes place in the prostate too, especially in the fibroblasts[62,63], while others could not confirm these findings[64].

As we have seen in the previous sections of this paper many of the biological processes of epithelial tissue are indirectly controlled by androgens through androgen-dependent mediators of stromal origin. Although the presence of estrogen receptors is still under debate[53] (immunocytochemical assays in some cases have shown no staining[65]) they appear to be more abundant in the stroma[53,66-68]. Similarly, by estrogenic stimulation the stroma could elicit an estrogenic effect on the epithelium by regulating the production of stromally-derived growth modulating factors, either stimulatory or inhibitory, and thereby exercising a paracrine influence on the adjacent epithelium. Indeed, a stimulation of the stromal compartment, in particular of the smooth muscle cells, could be observed after estrogen treatment[69]. Here, interesting theories have been proposed to explain the observation that estrogens are capable of transforming prostatic smooth muscle cells into 'activated' smooth muscle cells[70,71]. This concept has been developed from earlier observations on vascular smooth muscle cells, where a 'synthetic' phenotype was recognized in the developing organism and a 'contractile' state in the adult. The former phenotype has a fibroblast-like appearance, divides, and secretes extracellular matrix components[72]. An ultrastructural study on this phenomenon in the dog's prostate revealed that in the case of diminished androgen levels and increased estrogen levels, signs of metabolic and structural alterations developed in the smooth muscle cells while similar alterations occurred in adjacent fibroblasts. Ultimately estrogen was identified as the compound inducing structural and functional dedifferentiation of prostate smooth muscle cells in castrated animals treated exclusively with estradiol[73]. Furthermore, estrogen-mediated dedifferentiation was also observed in castrated dogs treated with an androgen that is readily metabolized to

estrogen. In the initial stages of this androstenedione treatment, alterations in smooth muscle cells were seen resembling those of the synthetic state of the vascular smooth muscle[74]. In another study it was shown that concurrent treatment with the aromatase inhibitor 4-OH androstenedione antagonized the estrogen-related effects on the prostate[75]. However, Zhao *et al.* were not able to show similar changes in the rat ventral prostate[76].

From these data it is tempting to speculate that a changed endocrine situation leads to an increased number of 'activated' or 'synthetic' smooth muscle cells, affecting certain stromal-epithelial interactions which usually operate during the development of the prostate and resulting in BPH[71]. However, comparative analyses of estrogen receptors (ER) in normal prostates, prostates causing no or only mild obstructive symptoms (operated on for prostate or bladder cancer), and large BPH tissues revealed low ER numbers in normal prostate, highest concentrations in non-malignant stroma of non-obstructive prostates, and no ER at all in stroma of obstructive prostates [77]. Brolin *et al.* also found no ER staining in eight BPH tissue samples tested[78]. The authors concluded that stromal growth in obstructive BPH may not be mediated via ER. However, it cannot be excluded that an increase of stromal ER concentration (as observed in non-obstructive prostates) is directly involved in induction of BPH, leading further prostatic growth thereafter into an estrogen independent state[77].

In connection with this subject it is interesting to note that it has been suggested that dietary phytoestrogens may have a role in influencing growth regulation of the prostate. This could explain the difference in the incidence of prostatic disease found in Caucasians and Oriental people[55].

### 2.5.2. Tissue interactions in carcinogenesis

Since it has been shown that stromal-epithelial interactions continue to be of fundamental importance from fetal periods into adulthood, it is clear that these interactions could play a significant role in carcinogenic processes. While only little direct evidence for this idea is available on the prostate itself, there are several reports on other organ-systems supporting this concept. Some of them are outlined in Table I. The first four examples demonstrate the 'normalizing' effect of embryonic and normal stroma on neoplastic cells indicating that removal of certain physiological and selective pressure constraints can reverse the expression of previously malignant phenotypes and restores the cell to its normal phenotype, even though genetic changes that may have occurred during (multistep) malignant transformation. The next two studies depicted in Table I show the proliferation-inducing influence of 'transformed' fibroblasts on normal epithelia. All examples given illustrate the profound effects the stromal environment can have on the epithelium.

In another series of papers it was shown that the hormonal carcinogenesis in the female

genital tract exhibits a progression of events in which the stroma is definitely implicated[6]. Normally, the vaginal epithelium is dependent on estrogen for proliferation and cornification. However, if mice are injected neonatally with estrogens the vaginal epithelium becomes permanently cornified and hyperplastic throughout life even in the absence of ovaries, adrenals and pituitaries. It has been shown that persistent vaginal cornification is indeed estrogen-independent and results from direct irreversible effects upon the vagina. This ovary-independent persistent hyperplastic state is well recognized as an early stage in a series of events that ultimately lead to vaginal carcinogenesis. Biochemical and autoradiographic data demonstrate the lack of estrogen receptors in the epithelium so, at the time of treatment, they could only be detected in the stroma. Thus, the induction of ovary-independent persistent vaginal cornification is elicited neonatally at a time when estrogen receptors are present solely in the mesenchyme, even though the resultant abnormal proliferative state is ultimately expressed within the epithelium. These findings are consistent with the studies of androgen action on Tfm/wild-type tissue recombinants (see earlier sections). Tissue recombinant studies with vaginal stroma (VS) and vaginal epithelium (VE) derived from treated (EVS ; EVE) and untreated mice (VS ; VE) demonstrated that the stroma continues to play an important role in maintenance of this abnormal state. The recombinants were grafted into ovariectomized hosts whose estrogen levels were so low as to result in atrophy of the host's vaginal epithelium. The EVS and EVE combination and the VS and VE combination responded as expected with hyperplasia (85 %) and atrophy (83 %), respectively. However, the incidence of vaginal epithelial hyperplasia was about 60 % for both the EVS and VE and the VS and EVE recombinants. These findings demonstrate that as a result of neonatal estrogen treatment (when no estrogen receptors are present in the epithelium) permanent irreversible effects are elicited in both stroma and epithelium, apparently through direct effects on the mesenchyme and indirect effects upon the epithelium. Prins recently found similar imprinting features after neonatal estrogen exposure resulting in suppression of prostate growth and reduction of prostate responsiveness to testosterone in adulthood. The role of the stroma herein was not investigated however[86].

The relationship between the systems described above and prostatic carcinogenesis is uncertain but there are important parallels between the prostate and the other systems mentioned. Indeed, several studies on stromal-epithelial interactions in prostate cancer support this concept. For instance, Fujii and coworkers showed the (fetal) mesenchyme-induced conversion of bladder carcinomas to glandular structures resembling prostatic carcinoma *in vivo*[87], while recently Chung and coworkers showed a marked inhibition of the growth of a hormone independent rat prostatic tumor cell line (Nb-102-pr) when grafted in combination with urogenital sinus mesenchyme. In some tumors urogenital sinus mesenchyme was found to

induce histomorphological changes toward more differentiated forms resembling that of the adult gland.[88,89]. These findings demonstrate the continuous regulating influence of stroma on the (neoplastic) epithelium.

**Table I.** Stromal-epithelial interactions in carcinogenesis

| *Tissue combination* | | *Observation* |
|---|---|---|
| mammary carcinoma cells | embryonic mammary mesenchyme | reduction of epithelial proliferation; more orderly epithelial architecture[79] |
| basal cell carcinoma cells | normal mesenchyme | complete loss of malignant phenotype, return to normal epithelial differentiation[80] |
| human cancer cell lines | human embryonic fibroblasts | (coculture) degeneration of tumor cells[81] |
| basal cell carcinoma-derived fibroblasts | normal epidermal cells | growth promoting activity[82] |
| bladder carcinoma cell line | urogenital sinus conditioned medium | decrease in cell proliferation; altered morphology, protein synthesis and secretion[83] |
| psoriasis derived connective tissue | normal epidermal cells | strong epidermal proliferation[84] |
| embryonic mammary mesenchyme grafted in situ | mammary tumor virus bearing mice | latency period of appearance of mammary tumors reduced[85] |

The idea that both the epithelium and the stroma must be considered as potential sites of action in carcinogenesis has gained relevance by the following reports. On the one hand, Pritchett and coworkers found that cocultures of epithelium derived from invasive bladder carcinoma with normal bladder stroma resulted in a remarkable increase in epithelial cell proliferation compared to epithelium derived from a (non-invasive) papillary bladder carcinoma or from a normal bladder[90]. These findings suggest a primary epithelial abnormality as a cause of 'aggressive' growth, rendering the cells more susceptible to growth promotion by the

normal fibroblasts. On the other hand however, it was shown by Hodges and colleagues that cell surface markers characteristic of bladder carcinoma can be experimentally induced in normal (untreated) bladder when it is associated with bladder stroma previously treated with a carcinogenic agent[91], showing again the profound effect the stroma can have on the epithelium. Later on Oishi and associates[92] reported the altered surface properties of fibroblasts associated with prostatic carcinoma, indicating that there is definitely 'something going on' in the stromal part of this epithelial neoplasm. Indeed it was observed by several authors that the fibroblasts of different primary tumors exhibit features reminiscent of their fully malignant neighboring cells[93]. Thompson and coworkers studied a mouse prostate reconstitution model, which allows the introduction of genes, singly or in combination, into cells derived from the urogenital sinus. These cells are then 'reconstituted' and grafted under the renal capsule. When *ras and myc* oncogenes were introduced into both the epithelial and the mesenchymal compartments, poorly differentiated adenocarcinomas resulted. Introduction of both oncogenes only into the epithelium produced mainly epithelial hyperplasia, while introduction of both oncogenes into the mesenchyme produced only mesenchymal dysplasia[94,95].

Recently, Chung and coworkers described the development of a mixed adenocarcinoma and fibrosarcoma as a result of inoculation of tumorigenic fibroblast and non-tumorigenic prostatic epithelium into the subcutaneous space of syngeneic rats[96,97]. The epithelial cells subcloned from these tumors remained non-tumorigenic. It was found that only the proliferation of fibroblasts could be stimulated by DHT *in vitro*, while the epithelial cells were not responsive. Consequently, the *in vivo* (mixed) tumors were androgen responsive as well as the uniform fibrosarcomas formed after inoculation of fibroblasts only. In this model, prostatic fibroblasts determined epithelial growth, androgen responsiveness, and tumorigenicity. In another study they found that these tumorigenic rat prostate fibroblasts also accelerated the growth and shortened the latency period of different human epithelial tumors when co-inoculated in athymic nude mice[98]. Other immortalized or transformed (non-tumorigenic) fibroblast cell lines were also capable of accelerating human epithelial tumor growth, resulting exclusively in carcinomas. An important and surprising observation was that lethally irradiated fibroblasts retained the potential to accelerate tumor growth. This result strongly suggests that components of the extracellular matrix may be able to initiate and/or promote epithelial proliferation during various stages of tumor formation. However, because viable fibroblasts were more efficacious, a continued production of tumor-promoting factors by the fibroblasts seems to be necessary to cause the maximal epithelial proliferation.

Gleave *et al.* co-inoculated nontumorigenic human prostate cancer cells (LNCaP) and various tissue-specific fibroblasts subcutaneously in athymic mice. LNCaP tumors were induced most consistently by human bone fibroblasts and prostate fibroblast cell lines[99]. These

observations illustrate the importance of stromal-epithelial interaction in prostate tumor growth and suggest that (tissue specific) extracellular matrix and paracrine-acting growth factors play a role in prostate cancer growth and metastasis. The findings also may explain why prostate cancer selectively metastasizes to the bone. *In vitro* studies have demonstrated the stimulation of prostate cancer cells by bone marrow derived factors[100]. Picard and coworkers reported the presence of growth factors in the conditioned medium of cultured embryonic fibroblasts that were capable of stimulating the growth and metastasis of a rat rhabdomyosarcoma cell line; another example of fibroblast-mediated tumorigenicity[101].

Even more intriguing than the above-mentioned data, indicating an involvement of stromal cells in the process of local malignant growth, are data pointing to deviations of fibroblasts at localizations unrelated to the tumor. Reports originating from several different laboratories indicate that skin fibroblasts from patients with common forms of epithelial cancers may display phenotypic characteristics generally associated with transformed or fetal cells or both[93,102]. In some cases it was found that tumor promoter alone induced neoplastic transformation, which may indicate that the fibroblasts existed in an initiated state. Schor and coworkers studied skin fibroblasts from patients with breast cancer, melanoma, polyposis coli, and retinoblastoma, and observed altered fetal-like migratory behaviors[103,104]. They were able to purify the migration-stimulating factor from the conditioned medium of these cells[105]. This phenomenon was more prominent in patients with a positive family history of breast cancer and was also seen in unaffected first-degree relatives of patients.

Schor and associates proposed two hypotheses on the relationship between malignant growth and generalized fibroblastic abnormalities[106]. First, the genetic or epigenetic defect expressed by the fibroblasts may be similarly expressed by the target epithelial cell population. Alternatively, it is conceivable that deviant fibroblasts are dysfunctional in the sense that normal inductive interactions with incipiently malignant cells are disturbed. The implication of the second mechanism in carcinogenesis would be momentous. The propensity to get certain types of cancer would be partly determined by genetic or epigenetic conditions of the mesenchymal tissues.

## 2.6. Discussion

In an impressive series of experiments, Cunha, Chung and others have shown the profound influence of stroma on the epithelium. Several lines of evidence support the concept that a multitude of epithelial features are regulated by androgens indirectly through androgen-dependent mediators of stromal origin, while a similar mechanism could be applicable to the

action of estrogens on the prostate. The ability of prostatic epithelium to respond to these inductors is manifested at all ages from fetal to adult.

Current models of steroid hormone action envisage a direct interaction of receptor-steroid complexes with genetic regulatory regions to control the expression of tissue-specific genes in adult epithelia. However, the mechanism of action of androgens must be expanded to take into account the interactions of both cell types that clearly act in concert to ensure the normal development and function of the prostate. Tenniswood[107] has proposed an interesting hypothesis about the role of stromal-epithelial interactions in the control of gene expression in the prostate which is outlined below and depicted in Figure 3. He presupposes the existence of a stimulation-feedback loop between the epithelial and stromal cells of the prostate. Three factors: two growth factors, namely a 'stromally derived growth factor' (SDGF) and an 'epithelially derived growth factor' (EDGF), and one inhibiting factor, 'epithelially derived inhibiting factor' (EDIF), together modulate gene expression in the prostate during development and adult function. In the normal adult gland the constitutive expression of EDIF by the epithelial cells would repress SDGF gene expression in the stromal cells. This would prevent replication in both cells enabling them to express continually the genes for steroid metabolism and prostate specific secretory function.

From this concept, it is also possible to explain the etiology of certain prostatic diseases as well as the heterogeneity of epithelial proliferation after androgen replacement. For instance, the EDIF loop may fail either due to loss of EDIF gene expression in the epithelial cells or to the insensitivity of the stroma to the effects of EDIF. This would lead to a continued synthesis of SDGF and the consequent uncontrolled replication in the tissue. This may be the case in BPH : the reawakening of 'embryonic features' could in fact be the continued inappropriate expression of these normal, regulatory genes. If the production of SDGF and EDGF is not coordinately regulated, the overproduction of one or the other would be expected to lead to hyperplastic structures that are either predominantly epithelial or stromal.

However this hypothesis does not explain carcinogenesis in the prostate. To do this we have to postulate an autocrine loop in the epithelium or another route of escape. The expression of the gene for an 'epithelially derived epithelium growth factor'(EDEGF) could very well (at least partially) be triggered or facilitated by stromal factors as we might conclude from the data presented in this chapter. Since cancer cells metastasize without their associated stroma this loop ultimately has to be 'disconnected' from (prostatic) stromal influences to become autostimulatory.

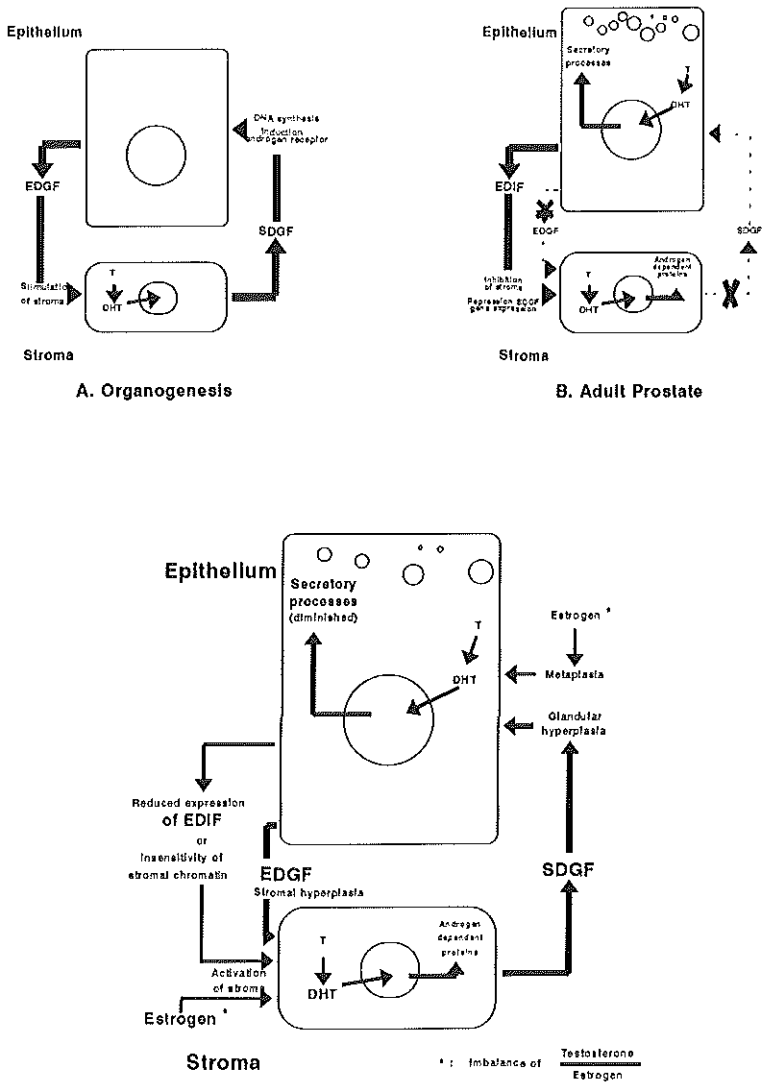


**Figure 3** The Tenniswood hypothesis[107][modified]. SDGF = stromally derived growth factor; EDGF = epithelially derived growth factor; EDIF = epithelially derived inhibiting factor.

The effects of DHT can be roughly divided into two categories: those responses required for the process of replication and those required for normal prostate function and secretion[107]. These processes may occur at the same time in the prostate[108]. However, when castrated rats are treated with androgen, replication ceases when the prostate reaches a predetermined size, whereas the synthesis of secretory proteins either continues[108] or commences[109]. This suggests that these two androgen-dependent processes (replication and transcription) are controlled in fundamentally different ways. Indeed, Thompson and associates were able to show that the growth and differentiation of the prostatic epithelium can be uncoupled[110]. Later on, Darbre and King have been able to separate the androgen-regulated proliferation of breast cancer cells from the androgen-related morphological changes, indicating that in breast cancer, too, the two parameters are not totally interdependent[111]. From this review we have learned that the androgen-induced proliferation of epithelium is possibly entirely mediated via the stroma. This idea was again supported by the observations made by Chang and Chung[112] on cocultures of rat prostatic cells. Analogous observations have been reported on the estrogen-induced proliferation of normal mammary epithelia[113,114]. Taking into account the fact that normal prostatic epithelial cells never have been shown to respond to androgen with proliferation *in vitro*[112,115-119], I would like to suggest that a proliferative response of cultured epithelial cells to sex steroids is a hallmark of transformation and thus restricted to cancer cells [120,121]. According to this theory the normal task of the androgen-receptor complex in the epithelium would be the induction of normal cytodifferentiation and secretion. However, the results of the tissue recombination experiments using wild-type mesenchyme plus Tfm epithelium raise serious questions concerning the validity of such theories. These recombinants appear to form normal prostatic tissue in terms of its morphology, proliferative response to androgens, and contents of prostatic protein, yet its epithelium is devoid of androgen receptors. Unfortunately, there is as yet no definite proof that these recombinants synthesize *all* of the androgen-dependent epithelial secretory proteins characteristic of normal prostate. This has to be resolved before we can draw definite conclusions regarding these tests.

The fact that in most patients prostate cancer is initially androgen-dependent, for this reason, does not inevitably mean that the primary deviation took place in the epithelial part of the tumor. Theoretically there are different pathways leading to this status:

1 A primary defect in fibroblasts, inducing (uncontrolled) androgen-mediated proliferation of epithelial cells (for instance by production of stromally derived growth factors or alteration of extracellular matrix);

2 A primary defect in epithelial cells, making them more sensitive to this androgen-induced proliferation mediated by the stroma; if we could culture them, such cancer cells would ( theoretically ) not be stimulated by androgens;

3 A primary defect in epithelial cells, causing them to proliferate in response to androgens; if we succeeded in culturing these cells, they would probably be sensitive to androgens.

The last alternative especially would apply to the androgen-responsive metastases frequently found in these patients. However, eventually androgen-independent cell populations appear and will take over the clinical course, resulting in endocrine-independent disseminated disease. As indicated before, this has to be due to an epithelial aberration, for these cell metastasize without their supporting stroma.

As can be concluded from these observations, the development of an androgen-independent prostate carcinoma is considered to be a multi-step event. Whether or not each step is caused by a mutation (in the epithelium) remains to be determined.

It is clear that much research has to be done before all questions will be answered. However, we have to be aware of the fact that a loss of coordination and alteration in the interactions between epithelial and stromal cells may play an important role in carcinogenesis. Unfortunately, detailed information on modes of action between cells of different types can only be obtained from *in vitro* systems. These models in their isolated stage may differ fundamentally from the complex pattern of *in vivo* interactions. If possible, the validity of the information obtained in such tests has to be confirmed by experiments performed on the *in vivo* system from which these tests originate. The essential difficulty was best put into words by Smithers[122]: " Reduction gains precision about parts but at each step loses information about the larger organization it leaves behind."

