Urol Res (1995) 23:119–125 © Springer-Verlag 1995

ORIGINAL PAPER

J.-E. Damber · A. Bergh · B. Assarsson · M. Gåfvels

Epidermal growth factor receptor content in rat prostatic adenocarcinoma: effects of endocrine treatment

Received: 15 June 1994 / Accepted: 24 January 1995

Abstract Epidermal growth factor receptor (EGF-R) was studied in Dunning prostatic cancer models using competitive binding assays and solution hybridization assay. EGF-R-binding capacity and mRNA were demonstrated in a hormone-sensitive R3327 prostatic tumor from both control and castrated animals while no such activity was found in the hormone-independent AT-1 tumors. Castration induced no quantitative changes in the EGF-R. Estrogen treatment induced a significant reduction of the binding capacity of EGF-R and its mRNA. It was concluded that EGF-R is present in the androgen-sensitive Dunning prostatic tumor models (R3327), but that the androgen-insensitive, undifferentiated AT-1 tumor lacks EGF-R expression. Endocrine treatment has no significant effect on the EGF-R in these tumor models.

Key words EGF-receptor · Dunning prostatic adenocarcinoma · Castration · Estrogen · Binding analysis · Hormone independency

J.-E. Damber (✉)
Department of Urology and Andrology, University of Umeå, S-901 85 Umeå, Sweden
Fax (+ 46) 90 125396

A. Bergh
Department of Pathology, University of Umeå, S-901 85 Umeå, Sweden

B. Assarsson · M. Gåfvels
Department of Physiology, University of Umeå, S-901 85 Umeå, Sweden

Prostate cancer growth is mostly androgen dependent, at least in the early stages, and this forms the basis for the endocrine treatment of this disease [15, 39]. However, sooner or later the tumor progresses to a more aggressive state and the growth becomes androgen-independent [32]. This transition is the major obstacle to a successful treatment outcome. The progression of prostate cancer into the hormone-independent state represents an intriguing multistep process of carcinogenesis and recent investigations have produced information which suggests that a variety of polypeptide growth factors may be involved in this process [5, 10, 36].

The epidermal growth factor receptor (EGF-R) is a membrane glycoprotein and binding of EGF or transforming growth factor alpha (TGF-α) to this receptor results in an intracellular response that can lead to mitosis by activation of the tyrosine kinase [7]. The amount of EGF-R appears to vary according to the cell type and stage of differentiation of many malignant tumors. For example, poorly differentiated bladder and breast tumors contain higher levels of EGF-R than other histological grades of these cancers [17, 31]. High-affinity EGF-Rs have been detected in normal rodent [35] and human [4] prostate tissue. In addition, EGF-R binding and mRNA levels have been found to be elevated in human prostate cancer compared with normal human prostate [4, 30]. However, it has also been shown that a depletion in the number of EGF-R-binding sites for prostatic cancer specimens correlates with the loss of differentiation of the tumor [26]. Thus, the relationship between the grade of differentiation of prostatic tumors and the expression of EGF-R appears to be somewhat different than with most other malignancies.

If the autocrine loop involving the EGF-R is important for prostatic cancer cell growth, an interrelationship between androgens and this loop appears to be logical. It has been shown that androgens upregulate

EGF-R expression in human prostatic cancer cell lines (LNCaP) in vitro [10, 34]. The growth of another cell line of prostate cancer such as DU-145 is stimulated by EGF [37] and this cell line, which is androgen-insensitive, expresses higher levels of EGF-R than the androgen-dependent LNCaP cell line [25]. In vivo, on the other hand, androgen ablation treatment appears to upregulate the expression of EGF-R, at least in the normal prostate [35].

In recent publications [19, 20], we have shown that estrogens probably promote direct inhibitory effects on the growth of the Dunning prostatic adenocarcinoma. The mechanism of action is not known, and the possible involvement of growth factors and their receptors ought to be examined. An interaction between estrogens and the regulation of EGF-R expression has been suggested in other systems [21].

