Chapter 5
DISCUSSION
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Human hyperplastic and malignant prostates were examined for amplified expression of growth factors and receptors employing a battery of specific antibodies. The primary objective was to determine which one of these is increased in pathological growth of prostate. The rationale for the selection of these growth factors and receptors in the present study was based on the literature evidence of their amplified expression in varieties of human malignancies including that of prostate.

Cells within a normal tissue undertake programs of growth and differentiation on the basis of signals received from the other cells in the tissue or organism. This network of intercellular signalling represents the basis for establishing and maintaining normal tissue architecture. Many of the signals passing between cells appear to be carried by growth factors which can influence cell growth in a positive or negative way. These growth factors are released in carefully measured amounts by some cells and are conveyed and bound to specific receptors displayed on the target cells. This binding in turn triggers a complex signal transduction cascade that ultimately affects the cells decision to grow or to remain quiescent. The process of tumorigenesis represents a disruption of this complex signalling network. As a result, a cell may loose dependence on exogenous mitogenic growth factor and develop an autonomus mode of growth control.

The expression of growth factors and receptors in prostatic tumours was looked for immunohistochemically, a method that has advantage over biochemical techniques that employ tissue homogenates for analysis and has the following inherent limitations.
Firstly, the type of cell/cells making the protein can not be ascertained, nor the subcellular location of it. By immunohistochemically differential observation can be made of what is present in the cancerous cells, apart from the normal cells invariably contained in a biopsy. This discrimination is lost, when homogenates be analysed.

The results of immunohistochemical examination indicate that the synthesis of some of these proteins are considerably enhanced in either benign prostatic hyperplasia or in carcinoma of prostate or in both. Epidermal growth factor, a single chain polypeptide has been implicated in the process of neoplastic transformation. It has been shown that EGF stimulates cell proliferation in cultured tumour cells (Carpenter and Cohen, 1979). It has been reported that normal human prostate produces EGF (Elson et al. 1984) and is mitogenic to human prostate cancer cells in culture (Peehl et al. 1989). Expression of this factor in the present study could only be demonstrated in a minority of benign tumours and in none of the malignant cases suggesting two possibilities. Firstly, either the tumour cells are not secreting this factor, or the methodology applied is not sensitive enough to detect low expression of this protein.

Transforming growth factor-βs, a more biologically complex family of growth factors also appear to be involved in growth disorders of the prostate. TGF-β operates bifunctionally, being inhibitory at lower and stimulatory at higher concentrations (Shain et al. 1990). Expression of high levels of TGF-β1 mRNA in PC3 and DU 145 cells as well as Dunning series of transplantable rat prostate carcinomas have been reported (Wilding et al. 1989; Steiner & Barrack, 1990). The antibody employed in the present study was directed against TGF-β2 which failed to detect the growth factor in
malignant prostates, the growth factor was detectable only in an occasional case of benign gland suggesting that the factor is uncommonly expressed in prostate tumours.

Both aFGF and bFGF have been reported to stimulate prostatic epithelial growth in culture (McKeehan et al. 1984). FGFs have been identified in many different tissues. FGF-basic has greater affinity to heparin, many times more potent a mitogen for fibroblast than aFGF and has a greater angiogenic potential. None of these growth factors could be detected in most of the cases studied, in contrast to the reported evidence of its expression in hyperplastic human prostates (Mansson et al. 1989; Mori et al. 1990). This could be due to the fact that in other studies, mRNA corresponding to FGFs were studied, whereas ours was an immunohistochemical approach. Although aFGF mRNA is abundant in the prostatic epithelium of immature animals, it disappears as the animals mature. Interestingly, expression of aFGF mRNA in the mesenchymal cells of androgen-responsive Dunning R3327 PAP prostate carcinoma (Isaacs 1987) lead to the speculation that the mesenchyme derived aFGF supports the growth of malignant epithelium in a paracrine manner. In vitro studies have confirmed that both aFGF and bFGF are mitogenic to epithelium and fibroblast cells of prostatic origin in culture (McKeehan et al. 1984 & 1987; Story et al. 1989). Further work is therefore indicated to determine whether the mRNA corresponding to FGFs are expressed in abnormal growth of prostate.