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# Immunocytochemical characterization of explant cultures of human prostatic stromal cells

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## Abstract

The study of stromal-epithelial interactions greatly depends on the ability to culture both cell types separately, in order to permit analysis of their interactions under defined conditions in reconstitution experiments. Here we report the establishment of explant cultures of human prostatic stromal cells and their immunocytochemical characterization. As determined by antibodies to keratin and prostate specific acid phosphatase, only small numbers (<5%) of epithelial cells were present in primary cultures; subsequent passaging further reduced epithelial cell contamination. Antibodies against intermediate filament proteins (keratins, vimentin, and desmin) and smooth muscle actin microfilaments demonstrated that the population of stromal cells cultured from benign prostatic hyperplasia and prostatic carcinoma differed with regard to their differentiation markers. Two contrasting phenotypes were identified in cultures derived from these two different lesions. One, exhibiting fibroblastic features, was predominant in cultures derived from benign lesions and the other, showing varying degrees of smooth muscle differentiation, was more abundant in carcinoma-derived cultures. These findings are indicative of a remarkable divergence in the stromal-epithelial relationships associated with these pathological conditions, and may provide us with a potential tool for studying these processes.

## Introduction

Control of cellular proliferation in the prostate involves a complex interaction of different cell types with soluble peptide growth factors, (steroid) hormones, and constituents of the extracellular matrix. The central role of urogenital sinus mesenchyme in prostatic ductal morphogenesis, epithelial growth, secretory cytodifferentiation and function has been clearly demonstrated. All these androgen-induced effects were shown to be mediated by mesenchyme in the perinatal period[1]. Further studies on tissue recombinants indicated that stromal-epithelial interactions have retained their integral role in the adult gland[2]. For example, although the androgen receptor is expressed in prostate epithelium, it is believed that the proliferative effect of androgen on epithelium in the adult gland is also mediated by the stroma[3,4].

Detailed studies require the capability to culture epithelial and stromal cells separately in order to permit reconstitution and analysis of their interactions under defined conditions. There have been several reports concerning primary culture of murine prostate epithelial[3] and stromal cells[4-7], while less information is available on *in vitro* models derived from human tissue. Although several human prostatic carcinoma cell lines have become available during the past decade[8-11], and methods were developed for primary culture of epithelial cells from normal and diseased prostates[12-16], only a few papers have been published about studies using cultured human prostatic stromal cells. No studies have been conducted so far regarding the characterization of these cells, which were tentatively designated as fibroblasts, a nomenclature mainly based on cell morphology.

Analysis of cytoskeletal proteins is commonly used to assess the differentiation state and the embryonic derivation of many cell types[17-20]. The availability of antibodies against the intermediate-sized filament proteins keratin, vimentin, and desmin has made it possible to distinguish between cells with epithelial, mesenchymal, and myogenic differentiation[18-22]. Further characterization of stromal cells can be achieved by analysis of actin isoform expression[23,24]. These developments have recently led to the recognition of a phenotypic heterogeneity among fibroblasts, which might be related to different biologic behaviors[24]. Fibroblastic cells have been found to express a repertoire of muscle differentiation features in physiologic as well as in pathologic conditions[24]. Many human epithelial tumors, for instance, are associated with the appearance of these so-called myofibroblasts that are believed to be responsible for the frequent, excessive collagen deposition and tissue contraction referred to as desmoplasia[25].

In this paper we describe the immunocytochemical characterization of explant cultures of human prostatic stromal cells. The presence of residual epithelial cells in these cultures was determined using antibodies to keratin and prostate specific acid phosphatase. Subsequently,

antibodies against other intermediate filament proteins and microfilament smooth muscle actin were used to investigate stromal cell cultures derived from benign prostatic hyperplasia (BPH) and prostate carcinoma (PC) in order to reveal possible phenotypic differences. Several cultures were tested at subsequent passages in order to monitor potential shifts in the expression of these markers during the course of subculturing.

## Materials and methods

### Human prostate tissues

Wedges of human prostate tissue were dissected from benign hyperplastic nodules and prostate cancers obtained by open prostatectomy or transurethral resection. After removal of portions of tissue for culture, the remaining prostate was fixed in formaldehyde and serially sectioned. The histology of hematoxylin- and eosin-stained sections immediately adjacent to tissue removed for culture was reviewed, and the findings were correlated with the findings in the corresponding cultures. All six prostatic malignancies were moderately differentiated adenocarcinomas, except for PC 302 and PC 313, which were both poorly differentiated carcinomas. Benign prostatic hyperplasia (BPH) tissue explants showed glandular hyperplasia in all seven explants, while in samples PA 557 and PA 558 an additional fibromuscular hyperplasia was noted.

### Tissue explants and subcultures

Tissue specimens were cut into small pieces of approximately 2 x 2 mm and placed in 60 mm petri dishes (Nunc) containing Earle's minimum essential medium (Gibco Europe, Breda, Netherlands) supplemented with 10 % fetal calf serum (FCS)(Hyclone Laboratories, Logan, Ut), 2 Mm glutamine, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (all from Gibco Europe). Cultures were maintained in a humidified incubator at 37°C in 5% $CO_2$/air. Medium was replaced twice a week. For subculture, cells were detached by trypsinization (0.05% containing 0.02% EDTA) (Gibco) and split in a 1:3 ratio as soon as monolayers became confluent.

### Immunostaining procedures

Cytospins of cultures were prepared after trypsinization. Following the preparation of a single cell suspension in culture medium, cells were washed and resuspended in phosphate buffered saline (PBS) at a final concentration of $10^5$ cells/ml. Subsequently, 0.1 ml of this suspension was spun down in a cytospin centrifuge for 10 min at 150 g. Cytospin preparations

were dried in air, fixed in cold acetone (-20°C) for 10 min, and left to dry at room temperature. In order to preserve cell morphology during the procedure, some of the stromal cell strains were seeded on object slides and stained in situ after 2-3 days of culture. Cytospins and fixed cell preparations were stored at -20°C until further use. As a control, skin fibroblasts (passage numbers 11-12) were treated similarly. Acetone-fixed BPH tissue sections were taken as positive controls for the applied antibodies.

**Table I. List of antibodies used.**

| *Antibody* | *Antigen (reference)* | *Source* | *Dilution* | *Supplier* |
|---|---|---|---|---|
| NCL-5D3 | Keratins 8, 18 & 19 -acinary epithelium [26] | mouse* | 1/10 | Euro-Diagnostics(Apeldoorn, NL) |
| RGE53 | Keratin 18 -luminal cells [17] | " | 1/10 | " |
| RKSE60 | Keratin 10 -squamous epithelium [21] | " | 1/10 | " |
| RCK103 | Keratin 5 -basal cells [27] | " | 1/5 | Dr. FCS Ramaekers, Dept. of Pathology University of Maastricht (Maastricht, NL) |
| α-PSAP | Prostate spec. acid phosph. -prostatic glandul. epith.[28] | rabbit** | 1/1,000 | Dept. of Pathology, Erasmus University (Rotterdam, NL) |
| F39.4 | Androgen receptor [29] | mouse* | 1/10,000 | " |
| 1A4 | α-Smooth muscle actin -smooth muscle cells [23] | " | 1/60,000 | Sigma (St Louis, USA) |
| Mon-3001-5 | Desmin -muscle cells | " | 1/15 | Sanbio (Uden, NL) |
| PVI | Vimentin [30] | rabbit** | 1/15 | Euro-Diagnostics (Apeldoorn, NL) |

*, monoclonal antibody; **, polyclonal antibody

The staining procedure included preincubation with blocking serum to reduce nonspecific staining. Nonimmune swine serum (DAKO, Denmark) was used for polyclonal antibodies, and nonimmune rabbit serum (DAKO) was used for monoclonal antibodies. Both sera were diluted ten times in PBS before use. Object slides were then incubated with the

specified antibody (see Table I) for 30 min at 37°C at the indicated dilution. We used rabbit antimouse peroxidase (DAKO) as secondary antibodies, diluted 1/100 and containing 2% nonimmune rabbit serum, and swine antirabbit peroxidase (DAKO), diluted 1/100, for monoclonal and polyclonal antibodies, respectively . All dilutions of sera or antibodies were prepared with PBS (Ph 7.2), except for polyclonal antibodies, which were diluted in PBS containing 5% bovine serum albumin (BSA). The peroxidase activity was visualized by DAB and hydrogen peroxide. All samples were counterstained with Mayer's hematoxylin.

**Cell counts**

Scoring of positively stained cells was done by one investigator, at 100x magnification. At least 500 cells were counted per sample. Differences between groups were considered statistically significant if a $P$ value $\leq 0.05$ was found using the Wilcoxon rank test (Mann-Whitney).

## Results

Explant cultures of prostatic tissue gave rise to an initial halo of epithelial cells within a few days. After approximately 1 week, the first strands of fibroblastic cells could be seen growing out of the tissue blocks. During the following weeks, epithelial cells became increasingly overgrown by stromal cells. Subculturing after trypsinization appeared to promote stromal cell growth and to reduce epithelial cell contamination. At low density, the stromal cells had a flattened irregular polygonal appearance, while at confluent culture they showed a more spindlelike morphology and formed dense aggregates of elongated cells into fan-shaped bundles (Fig.1). No morphological differences were observed between cultures derived from malignant and nonmalignant lesions. Doubling times of the established stromal cell strains varied between two and four days.

In order to assess the extent of epithelial contamination of stromal cell cultures, epithelium-specific antibodies were applied to cytospin preparations and to cultures grown on glass slides. After three or more passages, prostate specific acid phosphatase, a marker of glandular epithelium known to be expressed *in vitro*[16], was not detectable in our cultures (Table II). Similarly, no androgen receptor (AR) positive cells were detected, even though in BPH tissue sections some of the stromal cell nuclei reacted with the AR-specific antibody (not shown). Since epithelial cells are known to express keratins as well during *in vitro* culture, keratin antibodies have been used to evaluate prostatic epithelial cell cultures derived from human tissues[15,16]. To confirm that the ratio of epithelial/stromal cells shifts in favor of the

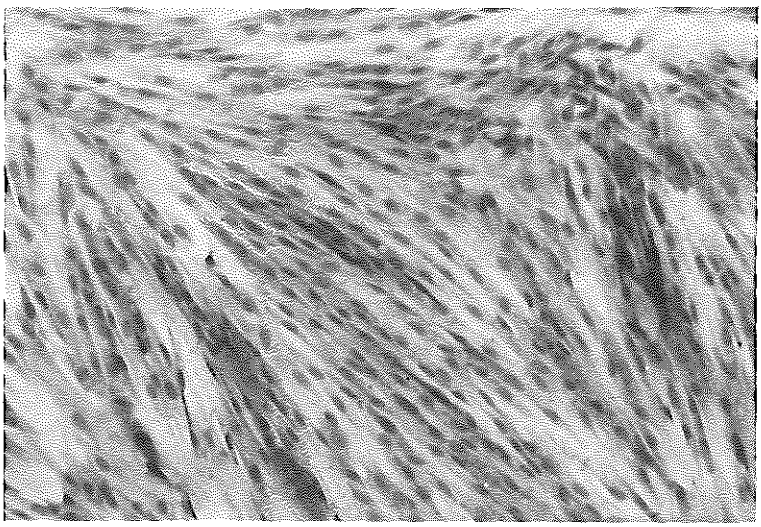

**Figure 1.** Stromal cell culture derived from malignant prostatic tissue (fifth passage). Giemsa staining, magnification 100x.

stromal cells with increasing culture time, primary cultures were harvested after 1 week when the initial outgrowth of solely epithelial-like cells was seen (PA 555 = positive control), other cultures were trypsinized after several weeks, giving the fibroblasts enough time to crowd out epithelial cells (PA 544, PA 546, PC 302, PC 303 = negative controls). The remaining cultures shown in figure 2 were trypsinized after an intermediate period of time; both fibroblasts and epithelial cells were recognized by phase contrast microscopy. Cytospins prepared from the cultures were stained with the NCL-5D3 antibody, which is specific for keratins of glandular epithelium. As a result, only a small fraction (1.0-4.2%) of the cells from long-term primary cultures showed a positive reaction with the NCL-5D3 antibody, whereas in early stage PA 555 (positive control) more than 98% of the cells were keratin positive (Fig.2). Primary cultures that were scored after 2-3 weeks showed intermediate levels of epithelial cell contamination. However, at subculture, the epithelial cell number rapidly decreased, as illustrated by culture PC 313 in Figure 3. To exclude the possibility that epithelial cells had escaped identification due to a change in chain-specific cytokeratin expression during *in vitro* growth, other keratin-specific antibodies were applied as well. As no staining was detected with these antibodies (Table II), it became unlikely that, for instance, morphologically atypical basal cells reactive with RCK103 or cells with squamous metaplasia reactive with RKSE60 were present.

**Table II. Immunocytochemical analyses of prostatic stromal cells cultured and fixed on object slides**

| *Antibody* | | *Control* | | *Stromal cell cultures*[1] |
|---|---|---|---|---|
| | | BPH-tissue | Skin-fibroblasts[2] | |
| **A** | **Epithelial markers** | | | |
| NCL-5D3 | Acinary epithelium | +[3] | - | - |
| RGE53 | Luminal cells | +[3] | - | - |
| RKSE60 | Squamous epithelium | - | - | - |
| RCK103 | Basal cells | +[4] | - | - |
| α-PSAP | Prostate specific acid phosphatase | +[3] | - | - |
| F39.4 | Androgen receptor | +[3,5] | - | - |
| **B** | **Stromal markers** | | | |
| 1A4 | α-Smooth muscle actin | +[5] | - | + |
| Mon-3001-5 | Desmin | +[5] | - | + |
| PVI | Vimentin | +[5] | + | + |

[1] At least 4 different cell 'strains' were tested at passage # 2-8; [2] Passage number 11-12; [3] Glandular epithelium; [4] Basal cells; [5] Stroma.

All six stromal cell strains tested, as well as control cultures of skin fibroblasts, were strongly stained by vimentin antibody (Table II). Cytospin preparations of four more carcinoma-derived and four BPH-derived cultures also contained over 95% vimentin-positive cells. Cytospins of the keratin positive epithelial outgrowth of explant PA 555, however, showed similar numbers of cells reacting with vimentin antibodies.

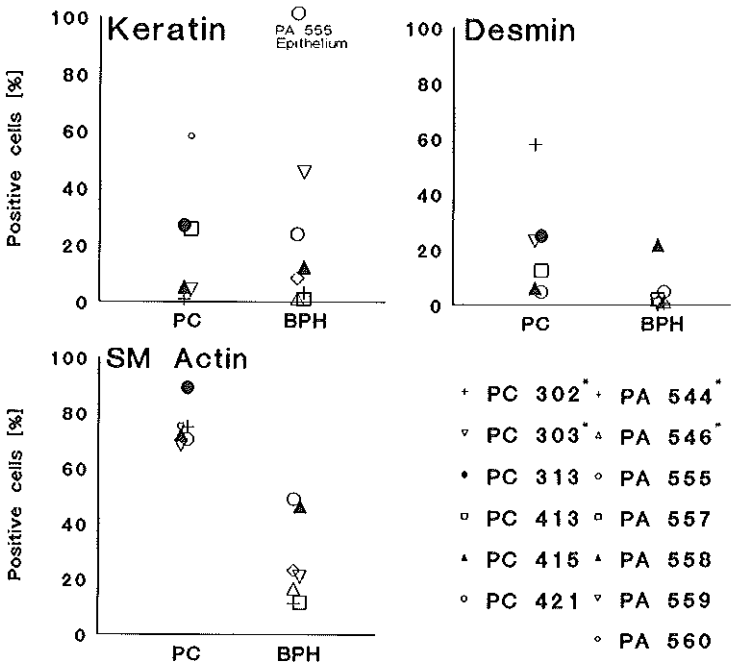


**Figure 2.** Immunocytochemical detection of keratins (NCL-5D3 antibody), desmin, and $\alpha$-smooth muscle (SM) actin in cytospins prepared from the first outgrowth (passage nr 1) of stromal cells from malignant (PC) and benign (BPH) prostatic tissue specimens in explant culture. *: left to crowd out epithelial cells. Desmin content PC > BPH: $p \leq 0.05$ and $\alpha$-SM actin content PC > BPH: $p \leq 0.01$ (Wilcoxon rank test, $n = 13$).

Using cells cultured on glass slides, desmin antibodies stained a fine network of mainly longitudinally running filaments covering the entire cytoplasm in all cell strains (Fig.4). In cytospin preparations, the average number of desmin containing cells was estimated to be 21.5% (median, 17.9; SD, 19.7) for carcinoma- and 4.6% (median, 1.4; SD, 7.7) for BPH-derived cultures (Fig.2). This difference in desmin positive cells proved to be statistically significant ($P \leq 0.05$) using the Wilcoxon rank test. The percentage of positively stained cells was relatively constant over several passages (Fig.3). To this end, cell strains were maintained in culture up to six months.

The 1A4 antibody, directed against $\alpha$-smooth muscle (SM) actin, stained more coarse

longitudinal fibrillar structures in the cytoplasm (Fig.4). In primary cultures, the average number of positive cells in carcinoma-derived cultures (mean, 75%; median, 73.6; SD, 7.4) was significantly larger ($P \leq 0.01$) than the number in BPH-derived stromal cell cultures (mean, 25.6%; median, 21.0; SD, 15.8). These differences, however, were only manifest during early passages. At subculture, the percentage of $\alpha$-SM actin positive cells in BPH-derived stromal cell cultures increased, whereas in carcinoma-derived cultures the number of positive cells generally declined (Fig.3).

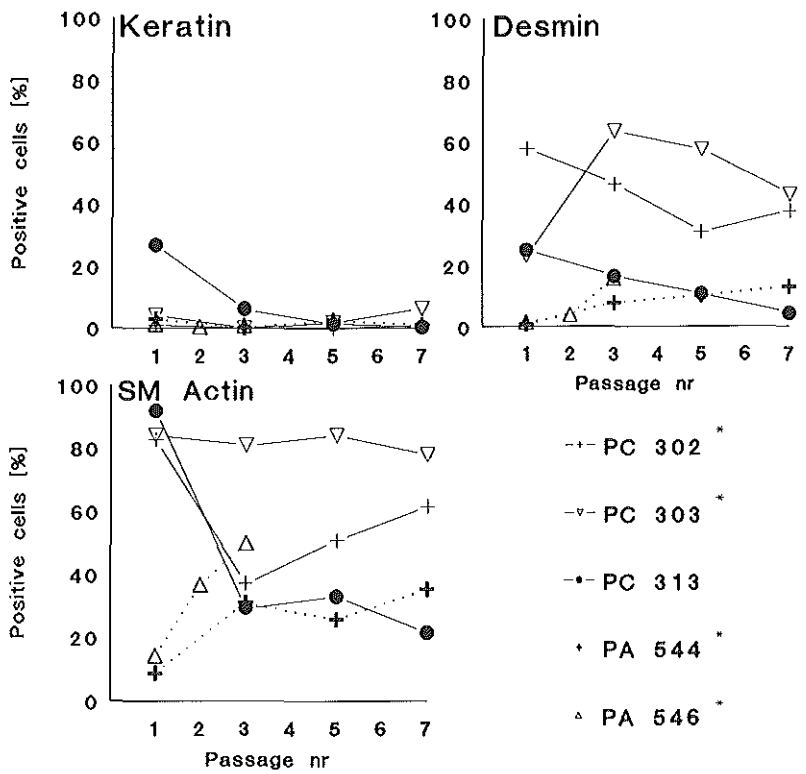


**Figure 3.** Immunocytochemical detection of keratins (NCL-5D3 antibody), desmin, and $\alpha$-smooth muscle actin in cytospins prepared from stromal cell cultures at subsequent passages of *in vitro* culture. *: left to crowd out epithelial cells.

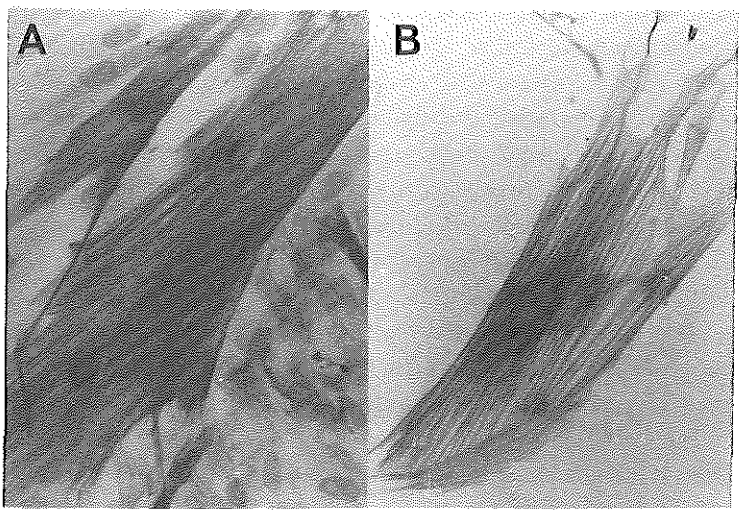


**Figure 4.** Immunocytochemical staining of desmin (A), and α-smooth muscle actin (B) in prostatic stromal cells cultured on object slides at low density. Magnification 400x.

## Discussion

The results presented in this paper show the relative ease with which stromal cell cultures can be obtained from prostatic tissue explants. After a few weeks, the rich culture medium selectively stimulates stromal cell proliferation and gives rise to an overgrowth of these cells at the cost of the initially appearing epithelial cells. As determined by immunocytochemical analysis of keratins in cytospins, such stromal cell outgrowths contained less than 5% epithelial cells during the first passage. Usually this low number of contaminating epithelial cells decreased even further in subsequent subcultures (Fig.3). In fact, epithelial cells were not detected by microscopy or cytokeratin staining after the second passage. It is needless to say, that the epithelial cell content of the first harvest depends on the time spent waiting for the stromal cells to crowd out the epithelial cells (see also Fig.2). Since trypsinization and subcultivation in our hands appeared to be selective in favor of the stromal cell population, subcultures were usually initiated at an early stage.