The present investigation was performed in order to elucidate some aspects of EGF-R regulation in the Dunning prostatic tumor system. Using different techniques, the expression of EGF-R in prostatic tumor tissue was related to grade of differentiation, androgen ablation therapy and estrogen treatment.

Materials and methods

Animals and tissue

Dunning R3327 prostatic tumors originating from Dr. N. Altman (Organ System Program of the National Cancer Institute, Miami, Fla., USA) were transplanted to Copenhagen × Fisher F_1 male rats [20]. Transplantation was performed bilaterally into each flank, when the animals were 10–11 weeks old, under anesthesia induced by i.m. injections of diazepam (Stesolid, Dumex, Denmark), together with i.m. injections of fentanyl citrate with fluanisone (Hypnorm, AB Leo, Sweden). This tumor subline is well characterized and androgen-sensitive [16]. Dunning AT-1 is a fast-growing, anaplastic tumor with a tumor doubling time of 2.1 days. It is characterized as androgen-independent, i.e., it grows in a similar way in both intact and castrated animals [16]. Copenhagen × Fisher F_1 male rats transplanted with one Dunning AT-1 tumor each were kindly provided by Dr. P.-I. Kristenssen, Kabi Pharmacia, Lund, Sweden and originated initially from Dr. J.T. Isaacs (Baltimore, Md., USA). The rats were housed in the local animal house under controlled temperature (+25 °C) and humidity (40–60%) on a 14-h light, 10-h dark schedule. The animals had free access to water and pelleted food.

Treatment

The treatment period began when the tumors reached similar sizes (approximately 1.5 cm^3). The rats bearing the Dunning R3327 PAP tumors were randomized into three groups with five to seven rats in each group: intact animals, castrated animals and one group of castrated rats treated with 50 µg estradiol-17β s.c. daily, starting on the day of castration. The castrated animals were injected daily with sesame oil s.c. as vehicle. Six weeks after the randomization, the animals were killed and the tumors were excised and pieces from each tumor were frozen in liquid nitrogen. Similarly, tumors from six animals bearing the AT-1 prostatic tumor were used.

EGF-R-binding assay

Approximately 1 g of each tumor was homogenized with an Ultra-turrax (TP 18/10) in 6 ml ice-cold 0.25 M sucrose using four 10-s periods allowing a 30-s cooling interval between bursts. After subsequent centrifugation for 15 min at 1000 *g*, the supernatant was saved and the pellet was resuspended in sucrose, rehomogenized and centrifuged as above. The two supernatants were then combined and centrifuged for 30 min at 17 000 *g*. The new supernatant was made to final concentrations of 0.1 mol/l NaCl and 1 mmol/l $MgSO_4$ and centrifuged at 105 000 *g* for 1 h in a Beckman ultracentrifuge. The pellet was resuspended in 4 ml 0.05 mol/l TRIS-HCl buffer (pH 7.4), mechanically homogenized and again centrifuged at 105 000 *g* for 1 h. The new pellet was suspended in TRIS-HCl, 1 ml/g tissue weight, and frozen at −70 °C until binding analysis.

One hundred-microliter aliquots of the membrane preparation were incubated for 16 h at +4 °C with increasing concentrations of human epidermal growth factor (hEGF) (0–100 ng) and a constant amount of ^{125}I-hEGF (approx. 6000 cpm, specific activity > 900 Ci/mmol, Amersham) in a final volume of 200 µl. After the incubation, a method using hydroxyapatite as described by Benraad and Foekens [1] was used to separate free and receptor-bound ligand. EGF-R-binding capacity was calculated according to the method of Scatchard after correction for the nonspecific binding. In order to test the specificity of the assay, increasing amounts of different growth factors were added to the incubation, and the effect on the EGF-binding curve was tested.

Synthesis of EGF-R probe and mRNA standard

The RNA probe used for solution hybridization and RNase protection assay was synthesized as described by Ekberg et al. [11]. The probe, a gift from Dr. J. Schlessinger (Israel), was a single-stranded antisense RNA probe synthesized by in vitro transcription of a 768-bp human EGF-R receptor cDNA (nct 2318-3085), according to the sequence published by Ullrich et al. [40]. Labeling of the RNA strand was performed in vitro by incorporation of ^{35}S-CTP (Amersham) in a reaction catalyzed by T7 polymerase (Promega) [29]. DNA template was removed by DNase I treatment (Boehringer, FRG), and the RNA probe was then purified with phenol/chloroform extraction and ethanol precipitation.