A fairly high percentage (25%) of prostatic tumour tissues in the present study gave positive reaction with anti-TGF-α. It was demonstrable in only one out of seven CaP tissues. TGF-α, a potent mitogen has been reported to be consistently associated with the pathological process of neoplastic
transformation in a variety of malignancies including those of liver (Liu et al. 1988; Raymond et al. 1989) mammary gland (Salomon et al. 1987) and pancreas (Smith et al. 1987; Ohmura et al. 1990). Could TGF-α play the role of a growth promoting factor in human prostatic tumours? Derynck et al (1987) have demonstrated the expression of messenger RNA for TGF-α and its receptor, EGFR in PC3 cells, a human androgen - independent prostate cancer cell line. More recently, to understand the physiological role of TGF-α and the consequences of its overproduction/overexpression, significant epithelial hyperplasia and focal dysplastic changes that resembled carcinoma in situ in the anterior prostate were observed in transgenic mice (Sandgren et al. 1990). These observations support the notion that TGF-α is a potent epithelial cell mitogen and an oncogenic protein and plays important role in cellular proliferation and neoplastic transformation.

The present study demonstrated moderate to high immunohistochemical reaction for EGFR in hyperplastic (72.2%) and malignant (50%) prostates. Although the reaction was predominantly observed in the epithelial and cancer cells, reaction of mild to moderate intensity was also observed in stromal cells suggesting the distribution of EGF receptor in different cell types. The EGF receptor is involved in action of both EGF and TGF-α. High affinity EGF receptors have been detected in rodent and human prostates (Maddy et al. 1987; Traish and Woritz, 1987; Davies and Eaton, 1989). There is some disagreement as to whether EGF receptor is increased or down-regulated in prostate cancer tissue compared to normal prostate. Assessment of mRNA level tends to support the former (Morris and Dodd, 1990). The EGF receptor is a transmembrane glycoprotein and in all the cases showed positive reaction, the receptor was demonstrated in the plasma membrane. The receptor has tyrosine kinase activity and is involved in cell
proliferation. The monoclonal antibody competitively blocks EGF binding to
the receptor, inhibits activation of receptor tyrosine kinase induced by EGF
and TGF-α and stimulates receptor internalization (Sato et al. 1983; Kawamoto et al. 1984; Gill et al. 1984). The monoclonal antibody also
inhibits cell proliferation induced by ligands, both in culture and in nude
mouse xenograft models (Kawamoto et al. 1983; Masui et al. 1984 & 1986).
These findings warrant exploration of the therapeutic potential of anti-EGFR
antibody or other agents that act in a comparable manner.

Expression of c-neu protein in human hyperplastic and malignant
prostate tissues has been studied to understand the pattern and frequency of its
expression. It was observed that a very high percentage of BPH and all
prostatic adenocarcinoma tissues showed positive reaction of varying intensity
for c-neu protein. Strong, moderate and weak positive reactions were
observed in 33.3% (12/36), 47.2% (17/36) and 13.8% (5/36) cases of BPH
patients, respectively. In a recent study employing a monoclonal antibody that
recognizes -COOH terminal, Mellon et al. (1992) demonstrated strong
positive reaction in about 18% of glands. Higher percentage of strongly
positive reactions as observed in the present study could possibly be
attributable to methodological difference like a longer incubation period
employing more diluted primary antibody was done in this study.