Mesenchymal cells are known to express vimentin as intermediate-sized filament

protein[18-22]. However, it was shown by others that nonmesenchymal cells can also express vimentin during *in vitro* culture[31]. Since we experienced this to be true for our primary cultures of prostatic epithelium (PA 555: 98% keratin-positive as well as 95% vimentin-positive cells), the results obtained with anti-vimentin antibodies are of limited value for the characterization of cultured prostatic (stromal) cells.

Fibroblastic cells, though traditionally considered to have a relatively common morphology in culture, are endowed with multiple functional properties; they play an essential role in the synthesis and regulation of extracellular matrix components, contribute to wound healing, and are involved in the pathogenesis of fibrotic processes[32], and probably in neoplasia[33]. Characterization of intermediate filament proteins and contractile proteins has shown to be a useful approach in differentiating fibroblastic phenotypes[23,34]. Defined subtypes have been found to express both vimentin and desmin *in vivo*, suggesting that some stromal cells are equipped with muscular elements and might participate in wound closure and visceral contraction[35,36]. For example, immunohistochemical studies have revealed that some testicular stromal cells have muscular phenotypic features, supporting the assumption that these myoid cells might influence the migration of sperm[35]. Recently, a remarkable morphological and functional similarity was demonstrated between these rat testes-derived peritubular cells, and rat prostatic stromal cells in culture[7]. Furthermore, the determination of cytoskeletal proteins in stromal cells associated with pathologic conditions has revealed a spectrum of phenotypes that have not yet been found in normal tissues[37]. On one hand, these findings support the possibility that certain epithelial neoplasias affect the growth and differentiation of fibroblasts present in their immediate vicinity. Fibroblasts associated with lung, breast, and colon carcinomas, for example, have been shown to express $\alpha$-SM actin[24,38]. These tumor-associated myofibroblasts are thought to be responsible for the desmoplastic response to neoplasias[24,37]. On the other hand, desmoplasia has been considered to arise in early stages of tumorigenesis[24]: this led to the search for mesenchymal cells with smooth muscle cell differentiation features in relation to epithelial proliferations, thought to predispose to malignant transformation. Cytoskeletal analysis of specimens from colon, breast, and uterus, indeed, has revealed that the presence of $\alpha$-SM actin-expressing mesenchymal cells may be a feature of premalignant lesions or intraepithelial neoplasia[37]. A prominent role of the stroma in the pathogenesis of BPH nodules in the human prostate was proposed by McNeal more than a decade ago[39]. Obviously, the immunocytochemical observations reported here carry great potential diagnostic and prognostic significance: if similar changes were to be reflected *in vitro*, this would provide us with a potential tool for studying these (pathologic) phenotypes in detail.

When we focus on our results regarding the initial outgrowth of the explants, a striking difference is noticed between cultures derived from BPH and prostatic carcinoma. Whereas

BPH-derived stromal cell cultures predominantly consisted of fibroblastic cells, stromal cells derived from malignant lesions showed smooth muscle differentiation, as was clearly demonstrated by the presence of smooth muscle α-actin protein in approximately 75% of the cells (Fig.2). This conclusion was further substantiated by the observation that these cultures also contained relatively high numbers of desmin positive cells (25%) compared to BPH-derived cultures (<5%). The observation that a substantially lower number of cells stained for desmin than for α-SM actin is in agreement with findings reported by Shapiro et al.[40]. These authors showed that the percent area density of actin positive tissue in prostate biopsies was twice as large as the area density of the antidesmin stained tissue compartment, which led to the authors' conclusion that antidesmin appears to underestimate the amount of prostate smooth muscle.

Immunocytochemical evaluation of serial passages revealed that in BPH-derived cultures, increasing numbers of cells stained positive for α-SM actin, whereas some of the carcinoma-derived cells became α-SM actin negative, resulting in a more even distribution of phenotypic markers in both culture types (Fig.3). One carcinoma-derived culture, however, retained a high level of α-SM actin expression during all seven passages tested. The pathological classification of the parental tumor did not provide any clues as to why these cells were able to maintain this highly differentiated state under circumstances in which most cell types show a tendency to dedifferentiate to a state bearing more general mesenchymal properties[41]. The shift in phenotype observed in our experiments might be due to the separation of stromal cells from the epithelium (which was still present inside the tissue explants) as a result of subculture. However, it is also possible that the changes observed were caused by extended cultivation as has been described by several authors[17,42,43]. On one hand, it has been shown that α-SM actin negative fibroblasts from breast tissue undergo smooth muscle differentiation after 4 days of cultivation[38]. On the other hand, vascular smooth muscle cells were shown to downregulate α-SM actin and thereby resemble myofibroblasts[44], as might have been the case in our cultures. It is therefore important to take into consideration in future *in vitro* studies that apparent fibroblasts may represent modulated smooth muscle cells[44]. In this respect it is interesting that cultured lens cells gradually develop α-SM actin, despite the fact that they do not contain this protein *in vivo*[24]. However, as the difference in phenotypes between BPH and carcinoma-derived cultures may gradually fade at subculture, it should be recommended to carry out (coculture) experiments with stromal cells from the earliest passage possible.

In conclusion, cultured human prostatic stromal cells are heterogeneous with regard to their differentiation markers. Two contrasting phenotypes, one exhibiting fibroblastic features and the other showing varying degrees of smooth muscle differentiation, were detected in

primary cultures derived from lesions with clinically different behavior. The outgrowth of different cell populations under similar culture conditions is indicative of a remarkable divergence in the stromal-epithelial relationship between these two pathological conditions and may provide us with an excellent tool for studying these processes. The reciprocal interaction of these stromal cells with normal epithelium, for instance, tested in coculture experiments, might help us unravel the complex mechanisms leading to benign and malignant neoplasia.

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# Negative control of epithelial cell proliferation by prostatic stroma

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## Abstract

The influence of prostatic stromal cells on the growth of the prostatic carcinoma cell lines PC-3 and LNCaP was examined. In a double layer soft agar system, clonal growth of both cell lines was inhibited by all prostatic stromal cell strains tested irrespective whether they were derived from malignant or non-malignant prostate tissue. Irradiated as well as quiescent stromal cells showed similar effects. The finding that growth was inhibited also in the presence of stromal cell conditioned medium suggests that the observed effect were mediated by a diffusible growth inhibiting factor. Organ-specific production was suggested by the observation that skin fibroblasts stimulated rather than inhibited PC-3 cell growth. These findings indicate a negative control of epithelial cell proliferation by prostatic stroma.

## Introduction

Maintenance of the differentiated structure and secretory function of the prostate is known to be regulated by testicular androgens. Because of the remarkable capacity to induce regenerative growth of the prostate gland in castrated animals, androgens were thought to act as a mitogen for prostatic cells. However, efforts made to demonstrate a direct effect of androgens on normal prostatic epithelial cells failed to show such a mitogenic response[1-4]. Following the observation that epithelium looses its growth capacity when separated from the stroma[5], it has become increasingly clear that both autocrine and paracrine factors produced by epithelial and stromal cells may play an important role in the local control of prostatic growth. The direct importance of embryonic urogenital sinus mesenchyme in prostatic ductal morphogenesis, epithelial growth, secretory cyto-differentiation and function has been demonstrated convincingly[6]. Subsequently, it was shown that mesenchyme was able to induce further epithelial growth in adult glands of intact mice in a dose-dependent fashion[7,8], indicating that stromal epithelial interactions have retained their integral role in the adult prostate[9]. For this reason, the development of cancer and other disease states is likely to involve a loss of coordination and alteration in these interactions[10]. McNeal[11] already suggested the reversion of prostatic stroma to an 'embryological state' inducing inappropriate epithelial proliferation in benign prostatic hyperplasia (BPH). Tenniswood[12] hypothesized the production in the prostate of three growth-modulating factors that are controlling growth and differentiation. He also suggested that the development of BPH may be caused by continued elevated expression of one or more of these factors.

Another intriguing observation is that, upon maturation, accessory sexual organs reach a typical size and weight which is characteristic for that particular organ[6]. Likewise, administration of testosterone to androgen deprived rats restores the size of the prostate to its normal precastration level and no overgrowth is induced even after prolonged administration[13]. Indeed, in intact adult rats androgen levels are high while prostatic epithelial cell proliferation is low and in balance with cell death[14]. The experiments performed by Cunha and Chung[8] indicated that epithelial growth and, therefore, absolute prostatic size was regulated by the amount of mesenchymal (stromal) tissue present. Several investigators have been searching for the molecular basis of this homeostatic constraint mechanism that curtails further increase in cell number once the gland has reached its normal size. Biochemical analyses have shown the presence of inhibitors in the prostate but, since these determinations were performed on tissue homogenates, it is still not clear whether these are produced in the stroma or the epithelium[15,16].

The present study further explored the hypothesis of a negative growth control by the

stroma. Using a two layer soft agar coculture system we observed an inhibition of epithelial cell growth by prostatic stromal cells. The possible role of this negative interaction is discussed.

## Materials and methods

### Cell lines

The human prostatic carcinoma cell lines PC-3 and LNCaP were used as a source of epithelial cells. The cells were maintained in monolayer culture and were used between passages 35-65 and 20-40, respectively. Human stromal cell strains were established from tissue specimens obtained at surgery or autopsy. Stromal cells were cultured from benign prostatic hyperplasia (BPH), prostatic carcinoma (PC) and normal prostatic tissue (NP). All cultures were used between the $4^{th}$ and $9^{th}$ passage. Human non-genital skin (H1) was used as source of non-prostatic fibroblasts.

### Culture media

In liquid monolayer culture, PC-3 cells were grown in RPMI 1640 (Gibco), supplemented with 7% fetal calf serum (FCS) (Commonwealth Serum Lab.), 2 Mm glutamin, penicillin and streptomycin (Gibco). Stromal cells were grown routinely in Eagle's MEM (Gibco) with 10% FCS, 2 Mm glutamin, and antibiotics. Conditioned media (CM) were collected from confluent cultures which were maintained in RPMI 1640 plus 10% FCS and supplements as specified above. After three days the medium was collected, centrifuged at 1900 g for 15 min. and stored at -80° C.

### Preparation of soft agar plates

A bottom layer of 3 ml, consisting of 0.5% agarose (Bio-Rad, low gel temperature agarose) in RPMI 1640 with 20% FCS and/or horse serum, and supplements as specified, was poured into petri dishes (Costar, 60 mm). This layer was allowed to solidify for 15 min at 4° C. Subsequently a top layer of 2 ml of 0.3% agarose in RPMI 1640 plus 20% FCS, containing the PC-3 or LNCaP cells, was poured over the bottom layer and left to set in the same manner.

In coculture experiments, varying numbers of stromal cells were seeded on the bottom of the dishes and incubated overnight in MEM with 10% FCS before preparation of the agarose layers. Cocultures with quiescent cells were obtained by directly suspending the stromal cells in the bottom layer agarose ('non anchored'). Viability of the cells was tested

with the Trypan-blue exclusion test. In experiments with CM, bottom layers were prepared from mixtures of CM and RPMI 1640 plus 20% FCS or phosphate buffered saline (PBS) in various proportions. The layers were solidified with 0.5% agarose as described above. The plates were incubated for 10-14 days in an atmosphere of 5% $CO_2$ in a humidified incubator at 37° C.

**Counting of colonies**

Plating efficiency (PE) was determined by counting the number of colonies of ≥ 200 $\mu$m in size within 20 squares of 25 $mm^2$ per dish. To this end, the petri dishes were placed on a grid and a binocular operation microscope with a graduated scale in one eye-piece was used (magnification: 50x). All counts were done by the same investigator (J.J.K.). Given values are means of triplicate cultures, except for controls (6 plates/test). The significance of difference between values of the experimental groups was calculated using Student's t test. Differences were considered statistically significant if the two sided p value was smaller than 0.05.

## Results

**Optimization of clonal growth**

The clonal growth of PC-3 in solidified medium was dependent on the serum content, the species from which the serum was derived, and the concentration of glutamin. No differences were observed when either F-12 or RPMI 1640 was used as the basal medium. A linear relationship between PE and FCS concentration was found in the range of 5 to 20% FCS. Partial or complete replacement of FCS by horse serum caused the PE to drop considerably. Although colonies in this medium were numerous, the majority of these was too small to be counted. In RPMI 1640, supplemented with 20% FCS and 2 Mm glutamin, clonal growth of PC-3 cells proved to be optimal and PE ranged from 25-35% in different experiments. LNCaP cells plated in the same medium had a PE of about 10%. The PE obtained when $5.10^3$ PC-3 cells were seeded in the top layer did not differ significantly from the PE scored with $10^4$ PC-3 cells.

**Effects of cocultured stromal cells in monolayer**

Clonal growth of PC-3 cells was inhibited by coculture with human prostatic stromal cells. In Figure 1 the effects on the PE of PC-3 cells, exerted by monolayers of stromal cells derived from normal prostate (NP), benign prostatic hyperplasia (BPH) and from prostatic carcinoma (PC) are shown. In general, there was an evident decline of the PE in the presence

of increasing numbers of stromal cells. The maximal level of inhibition observed amounted to 40% of the PE obtained in the absence of stromal cells. At lower cell densities, stromal cells derived from NP and BPH, showed a tendency to stimulate, while those derived from PC inhibited clonal growth at the same initial cell numbers. At higher cell numbers, no clear differences were observed between stromal cells derived from normal prostate, prostate carcinoma and BPH.

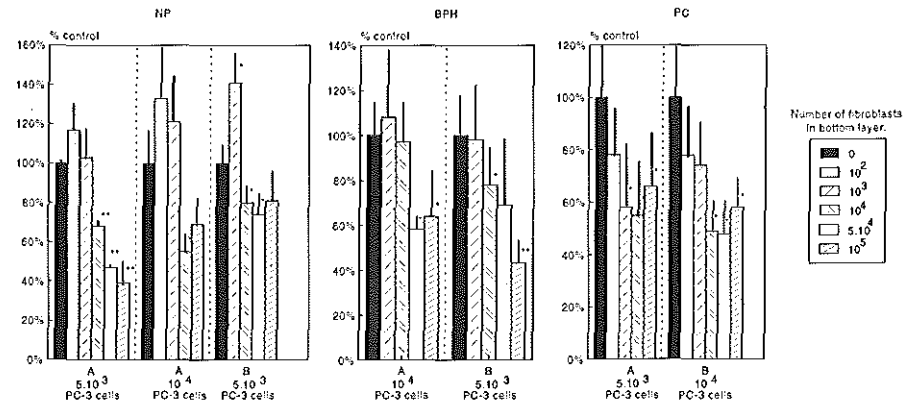


**Figure 1.** Effect of cocultured fibroblasts (anchored) on the plating efficiency of PC-3 cells. In a two layer soft agar assay, the indicated number of fibroblasts, derived from normal prostate (NP), benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC), was allowed to attach overnight to the bottom of the petri dish. Subsequently, the bottom layer and then the top layer, containing the indicated number of PC-3 cells, were prepared. A and B: indicates fibroblast cultures derived from different donors with the indicated pathology. All given values are means of triplicate cultures, except for controls (no fibroblasts inoculated) where six plates were counted. * : $p \leq 0.05$ ; ** : $p \leq 0.01$.

In order to exclude the possibility that the inhibition was due to depletion of the medium and/or accumulation of toxic breakdown products, 3 ml of fresh liquid medium (RPMI 1640 + 20% FCS) was added on top of the solid medium and changed twice a week. Figure 2 shows that no significant changes in clonal growth were induced by this treatment suggesting that the inhibition was caused by a diffusible inhibiting factor secreted by the stromal cells.

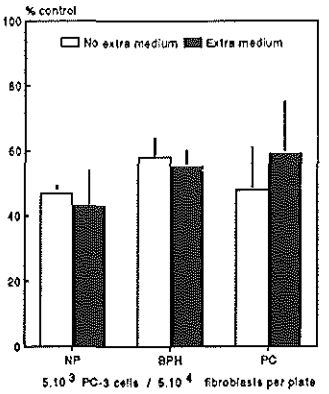


**Figure 2.** Influence of extra medium on plating efficiency of PC-3 cells cocultured with fibroblasts (anchored). On top of the upper layer, 3 ml of fresh medium was added and changed twice a week. Controls were the same as in figure 1. Bars are means + S.D.

### Effect of irradiated, and quiescent stromal cells

To study the effect of non-proliferating stromal cells on the clonal growth of PC-3 cells, cells were either irradiated with 15 Gy before preparation of the agarose layers or directly suspended in the bottom layer. In the latter case, the 'non-anchored' fibroblasts were still viable but remained quiescent; no colonies were formed. In both types of experiments, growth of PC-3 cells was inhibited in proportion to the number of stromal cells present and to the same extent as in the presence of growing stromal cells (Figure 3). These results may indicate that secretion of the putative inhibiting factor is not dependent on cell proliferation.

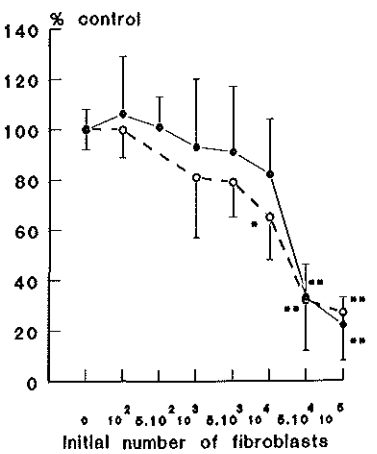


**Figure 3.** Effect of an irradiated monolayer of stromal cells [-●-] and quiescent (non-anchored) stromal cells [-O-] on the plating efficiency of PC-3 cells. Both cultures were obtained from BPH tissue. Inoculum: irradiated -$10^4$ PC-3 cells ; non-anchored - $2.5.10^4$ PC-3 cells. * : $p \leq 0.05$ ; ** : $p \leq 0.01$; N=4.

### Effects of conditioned medium

In order to investigate the secretion of this factor further, conditioned media of stromal cell monolayer cultures were collected and tested for inhibitory activity. Media from NP, BPH and PC-derived stromal cells caused a dose dependent inhibition of the clonal growth of PC-3 cells (Figure 4). Similar experiments performed with LNCaP cells gave essentially the same results (Figure 5). In these tests conditioned media were diluted with PBS; replacement of PBS by RPMI 1640 did not affect the PE of the PC-3 or LNCaP cells. Although the maximal levels of inhibition caused by conditioned media were higher than those found with cocultured stromal cells, the differences between the media conditioned by fibroblasts from different sources appeared to be similar in both experiments. CM of PC-3 cells showed no effect on the PE. Inhibition by CM obtained from non-confluent cultures was significantly less than that reached with CM from the same cultures after attaining confluence (not shown).

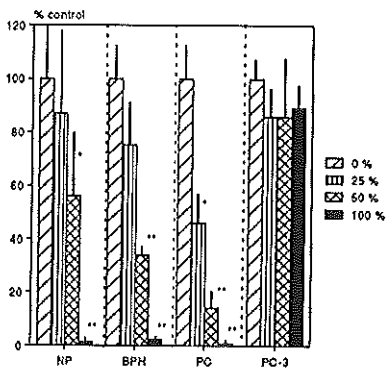


**Figure 4.** Inhibition of clonal growth of PC-3 cells by conditioned medium. Conditioned media were collected from monolayer cultures of stromal cells derived from normal prostate (NP), benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC). After centrifugation they were diluted with PBS to obtain the indicated content of CM. The figure shows the plating efficiency compared to the control dishes containing exclusively PBS in the bottom layer. Inoculum: $5.10^3$ PC-3 cells. The right part of the figure shows the effect of conditioned medium obtained from PC-3 cells on its own plating efficiency. Vertical lines represent S.D. * : $p \leq 0.05$ ; ** : $p \leq 0.001$.; N=4.

### Effect of non-prostatic fibroblasts

To test organ specificity of secretion of the proposed growth inhibiting factor, CM from human non-genital skin fibroblasts (H1) was collected and tested. Conditioned media from early passage number H1 fibroblasts showed stimulation up to 300% above control values (not shown). However, only fresh CM was shown to be mitogenic whereas stimulation was

lost upon freezing. Cocultured H1 fibroblasts, tested in the same way as prostatic stromal cells (monolayer), exerted a striking stimulation of the growth of PC-3 cells (Figure 6). Inhibition of clonal growth by BPH stromal cells was detectable at an initial cell number of $10^4$. With this number of skin fibroblasts a (statistically) significant stimulation was seen reaching a final level up to 100% above the control values when $10^5$ cells were seeded.

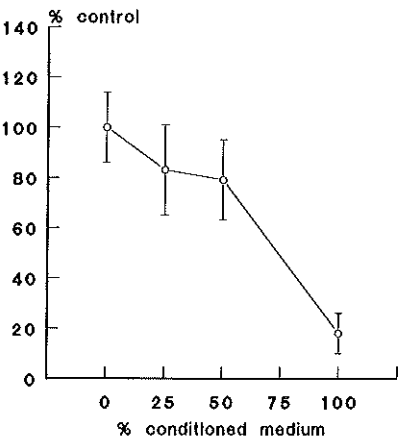


**Figure 5.** Influence of conditioned medium on the plating efficiency of LNCaP cells. Conditioned medium from BPH fibroblasts was tested as in figure 4. Inoculum: $5.10^3$ LNCaP cells. Vertical lines indicate $\pm$ S.D. N=4.

## Discussion

From the present study it can be concluded that stromal cells, isolated from the human prostate and grown *in vitro*, secrete a diffusible product that inhibits the clonal growth of the prostatic carcinoma cell lines PC-3 and LNCaP. Organ specific secretion is suggested by the observation that skin fibroblasts were stimulatory in these tests. Although the limited number of different prostatic stromal cell strains tested does not allow definite conclusions, it was noticed that carcinoma derived stromal cells inhibited PC-3 cell growth at lower numbers of cocultured cells than did those obtained from BPH specimens or normal prostates. At these low cell numbers, stromal cells from non-malignant prostates tended to stimulate clonal growth. However, at higher cell numbers no striking differences were observed between the maximal levels of inhibition reached. These observations strongly suggest the presence of a negative growth control of prostatic epithelium by the stroma.