A nonradioactive sense RNA standard was synthesized in the same way as the antisense RNA probe, using a construct where the 768-bp fragment was inserted in the opposite direction in the plasmid. The amount of sense RNA was determined by spectrophotometry.

Preparation of total RNA and total nucleic acid

Total RNA from human placenta, Dunning tumors and rat liver was prepared as described by Chirgwin et al. [3]. Tissue was homogenized with a Polytron (3 × 10 s at medium setting) in a SET buffer containing 1% sodium dodecyl sulfate (SDS), 10 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM TRIS-HCl (pH 7.5). Total nucleic acid (TNA) was then prepared by digestion of the homogenized tissue with proteinase K and subsequent extraction with phenol/chloroform [8]. The nucleic acid was then precipitated with ethanol and dissolved in SET buffer.The amount of DNA was determined by fluorometry measurements.

Solution hybridization assay

To quantify EGF receptor mRNA, the ^{35}S-labeled human antisense RNA probe was hybridized to TNA samples overnight at 70 °C. The

solution was then treated with RNase A1 (Sigma) and T1 (Boehringer), and precipitated with 6 mol/l trichloroacetic acid according to Ekberg et al. [11]. Precipitates were collected on Whatman GF/C filters (Clifton, N.J., USA), which were then washed with 4% trichloroacetic acid in 5 mmol/l $Na_4P_2O_7$. The hybrid was solubilized in 1 ml Soluene 350 (Packard) and counted in a scintillation counter. To quantify the concentration of EGF receptor mRNA in the samples, the antisense probe was in a parallel set of samples hybridized to known concentrations of sense RNA strands (see above). The abundance of EGF receptor mRNA in the unknown samples was expressed as fmol message/mg DNA.

RNase protection assay

RNase protection assay was performed as described by Sambrook et al. [33] by hybridizing 100000–750000 cpm ^{35}S-labeled antisense RNA probe overnight to increasing amounts of unlabeled sense RNA (0–15 fmol) or increasing amounts of total RNA (0–50 µg), prepared from human placenta and Dunning tumors. Hybridization were performed for 17–19 h at 70 °C in 40 mM PIPES (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl, and 25% formamide. Digestion was performed for 60 min at 37 °C, and protected material was purified by phenol/chloroform extraction and ethanol precipitation. The protected material was then dissolved and fractionated by electrophoresis on a 7% polyacrylamide/6 M urea mini-gel (Bio-Rad) with a parallel RNA size standard (BRL RNA ladder, Gaithersburg, Md., USA) according to Sambrook et al. [33]. RNase-protected material was displayed by autoradiography on Kodak XAR-5 film and molecular weights determined according to the migration distance of the ethidium bromide stained RNA standard.

Statistics

Values are expressed as mean ± SEM. Comparisons between groups were performed using the Mann-Whitney U-test. A *P*-value less than or equal to 0.05 was considered statistically significant.

Results

Using competitive binding assays, specific high affinity binding sites for EGF were demonstrated in the Dunning R3327 prostatic tumors. A typical binding curve and a Scatchard analysis are shown in Fig. 1. The binding was specific for EGF and TGF-α since other growth factors could not displace radioactive EGF ligand from the receptor (Fig. 2). No specific EGF-R-binding activity could be demonstrated in the AT-1 tumors. The EGF-R-binding capacities for the different treatment groups of R3327 prostatic tumors are shown in Fig. 3. As seen, castration induced no changes in the binding capacity, while estrogen treatment reduced the number of binding sites significantly.