HER-2/c-neu gene represents a family of cellular genes involved in
cell growth and differentiation. Although the amplification and overexpression
of c-neu proto-oncogene has been reported in several human malignancies
particularly in breast cancer with poor prognosis (Semba et al. 1985; Yokota
et al. 1986; Tal et al. 1988; Slamon et al. 1989; Schneider et al. 1989; Weiner
et al. 1990; Wright et al. 1990; Berchuck et al. 1991), the present study is
amongst the few observations on its presence in prostatic tumours. c-neu oncoprotein belongs to the EGF receptor family and normally has a membrane localization. However, since the monoclonal antibody employed for immunohistochemical investigation recognized the COOH terminal of the protein, the localization observed in many cases are of transmembrane cytoplasmic portion. In a recent study, using a polyclonal antibody against the internal domain and a monoclonal antibody recognizing external domain of p185\textsuperscript{neu}, cytoplasmic staining was most frequently observed in BPH and CaP tissues (Ware \textit{et al.} 1991). Cytoplasmic localization could be explained by the fact that cytoplasm is the site for synthesis of this membrane protein before it is transported to the plasma membrane or the consequence of internalization of receptor following ligand binding. It is recognized that a high percentage of prostatic tumours are not dependent on androgens. There may be an involvement of growth factors that operate by autocrine, juxtacrine or paracrine fashion. The overt expression of the c-neu receptor protein in prostatic tumours as observed in the present study raises the question of the nature of the corresponding growth factor binding with this receptor protein. Although, the true ligand for this receptor is not known, several putative ligands, such as heregulin, \textit{neu} differentiation factor (NDF), a 30 kDa transforming growth factor - alpha (TGF-\alpha) like glycoprotein (gp 30), a \textit{neu} protein - specific activating factor (NAF) and a polypeptide (p25) have been described (Lupu \textit{et al.} 1990; Dobasshi \textit{et al.} 1991; Tarakhovsky \textit{et al.} 1991; Holmes \textit{et al.} 1992; Wen \textit{et al.} 1992). The causal relationship between the induction of the corresponding growth factor and receptor protein may provide insights into proliferation of the prostate. This protein was present in all cases of prostatic adenocarcinoma biopsies in our study and also in majority of BPH cases. Thus the effect of a specific antibody reacting with this protein on the
growth of two tumour cell lines, LNCaP and DU 145 was undertaken. However, the presence of this murine monoclonal antibody directed against the extracellular domain of c-neu protein failed to influence the growth of LNCaP and DU 145 cells in vitro. In contrast, cytotoxic effects of a few c-neu monoclonal antibodies directed against the various antigenic determinants of external domain of p185 neu, on other cancer cells particularly of breast cancer origin has been reported in cultures and in vivo (Drebin et al. 1986 & 1988; Hudziak et al. 1989).

Prostate specific acid phosphatase is a useful prostatic marker and immunohistochemical demonstration of PSAP has been useful in determining the prostatic origin of metastatic adenocarcinomas (Nadji et al. 1980; Lippert et al. 1982). While PSAP is claimed to have 100% specificity and sensitivity to prostatic cancers (Nadji et al. 1980) others have documented negative staining for PSAP (Jobsis et al. 1978). In the present study, PSAP could be demonstrated immunohistochemically in many (73.7%, BPH and 57.1%, CaP) cases but not in all. A similar type of observation has been made earlier by others (Fishleder et al. 1982; Ellis et al. 1984; Mori and Wakasugi, 1985), wherein PSAP was demonstrated immunohistochemically in benign hyperplasia in more number of cases than the cancerous prostates. The present observation is concordant with the lesser activity of this enzyme in malignant prostates as described previously (Lippert et al. 1982; Bates et al. 1982). Electron microscopy suggested that the low level of PSAP in malignant prostates presumably results from changes in cellular translocation. This regulatory failure of secretion kinetics may appear to be associated with malfunction of microfilaments and microtubules as a component of cell cytoskeleton in anaplastic prostate cells (Mori and Wakasugi, 1985). Elevated
level of PSAP in serum was however, observed in a small number of BPH patients and in none of the CaP cases. Increase in serum PSAP has been reported in highly anaplastic prostatic carcinomas but not in well differentiated adenocarcinoma of prostate (Pontes et al. 1981). All the BPH patients with high PSAP levels in serum however had strong immunostaining in tissues. Moreover, in several other cases where the tissue reaction was stronger the serum value was within normal limits. In ten hyperplastic and three cancer tissues, PSAP could not be demonstrated, histochemically, even though, serum PSAP levels in these patients were within normal range. Immunohistochemical studies showed an increase in the content of prostate specific antigen in benign (94.6%) and cancerous (85.7%) prostatic tissues in comparison to the normal prostates. While some reports (Nadji et al. 1980; Nadji et al. 1981) claimed 100% sensitivity for PSA in prostate cancer, this study confirm the findings of others (Jobsis et al. 1978; Bentz et al. 1982; Stein et al. 1982) that majority but not all prostatic adenocarcinomas do not stain for PSA. In six out of seven cancerous prostates, PSA was localized in the cytoplasm, as well as in the cell membrane. Intramembranous presence may be explained by the localization of the protein in the membrane before its extracellular transport. On the other hand the presence of PSA in the cell membrane could also represent the receptor bound protein.