The idea that stroma not only induces a mitogenic response but can also act as a rein on the epithelium is supported by observations made on other organ systems. Negative

regulators of cell growth have been demonstrated in other fibroblast cultures[17-19]. Several coculture experiments as well as *in vivo* studies show the controlling influence of the stroma on the epithelial compartment[10,20-24]. Similar experiments carried out on the male genital tract fit in with these findings. Rowley & Tindall[25] found that diffusible factors produced by fetal urogenital sinus decreased the proliferation of bladder carcinoma cells in vitro. Recently Chung and coworkers[26] showed a marked inhibition of the growth of a hormone independent rat prostatic tumor cell line (Nb-102-pr) when grafted in combination with urogenital sinus mesenchyme. Indeed, those findings indicate that, in the urogenital tract, mesenchymal cells are capable of inhibiting epithelial cell proliferation.

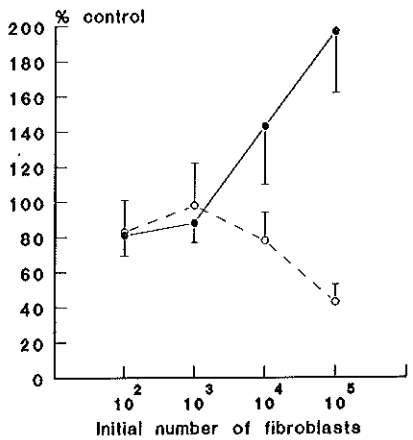


**Figure 6.** Plating efficiency of PC-3 cells cocultured with stromal cells (anchored) from different tissues. Dishes were prepared as indicated in Figure 1. BPH: stromal cells derived from benign prostatic hyperplasia; H1: fibroblasts derived from non-genital skin. Inoculum: $5.10^3$ PC-3 cells; N=4.

Several investigators have studied the stromal-epithelial interactions in the prostate *in vitro* but their reports were mainly focused on the mitogenic influence of the stroma[27,28]. Kirk and associates[29] showed an inhibition of PC-3 cell growth in a double layer soft agar system by lung fibroblasts, but so far no reports have been published on stromally derived epithelial cell growth inhibitors in the prostate. Kabalin et al.[28] inoculated prostatic epithelial cells directly on a monolayer of prostatic fibroblasts and reported a striking stimulation of the clonal growth, especially at lower numbers of fibroblasts seeded. Interesting however, are the results they obtained by seeding higher numbers of fibroblasts. Cocultures containing more than $10^4$ fibroblasts showed a marked inhibition of epithelial cell growth. These observation fit in remarkably well with our own findings.

The presence in the prostate of TGF-ß, a well known inhibitor of epithelial cell growth[30], has been demonstrated by the presence of their mRNAs[15,16]. *In vitro* studies have

implicated TGF-ß as a potent inhibitor of prostatic epithelial cells, both normal and malignant[31]. Kyprianou and Isaacs[15] showed a (40-fold) increase of TGF-ß mRNAs four days after castration. Androgen administration to 4-day castrated rats led to a marked decrease in TGF-ß mRNA to a level comparable to its constitutive expression obtained in intact control animals, showing that expression of TGF-ß is under negative androgenic regulation. Since these studies were performed on whole tissue homogenates, no conclusions regarding the source of TGF-ß can be drawn from these tests. However, prostatic carcinoma cells have been shown to secrete TGF-ß[32,33], while the secretion of TGF-ß by fibroblasts from other organs is well documented too[34]. The work presented here shows that prostatic stroma also is capable of secreting negative mediators of epithelial cell growth. During embryological development androgen receptors are only present in the mesenchyme[35] indicating that all the androgen-induced events, like morphogenesis, proliferation and secretory cytodifferentiation, are mediated via the stroma[6]. Likewise, Chang and Chung[3] showed that androgen-induced epithelial proliferation in the adult rat prostate is mediated entirely through the stroma too. At this point it is tempting to speculate upon a similar role of the stroma in the castration-induced involution of the gland. Indeed, since castration-induced cell death mainly takes place in the epithelial compartment and the ratio of epithelial to stromal cells changes in favor of the stromal part, it is more obvious to assume that the increase in TGF-ß mRNA expression, an active process, takes place in the less affected cell type. Moreover, the fragmentation of genomic DNA of the epithelial cells, shown to be an early event in programmed cell death[36], argues against the possibility that a 40-fold rise in TGF-ß mRNA level on day 4 is caused by transcription of epithelial DNA. Definite answers however, can only be expected from studies using *in situ* hybridization techniques with TGF-ß specific probes.

From this point of view it would be very attractive to explain excessive i.e. neoplastic growth by a failure in this system which may normally regulate prostatic size. It could, for instance, be caused by a mutation in the epithelium rendering it insensitive to these factors[37]. This would also explain why tumors are able to metastasize without their stroma. However, we are well aware of the fact that the experiments reported here were done *in vitro* and cannot be extrapolated directly to the *in vivo* situation. More likely, prostatic neoplasia is due to an imbalanced regulation of the reciprocal stromal-epithelial interactions, where inhibitory and mitogenic signals normally act in concert. The observations on the process of castration-induced cell death strengthen the importance of our findings. Further efforts will be made to unravel the identity of this stromally derived epithelium inhibiting factor.

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# Inhibition of prostatic epithelial cell proliferation by a factor secreted specifically by prostatic stromal cells

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## Abstract

Stromal cells from the prostate were recently shown to inhibit clonal growth of the prostatic carcinoma cell lines PC-3 (hormone-independent) and LNCaP (hormone-sensitive) in coculture. The present study revealed that stromal cell-conditioned medium strongly inhibited proliferation of PC-3 and LNCaP cells when grown in monolayer culture. Antiproliferative activity was found to be reversible, and was produced specifically by prostatic stromal cells and not by stromal cells derived from skin, foreskin, uterus, kidney, and Wilms' tumor. Inhibition was not species-specific, since the cell lines AT-2.1 and MATLyLu, derived from the Dunning rat prostate tumor, were also sensitive. No inhibition, however, occurred on breast and renal carcinoma cell lines, suggesting a prostate-specific action. The putative inhibiting factor(s) could be concentrated and partially purified by ammonium sulfate precipitation. The possible role in stromal control of epithelial cell proliferation is discussed.

## Introduction

Since the observation was made that the epithelium loses its growth capacity when separated from the stroma[1], it has become increasingly clear that stromal-epithelial interactions play an important role in the local control of prostatic growth. The importance of embryonic mesenchyme in prostatic ductal morphogenesis, and epithelial cell growth and differentiation, has been demonstrated by a unique series of tissue-recombinant experiments[2]. Several observations have indicated that these interactions might have retained their integral role in the adult prostate as well[2,3]. It is for this reason that the development of cancer and other disease states is thought to involve a loss of coordination in such interactions. McNeal[4] has already suggested the reversion of prostatic stroma to an 'embryological state', inducing inappropriate epithelial proliferation in benign prostatic hyperplasia (BPH).

Upon maturation, accessory sexual organs reach a typical size and weight which is characteristic for those particular organs[2]. Likewise, administration of testosterone to androgen-deprived rats restores the size of the prostate to its normal precastration level without inducing overgrowth, even after prolonged administration[2]. At that stage, epithelial cell proliferation is low and in balance with cell death[5]. Experiments performed by Cunha and Chung[6] showed that epithelial cell growth and, therefore, absolute prostatic size was regulated by the amount of mesenchymal (stromal) tissue present. During embryological development, androgen receptors are present only in the mesenchyme, indicating that all androgen-induced events are mediated via the stroma[2,3]. Furthermore, it was also shown that in the adult rat prostate, the proliferative response to androgens was mediated through the stroma[7]. At this point it is tempting to speculate upon a similar role of the stroma in castration-induced involution of the gland. Androgen-induced stromally derived inhibitors of epithelial cell growth would participate in keeping the delicate balance between cell proliferation and programmed cell death. The observation that rat and human prostatic epithelial cells grown *in vitro* are able to proliferate in serum-free medium without the presence of androgens[8-11], while androgen ablation *in vivo* induces a marked regression of the glandular epithelium[5], indicates a major role for the stroma in a negative control of epithelial cell proliferation. Several investigators have been searching for the molecular basis of this homeostatic constraint mechanism that curtails further increase in cell number once the gland has reached its predetermined size. Biochemical analyses have shown the presence of (polypeptide) growth inhibitors in the prostate but, since these determinations were performed on whole-tissue homogenates, it is still not clear whether these are produced by the stroma or the epithelium[12,13].

The present study further explores the hypothesis of a negative control by the stroma. Using a two-layer soft agar assay, we previously observed an inhibition of the clonal growth of

human prostate tumor epithelial cell lines PC-3 (hormone-independent) and LNCaP (hormone-sensitive) by cocultured prostatic stromal cells[14]. To elucidate this interaction further, we established a number of stromal cell cultures from human prostatic carcinomas as well as benign prostatic hyperplasia nodules, and investigated the influence of conditioned medium (CM) of these cultures on the anchorage-dependent growth (monolayer cultures) of different epithelial cell lines from human and murine origin. Likewise, several nonprostatic stromal cell cultures were initiated, and their CM tested. The results demonstrated that only prostatic stromal cells accumulate inhibitory activity in the (conditioned) medium. Anchorage-dependent as well as anchorage-independent growth of prostatic carcinoma cell lines was inhibited in a dose-related fashion. The possible role of the stroma in a negative control of epithelial cell proliferation is discussed.

## Materials and methods

### Stromal cell cultures

Histologically proven tissue specimens from benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC), obtained by surgery, were cut into pieces (approximately 1 mm x 2 mm) and placed in 35 mm petri dishes (Nunc) containing 1.5 ml basal medium: Earle's Minimum Essential Medium (Gibco Europe, Breda, the Netherlands) supplemented with 10% Fetal Calf Serum (FCS) (Biological Industries, Beth Haemak, Israel), 2 Mm glutamine, penicillin, and streptomycin (all from Gibco Europe). Cultures were maintained in a humidified incubator at 37°C in 5% $CO_2$/air. Medium was replaced twice a week. The initial halo of epithelial cells grown from these explant cultures became overgrown with fibroblast-like cells within several weeks. Subsequently, cells were detached by trypsinization (0.05% in 0.02% EDTA)(Gibco Europe) and split in a 1:3 ratio every 2-3 weeks. In order to minimize the number of epithelial cells in our cultures (none were detected morphologically), we only used prostatic stromal cells of passage numbers 4-9 in this study. Using the monoclonal antibody NCL-5D3 (Organon, Oss, the Netherlands) reacting with Keratins 8, 18, and 19, we found the number of positive cells in these cultures (BPH as well as PC) varying between 0-5%, indicating that nearly all cells were of nonepithelial origin. Stromal cell cultures from skin, foreskin, uterus, kidney, and Wilms' tumor specimen were established in the same way.

### Cell lines.

The prostatic carcinoma cell line PC-3, obtained from Flow Laboratories (Irvine, Scotland) and maintained in basal medium, was used between passage numbers 30-45. LNCaP-

FGC cells (used at passage numbers 65-70), originally made available to us by Dr. J. Horoszewicz (Buffalo, NY), and the Dunning rat prostate tumor cell lines AT-2.1 and MATLyLu, kindly provided by Dr. J.A. Schalken (University of Nijmegen, The Netherlands), were all cultured in RPMI 1640 (Gibco Europe) supplemented with 10% FCS, glutamine and antibiotics. The renal carcinoma cell line RC-21, established at our laboratory, was cultured in basal medium. The breast carcinoma cell line MCF-7 (pass. nr. 330), generously given by Dr. P.M.J.J. Berns (Daniël den Hoed Cancer Center, Rotterdam, The Netherlands), was cultured in RPMI 1640 (Gibco Europe) containing 10% FCS, 2 μg/ml insulin (Sigma, St Louis, MO), glutamine, and antibiotics. All cultures were shown to be free of mycoplasma contamination by staining with Bisbenzimide (Hoechst dye 33258) obtained from Sigma.

**Collection of conditioned medium (CM)**

Prostatic stromal cell conditioned medium (≈ 0.2 ml/cm$^2$) was collected twice a week from confluent monolayers. After centrifugation (6,000 g; 20 min; 4°C), CM was stored at -20°C until further use.

**Growth inhibition assay**

Inhibition of anchorage-dependent cell growth was determined by means of a colorimetric assay based on the reduction of a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), to a colored formazan product (maximum absorption at 560 nm) by mitochondrial enzymes present only in living, metabolically active cells[15]. Results of these MTT-tests performed on prostatic epithelial cell lines were shown to correlate well with those obtained with [$^3$H]-thymidine incorporation assays and with direct DNA measurements[11,15]. Cells were harvested by trypsinization, resuspended in fresh culture medium, and plated in a volume of 0.1 ml per well in 96-well microtiter plates (Costar). Inoculum: 2,000 PC-3 cells per well. The next day (day 0), 0.1 ml of sample was added, resulting in a total volume of 0.2 ml, with a 50% dilution of the sample and a (basal) FCS concentration of 5%. At day 3, 30 μl of a 5 mg/ml solution of MTT (Sigma) in PBS was added to each well. After a 4-hr incubation at 37°C in 5% $CO_2$/air, the medium was carefully drawn off and the purple dye was dissolved in 0.1 ml DMSO (Merck, Darmstadt, Germany) containing 12.5% of a 0.1 M Sörensen buffer Ph 10.5. Plates were placed on a plate shaker for 5 minutes, and the absorbance at 560 nm was read using a Flow Titertek Multiskan plate reader. Unless otherwise stated, eight replicate wells were used for each sample. Wells containing medium but no cells served as blanks. Results are expressed as percentage of maximal growth from day 0, obtained in fresh basal medium. To determine the number of cells at this point, one extra plate was used and (16 wells) read at day 0.

### Growth curves

Growth curves (Figure 1) were determined in the presence of CM previously dialyzed (Spectrapor 3-cutoff: 3500 Dalton; Spectrum Medical Ind., Los Angeles, CA) against basal medium without serum. In order to avoid the formation of a confluent monolayer, PC-3 cells were plated at a density of 1,000 cells/well. MTT-tests were performed on separate plates at the days indicated.

### Production of inhibitory activity

Stromal cells were plated in 24-well plates in 0.6 ml ($\approx$ 0.3 ml/cm$^2$) of basal medium and left to attach overnight. All wells were changed to fresh medium twice a week (days 1, 4, 8, and 11). At the days indicated, CM was harvested from two wells, pooled, and stored at -20°C after centrifugation. Subsequently, both monolayers were trypsinized and viable cells counted after addition of Trypan-blue. The average of these cell counts is given. After all samples were collected, inhibition of PC-3 cell growth by the respective CM was tested by means of the MTT-assay.

### Concentration and partial purification CM

CM of different passages was pooled and fractionated by ammonium sulfate precipitation. This was performed through a stepwise increase in the level of saturation by adding solid ammonium sulfate (Sigma) to the medium at 0°C under continuous stirring[16]. At every 10% rise, the solution was centrifuged (10,000 g; 4°C; 20 min) and the pellet dissolved in PBS (Gibco). During this procedure, Ph did not drop below 6.5. Samples from supernatant and pellet were extensively dialyzed (cutoff: 3.5 kD) at 4°C against PBS and MEM, respectively, and stored at -20°C after sterilization through a 0.45 $\mu$m membrane (Schleicher & Schuell, 's-Hertogenbosch, The Netherlands). Osmolarity was checked to be 275-325 mOsm, using a Roebling osmometer (Vogel, Giessen, Germany).

### Protein determination

Protein concentrations were measured with the Bio-Rad Protein Assay Kit (Bio-Rad Labs, Veenendaal, The Netherlands). A 1:1-mixture of the albumin and globulin solutions was used as a standard.

### Statistical analysis

All statistical comparisons were made using Student's *t* test analyses. Differences were considered statistically significant if the two-tailed *P* value was smaller than 0.05.

## Results

### Effect of prostatic stromal cell CM on PC-3 cells in monolayer cultures

Anchorage-independent growth of human prostatic tumor epithelial cell line PC-3 was reported to be inversely correlated with the number of human prostatic stromal cells present in coculture experiments. Furthermore, it was shown that stromal cell CM inhibited clonal growth of the hormone-independent PC-3 cells as well as the hormone-sensitive prostatic carcinoma cell line LNCaP[14]. As is depicted in Figure 1A, the anchorage-*dependent* culture (monolayer) of PC-3 cells was also inhibited by prostatic stromal cell CM. Growth inhibition, as determined by the MTT-test, of up to 45% was observed on the fourth day, as compared to growth in basal medium. At day 1 and day 2 the proliferation in CM was 53 and 55% of the controls, respectively. Growth in phosphate buffered saline (PBS) was better than that found in dialyzed CM, indicating that the inhibition was not merely due to depletion of nutrients or mitogens from the medium, but was caused by diffusible growth inhibiting factor(s) present in the CM. Replacing CM with basal medium after 1 day incubation resulted in a growth rate similar to that of controls (Figure 1B). At that time, CM had decreased cell number to 50% of the control, however, no 'catch-up' growth was observed. Similar observations were made when media were changed at day 3. These findings indicate that inhibition was reversible after removal of the antiproliferative stimulus.

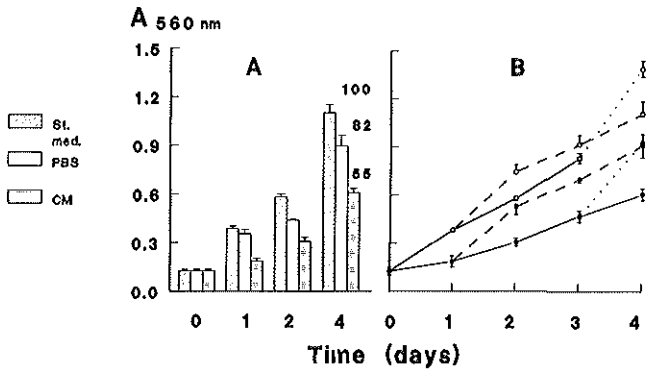


**Figure 1.** A: Inhibition of PC-3 cell growth by prostatic stromal cell CM. The left axis gives the absorbance at 560nm as determined by the MTT test. Data labels on the right indicate the percentage of growth compared to proliferation in basal medium. B: Reversibility of inhibition. At day 1 or day 3, half of the cultures were changed to fresh basal medium. Cultures initiated with CM, -●-. Cultures initiated with basal medium, -o-. Continuous lines, no medium change. Dashed lines, medium change at day 1. Dotted lines, medium change at day 3. Inoculum: 1,000 cells/well. *Error bar*: SD.

**Production of inhibitory activity by prostatic stromal cells.**

Figure 2, illustrating the 'kinetics' of production, demonstrates the positive correlation between the number of stromal cells present and the level of inhibition induced by their respective CM. About 30,000-40,000 cells/cm$^2$ were needed to induce a statistically significant inhibition (more than 15%) of PC-3 cell growth. This level of inhibition was reached at day 2 and day 7, respectively, in cultures plated at high and low density. However, at higher cell densities ($\geq$50,00 cells/cm$^2$), the correlation between cell number and inhibition no longer existed. Both cultures became density-arrested at the end of the second week. At this point, the graph shows that inhibitory activity still accumulated in time, illustrating its independence of proliferation as reported earlier[14]. However, despite a clear difference in cell number, the CM caused equal levels of inhibition. Although the activity of the CM still appeared to be rising after four days, we considered this to be the maximal period for conditioning, for two reasons. First, after four days without medium refreshment, the stromal cells appeared less viable. Some cells detached from the substratum and were washed away with the medium, as is seen on day 8. Second, maximal activity, present in medium covering a confluent monolayer of stromal cells, usually did not exceed the 45% level, irrespective of whether it was conditioned for four days or longer. The fact that concentrating the CM enabled us to obtain higher levels of inhibition (see below), indicated that this critical level was not due to limitations in our test system.

In order to find out if the passage number of the stromal cell cultures affected activity, several cultures were initiated and maintained for several months. As is depicted in Table IA, levels between 35-45% inhibition were consistently found in CM from nearly all stromal cell cultures derived from both malignant (PC) and nonmalignant (BPH) prostates. However, no clear-cut differences in activity were seen between CM of subsequent passages.

**Production of inhibitory activity by non-prostatic stromal cell cultures.**

Table IB lists the growth of PC-3 cells in CM obtained from stromal cell cultures derived from skin, foreskin, normal uterus, Wilms' tumor, and normal kidney tissue. Cultures were initiated, subcultured, and CM tested in essentially the same way as prostate-derived cultures. No biologically significant inhibition of PC-3 cell growth occurred, especially if it is taken into account that 5-10% less growth inhibition was observed when depleted nutrients were replaced by dialyzing these samples against medium (not shown). These data demonstrated that induction of inhibitory activity was confined to prostatic stromal cells, strongly suggesting an organ-specific production.

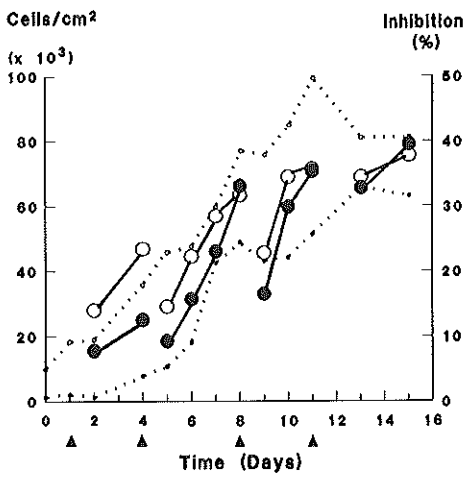


**Figure 2.** Production of inhibitory activity by prostatic stromal cells. Cells were plated in 24-well plates at two densities: a) 10,000 cells/cm$^2$ (open symbols) and b) 1,000 cells/cm$^2$ (closed symbols). Medium was changed twice a week (▲). At the days indicated, samples were taken and cell number counted (two wells). Left axis gives the number of stromal cells per cm$^2$ represented by the dotted lines. Solid lines represent the inhibition of PC-3 cell growth by the respective CM as shown on the right axis. As a control, basal medium was incubated without cells, centrifugated and stored as CM. Levels of significance were calculated using these control values. Inhibition $>15\%$ was statistically significant ($P \leq 0.05$), while values $>20\%$ were highly significant ($P \leq 0.005$).