In order to characterize the solution hybridization assay, control experiments were performed by incubating known amounts of in vitro synthesized sense RNA with a constant amount of ^{35}S-labeled antisense RNA. Figure 4 shows a dose-radioactivity relationship between amount of sense RNA added and the radioactivity protected from RNase treatment. Linearity of the assay is apparent in the 0- to 5-fmol range. On

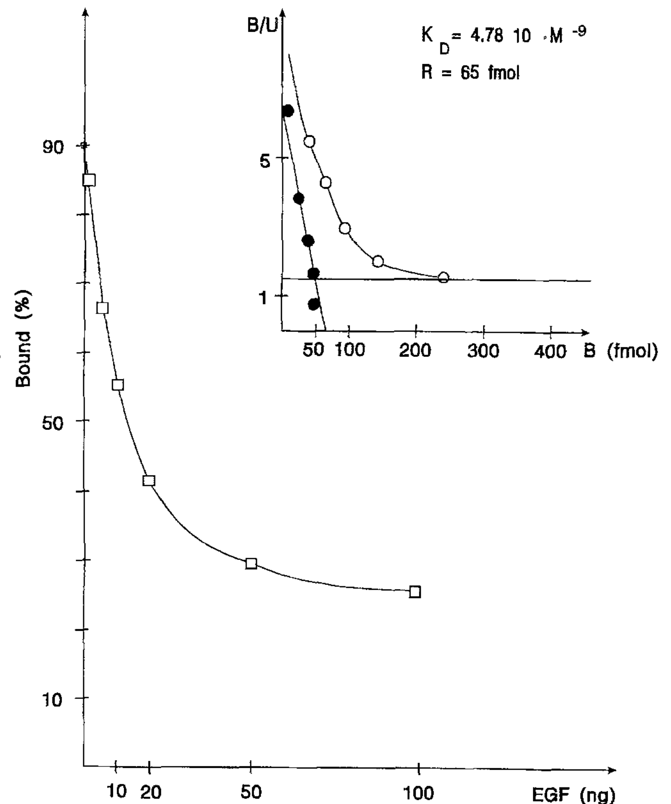

Fig. 1 Binding curve and Scatchard plot of specific hEGF binding to Dunning R3327 prostatic adenocarcinoma

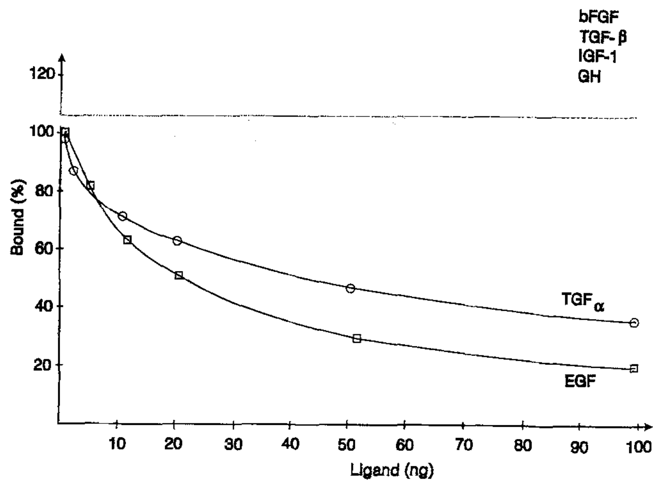

Fig. 2 Specificity of ^{125}I-hEGF binding to Dunning R3327 prostatic adenocarcinoma. Bound defined as 100% when no competitor was added. □ unlabeled hEGF, ○ unlabeled TGF-α. *Shaded area* represents bFGF, TGF-α, IGF-1 and GH

denaturating polyacrylamide gels, RNase-protected material was shown to be less than 0.8 kb in size, with the most marked band slightly below 0.8 kb and corresponding to the actual full length of the labeled antisense RNA. A dose-radioactivity relationship was also demonstrated on the autoradiograms (Fig. 4, inset lanes 1–3). The probe not undergoing RNase treatment