Moderate to strong immunocytochemical localization of PSA was observed in 94.6% cases of hyperplastic prostates. In two BPH tissues, it could not be demonstrated as the prostatic chips were histologically composed of fibromyomatous tissues with no evidence of acinar epithelium. The serum level of PSA in the present study fairly correlates with the immunohistochemical demonstration. In four CaP patients (80%) and 23 BPH patients (65.7%), PSA level in the serum was elevated. In an earlier study
when combined measurements of both PSA and PSAP were performed, 83.3% of stage A and 94% of stage D patients had increased serum PSA and/or PSAP, but high levels for one or both markers were also reported in 48% of patients with BPH (Kuriyama et al. 1982). Elevated serum PSA has also been reported from 30 to 50% of patients depending on the size of the prostates and degree of obstruction, whereas increase in the level has been reported in 25 to 92% of patients with prostate cancer depending on tumour volume (Stamey et al. 1987; Hudson et al. 1989; Partin et al. 1990).

PSA is a 34 kDa serine protease belonging to kallikrein gene family. It is present in seminal plasma, where it is believed to have a proteolytic action on semen coagulum (Lilja 1985; Lee et al. 1989). Kallikreins are however observed in various tissues and fluids like mandibular salivary glands, pancreas, blood, urine etc. Some of these have been shown to be involved in the post-transcriptional processing of polypeptide precursors to their biologically active forms by limited proteolysis (Isackson et al. 1987; MacDonald et al. 1988). Whether the prostate specific kallikrein, PSA has similar role in converting progrowth factors into mature form is not known but can be speculated. Further research is necessary to know the biological role of this protein. At present what is known is the broad correlation between the serum level of PSA and prostate tissue mass which is the most reliable clinical indicator for prostatism. In the present study many patients had elevated serum PSA values. In a recent study by Henttu et al (1992) it has been demonstrated that androgens stimulate the expression of PSA gene in LNCaP cells.

In order to have an insight into the possible role of PSA on growth of prostatic carcinoma cells, the study was carried out in vitro and in vivo using...
established prostate cancer cell lines. Androgen - dependent (LNCaP) and androgen - independent (DU 145) human prostate cancer cells, both tested and found positive for PSA, were exposed to antibodies generated against PSA. Anti-PSA antibodies had profound effect on the growth and viability of these cells. This effect was caused in a dose dependent manner on both androgen-dependent and androgen-independent cells, however the former was found to be more sensitive. The cytotoxic effect was not due to any non-specific components of the goat serum as preimmune serum manifested no action. Antibodies against another unrelated antigen, nuclear polyhedrosis baculovirus raised in the same species exercised also no inhibitory effect. The cytotoxic effect of the antibody was absorbable by PSA implying thereby that antibodies directed against PSA were exercising the nocive action.

The cytotoxic action of antibody appears cell specific. Parallel experiments employing laryngeal carcinoma cell line (HEp-2) clearly demonstrated that anti-PSA antibodies had no effect on the viability and multiplication of these cells. These observations indicate that anti-PSA antibodies acted on PSA secreting cells, LNCaP and DU 145, but not on non-PSA secretory tumour cells like HEp-2.

Being given that the antigen targeted by these antibodies is exclusive to prostate, it may be expected that immunotherapy with monospecific antibodies would have targeted action at the prostate cells. In order to verify this hypothesis, the study was extended in vivo to transplantable DU 145 tumours in athymic nude mice. The results support a role for PSA in the in vivo genesis of DU 145 prostate tumour. Anti-PSA antibodies at adequate concentrations prevented the growth of the tumour mass when implanted carcinoma cells were exposed to antibodies prior to implantation. DU 145
cells exposed to saline prior to implantation and subsequently treated with saline grew into huge tumours further substantiate the specificity of the effect of administration of anti-PSA antibodies. The fact that the anti-PSA antibody treatment was effective specifically in the PSA producing cells was demonstrated by the ineffectiveness of similar treatment on the growth of tumours induced by PSA-nonsecreting cells, HEp-2.