**Concentration and partial purification of CM.**

In order to concentrate inhibitory activity, we performed an ammonium sulfate precipitation of CM. Figure 3 demonstrates that nearly all inhibiting activity was found in the pellet formed between 40-50% saturation. As a result, the remaining supernatant no longer had a negative effect on cell growth. These findings indicate that the inhibition was caused by precipitable factor(s) in the CM and was not due to depletion of nutrients or growth stimulating factors. Approximately 25% of the total amount of protein originally present in the CM was delivered in the concentrated fraction, while 75% could be discarded. Essentially the same results were obtained when the number of steps was reduced (i.e., 0-40%, 40-50%, and 50-100% saturation). Serial dilution curves of such a preparation are shown in Figure 4. Inhibition of PC-3 cell growth reached its maximum of approximately 60% compared to controls, at a protein concentration of 2 mg/ml. Using other, more concentrated preparations, inhibition levels of up to 90% were found at protein concentrations of about 4 mg/ml. In a

**Table I. Inhibition of PC-3 cell growth by CM from different stromal cell cultures***

A *Prostate derived stromal cell cultures.*

| Culture | Pass nr | Number of CM tested | % Inhibition [average] |
|---|---|---|---|
| PA 517 | 5 | 11 | 40 |
| PA 518 | 4 | 4 | 30 |
| | 6 | 9 | 40 |
| | 7 | 7 | 38 |
| | 8 | 14 | 37 |
| PA 519 | 6 | 11 | 45 |
| PA 520 | 4 | 8 | 39 |
| | 5 | 13 | 40 |
| | 6 | 9 | 41 |
| | 7 | 25 | 48 |
| PA 526 | 3 | 5 | 39 |
| | 4 | 8 | 39 |
| | 5 | 6 | 39 |
| PA 527 | 3 | 15 | 35 |
| | 4 | 13 | 38 |
| PC 239 | 4 | 5 | 36 |
| | 5 | 6 | 32 |
| | 6 | 13 | 35 |
| | 7 | 35 | 37 |
| PC 242 | 3 | 3 | 40 |
| | 4 | 7 | 40 |
| | 5 | 37 | 43 |
| PC 245 | 4 | 10 | 38 |
| | 5 | 10 | 42 |
| PC 259 | 4 | 13 | 32 |

B *Non-prostatic stromal cell cultures.*

| Culture | Pass nr | Number of CM tested | % Inhibition [average] |
|---|---|---|---|
| HF 10.3 | 9 | 9 | 8 |
| Skin | 10 | 24 | 18 |
| | 11 | 13 | 13 |
| FS 1 | 3 | 2 | 7 |
| Foreskin | 4 | 6 | 14 |
| Ut 1 | 3 | 29 | 13 |
| Uterus | | | |
| WT 3 | 4 | 9 | 17 |
| Wilms' tumor | | | |
| WT 7 | 5 | 7 | 9 |
| Wilms' tumor | | | |
| NN 1 | 3 | 3 | 16 |
| Kidney | 4 | 3 | 9 |
| | 5 | 5 | 18 |

* Cultures were initiated and subcultured as described in Materials and Methods. Twice a week, CM from confluent monolayers was harvested and fresh medium given. Centrifuged CM was stored at -20°C. Within 4 weeks, the antiproliferative effect of these undialyzed samples was measured.
PA, Benign prostatic hyperplasia; PC, Prostate cancer.

comparative experiment, bovine serum albumin (BSA) was shown to interfere with cell growth only at higher concentrations: approximately 10% inhibition at 6 mg/ml. These findings indicate that the observed inhibition of semipurified inhibitor was not merely due to a high protein content. Basal medium and skin-derived stromal cell CM were fractionated by the same procedure in order to investigate the possibility that the observed effects were caused by concentration and/or activation of serum-derived growth inhibitors. The effect of these preparations on cell growth (tested at concentrations up to 6 mg/ml) did not differ significantly from control cultures, again indicating that inhibition caused by prostate stromal cell CM was not due to a high protein concentration.

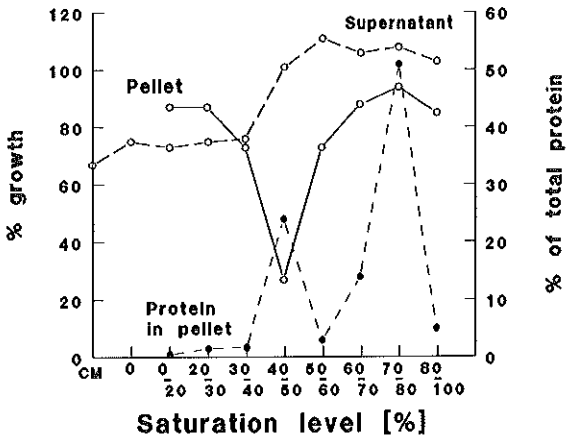


**Figure 3.** Concentration of inhibitory activity by ammonium sulfate precipitation. The saturation level was increased stepwise and the effect of pellet and remaining supernatant tested on PC-3 cell growth after dialysis (open dots and left axis). The amount of protein in the pellet is given as a percentage of the total protein present in the original solution (closed dots and right axis).

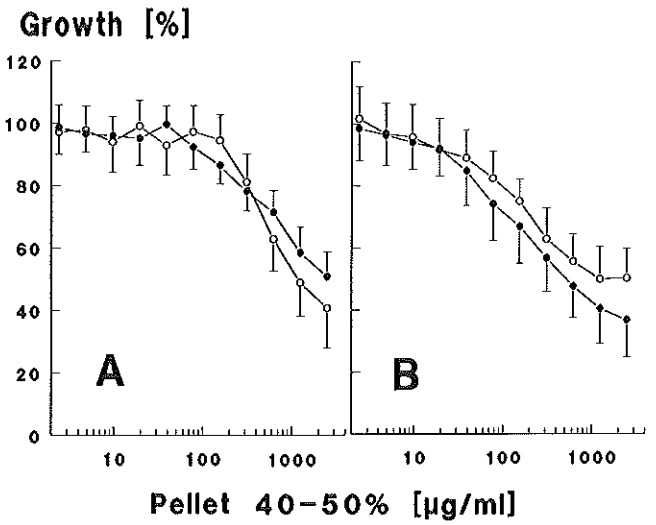


**Figure 4.** Serial dilutions of concentrated CM (Pellet 40-50%) on (A) human prostatic carcinoma cell lines PC-3 (-O-) and LNCaP (-●-), and (B) cell lines from the Dunning tumor (rat prostate) AT2.1 (-O-) and MATLyLu (-●-). Inoculum: PC-3, 2,000; LNCaP, 5,000; AT2.1 and MATLyLu, 2,000 cells/well. Bars, SD.

**Inhibition of epithelial cell lines.**

As shown in Figure 4A, different human prostatic carcinoma (epithelial) cell lines were inhibited when grown in monolayer culture. Anchorage-dependent growth of both the hormone-insensitive cell line PC-3 and the androgen-responsive cell line LNCaP was inhibited in a dose-related fashion. Figure 4B illustrates that similar results were obtained with epithelial cell lines AT-2.1 and MATLyLu, derived from the Dunning rat prostate tumor. These findings indicate that the growth-inhibiting factor present in the CM did not have a species-specific effect. Preliminary tests on primary epithelial cell cultures (second passage) derived from BPH and prostatic carcinoma, showed these cells to be inhibited as well. Growth assays performed on the human breast carcinoma cell line MCF-7 as well as on the human renal carcinoma cell line RC-21 did not show inhibitory activity (Figure 5). These findings suggest that CM-derived inhibition may be prostate-specific.

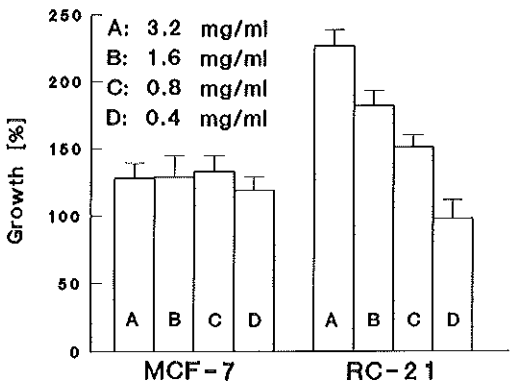


**Figure 5.** Serial dilutions of concentrated inhibitor on the breast carcinoma cell line MCF-7 and the renal carcinoma cell line RC-21. Inoculum: MCF-7, 3,000; RC-21, 10,000 cells/well. Bars, SD.

## Discussion

Cellular proliferation in the prostate is controlled by a complex interaction of different cell types with soluble peptide growth factors, (steroid) hormones, and constituents of the

extracellular matrix. It is likely that the array of peptide factors which play a role in the regulation of cell attachment and proliferation affects these processes through both positive and negative control mechanisms. Several epithelial cell growth-promoting peptides have been identified in the prostate and in prostate-derived epithelial cell cultures[17,18]; some of them have been positively identified as also being produced by stromal cells, suggesting a role in stromal-epithelial interactions[19-21]. Following the observation that urogenital sinus mesenchyme plays a major role in the androgen-induced development of the gland[2], stroma has been recognized as a potential site of disregulation of epithelial cell growth, which may eventually lead to neoplasia[22]. The fact that prolonged androgen administration to castrated animals does not induce the gland to grow beyond its predetermined size[2], together with the observation that withdrawal of androgens decreases epithelial cell number only in the *in vivo* situation where stroma is present[8,5], suggests a role for stromally-derived epithelial cell growth inhibitors in the prostate.

The results presented in this paper suggest that prostatic stromal cells secrete a diffusible epithelial cell growth inhibitor(s). Anchored, as well as nonanchored, growth of the hormone-independent prostatic carcinoma cell line PC-3 and the androgen-responsive prostatic carcinoma cell line LNCaP was affected. Primary epithelial cell cultures from BPH and prostate cancer appeared to be sensitive as well. Accumulation of antiproliferative activity in the CM was positively correlated with cell number and confined to prostate-derived stromal cells. This organ-specific production appears to be a unique property, for it has not been described before in prostate research. Characterization currently underway shows that the putative factor appears to be a protein with a molecular weight $>3.5$ kD, sensitive to reducing agents, indicating the presence of S-S bridges that are needed for biological activity[23]. Several aspects concerning the production are still under investigation. For the present, serum-derived factors are required for optimal production and/or secretion. Preliminary results in our hands failed to show significant effects of steroids (testosterone, dihydrotestosterone, or estradiol) in this respect, however.

That stroma not only induces a mitogenic response, but can also act as a 'brake' on the epithelium, is suggested by observations made on other organ systems. Negative regulators of cell growth have been demonstrated in other fibroblast cultures[24-26]. Several coculture experiments as well as *in vivo* studies showed the controlling influence of the stroma on the epithelial compartment[27], ranging from induction of differentiation[28,29], to tumor cell degeneration[30]. Similar experiments carried out on the male genital tract fit in with these findings. Rowley and Tindall[31] found that diffusible factors produced by fetal urogenital sinus decreased the proliferation of bladder carcinoma cells *in vitro*. Recently, Chung *et al.*[32] showed a marked growth inhibition of a hormone-independent rat prostatic tumor cell line (Nb-102-pr)

when grafted in combination with urogenital sinus mesenchyme. Hayashi and Cunha[33], performing similar experiments on Dunning R3327 rat prostate tumor lines, demonstrated a reduction in epithelial cell growth rate and an apparent reduction or loss of tumorigenesis of these cells. Indeed, those findings indicate that, in the urogenital tract, mesenchymal cells are capable of inhibiting epithelial cell proliferation.

Several investigators have studied stromal-epithelial interactions in the prostate *in vitro*, but their reports were mainly focused on the mitogenic influence of the stroma[34-36,19,21]. Kirk *et al.*[37] showed an inhibition of PC-3 cell growth by lung fibroblasts, using a serum-dependent soft agar assay. So far, no reports have been published by other groups on stromally-derived epithelial cell growth inhibitors in the (adult) prostate. Remarkably, however, all studies demonstrating stromal stimulation of prostatic epithelial cell growth were performed under serum-free conditions. Regarding the lack of a growth stimulatory response of the tumor cell lines to our fibroblast-conditioned medium, one might suggest that such an effect might have been masked by the relatively high serum content (5% FCS) maximally stimulating cell proliferation. However, this is contradicted by the fact that MTT tests were performed under conditions that still allowed stimulation of growth, e.g., in the presence of higher serum concentrations.

During the past decade, much effort has been put into the development of systems for serum-free culture of prostatic cells. Indeed, a serum-free environment is ideal for testing as well as identification of regulatory peptides. However, as a consequence of this simplification, certain information may be lost, as demonstrated by the results presented in this report. These considerations bring to light a crucial dilemma in oncological research, as stated by Smithers: "Reduction gains precision about parts but at each step loses information about the larger organization it leaves behind"[38]. The glandular cells in the prostate receive nutrients, growth-modulating peptides, trophic nerve factors, and (steroid) hormones that have traversed the endothelial cells, stromal cells, extracellular matrix, and the acinar basement membrane. Consequently, the stromal compartment has ample opportunity to modify epithelial cell microenvironment. It is conceivable that (perhaps unknown) serum-derived factors may very well participate in this, for instance, by modifying stromal secretions, as was shown for androgen-induced epithelial cell proliferation in the rat prostate[7]. Thus, tests performed in the presence of serum or other undefined supplements, such as bovine pituitary extract, will provide us with useful information about physiological and pathophysiological processes, and are essential to basic oncological research. However, these supplements will eventually hamper identification and isolation of the substance(s) of interest.

So far, TGF-ß is the only well-known epithelial cell growth inhibitor that has been identified in the prostate[24,39]. Other inhibitory peptides that have been associated with the

prostate[11,40,41] are not likely candidates for elaborating antiproliferative activity in our tests. The presence of TGF-ß mRNA has been demonstrated in human prostatic tissue[13] as well as in rat ventral prostate[12]. *In vitro* studies have implicated TGF-ß as a potent growth inhibitor of prostatic epithelial cells, both normal and malignant[42]. Kyprianou and Isaacs[12] showed a 40-fold increase of TGF-ß mRNAs four days after castration. Androgen administration to four-day castrated rats led to a marked decrease in TGF-ß mRNA, to a level comparable to its constitutive expression obtained in intact control animals, showing that expression of TGF-ß is under negative androgenic regulation. *In vitro* and *in vivo* experiments showed TGF-ß to induce programmed cell death of rat ventral prostate glandular cells[43]. Since these studies were performed on whole tissue homogenates, no conclusions regarding the source of TGF-ß can be drawn from these tests. However, prostatic carcinoma cells have been shown to secrete TGF-ß[39], while the secretion of TGF-ß by fibroblasts from other organs is also well documented[24]. The work presented here shows that prostatic stroma is also capable of secreting negative mediators of epithelial cell growth. Since castration-induced cell death mainly takes place in the epithelial compartment, and the ratio of epithelial to stromal cells changes in favor of the stromal part, it is tempting to assume that the increase in TGF-ß mRNA expression, an active process, takes place in the less affected cell type. Definitive answers however, can only be expected from studies using *in situ* hybridization techniques with TGF-ß-specific probes.

From this point of view it would be very attractive to explain excessive (i.e., neoplastic) growth by a failure in this system which may normally regulate prostatic size. It could, for instance, be caused by a mutation in the epithelium, rendering it insensitive to these factors[44]. Recently, elevated levels of TGF-ß immunoreactivity have been associated with prostate cancer[45]. These observations may reflect insensitivity of cancer cells, possibly associated with a resulting failure in the negative feedback loop that controls its production and secretion by the stroma.

In summary, the data presented here demonstrate that prostatic stromal cells produce an epithelial cell growth-inhibiting activity that may share some properties with TGF-ß. However, especially the results regarding the organ-specific production and action appear to distinguish this putative factor from the TGF betas, and strengthen the importance of our findings. It would be very interesting to investigate whether this factor is the major 'brake' on the prostate, controlling epithelial proliferation and thus prostatic size. Further efforts will be made to unravel the identity of this prostatic stroma-derived growth inhibitor. Studies are already in progress about the relationship of this factor(s) to the type ß Transforming Growth Factors mentioned above.

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# Stromal inhibition of prostatic epithelial cell proliferation not mediated by Transforming Growth Factor ß

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## Abstract

The paracrine influence of prostatic stroma on the proliferation of prostatic epithelial cells was investigated. Stromal cells from the human prostate were previously shown to inhibit anchorage-dependent as well as anchorage-independent growth of the prostatic tumor epithelial cell lines PC-3 and LNCaP. Anti-proliferative activity, mediated by a diffusible factor in the stromal cell conditioned medium, was found to be produced specifically by prostatic stromal cells. In the present study the characteristics of this factor were examined. It is demonstrated that prostate stroma-derived inhibiting factor is an acid- and heat-labile, dithiothreitol-sensitive protein. Although some similarities with type beta transforming growth factor (TGF-ß)-like inhibitors are apparent, evidence is presented that the factor is not identical to TGF-ß or to the TGF-ß-like factors activin and inhibin. Absence of TGF-ß activity was shown by the lack of inhibitory response of the TGF-ß-sensitive mink lung cell line CCL-64 to prostate stromal cell conditioned medium and to concentrated partially purified preparations of the inhibitor. Furthermore, neutralizing antibodies against TGF-ß1 or TGF-ß2 did not cause a decline in the level of PC-3 growth inhibition caused by partially purified inhibitor. Using Northern blot analyses, we excluded the involvement of inhibin or activin. It is concluded that the prostate stroma-derived factor may be a novel growth inhibitor different from any of the currently described inhibiting factors.

## Introduction

Control of cellular proliferation in the prostate involves a complex interaction of different cell types with soluble peptide growth factors, (steroid) hormones and constituents of the extracellular matrix. It is likely that the array of peptide factors which play a role in the regulation of cell proliferation and differentiation affects these processes through both positive and negative control mechanisms. The direct importance of embryonic mesenchyme as a mediator of the androgen-induced prostatic ductal morphogenesis, epithelial growth, secretory cyto-differentiation and function has been demonstrated convincingly[1]. Subsequently, it was shown that these interactions may have retained their integral role in the adult prostate[1,2]. It is an intriguing observation that, upon maturation, accessory sexual organs reach a typical size and weight which is characteristic for that particular organ[1]. Likewise, administration of testosterone to androgen-deprived rats restores the size of the prostate to its normal precastration level without inducing overgrowth, even after prolonged administration[1]. At that stage, epithelial cell proliferation is low and in balance with cell death[3]. Several investigators have been searching for the molecular basis of this homeostatic constraint mechanism that curtails further increase in cell number once the gland has reached its predetermined size.

So far, TGF-ß is the only well-known epithelial cell growth inhibitor that has been identified in the prostate[4,5]. Its presence in prostatic tissue has been demonstrated by Northern blot analyses[6,7], and elevated levels of TGF-ß have recently been associated with prostate cancer[8]. *In vitro* studies have implicated TGF-ß as a potent inhibitor of prostatic epithelial cells, both normal and malignant[9-11]. As these cells were shown to secrete TGF-ß themselves[12,13], an autocrine mode of action is suggested. *In vivo*, TGF-ß has been shown to act mainly on the rate of prostatic glandular cell death, while its expression appeared to be under negative androgenic regulation[14]. Since these determinations were performed on whole tissue homogenates, it is still not clear whether TGF-ß is produced by the stroma or the epithelium. A recent immunohistochemical study[15] demonstrated immunoreactivity to TGF-ß1 antibodies in epithelial and mesenchymal cells, but unfortunately this kind of experiment does not reveal the site of synthesis of the proteins. However, the observation that rat and human prostatic epithelial cells grown *in vitro* can proliferate in serum-free medium without the presence of androgens[16-18], while androgen ablation *in vivo* induces a marked regression of the glandular epithelium[3], indicates a major role for the stroma in the negative control of epithelial cell proliferation. A number of investigators have studied stromal-epithelial interactions in the prostate *in vitro*, but their reports mainly focused on the mitogenic influence of the stroma[19-23]. No further reports from other groups have been published on stromally derived epithelial cell growth inhibitors in the adult prostate. Remarkably, all studies demonstrating stromal

stimulation of prostatic epithelial cell growth were performed under serum-free conditions. However, Kirk *et al.* showed an inhibition of PC-3 cell growth by lung fibroblasts, using a serum-dependent soft agar assay[24]. Using a similar assay, we previously observed inhibition of the clonal growth of prostatic carcinoma cell lines PC-3 (hormone independent) and LNCaP (hormone responsive) by cocultured prostatic stromal cells[25]. Rowley reported growth inhibition of a bladder carcinoma cell line by medium conditioned by urogenital sinus explants in the presence of 5% FCS and 5% (synthetic) Nu-medium[26]. Later, it was shown that antiproliferative activity was produced by a fibroblastoid cell strain probably derived from urogenital sinus mesenchyme[27]. Although cells were shown to express urogenital sinus-derived inhibitory factor (UGIF) activity for up to 5-7 days in unsupplemented Dulbecco's Modified Eagle Medium (DMEM, changed daily), cultures failed to survive under these unphysiological conditions. The presence of the UGIF was tested in a serum-containing assay. Using physicochemical, biological, and immunological methods UGIF was demonstrated to differ from known growth inhibiting factors including TGF-ß. Using the same bioassay, however, no activity could be found in chemically defined medium developed for long-term culture that was conditioned in exactly the same manner. Only addition of steroid hormone caused the fibroblastoid cells to produce inhibiting activity that at the end was identified as being caused by activated TGF-ß and not by UGIF[28]. Although a serum-free environment is ideal for testing as well as identification of regulatory peptides, certain information may be lost, as suggested by the findings reported by Kirk[24], Rowley[28], and the results presented here. The prostatic stromal cell derived antiproliferative activity we found was mediated by a diffusible factor present in serum-containing conditioned medium (CM) of stromal cells from neoplastic lesions of (adult) prostates[25,29,30]. In the present report we show that this factor is different from TGF-ß, its related peptides inhibin and activin, and from other inhibitors previously described. Awaiting further identification, we provisionally and operationally refer to this putative inhibiting factor as "prostate-derived Epithelium Inhibiting Factor": **p-EIF**.