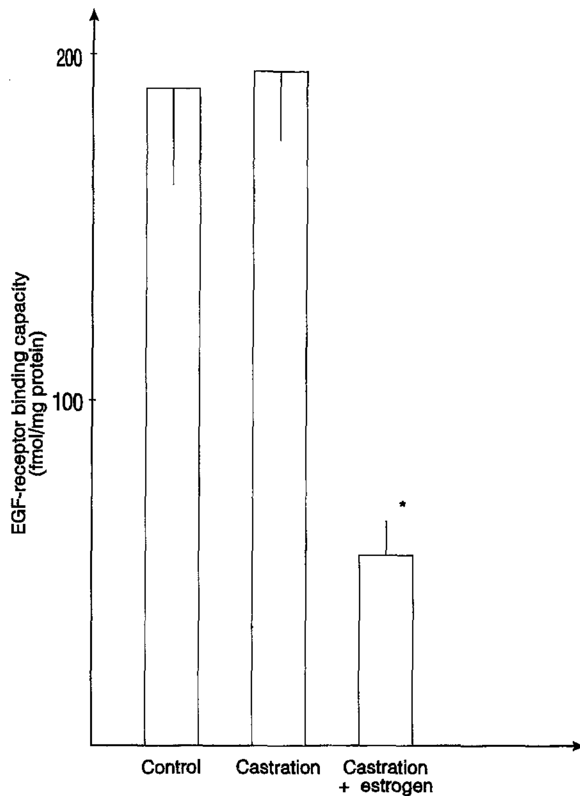

Fig. 3 EGF-R-binding capacities of Dunning R3327 prostatic adenocarcinoma after different treatments. Estrogen treatment induced a significant reduction of the EGF-R-binding capacity. *Each bar* represents the mean (± SEM) of seven to nine tumors. * Significantly reduced compared with control ($P < 0.0001$) and castration ($P < 0.002$)

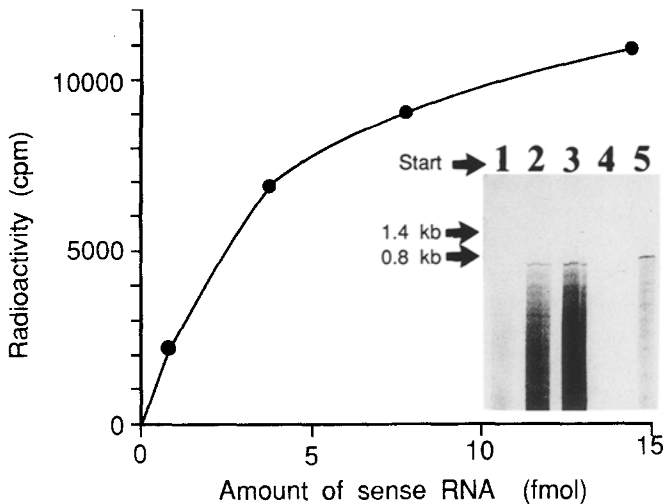

Fig. 4 Increasing amounts of in vitro synthesized sense RNA standard were incubated with a constant amount of ^{35}S-labeled human EGF-R antisense probe (20000 cpm) in the solution hybridization assay and the radioactivity of RNase-protected material was measured by scintillation counting. RNase-protected material was displayed on autoradiograms (*inset*) by hybridizing increasing amounts of sense RNA with 750000 cpm ^{35}S-labeled EGF-receptor antisense probe, in an RNase protection assay. *Lanes 1–3* show increasing amounts of sense RNA (0.8, 3.8 and 7.8 fmol, respectively), *lane 4* shows lack of signal for RNase-treated probe only, while *lane 5* shows a non-RNase-treated probe