The tumouricidal effect of the anti-PSA antibody was tested in preformed tumours. Necrosis of tumour could be seen in higher dose groups tested. The zone of necrosis widened as the treatment was continued further. While growth of the saline treated control tumours progressed with time confirming the fact that PSA contributes to the growth of these tumours. The necrotic action was exercised specifically on tumour cells as supporting stromal cells, those of host origin were not killed by the treatment. The antiproliferative effect of anti-PSA antibody was microscopically characterized by cytoplasmic granulation and vacuolation, condensation of nuclear chromatin, disintegration of plasma membrane that finally leads to fragmentation of cells. Complement dependent lysis of the cells by the antibodies is not excluded, although antibodies themselves may be inactivating the bioactivity of PSA. Inactivation of complement in the anti-PSA serum by exposing the antiserum to 56°C for 30 minutes still left the serum to induce cytotoxic effect, albeit of a lower degree. Addition of exogenous complement augmented cytotoxicity of antibodies.

The anti-PSA antibodies appear to exercise a lethal cytotoxic action on both androgen-sensitive and androgen-insensitive prostate cancer cell lines. The mechanism by which this action is exercised require further study, be this complement mediated cytolysis or cytotoxicity exercised by another mode.
Cells exposed to antibodies for two hours or more do not grow on transfer to fresh medium. The potential use of anti-PSA antibodies for therapeutic purposes is indicated by the present study. However appropriate toxicological studies in suitable animal modal will be necessary. Figure 19 clearly demonstrated the presence of PSA in appreciable amounts in hyperplastic and malignant cases. A number of other human organs examined such as liver, lung, kidney, pancreas, testis, heart etc., did not stain for PSA. The antibody should therefore be acting specifically on the benign hypertrophic or hyperplastic and malignant prostates.

Besides passive use of antibodies, active immunization against PSA can be envisaged. In view of the immunological crossreactivity between the human PSA and the monkey PSA, these studies can be undertaken in monkeys. It has been possible to develop vaccines against "self" proteins such as human chorionic gonadotropin (hCG) (Talwar 1984; Talwar and Sad, 1990; Talwar et al. 1976,1990,1992,1992a & 1994; Srinivasan et al.1993) and LHRH (Shastri et al. 1981; Talwar et al. 1984). A similar strategy could be followed to make an anti-PSA vaccine.

The hypothalamic neurohormone, LHRH releases luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the adenohypophysis. Active immunization with LHRH demonstrated potential inhibitory effects on the pituitary - gonadal axis with resultant decrease in serum LH and FSH levels and marked functional and morphological changes of gonads and accessory sex organs in both sexes in a number of species including rats (Fraser et al. 1974 & 1982; Jayashankar et al. 1989; Giri et al. 1990 ; Rovan et al. 1992), rabbits (Arimura et al. 1973), ewes (Clarke et al. 1978), marmosets (Hodges and Hearn, 1977) and monkeys (Chappel et al. 1980; Giri
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et al. 1991). One of the basis of therapies for prostatic adenocarcinoma is cutting the source of androgens either by castration or by newer therapeutic agents. In a recent comparative study, employing anti-LHRH vaccine made by our institute, atrophic effect on prostate, a consequence of depletion of circulating testosterone, was found to be more pronounced than castration (Rovan et al. 1992). Keeping this in mind and having access to the bioeffective monoclonal antibody generated against LHRH (Talwar et al. 1985), we conducted experiments to evaluate the role of anti-LHRH antibody on the growth of prostatic cancer cells in vitro. The antibody was cytotoxic in a dose dependent manner to both androgen dependent and androgen independent cells. The cytotoxic effect was more pronounced in androgen-independent DU 145 cells. However, complete killing of cells could not be achieved at the dose tested. The anticellular effects of antibodies have confirmed that prostate cancer cells are directly sensitive to anti-LHRH antibodies. However, further studies are necessary to establish the role of this decapeptide on the growth and proliferation of malignant prostate cells. The combined use of anti-PSA and anti-LHRH therapies may improve the prevailing non-surgical therapeutic modalities for this major cancer of male reproductive tract.