## Materials and methods

### Stromal cell cultures

Surgically obtained tissue specimens from histologically proven benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC) were cut into pieces (approximately 1 mm x 2 mm), and placed in 35 mm petri dishes (Nunc) containing 1.5 ml basal medium: Earle's Minimum Essential Medium (Gibco Europe, Breda, The Netherlands) supplemented with 10% FCS (Biological Industries, Beth Haemak, Israel), 2 Mm glutamine, penicillin and

streptomycin (all from Gibco). Cultures were maintained in a humidified incubator at 37°C in 5% $CO_2$/air. Medium was replaced twice a week. The initial halo of epithelial cells grown from these explant cultures became overgrown with fibroblast-like cells within several weeks. Subsequently, cells were detached by trypsinization (0.05% trypsin in 0.02% EDTA)(Gibco, Europe) and split in a 1:3 ratio every 2-3 weeks. In order to minimize the number of epithelial cells in our cultures we used only prostatic stromal cells of passage number 4-9 in this study. Using the monoclonal antibody NCL-5D3 (Organon, Oss, The Netherlands) reacting with Keratin 8, 18, and 19, we found that the number of positive cells in these cultures (BPH as well as PC) never exceeded 5%, indicating that nearly all cells were of nonepithelial origin[31].

**Cell lines**

The prostatic carcinoma cell line PC-3, obtained from Flow Laboratories (Irvine, UK) and maintained in basal medium, was used between passage numbers 35 and 45. LNCaP-FGC cells (used at passage numbers 65-70), originally made available to us by Dr. J. Horoszewicz (Buffalo, NY), were cultured in RPMI 1640 (Gibco Europe) supplemented with 10% FCS, glutamine and antibiotics. The Mink lung cell line MLCCL 64[32] was obtained from the NIH (Frederick, MO, USA) and cultured in DMEM-high glucose (Gibco Europe) supplemented with 7.5% FCS, and antibiotics. All cultures were shown to be free of mycoplasma contamination by staining with Bisbenzimide (Hoechst dye 33258) obtained from Sigma (St Louis, MO, USA).

**Collection of conditioned medium**

Prostatic stromal cell conditioned medium ($\approx$ 0,2 ml/cm$^2$) was collected twice a week from confluent monolayers (approximately 60-100.10$^3$ cells/cm$^2$). After centrifugation (6,000 g; 20 min; 4°C), CM was stored at -20°C until further use.

**Concentration and partial purification**

CM of different passages was pooled and fractionated by ammonium sulfate precipitation. This was performed through a stepwise increase in the level of saturation by adding solid ammonium sulfate (Sigma) to the medium at 0°C under continuous stirring[33]. At every 10% rise the solution was centrifuged (10,000 g; 4°C; 20 min) and the pellet dissolved in PBS (Gibco). Samples from supernatant and pellet were extensively dialyzed (Spectrapor 3-cutoff: 3500 Dalton; Spectrum Medical Ind., Los Angeles, CA, USA) at 4°C against PBS and MEM, successively, and stored at -20°C after sterilization through a 0.45 $\mu$m membrane (Schleicher & Schuell, 's-Hertogenbosch, The Netherlands). Osmolarity was checked to be 275 - 325 mOsm, using a Roebling osmometer (Vogel, Giessen, Germany). Using this 'first-step'

purification procedure, 75% of contaminating protein could be discarded[30].

**Physicochemical characterization**

Concentrated and partially purified CM was diluted with PBS to a protein concentration of 2 mg/ml. Trypsin sensitivity was tested by incubation with trypsin (T 8003, Sigma) at a concentration of 100 μg/ml, for 2 hours at 37°C, followed by the addition of soybean trypsin inhibitor (STI) (T 9003, Sigma) at a final concentration of 200 μg/ml. As a control, equal amounts of trypsin and STI were pre-incubated in PBS for 30 minutes at 20°C, and subsequently incubated with the samples for an additional 2 hours at 37°C.

The reducing agent, dithiothreitol (DTT) (Sigma) was added to each sample at a final concentration of 130 Mm. Incubation was performed on a rocker platform for 60 minutes at 20°C. Control samples were incubated without DTT.

Acid treatment was performed by acidifying samples to Ph 1.5 with 2 M hydrochloric acid for 1 h at 20°C, then neutralizing by 2 M sodium hydroxide. Control samples were adjusted to the same volume as test samples, using 2 M sodium chloride.

Heat sensitivity was tested in the presence of 100 Mm Hepes, at 56°C for 30 minutes, or at 100°C for 3 minutes. Samples were incubated in closed glass tubes in a rocking water bath.

Controls for all tests mentioned above were obtained by similar treatment of basal medium (diluted to a protein concentration of 2 mg/ml). After incubation, all samples were subsequently dialyzed at 4°C against PBS, and MEM, and sterilized by filtration over a 0.45 μm pore membrane. For determination of growth inhibition (MTT-test), 180 μl of sample and 20 μl of FCS were added at day 0.

Stability at a range of temperatures was tested by heating aliquots of CM for 5 minutes at indicated temperatures. In order to keep the pH at the same level throughout the incubation, Hepes was added to a concentration of 100 mM. As a control, basal medium and serum-free basal medium were treated likewise. Samples were subsequently dialyzed against PBS and MEM and the inhibition of cell growth was tested after sterile filtration, using the MTT-test or the [$^3$H]-thymidine incorporation assay.

**Neutralization tests**

TGF-ß1 from human platelets and TGF-ß-1 neutralizing polyclonal antibodies raised in rabbits were prepared at the Hubrecht laboratory, Utrecht, The Netherlands[34]. Porcine platelet TGF-ß2 was obtained from Sandoz (Basle, Switzerland), while TGF-ß2 specific (neutralizing) antibodies, also from rabbit, were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Concentrated, semipurified inhibitor was diluted to 1 mg/ml, preincubated with antibody (30 μg/ml) for 1 h at 37°C, and subsequently added to wells containing PC-3 cells

(MTT test). Controls were obtained by pre-incubation with non-immune serum IgG. Activity of immune serum IgG was tested by incubation of TGF-ß1 (10 ng/ml in MEM 0.1% BSA) with equal amounts of antibody. Tests with TGF-ß2 specific antibodies were performed with 80 μg/ml antibody, and for controls 20 ng/ml TGF-ß2 was used. Optimal amounts of antibody (saturable activity) were determined by incubating inhibitor with different amounts of antibody.

**Protein determination**

Protein concentrations were measured with the Bio-Rad Protein Assay Kit (Bio-Rad Labs, Veenendaal, The Netherlands). A 1:1-mixture of the albumin and globulin solutions was used as a standard.

**Growth inhibition assays**

Inhibition of cell growth was determined by means of a colorimetric assay based on the reduction of a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), to a colored formazan product (maximum absorption at 560 nm) by mitochondrial enzymes present only in living, metabolically active cells[35]. Cells were harvested by trypsinization, resuspended in fresh culture medium, and plated in a volume of 0.1 ml per well in 96-well microtiter plates (Costar). Inoculum: PC-3, 2,000 per well; LNCaP, 5,000 per well; MLCCL-64, 1,500 per well. The next day (day 0), 0.1 ml of sample was added, giving a 50% dilution of the sample. At day 3, 30 μl of a 5 mg/ml solution of MTT (Sigma) in PBS was added to each well. After a 4 h incubation at 37°C in 5% $CO_2$/air, the medium was carefully sucked off and the purple dye was dissolved in 0.1 ml DMSO (Merck, Darmstadt, Germany). Plates were placed on a plate shaker for 5 minutes, and the absorbance at 560 nm was read using a Flow Titertek Multiskan plate reader. Unless otherwise stated, eight replicate wells were used for each sample. Wells containing medium but no cells served as blanks. Results are expressed as percentage of maximal growth from day 0, obtained in fresh basal medium. To determine the number of cells at this point, one extra plate was used and (16 wells) measured at day 0.

[$^3$H]-Thymidine incorporation was measured using 5,000 MLCCL-64 cells per well in 24-well plates (Nunc). The Mink lung carcinoma cell line CCL-64 is known to be very sensitive to TGF betas[13,36]. Cells were plated on day 1 in 1 ml DMEM (Gibco) supplemented as described. After 4 h, 0.1 ml of sample was added. At day 3, 0.5 μCi [$^3$H]-thymidine (Amersham, UK) in 0.1 ml Ham's F12 medium was added to each well and plates were incubated for another 16 h. Monolayers were washed 4 times with PBS, then fixed in methanol for 15 min at room temperature and dried in air. Cells were then dissolved in 1 ml 1M NaOH (30 min, 37°C), transferred to a scintillation vial and radioactivity was counted.

### Northern blot analyses

After removal of culture medium, stromal cells were frozen in solid $CO_2$/ethanol, and stored at -80°C. Total RNA was isolated using an acid guanidinium thiocyanate-phenol-chloroform extraction procedure[37]. Of each sample, 40 μg of total RNA was denatured in formamide/formaldehyde at 55°C for 15 min, before electrophoresis on denaturing 1% agarose/formaldehyde gels. After electrophoresis, RNA was blotted on Hybond N+ Amersham, UK) by diffusion. Filters were baked for 2 h at 80°C, and subsequently prehybridized for 2 h at 42°C in a hybridization solution containing 50% formamide, 9% w/v dextran sulphate, 10x Denhardt's [1x Denhardt's contains 0.02% w/v/ Ficoll, 0.02% w/v polyvinyl pyrrolidone, 0.02% w/v BSA], 5x SSC (1x SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 mM sodium phosphate (pH 6.8), and 100 μg/ml denatured salmon sperm DNA. Probes for hybridization were labelled with $^{32}P$ by random oligonucleotide labelling[38], denatured in boiling water for 5 min and added directly to the hybridization solution. Following 48 h of hybridization at 42°C, filters were washed to a final stringency of 0.1x SSC, 0.1% SDS, 50°C. Filters were exposed to Amersham Hyperfilm-MP (Amersham, UK) at -70°C for various lengths of time, using an intensifying screen. Probes used were described by Esch[39] and Derynck[40]. As a control for equal amounts of RNA, a hamster actin cDNA probe was used.

### Statistical analysis

The statistical significance of differences between individual treatment groups was calculated using Student's *t* test. Differences were considered statistically significant if the two-tailed *P* value was smaller than 0.05.

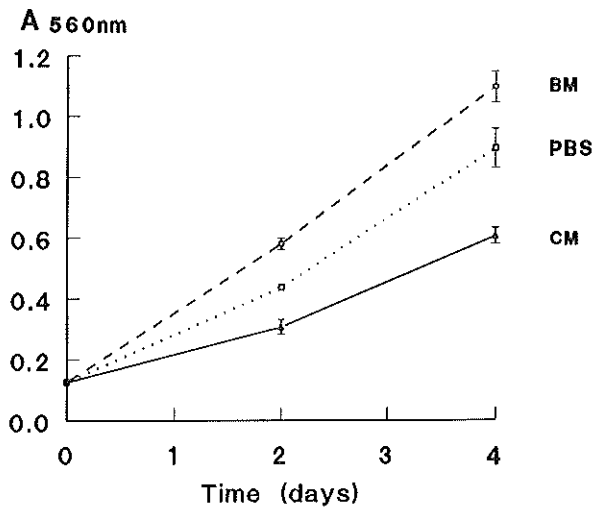


**Figure 1.** Inhibition of PC-3 cell growth by prostatic stromal cell CM. The Y-axis gives the absorbance at 560 nm as determined by the MTT test (see Material and Methods). All samples were dialyzed against basal medium without FCS. BM, basal medium; CM, Conditioned medium. Error bars, SD.

## Results

### Effect of prostatic stromal cell CM and partially purified inhibitor on the growth of prostatic tumor epithelial cells

We previously reported the inhibition of anchorage-independent growth of the hormone-insensitive prostatic carcinoma cell line PC-3 as well as the hormone-responsive prostatic carcinoma cell line LNCaP, by cocultured prostatic stromal cells[25]. Figure 1 shows that CM from prostatic stromal cells was capable of inhibiting the growth of PC-3 cells in monolayer cultures up to approximately 50%. Growth in PBS was significantly better than that found in dialyzed CM, indicating that the inhibition was not due to depletion of nutrients or of growth promoting factors.

Concentration and partial purification of CM resulted in a potent solution reaching inhibition levels up to 80-90% at protein concentrations less than 5 mg/ml. In a comparative experiment, BSA was shown to interfere with cell growth only at higher concentrations, demonstrating that the observed inhibition of semi-purified inhibitor was not merely due to a high protein content. As a control, basal medium and CM from skin fibroblasts were fractionated in a similar way. No inhibiting activity was found in these preparations. As can be seen in the dilution curves in Figure 2, LNCaP cells are almost equally sensitive to the antiproliferative activity as PC-3 cells. Preliminary studies on primary cultures of BPH and prostatic carcinoma epithelium showed these cells to be responsive too.

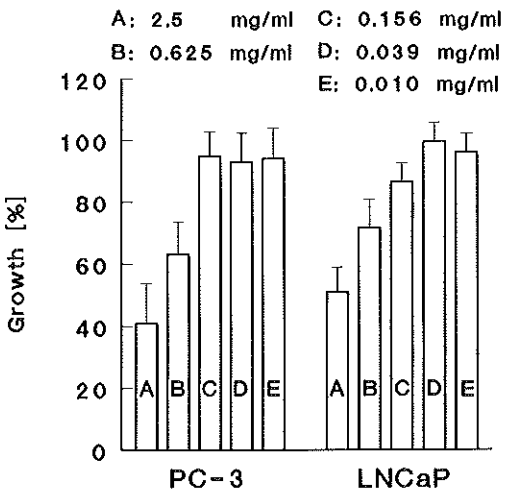


**Figure 2.** Serial dilutions of partially purified inhibitor on human prostatic carcinoma cell lines PC-3 and LNCaP, as determined by means of the MTT-test. Growth is given as percentage of growth obtained in fresh basal medium. Error bars, SD.

**Biochemical characterization**

The inhibitory activity of partially purified p-EIF preparations was tested under different conditions in an attempt to elucidate the nature of the putative inhibitor. The fact that the inhibitor could be precipitated by ammonium sulfate already suggested that a protein is involved. This idea was supported by the observation that trypsin digestion resulted in a statistically significant reduction in inhibitory activity (Figure 3A). Treatment with DTT resulted in a complete disappearance of activity (Figure 3B), demonstrating the requirement of S-S bridges for biological activity. Acid treatment of partially purified inhibitor significantly reduced the inhibiting activity (Figure 3C). Surprisingly, treatment of basal medium resulted in the generation of inhibiting activity, which may explain the observation that inactivation of the stromal cell-derived inhibitor did not restore growth to 100%, although inactivation of growth-promoting substances might as well have contributed to this effect. Figure 3D shows that treatment of the concentrated fraction of inhibitor at 56°C for 30 minutes resulted in a small, but statistically significant, decrease in antiproliferative activity. This observation suggested p-EIF to be heat labile. However, heating at 100°C restored the inhibiting activity to

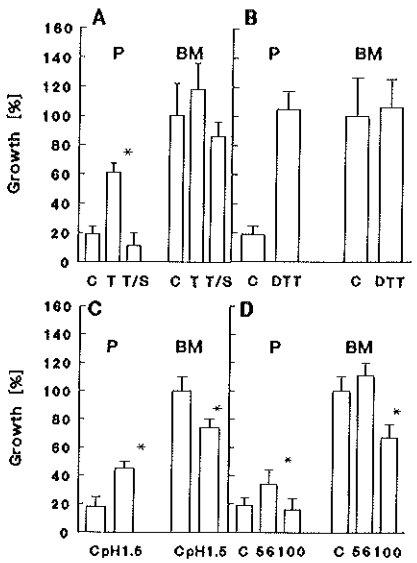


**Figure 3.** Physicochemical characterization of prostatic stromal cell derived inhibitor partially purified by ammonium sulfate precipitation. Inhibitor (P) and basal medium (BM) were treated with **A**: trypsin (T); **B**: dithiothreitol (DTT); **C**: acid (pH 1.5); **D**: Heat (56°C and 100°C). C (control), initial solution of inhibitor or basal medium; T/S, Trypsin preincubated with soybean trypsin inhibitor (for details see Materials an Methods); Bars, SD; *, significantly different from control.

levels found before treatment, while similar effects were seen after heating basal medium. In order to look at this aspect more carefully, we performed similar experiments on CM, using a wider range of temperatures. The increasing levels of inhibition generated in basal (serum-containing) medium by treatment at higher temperatures are clearly demonstrated (Figure 4A). However, a tendency of the stromal cell inhibitor to lose its inhibiting capacity (observed after heating at 56°C and 70°C) was noted.

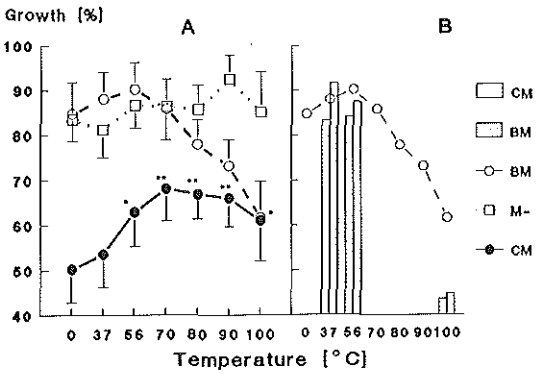


**Figure 4.** Induction of inhibitory activity by heat exposure. Samples of CM, basal medium (BM) including 10% FCS, and basal medium without serum (M-), were subjected to the temperatures indicated. Activity of samples was tested **A)** on PC-3 cells using the MTT test (lines); and **B**, in the TGF-ß assay on MLCCL-64 cells measuring[$^3$H]-thymidine incorporation (bars), showing activation of latent TGF-ß after heating at higher temperatures. For comparison, the curve for basal medium tested on PC-3 cells is shown again in graph B. Growth is given as the percentage of control cultures grown in fresh basal medium. Levels of significance were calculated in relation to the untreated CM: *, $P \leq 0.05$; **, $P \leq 0.01$; Bars (graph A), SD.

## Northern blot analysis

To explore the possibility that TGF-ß or TGF-ß-like substances were the active component in stromal cell-derived medium, Northern blot analyses were used to examine the possible expression of mRNAs coding for these compounds in stromal cells. Confluent cultures of stromal cells from BPH, Wilms' tumor and normal kidney tissue were used. TGF-ß1 mRNA was shown to be present in all four cultures investigated (Figure 5). However, since stromal cell CM from Wilms' tumor and normal kidney tissue did not inhibit PC-3 cell growth[30], this does not necessarily imply that sufficient amounts of (active) TGF-ß are secreted into the medium.

We also examined the involvement of activin and inhibin in cultured prostatic stromal cells. As shown in Figure 5, no ß-A/B chain or $\alpha$-chain mRNAs were detected in prostate derived stromal cell cultures, indicating that neither activin nor inhibin was synthesized in these cells. Hybridization occurred only with the probe for the ß-A chain and was restricted to cultures derived from normal kidney tissue. The presence of ß-A mRNA in human kidney fibroblasts, however, has not been reported before[41].

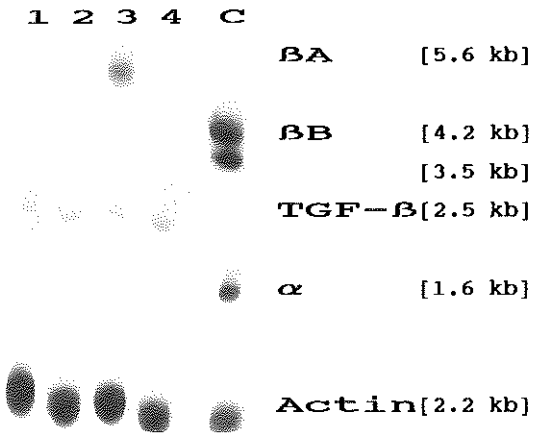


**Figure 5.** Northern blot analysis of total RNA from stromal cell cultures derived from Wilms' tumor (lane 1), tissues from two different BPH patients (lane 2 and 4), and normal kidney tissue (lane 3). C: Sertoli cells, known to express inhibin mRNAs, were used as a control. ßA, ßB: activin/inhibin subunits; $\alpha$: inhibin $\alpha$-subunit.

### Bioassay using MLCCL-64 cells for detection of TGF-ß activity

To test the production of active TGF-ß by prostate derived stromal cell cultures, we used a bioassay with the TGF-ß-sensitive mink lung carcinoma cell line MLCCL-64. For comparison, the inhibition of PC-3 cell growth by TGF-ß and partially purified inhibitor was also determined. Both TGF-ß1 and TGF-ß2 were found to inhibit PC-3 cell growth (Figure 6A). The concentrated solution of inhibitor had to be diluted 10 times to cause an inhibition similar to that of 5 ng/ml TGF-ß. As might be expected, TGF-ß1 and TGF-ß2 had a stronger antiproliferative effect on MLCCL-64 cells than on PC-3 cells (Figure 6B). However, no significant inhibition of MLCCL-64 cell growth was observed with CM or with serial dilutions

of the partially purified inhibitor. These data demonstrate that CM-mediated inhibition of PC-3 cell growth was not caused by TGF-ß. Those findings were confirmed by the highly sensitive [$^3$H]-thymidine incorporation assay on MLCCL-64 cells that appeared to be slightly stimulated by fresh frozen CM as well as by partially purified p-EIF (not shown). However, after prolonged storage (more than 2 months) CM tended to gain some inhibitory activity on both cell types compared with fresh basal medium, probably as a result of activation of latent TGF-beta.