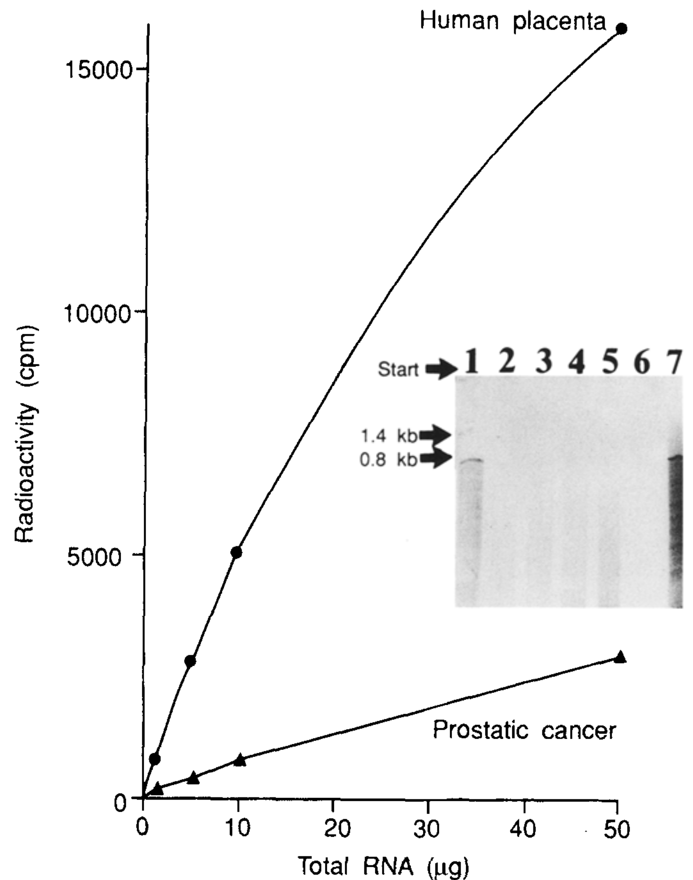

Fig. 5 Increasing amounts of total RNA from human term placenta and Dunning tumor from intact non treated rats were hybridized with 20000 cpm of ^{35}S-labeled human EGF-R antisense probe in the solution hybridization assay. RNase-protected material was also demonstrated by autoradiography (*inset*), after hybridizing increasing amounts of total RNA from Dunning tumors (lanes 2 and 3 containing 25 and 50 μg total RNA) and human placenta (lanes 4 and 5, with 25 and 50 μg, respectively) with 750000 cpm ^{35}S-labeled human EGF-R probe in the RNase protection assay. *Lane 1* shows protected material from 0.8 fmol sense RNA incubated with the RNA probe, *lane 6* shows RNase-treated probe and *lane 7* shows a non-RNase-treated probe

(Fig. 4, lane 5) appears slightly bigger on the gel due to the conservation of bases within the polylinker.

The ability of the solution hybridization assay to determine differences in amounts of EGF receptor mRNA in biological samples was determined by incubation of increasing amounts of total RNA from human placenta and Dunning tumors with the same amount of labeled anti-sense RNA. A clear dose-radioactivity relationship could be demonstrated for different amounts of total RNA added (Fig. 5). RNase-protected material from hybridizations containing total RNA extracted from human placenta and Dunning tumors respectively (Fig. 5, inset lanes 2–5) could be demonstrated on autoradiograms of polyacrylamide/urea gels. The actual difference in total RNA input

Table 1 EGF-R mRNA in different tissues of Copenhagen-Fischer rats bearing the Dunning prostatic adenocarcinoma (R3327 or AT-1) using solution hybridization assay. Human placenta was used as a positive control. Values are given as mean ± SEM of six to seven observations (ND, not detectable)

Tissue	EGF-R mRNA (fmol/mg DNA)
Human placenta	69.1
Rat liver	55.7
Dunning, R3327, control	11.9 ± 2.3
Dunning, R3327, castrated	11.1 ± 1.3
Dunning, R3327, castrated, estrogen	7.9 ± 1.0*
Dunning, AT-1, control	ND

* $P < 0.05$ compared with control and castrated Dunning R3327 prostatic tumors.

was not clear on the autoradiograms, probably due to the poor sensitivity of that method.

The amount of EGF-R mRNA in the Dunning prostatic adenocarcinoma (R3327 or AT-1) was determined by solution hybridization assay (Table 1). The Dunning tumors of the rats treated with castration + estrogen showed significantly lower levels than control ($P \leq 0.05$), and castrated only rats ($P \leq 0.05$). The control rats bearing Dunning AT-1 contained no detectable EGF-R mRNA.