Using this [$^3$H]-thymidine incorporation assay on MLCCL-64 cells increasing amounts of active TGF-ß were also detected in samples from basal medium and from CM, after heating (Figure 4B).

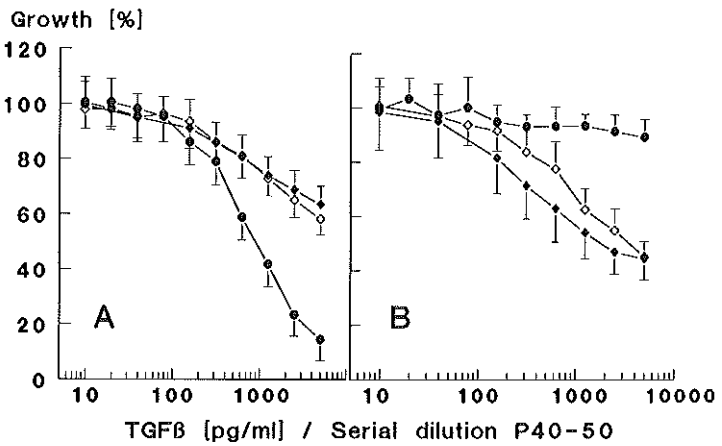


**Figure 6.** Serial dilutions of TGF-ß1 (-◊-) TGF-ß2 (-♦-) and partially purified inhibitor (-●-) on A) PC-3 cells, and B) MLCCL-64 cells, tested in the MTT-assay. TGF-ßs were diluted in MEM containing 0.1% BSA. The serial (2-fold) dilutions of inhibitor (Pellet 40-50%) were prepared in MEM. Highest concentration: 1250 µg/ml. Results are given as percentage of growth of control cultures grown in fresh basal medium. Bars, SD.

### Effect of TGF-ß neutralizing antibodies

In order to confirm the absence of active TGF-ß, we performed MTT-tests on PC-3 cells using neutralizing antibodies against TGF-ß1 and TGF-ß2. As shown in Figure 7A, pre-incubation of TGF-ß1 with polyclonal antibodies raised against purified TGF-ß1, neutralized its effect completely. Those antibodies, however, did not cause a significant decline in the level of inhibition induced by partially purified p-EIF. Tests with TGF-ß2-specific antibodies gave essentially the same results (Figure 7B). These findings, again, demonstrated that the putative

inhibitor present in the CM is different from TGF-ß. Since PC-3 cells are known to secrete predominantly TGF-ß2 in their CM[12], we were also interested in the influence of TGF-ß2 antibodies on the growth of PC-3 cells themselves. Figure 7B shows that these antibodies did not improve the growth of the PC-3, indicating that there was no active 'autocrine' TGF-ß2 present that could be recognized by the antibodies.

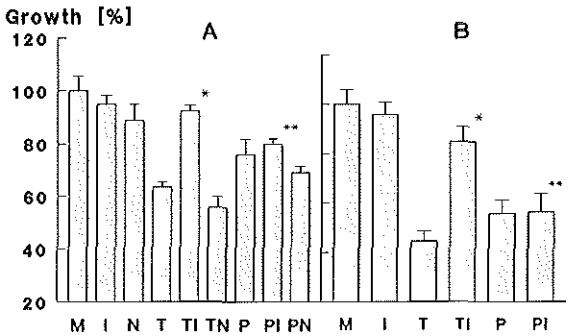


**Figure 7.** Effect of TGF-ß neutralizing antibodies on growth inhibition induced by prostatic stromal cell-derived inhibitor. **A)** TGF-ß1 antibodies. **B)** TGF-ß2 antibodies. Solutions of immune serum (**I**), nonimmune serum (**N**), TGF-ß1 (**T** in graph **A**) and TGF-ß2 (**T** in graph **B**) were prepared in MEM containing 0.1% BSA (**M**). Preparations of partially purified inhibitor (**P**) were diluted in the same solution. TGF-ß and stromal cell inhibitor were mixed with equal amounts of antibody and all samples were preincubated for 1 h at 37°C before they added to the test. Bars, SD ($n$ = 6). *, **T+I** is significantly different from **T** and not different from **I**; **, **P+I** is not significantly different from **P** and different from **I**.

## Discussion

Following the observation that epithelium loses its growth capacity when separated from the stroma[42], it has become increasingly clear that both autocrine and paracrine factors produced by epithelial and stromal cells play an important role in the local control of prostatic growth. It has been demonstrated that urogenital sinus mesenchyme plays a major role in the (androgen-induced) development of the gland[1]; subsequently it was shown that these interactions may have retained their integral role in the adult prostate[1,2,43]. These observations indicated that the development of cancer and other disease states is likely to involve a loss of coordination or other alterations in such interactions. McNeal has suggested the reversion of

prostatic stroma to an 'embryological state' inducing inappropriate epithelial proliferation in benign prostatic hyperplasia[44]. Tenniswood hypothesized the existence of two growth-stimulating factors and one growth inhibitor, produced in stroma and epithelium, that control growth and differentiation. He also suggested that the development of BPH may be caused by continuously elevated expression of one or more of these factors[45]. Indeed, several epithelial cell growth-promoting peptides have been identified in the prostate and in prostate-derived epithelial cell cultures[46,47], and some of these have been positively identified as being produced (also) by stromal cells, suggesting a role in stromal-epithelial interactions[21,23,48].

The fact, however, that prolonged androgen administration to castrated animals does not induce the gland to grow beyond its predetermined size[49], together with the observation that withdrawal of androgens decreases epithelial cell number only in the *in vivo* situation where stroma is present[3,16], suggests a role for stromally derived epithelial cell growth inhibitors. To date, the only well-studied epithelial cell growth inhibitor identified in the prostate is TGF-ß[4]. The transforming growth factor ß family includes a group of closely homologous proteins[50]. Three distinct molecular forms, designated TGF-ß1, TGF-ß2 and TGF-ß3, have been identified in mammals[5]. Most cells, including stromal cells, secrete TGF-ß as a latent complex which can be activated by treatment with heat, acid or proteases such as plasmin[28,51]. Also, commercially available sera have been shown to contain latent TGF-ß[52]. The expression and activation appears to be influenced by steroid hormones[7,19], as was shown also for urogenital sinus mesenchyme[28]. The expression of TGF-ß mRNA in human prostatic tissue [TGF-ß1 and ß2][6] as well as in rat ventral prostate [TGF-ß1][7] has been demonstrated by Northern blot analyses. Recent immunohistochemical studies of diseased human prostates demonstrated TGF-ß1 immunoreactivity in both epithelial and mesenchymal cells[15]. *In vitro* studies with TGF-ß1 have shown that this factor inhibits proliferation of prostatic carcinoma cell lines PC-3, DU 145 and LNCaP[10,11,53], while PC-3 cells were found to secrete latent TGF-ß (predominantly ß-2) in the culture medium[12,13].

Two other members of the TGF-ß family of growth and differentiation factors are activin and inhibin[50]. Both factors were previously shown to compete for TGF-ß binding to pituitary tumor cells[54]. Furthermore, TGF-ß-like properties of activin in developmental biology were recently demonstrated[55]. Both activin and inhibin can interfere with cell growth[56,57], and inhibin-like proteins were demonstrated in seminal plasma and prostate tissue[58].

Using a two layer soft-agar system, we previously observed an inhibition of the clonal growth of prostatic carcinoma cell lines by prostatic stromal cells. It was shown to be mediated by a diffusible factor present in the CM[25,30] which we referred to as p-EIF[29]. In the present work we investigated the possibility that p-EIF is identical to one of the well-know epithelial growth inhibitors mentioned above. Since TGF-ß mRNAs were expressed in our stromal cell

cultures (Figure 5) active TGF-ß could have been produced (among latent TGF-ß) by the stromal cells[28]. On the other hand, the active fraction of inhibitor might consist of 'fibroblast-activated' latent TGF-ß, for instance, present in the serum[52,59]. Our data show that p-EIF has several properties in common with TGF-ß, including reversibility of inhibition[30,60] and sensitivity to trypsin and reducing agents. Elaboration of inhibiting activity under acidic conditions (Figure 3C) is known to occur in solutions containing latent TGF-ß[51]. From the observations described above we may conclude that latent TGF-ß was derived from the serum present during conditioning and/or secreted by the stromal cells themselves. Since activation was observed in stability tests performed on concentrated partially purified inhibitor preparations, we have to conclude that latent TGF-ß co-precipitated with p-EIF, at 40-50% ammonium sulfate saturation. Similar considerations apply to heat treatment (Figure 3D). However, the tendency of the inhibitor to lose its inhibiting capacity upon heating (significant at 56°C and 70°C), was probably obscured at higher temperatures by the generation of active TGF-ß from the solution (Figure 4). This observation appeared to discriminate our inhibitor from the TGF-ß family of growth modulating factors. However, since the sample is not highly purified yet, we realize that the possibility of co-precipitation of active inhibitor adhering to other denatured products cannot be ruled out. Nevertheless, further strong biological and immunological evidence was provided demonstrating that p-EIF is different from these inhibitors. In particular the observation that the antiproliferative effect of semipurified p-EIF was not altered by TGF-ß1 and TGF-ß2 neutralizing antibodies (Figure 7) ruled out the presence of active TGF-ß1 and TGF-ß2 in these preparations. Absence of inhibition of MCF-7 cells[30], sensitive to all three TGF-ßs[61,62], supported this conclusion. In addition, the lack of an inhibitory effect on MLCCL-64 cell growth (Figure 6) argued strongly against the action of TGF-ß1, ß2 or ß3[61].

Recently, growth inhibition of a bladder carcinoma cell line as well as PC-3 cells by serum-containing medium conditioned by a fibroblastoid cell strain subcultured from urogenital sinuswas reported[26,27]. Physicochemical properties of the reported factor (UGIF) suggest that it is different from that found in the present study. However, it should be kept in mind that in both cases tests were performed on crude preparations limiting the value of these observations. Limonti reported the antiproliferative effect of luteinizing hormone-releasing hormone (LHRH) agonists on LNCaP cells. Specific binding sites were demonstrated, while receptor as well as peptide levels were thought to be negatively regulated by androgens[63]. However, since stromal cell-derived inhibitory activity was not lost upon dialysis (cutoff 3.5 kD), these peptides can be ruled out as candidates for the reported inhibitory activity on the basis of their molecular size. Interferons (IFN) have also been shown to inhibit growth of prostatic epithelial cells[64,65], and, among other cell types, fibroblasts have been recognized as a rich source of interferon

(arbitrarily called IFN-ß)[66]. We believe, however, this is not a likely candidate either, for two reasons. First, untreated fibroblasts produce subeffective concentrations of IFN, and only treatment with 'inducers' (e.g., virus infection or double-stranded RNA chains) leads to the secretion of detectable amounts of IFN-ß in the CM. Second, LNCaP cells were shown not to be inhibited by IFN-ß[11], while we demonstrated inhibition of anchorage-dependent and anchorage-independent growth of LNCaP cells by prostatic stromal cell CM.

In conclusion, the data presented in this paper demonstrate that adult prostate derived stromal cells cultured from neoplastic lesions, produce a unique factor, tentatively called 'prostate-derived Epithelium Inhibiting Factor'(p-EIF). On the basis of its spectrum of biological activity as well as its physicochemical and immunological properties, p-EIF can be discriminated from previously described growth inhibitors, including the TGF-ß-related proteins inhibin and activin[50], and from interferon beta[11]. The organ-specific production and the lack of inhibition on all non-prostatic epithelial cell lines tested, emphasize the importance of this inhibitor[30]. It would be very interesting to investigate whether this factor is the major 'brake' on the prostate, controlling epithelial cell proliferation and thus prostatic size. Several aspects concerning the production of p-EIF are still under investigation. Preliminary data suggest that serum-derived factors are required for optimal production and/or secretion. Preliminary results did not show significant effects of steroids (testosterone, dihydrotestosterone, or estradiol) in this respect however. Further knowledge of the factors influencing its production will facilitate purification and characterization. Antibodies raised against the purified factor would provide us with an excellent tool for studying its role in normal physiology and its possible involvement in the development of neoplasia.

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# Chapter 7

# Summary and concluding remarks

## 7.1. Introduction

In terms of incidence prostate cancer has surpassed lung cancer as the leading cancer in men in the United States[1]. A better understanding of the mechanisms which regulate prostatic growth in the normal and neoplastic prostate may facilitate the clinical manipulation of aberrant prostatic growth. Since the epithelium of the prostatic glandular acini is surrounded by a well-developed fibromuscular stroma, cell-to-cell communication has been implicated in the regulation of prostatic growth.

As reviewed in Chapter 2 of this thesis, paracrine regulation of prostatic growth was first suggested by Franks *et al.*[2] after observing a lack of growth capacity of epithelia which had been separated from the stroma. Tissue recombination studies using fetal stroma (mesenchyme) of the urogenital sinus and (adult) bladder epithelium revealed the appearance of prostatic epithelial morphogenesis indicating that the epithelial differentiation is based on instructive induction by the stroma rather than on irreversibel commitment of the epithelial cells[3]. The growth and thus the size of the 'neo-prostate' was found to be stroma (cell) density-dependent[4]. Tissue recombination studies with testicular feminization and wild type tissues have shown that the target of androgen action is the mesenchyme, which in turn mediates the growth of adjacent epithelial cells[3]. It was shown that in adult rat prostate androgens mediate the secretion of stromally-derived growth factors which stimulate epithelial proliferation[5,6].

Another intriguing observation is that, upon maturation, accessory sexual organs reach a typical size and weight which is characteristic for that particular organ[3]. Likewise, administration of testosterone to androgen deprived rats restores the size of the prostate to its normal precastration level and no overgrowth is induced even after prolonged administration or the use of supraphysiological doses[7]. Indeed, in intact adult rats androgen levels are high while prostatic epithelial cell proliferation is low and in balance with cell death[8]. Several investigators have been searching for the molecular basis of this homeostatic constraint mechanism that curtails further increase in cell number once the gland has reached its normal size. The observation, however, that normal rat and human prostatic epithelial cells grown *in vitro* (i.e. without stroma) are able to proliferate in serum-free medium without the presence of androgens[9-12], while androgen ablation in the presence of connective tissue induces a marked regression of the glandular epithelium[8], warrants the search for stromally-derived epithelium inhibiting factors in the prostate. That stroma not only induces a mitogenic response, but can also act as a 'brake' on the epithelium, is suggested by observations made on other organ systems. Negative regulators of cell growth have been demonstrated in other fibroblast cultures[13-15]. Several coculture experiments as well as *in vivo* studies showed the controlling influence of the stroma on the epithelial compartment, ranging from induction of

differentiation[16,17] to tumor cell degeneration[18]. Similar experiments carried out on the male genital tract fit in with these findings. Rowley and Tindall[19] found that diffusible factors produced by fetal urogenital sinus decreased the proliferation of bladder carcinoma cells *in vitro*. Recently, Chung *et al.*[20] showed a marked growth inhibition of a hormone-independent rat prostatic tumor cell line (Nb-102-pr) when grafted in combination with urogenital sinus mesenchyme. Hayashi and Cunha[21], performing similar experiments on Dunning R3327 rat prostate tumor lines, demonstrated a reduction in epithelial cell growth rate and an apparent reduction or loss of tumorigenesis of these cells. Indeed, those findings indicate that, in the urogenital tract, mesenchymal cells are capable of inhibiting epithelial cell proliferation.

## 7.2. Characterization of explant cultures of human prostatic stromal cells

Detailed studies of stromal-epithelial interactions are facilitated by the availability of separately cultured epithelial and stromal cells, permitting reconstitution and analysis of their interactions under defined conditions. There have been several reports concerning primary culture of murine prostate epithelial [9] and stromal cells [5,22-24], while less information is available on *in vitro* models derived from human tissue. Although several human prostatic carcinoma cell lines have become available during the past decade [25-28], and methods were developed for primary culture of epithelial cells from normal and diseased prostates [10,29-32], only a few papers have been published about studies using cultured human prostatic stromal cells. So far, no studies have been conducted regarding the characterization of human prostatic stromal cells. These cells were tentatively designated as fibroblasts, a nomenclature mainly based on cell morphology. Since we planned to perform *in vitro* experiments on stromal-epithelial interactions using human prostatic 'fibroblasts' as stromal cells, we decided to analyze these cultures first.

Stromal cell cultures derived from prostate cancer tissue and from BPH nodules were characterized immunocytochemically using antibodies against the intermediate-sized filament proteins keratin, vimentin, and desmin, and antibodies against smooth muscle actin microfilaments[33,34]. As shown in Chapter 3, the stromal cell populations derived from BPH and prostatic carcinoma differed with regard to their differentiation markers. Whereas BPH-derived stromal cell cultures predominantly consisted of fibroblastic cells, stromal cells derived from malignant lesions in addition showed smooth muscle differentiation (myofibroblast). The outgrowth of different cell populations under similar culture conditions is indicative of a remarkable divergence in the stromal-epithelial relationship between these two pathological conditions with clinically different behavior; this may provide an excellent tool for studying

these processes. However, as the difference in phenotypes between BPH and carcinoma-derived cultures may gradually fade at subculture, it should be recommended to carry out (coculture) experiments with stromal cells from the earliest passage possible. Unfortunately, the nature of our experiments did not permit us to use very low passage number cells under all circumstances. Since the experiments required large numbers of stromal cells, these cells had to undergo several population doublings before sufficient numbers were obtained; they had to be subcultured at least four times. At passage number four, however, the immunocytochemically determined phenotypic difference between 'malignant' and 'nonmalignant' stromal cells was lost. This may explain why we have not been able to notice any significant differences in inhibitory activity between tests performed on BPH-derived or prostatic carcinoma-derived stromal cell CM. Although in coculture experiments (chapter 4) at low stromal cell densities a somewhat stronger inhibion was observed using carcinoma derived stromal cells, at higher cell numbers no striking differences were observed between the maximal levels of inhibition reached by the two stromal cell types. Likewise, besides the tendency to obtain a similar phenotype at subculture, the lack of a significant difference in level of activity of CM from malignant and nonmalignant stromal cell cultures may as well be due to the high cell density needed for production of potent CM.

## 7.3. Negative control of epithelial cell proliferation by prostatic stroma

Initially, the influence of prostatic stromal cells on the growth of the prostatic carcinoma cell lines PC-3 (hormone independent) and LNCaP (hormone dependent) was examined using a double layer soft agar system which mechanically separates both cell types. Clonal growth of both cell lines was inhibited by all prostatic stromal cell strains tested irrespective of whether they were derived from malignant or non-malignant prostate tissue. Irradiated as well as quiescent stromal cells showed similar effects indicating that the activity is not dependent on cell proliferation. Inhibition was shown to be mediated by a diffusible product(s) present in the conditioned medium of the stromal cells. Organ specific secretion is suggested by the observation that skin fibroblasts were stimulatory in these tests.

To elucidate this interaction further, we established a number of stromal cell cultures from human prostatic carcinomas as well as benign prostatic hyperplasia nodules, and investigated the influence of conditioned medium of these cultures on the anchorage-dependent growth (monolayer cultures) of different epithelial cell lines from human and murine origin (chapter 5). Likewise, several nonprostatic stromal cell cultures were initiated, and their CM tested. Using the 'MTT-test', a convenient semi-automated assay, antiproliferative activity was

found to be reversible, dose-dependent, and produced specifically by prostatic stromal cells and not by stromal cells derived from skin, foreskin, uterus, kidney, and Wilms' tumor. Inhibition was not species-specific, since the cell lines AT-2.1 and MATLyLu, derived from the Dunning rat prostate tumor, were also sensitive. Breast and renal carcinoma cell lines were not inhibited, however, suggesting a prostate-specific action. The putative inhibiting factor(s) could be concentrated and partially purified by ammonium sulfate precipitation indicating that protein structures are involved. This idea was confirmed by the observation that after trypsin digestion inhibitory activity was lost. Further physicochemical characterization showed a molecular weight > 3.5 kD, and a sensitivity to reducing agents, indicating that the presence of S-S bridges is needed for biological activity. It was also suggested that the putative inhibitor may be acid- and heat labile.

Transforming growth factor-ß is the only well-known epithelial cell growth inhibitor that has been identified in the prostate[13,35]. The presence of TGF-ß mRNA has been demonstrated in human prostatic tissue[36] as well as in rat ventral prostate[37]. *In vitro* studies have implicated TGF-ß as a potent growth inhibitor of prostatic epithelial cells, both normal and malignant[38]. Kyprianou and Isaacs[37] showed a 40-fold increase of TGF-ß mRNAs four days after castration. Upon androgen administration to four-day castrated rats levels returned to precastration values, showing that expression of TGF-ß is under negative androgenic regulation. *In vitro* and *in vivo* experiments showed TGF-ß to induce programmed cell death of rat ventral prostate glandular cells[39]. Since these studies were performed on whole tissue homogenates, no conclusions regarding the source of TGF-ß can be drawn from these tests. However, prostatic carcinoma cells have been shown to secrete TGF-ß *in vitro*[35], while the secretion of TGF-ß by fibroblasts from other organs is also well documented[13]. Since castration-induced cell death exclusively takes place in the epithelial compartment, and the ratio of epithelial to stromal cells changes in favor of the stromal part, it is tempting to assume that the increase in TGF-ß mRNA expression, an active process, takes place in the less affected cell type. Moreover, the fragmentation of genomic DNA of the epithelial cells, shown to be an early event in programmed cell death[40], may argue against the possibility that a 40-fold rise in TGF-ß mRNA level on day 4 is caused by transcription of epithelial DNA. Definite answers however, can only be expected from studies using *in situ* hybridization techniques with TGF-ß specific probes.