Discussion

The results of the present study suggest that the hormone-dependent Dunning R3327 prostatic tumors contain receptor proteins with affinity for EGF and TGF-α. This is in line with the demonstration of EGF-R binding in human prostate cancer [4, 30] and in different human prostatic cancer cell lines in vitro [10, 37]. The binding characteristics corresponded to similar high-affinity binding sites to those demonstrated in the human prostate [12, 26]. The number of binding sites was reported to be higher in the Dunning tumor than in the rat ventral prostate [35]. In the human and mouse prostate [22, 23, 26], the occurrence of two classes of EGF-binding sites has been suggested. However, the interpretation of more than one binding site may be related to difficulties in analyzing binding data when employing Scatchard analysis as discussed by Habib and Chisholm [14] and, furthermore, the occurrence of two classes of EGF-binding site is not a uniform finding [24, 26].

There was no effect of castration whatsoever on the EGF-R-binding capacity or mRNA level in the Dunning R3327 prostatic tumor. The relationship between the androgenic signal and the regulation of the EGF-R is therefore obscure in this tumor model. These findings are also contradictory to the clear-cut upregulation of EGF-R in the rodent prostate [35], and it has been suggested [38] that androgen deprivation might promote EGF-R induction and thereby prevent complete prostatic regression after castration. It has also been reported that EGF-R of the prostatic cell line LNCaP cells is upregulated by androgen [34]. Furthermore, an inverse relationship between the concentration of EGF-R and androgen receptors has been shown for human carcinomatous prostate [4]. Whether these differences when compared with the present findings in the Dunning tumor are related to species, normal tissue versus cancer tissue, or a preponderance of different cell types is at present unknown.

We found no indications for the presence of EGF-Rs in the undifferentiated, anaplastic Dunning AT-1 prostatic tumor. This finding is in line with other studies of prostate cancer demonstrating that neoplastic tissue has a reduced EGF-R-binding capacity compared with benign tissue [13, 26, 28]. On the other hand, Eaton et al. [9] detected significantly higher concentrations of EGF-R in carcinoma relative to benign prostatic hyperplasia in a transurethral prostatectomy specimen, and the expression of EGF-R mRNA has been demonstrated in the androgen-independent PC3 human prostatic cancer cell line [6]. The number of EGF-binding sites for androgen-insensitive cells was ten fold greater than that expressed in an androgen-responsive cell line [25]. Thus, the progression of prostatic cancers to an androgen-independent state may be due to a loss of androgen regulation of growth factors and their receptors, but at least in the Dunning tumor system this process appears not to involve the EGF-R loop. The possibility that other growth factor systems are involved in the progression to androgen independence remains an intriguing area of research [27, 41].

There are several lines of evidence that estrogens may promote direct effects on prostatic cancer growth [18]. The possible involvement of growth factors and their receptors in promoting these effects of estrogen cannot be ruled out. In the present study we found a downregulation of EGF-R-binding capacity and mRNA expression after treatment of the rats bearing the Dunning R3327 prostatic tumor with estrogens. The reduced EGF-R-binding capacity/mg protein can thus be explained both by the reduction of the epithelial cell number known to take place after estrogen treatment [19], and a decreased EGF-R expression per cell.

To summarize, the present study demonstrates high-affinity binding sites for epidermal growth factor in the Dunning R3327 prostatic adenocarcinoma. Furthermore, the EGF-R content appears not to be influenced by androgens since no effect was observed after castration. In the Dunning prostatic tumor system a downregulation of EGF-R takes place in the androgen-insensitive, dedifferentiated AT-1 tumor. The importance of this finding for the understanding of tumor progression into androgen independency is at present unknown.

Acknowledgements The present study was supported by grants from the Swedish Cancer Society (project no. 1760), Maud and Birger Gustafssons Foundation, the Sahlbergs Foundation, the Medical Faculty of Umeå, the Lions Research Foundation, Umeå, and the Swedish Medical Research Council (project no. 5653). Mrs. Christina Sandström, Mrs. Gunvor Hellström and Mrs. Birgitta Ekblom are thanked for their skillful technical assistance.

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