From this point of view it would be attractive to explain excessive (i.e., neoplastic) growth by a failure in the negative control system which may normally regulate prostatic size. It could, for instance, be caused by a change in the epithelium, rendering it insensitive to these factors[41]. Recently, elevated levels of TGF-ß immunoreactivity have been associated with prostate cancer[42]. These observations may reflect insensitivity of cancer cells, possibly

associated with a resulting failure in the negative feedback loop that controls its production and secretion by the stroma. A recent immunohistochemical study[43] demonstrated immunoreactivity to TGF-ß1 antibodies in epithelial and mesenchymal cells, but unfortunately this kind of experiments also does not reveal the site of synthesis of the proteins.

At this stage in our research it was conceivable that the stromal cell derived inhibition was caused by the action of TGF-ß. However, our latest experiments excluded this possibility (chapter 6). Although some similarities with type ß transforming growth factor-like inhibitors were apparent, it was concluded that the factor is not identical to TGF-ß or to the TGF-ß-like factors activin and inhibin for the following reasons. First, using MTT-tests as well as [$^3$H]-thymidine incorporation assays, the absence of TGF-ß activity was shown by the lack of inhibitory response of the TGF-ß-sensitive mink lung cell line CCL-64 to prostate stromal cell conditioned medium and to concentrated, partially purified preparations of the inhibitor. Furthermore, neutralizing antibodies against TGF-ß1 or TGF-ß2 did not cause a decline in the level of PC-3 growth inhibition caused by partially purified inhibitor. Northern blot analyses excluded the involvement of inhibin or activin.

## 7.4. General conclusion

The data presented in this thesis demonstrate that adult-prostate derived stromal cells cultured from neoplastic lesions, produce a unique factor, tentatively called "prostate-derived Epithelium Inhibiting Factor": p-EIF. On the basis of its spectrum of biological activity as well as its physicochemical and immunoreactive properties, p-EIF can be discriminated from previously described growth inhibitors, including the TGF-ß-related proteins inhibin and activin[44], and from interferon-ß[12]. It would be very interesting to investigate whether this unique factor is the major 'brake' on the prostate, controlling epithelial cell proliferation and thus prostatic size. The organ-specific production and the lack of inhibition on the non-prostatic epithelial cell lines tested, suggest future organ-specific therapeutic potency.

## 7.5. Discussion

In this thesis the hypothesis of a negative control of prostatic epithelial cell proliferation by its stroma was investigated. The aforementioned idea was partly based on the observation that (normal) epithelial cells without stroma (i.e., *in vitro*) are able to proliferate in the *absence* of androgens, while androgen withdrawal in the intact organ or organ explant

culture leads to a massive reduction in epithelial cell number. One has to realize, however, that theoretically there are more options leading to this status. For instance, a cessation of androgen dependent secretion of stromally-derived epithelial growth stimulating factors in the intact organ may induce a similar cascade of involutional events. Cultures of normal prostatic murine- and human epithelial cells are not stimulated in their growth by androgens; the *in vitro* proliferation could then be caused by growth factors present in the culture medium that *in vivo* would not be able to reach the epithelial compartment as will be discussed later. However, the discovery of epithelial cell inhibiting activity in stromal cell conditioned medium as described in this thesis, is favoring the first-mentioned hypothesis.

A number of investigators has studied the stromal-epithelial interactions in the prostate *in vitro*, but their reports mainly focused on the mitogenic influence of the stroma[45-49], supporting the second idea described above. No further reports from other groups have been published on stromally derived epithelial cell growth inhibitors in the adult prostate. Remarkably, all studies demonstrating stromal stimulation of prostatic epithelial cell growth were performed under serum-free conditions. Kirk, however, showed an inhibition of PC-3 cell growth by lung fibroblasts, using a serum-dependent soft agar assay[50]. Rowley reported growth inhibition of a bladder carcinoma cell line by medium conditioned by urogenital sinus explants in the presence of 10% FCS[19]. Later on, it was shown that antiproliferative activity was produced by a fibroblastoid cell strain probably derived from urogenital sinus mesenchyme[51]. The presence of the 'Urogenital sinus derived Growth Inhibitory Factor' (UGIF) was tested in a serum-containing assay. Using physicochemical, biological, and immunological methods, UGIF was demonstrated to differ from known growth inhibiting factors including TGF-ß. Using the same bioassay, however, no activity could be found in chemically defined medium that was conditioned in exactly the same manner. Only the addition of steroid hormone caused the fibroblastoid cells to produce inhibiting activity that was ultimately identified as being caused by activated TGF-ß and not by UGIF[52]. During the past decade, much effort has been put into the development of systems for serum-free culture of prostatic cells. Indeed, a serum-free environment is ideal for testing of regulatory peptides as well as for their identification. However, as a consequence of this simplification, certain information about other growth regulating pathways may be lost as suggested by the results presented here and the findings reported by Kirk[50] and Rowley[52]. These considerations bring to light a crucial dilemma in oncological research, "Reduction gains precision about parts but at each step loses information about the larger organization it leaves behind"[53]. The glandular cells in the prostate receive nutrients, growth-modulating peptides, trophic nerve factors, and (steroid) hormones that have traversed the endothelial cells, stromal cells, extracellular matrix, and the acinar basement membrane. Consequently, the stromal compartment has ample

opportunity to modify epithelial cell microenvironment. It is conceivable that (perhaps unknown) serum-derived factors may very well participate in this, for instance by modifying stromal secretions, as was shown for androgen-induced epithelial cell proliferation in the rat prostate[5]. Thus, tests performed in the presence of serum or other undefined supplements, such as bovine pituitary extract, will provide us with useful information about physiological and pathophysiological processes, and are essential to basic oncological research. However, these supplements will eventually hamper identification and isolation of the substance(s) of interest.

So far, transforming growth factor-ß is the only well-known epithelial cell growth inhibitor that has been identified in the prostate[13,35], and the secretion of TGF-ß by fibroblasts from other organs is also well documented[13]. Although p-EIF shares some similarities with type ß transforming growth factor (TGF-ß)-like inhibitors, evidence has been presented that the factor is not identical to TGF-ß or to the TGF-ß-like factors activin and inhibin. It is concluded that prostate stroma-derived factor may be a novel growth inhibitor different from any of the currently described inhibiting factors.

## 7.6. Future research

A next step in the study of this factor would be a further biological characterization; it would be advisable to test its activity on a larger number of nonprostatic epithelial cell types to make sure that, besides the production, also the action is prostate specific. If so, this would be a great stimulus to prostate research, and might give rise to new developments in the selective treatment of prostatic neoplasia. The effect of p-EIF on primary cultures of prostatic epithelium from BPH or PC, for instance, should be investigated thoroughly as well as its effect on the stromal cells themselves in order to find out whether it is autostimulatory or inhibitory. The result may provide more information on its role in normal physiology and its possible involvement in the development of neoplasia. Although it may end up rather time consuming, efforts should be put into the purification of the inhibitor. As an example, the purification of inhibin should be kept in mind; after the introduction of the concept of inhibin, more than half a century passed before significant progress in the purification of gonadal inhibin was reported. Among other causes, this was thought to be due to the aberrant behavior of the molecule when conventional techniques were used[54]. In general, the isolation of substances that are inhibitory in bio-assays is more difficult than purifying agents with a positive biological response, for every time the target cells show a reduced proliferation rate it has to be verified that it is really caused by the substance of interest and not the result of, for instance, cell toxic contaminants in the solution, a changed osmolarity, or a (very) high protein

content in the sample.

For the time being, however, the presence of relatively large amounts of serum in the conditioned medium appears as an insurmountable problem. Purification at this stage can be considered as 'looking for a needle in the haystack', and should be postponed until the production of p-EIF has been realized under more appropriate conditions. Preliminary data suggest that serum-derived factors are required for optimal production and/or secretion. Preliminary results however, failed to demonstrate significant effects of steroids (testosterone, dihydrotestosterone, or estradiol) in this respect. Further knowledge of the factors influencing its production may eventually facilitate purification and characterization. Antibodies raised against the purified factor would provide us with an excellent tool for studying its role in normal physiology and its possible involvement in the development of neoplasia.

It should be noted that it is possible that, at the end, *no* physiological or pathophysiological role for this substance can be defined. One might assume that the very artificial circumstances like *in vitro* culture may lead to expression of previously 'hidden' genes resulting in the production and secretion of factors not seen *in vivo*. Like the altered intermediate sized filament expression after *in vitro* culture reported in this thesis (chapter3), it is conceivable that the production of inhibitory substances might be a similar unphysiological response to the unphysiological conditions. But even in that case it deserves our full attention because any agent that is capable of inhibiting prostatic epithelial (cancer) cells should be considered as a future treatment option in urological oncology.

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# Samenvatting

De prostaat maakt deel uit van de mannelijke geslachtsorganen. De klier produceert een belangrijk deel van het semen en is gelegen rond de urinebuis aan de basis van de urineblaas in het kleine bekken. Een vergroting van het orgaan kan aanleiding geven tot klachten en kan berusten op een goedaardige vergroting, ook wel BPH genoemd, of op prostaatkanker. Een wezenlijk verschil tussen beide vormen van overmatige groei is dat de kwaadaardige afwijking kan metastaseren ('uitzaaien') naar andere organen en op deze wijze aanleiding kan geven tot de dood van de patiënt. Na longkanker is het de belangrijkste oorzaak van kankersterfte in de Westerse wereld: per jaar worden in Nederland ruim 4.000 nieuwe gevallen ontdekt terwijl jaarlijks ruim 2.000 patiënten aan de ziekte bezwijken. Als behandeling van het prostaatcarcinoom in een vroeg stadium (niet gemetastaseerd) kan operatie of radiotherapie (bestraling) toegepast worden. Een niet onaanzienlijk deel van de patiënten wordt gediagnostiseerd op een moment dat er reeds metastasen aanwezig zijn. Het merendeel van hen reageert aanvankelijk goed op 'hormonale behandeling' hetgeen d.m.v. een medische of chirurgische castratie wordt uitgevoerd. Na verloop van tijd wordt het prostaatcarcinoom echter ongevoelig voor deze behandeling en zet het proces van tumorgroei zich voort, waarna tot op heden nog slechts palliatieve (symptoom-bestrijdende) ondersteuning rest. De oorzaken die aan de goedaardige en maligne nieuwvorming ten grondslag liggen, alsmede de mechanismen die progressie tot hormoon-onafhankelijkheid van prostaatkanker bewerkstelligen zijn vooralsnog onopgehelderd. Men vermoedt dat vele factoren een rol spelen bij het ontstaan hiervan, waaronder erfelijke aanleg, hormonale invloeden en de voeding. Met het werk beschreven in dit proefschrift werd getracht een bijdrage te leveren aan het in kaart brengen van de factoren welke de groei van prostaatcellen kunnen beïnvloeden.

De embryonale ontwikkeling en de (latere) groei en functie van de prostaat zijn afhankelijk van de aanwezigheid van mannelijke hormonen (androgenen). In *hoofdstuk 2* wordt een literatuuroverzicht gegeven van een aantal belangrijke (dier)experimenten welke tot een verdieping van ons inzicht in de hormonale regulatie van de prostaat(groei) hebben geleid. De klier bestaat zoals de meeste klieren voor het grootste deel uit bindweefsel (stroma) en klierweefsel (glandulair, epitheliaal weefsel). Uit de genoemde experimenten is gebleken dat de regulering van de door mannelijke hormonen geïnduceerde embryonale groei en ontwikkeling verloopt via het stroma; indien er geen hormoonreceptor aanwezig is in het bindweefsel zal geen prostaat aangelegd worden. Door nader onderzoek werden eveneens aanwijzingen verkregen die erop duiden dat ook in de volwassen prostaat belangrijke androgene (groei)effecten via het stroma kan verlopen. Anderszins kan men vermoeden dat omgekeerd ook de afname (involutie) van de klier(grootte), bijvoorbeeld t.g.v. hormoononttrekking, evenzo door het stroma gereguleerd kan worden. Ook het feit dat de klier onder invloed van hormonen (in de puberteit) uitgroeit tot een bepaalde maximum grootte en dat er ondanks de

blijvende aanwezigheid van androgenen geen verdere (overmatige) volumetoename resulteert, zou middels een negatieve controle (remmende invloed) vanuit het stroma verklaard kunnen worden. De negatieve controle zou kunnen bestaan uit een afname van positieve groei-effecten danwel als een toename van groei-remmende factoren. De meeste experimenten *in vitro* (m.b.v. celkweken) laten een groei-stimulerende invloed zien van gekweekte bindweefselcellen op epitheelcellen van de prostaat.

In dit proefschrift worden experimenten beschreven welke werden uitgevoerd met kweken van humane stromale cellen van de prostaat en de bekende *in vitro* tumorcellijnen van de menselijke prostaat: PC-3 en LNCaP, alsmede de Dunning (rat)prostaat-tumorcellijnen MATLyLu en AT-2.1. De stromale cellen werden gekweekt van ziek prostaat weefsel afkomstig van patiënten met BPH en van patiënten met een prostaatcarcinoom. Een goede karakterisering van deze kweken van humane stromale cellen was nog niet in de literatuur gemeld. In *hoofdstuk 3* wordt de immunocytochemische karakterisering besproken. Hiertoe werden antilichamen gebruikt welke gericht zijn tegen specifieke eiwitkomponenten van het celskelet ('intermediate sized filament proteins'): keratine, desmine en vimentine. Aanwezigheid van deze eiwitten is karakteristiek voor cellen afkomstig van respectievelijk epitheel, spier- en bindweefsel. Tevens werd gekleurd met antistoffen tegen actine afkomstig van gladde spiercellen. Door het tellen van het aantal aangekleurde cellen in een bepaalde kweek (van een bepaalde patiënt) werd duidelijk dat d.m.v. de toegepaste, relatief eenvoudige, methode van 'explant-culture' reeds in de eerste passage ('kweekronde') een vrij zuivere kweek van stromale cellen kon worden verkregen: $< 5\%$ contaminatie met epitheliale cellen. In de volgende (sub)cultures nam dit percentage snel nog verder af. Opmerkelijk was de bevinding dat kweken afkomstig van de maligne afwijking beduidend meer cellen bevatten met eigenschappen van gladde spiercellen dan de BPH cultures. Dergelijke cellen, ook wel aangeduid als myofibroblasten, werden ook gemeld aanwezig te zijn in andere tumoren; ze zouden verantwoordelijk zijn voor de desmoplastische reactie zoals die beschreven is bijvoorbeeld in samenhang met het mammacarcinoom (borstkanker). Ondanks het verschil in fenotype ('uiterlijk') van de kweken van BPH en prostaatcarcinoom werd bij de in dit proefschrift beschreven proeven géén duidelijk verschil gevonden in de uitkomsten van de experimenten. Dit zou (mede) verklaard kunnen worden uit het feit dat het (immunocytochemische) verschil tussen beide celkweektypen afnam tijdens het doorkweken (na enkele passages) in combinatie met feit dat voor het verkrijgen van voldoende cellen het opkweken gedurende minimaal drie passages onvermijdelijk was.

*Hoofdstuk 4* beschrijft het effect dat stromale cellen hebben op de groei van de genoemde humane epitheliale cellijnen van de prostaat. Hiertoe werden de epitheelcellen in een tweelagig 'soft-agar' testsysteem samengebracht met de bindweefselcellen. In dit systeem zijn

de celtypen ruimtelijk, doch niet (bio)chemisch, van elkaar gescheiden. Bij deze tests die werden uitgevoerd in de aanwezigheid van serum (een rijk voedingsmedium) bleek de groei van het epitheel geremd te worden, de mate van remming was (binnen bepaalde grenzen) evenredig met het aantal meegekweekte stromacellen. Nadere analyse suggereerde de aanwezigheid van een groeiremmende stof in het kweekmedium (geconditioned medium: CM) van de stromacellen.

Door het gebruik van een meer handzame test (MTT-test), waarbij de epitheelcellen worden gekweekt in multiwell platen en de hoeveelheid cellen aan het einde van een groeitest (semi-)automatisch kan worden bepaald, werd het mogelijk meerdere monsters tegelijkertijd te testen. In *hoofdstuk 5* wordt aangetoond dat naast de 'non-anchorage dependent growth' (groei in agar) van de epitheelcellen ook de 'anchorage dependent growth' (groei op de bodem van weefselkweekmateriaal) wordt geremd door CM van stromacellen. De proliferatie van zowel de hormoonafhankelijke LNCaP cellijn als de hormoonongevoelige PC-3 cellijn neemt daarbij af. Het effect bleek reversibel, en werd uitsluitend gevonden in het CM van stromacellen van de prostaat: bindweefselcelkweken afkomstig van huid, preputium (voorhuid), uterus (baarmoeder), nier en Wilms' tumorweefsel lieten geen significante remming zien in de tests. Het effect bleek niet (dier)soortspecifiek daar ook de Dunning prostaattumor-cellijnen MATLyLu en AT-2.1, afkomstig van de rat, werden geremd in hun groei. De aanwezigheid van een specifieke groeiremmende factor (inhibitor) werd mede ingegeven door het feit dat het inhibitoire effect kon worden geconcentreerd door het uit het (serumhoudende) geconditioneerde medium neer te slaan (precipiteren); op deze wijze kon, door de factor opnieuw in een weinig medium op te lossen, een zeer potent concentraat bemachtigd worden (tot 90% groeiremming in de MTT-test). Bij deze procedure gelukte het tevens om ca 75% van de overtollige (serum)eiwitten kwijt te raken.

De verklaring voor het gegeven dat de meeste artikelen over dit onderwerp een stromale stimulering van het epitheel beschrijven lijkt te moeten worden gezocht in het al of niet aanwezig zijn van serum bij de proeven hetgeen in de laatste hoofdstukken wordt bediscussieerd. Samengevat lijkt het dat de inhibitor uitsluitend wordt geproduceerd in aanwezigheid van serum. Men moet zich daarbij ook realiseren dat *in vivo* met name de bindweefselcellen in nauw contact staan met het bloed en lymfevocht, en dat bij het ontberen hiervan een minder fysiologische omgeving resulteert. Anderzijds kan de aanwezigheid van serum het identificeren, en met name het isoleren van groei-modulerende factoren ernstig hinderen. In dit verband geldt het adagium: "Reduction gains precision about parts but at each step loses information about the larger organization it leaves behind" (Smithers, referentie 122, hoofdstuk 2).

Uit *hoofdstuk 6* blijkt dat de in dit werk gevonden remmende factor zich onderscheidt

van andere, reeds bekende inhibitoren. Fysico-chemisch onderzoek toonde dat de factor gevoelig is voor reducerende stoffen en proteolytische enzymen hetgeen de aanwezigheid van eiwitstructuren met daarin disulfidebruggen demonstreert, die nodig zijn voor biologische activiteit. Daarnaast leek er sprake van zuur- en hitte-instabiliteit. Met name de laatste twee eigenschappen differentiëren de factor van de veel beschreven Transforming Growth Factor ß (TGF-ß) en zijn subtypen. Ondanks het feit dat commercieel verkrijgbaar (runder)serum ook latent TGFß bevat, kon middels biologische tests op de voor TGFß zeer gevoelige CCL-64 cellijn alsmede immunologische proeven met neutraliserende antistoffen het onderscheid aannemelijk worden gemaakt. Vooralsnog werd de factor vanaf dit moment aangeduid met p-EIF: prostate-derived Epithelium Inhibiting Factor.

Geconcludeerd wordt dat het hier wellicht handelt om een nieuwe groei-modulerende factor die mede gezien de prostaat-specifieke produktie uniek genoemd mag worden. Verder onderzoek lijkt ten zeerste gerechtvaardigd.

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|---|---|
| aFGF | acidic fibroblast growth factor |
| AR | androgen receptor |
| bFGF | basic fibroblast growth factor |
| BPH | benign prostatic hyperplasia |
| BSA | bovine serum albumin |
| CM | Conditioned medium |
| DAB | 3,3'-diaminobenzidine |
| DHT | dihydrotestosterone |
| DMEM | Dulbecco's modified Eagle medium |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleïc acid |
| DTT | dithiothreitol |
| ECM | extracellular matrix |
| EDGF | epithelially derived growth factor |
| EDIF | epithelially derived inhibiting factor |
| EDTA | ethylene-diamino-tetra-acetate |
| EGF | epidermal growth factor |
| ER | estrogen receptors |
| EVE | estrogen treated vaginal epithelium |
| EVS | estrogen treated vaginal stroma |
| FCS | fetal calf serum |
| Gy | gray |
| IFN | interferon |
| KGF | keratinocyte growth factor |
| LHRH | luteinizing hormone-releasing hormone |
| MEM | minimum essential medium |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| NGF | nerve growth factor |
| NP | normal prostatic tissue |
| PA | prostate adenoma $\approx$ benign prostatic hyperplasia |
| PBS | phosphate buffered saline |
| PC | prostate carcinoma |
| PE | plating efficiency |
| p-EIF | prostate-derived epithelium inhibiting factor |
| RNA | ribonucleïc acid |
| SD | standard deviation |
| SDGF | stromally derived growth factor |
| SM | smooth muscle |
| STI | soybean trypsin inhibitor |
| Tfm | testicular feminization |
| TGF-ß | transforming growth factor-beta |
| UGIF | urogenital sinus-derived inhibitory factor |
| VE | vaginal epithelium |
| VS | vaginal stroma |

# Nawoord

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**Concentration and purification of poliovirus by immune adsorption on immobilized antibodies.**

P. van der Marel, A.L. van Weezel, A.G. Hazendonk and A. Kooistra.

*Develop Biol Standard, 46:267-271, 1980.*

**K-ras Oncogene Activation as a Prognostic Marker in Adenocarcinoma of the Lung.**

R.J.C. Slebos, R.E. Kibbelaar, O. Dalesio, A. Kooistra, J. Stam, C.J.L.M. Meyer, S.S. Wagenaar, R.G.J.R.A. Vanderschueren, N. van Zandwijk, W.J. Mooi, J.L. Bos en Sj. Rodenhuis.

*N Engl J Med, 323:561-565 ,1990.*

# Curriculum vitae

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| mei 1995 | **Pharmacia Scientific Award 1995**<br>Toegekend op basis van het werk beschreven in hoofdstuk 3 van dit proefschrift. |