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ANDROGEN-INDEPENDENT BASAL CELL RE-EPITHELIALIZATION, c-erbB-2 mRNA EXPRESSION AND ANDROGEN-DEPENDENT EGFr mRNA EXPRESSION IN BENIGN PROSTATIC HYPERPLASIA EXPLANT CULTURES TREATED WITH FINASTERIDE

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We have analyzed the effects of the 5α-reductase inhibitor, finasteride (MK906), on the mRNA expression of the epidermal growth factor receptor and c-*erb*B-2 genes, in benign prostatic hyperplasia explant cultures treated with testosterone and with testosterone plus finasteride. A decrease of the epithelial cell content and an androgen-independent basal cell re-epithelialization was observed during the first 10 days of culture, suggesting a role of basal cells as stem cells involved in androgen-independent epithelial regeneration. Using a semi-quantitative reverse transcription polymerase chain reaction technique, we observed a significant decrease in expression of the epidermal growth factor receptor in the cultures treated with finasteride whereas no effect of finasteride on c-*erb*B-2 transcription was detected, although the expression of both genes was increased by dihydrotestosterone. *Int. J. Cancer* 76:519–522, 1998.

Benign prostatic hyperplasia (BPH) is one of the most common pathologies in aging men in the Western world, affecting 80% of men over 70 years old (Bostwick *et al.,* 1992). It is well established that prostate growth is dependent on androgens, since androgen withdrawal markedly reduces prostatic size (Bosch *et al.,* 1989), and men with 5α-reductase deficiency have a poorly developed prostate (Imperato-McGinley *et al.,* 1974). Thus, to treat patients with BPH, inhibition has been attempted of the intracellular enzyme 5α-reductase type 2, which metabolizes testosterone (T) to dihydrotestosterone (DHT), the androgen considered to be mainly responsible for prostatic growth. Among the 5α-reductase inhibitors, finasteride (MK 906) has been demonstrated to be an effective inhibitor of 5α-reductase type 2 activity (Rittmaster *et al.,* 1989; Spera and Lubrano, 1996; Stoner, 1996), and its effect has been assessed in men with BPH (McConnell *et al.,* 1992).

It is well known that proto-oncogenes can be activated by different processes, including androgen stimulation; once activated, they trigger uncontrolled cellular growth and transformation, as has been established in several human tumors (De Vita *et al.,* 1990). However, when compared with other processes, the involvement of activated cellular genes in the development of BPH remains poorly understood. Overexpression of EGFr and c-*erb*B-2 have been assessed in BPH as well as in prostate cancer and cell lines (Mellon *et al.,* 1992; Ching *et al.,* 1993; Lubrano *et al.,* 1993; Myers *et al.,* 1993; Harper *et al.,* 1995; Peehl, 1996), and overexpression of c-*erb*B-2 correlated with distant metastasis and poor prognosis in prostate carcinoma patients (Fox *et al.,* 1994).

In view of the important role of EGFr and c-*erb*B-2 in prostate growth and in neoplastic transformation, and of the fact that it is not clear whether or not finasteride can decrease the mRNA levels of EGFr and c-*erb*B-2 in BPH, we performed this study using a three-dimensional organ culture model and semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

MATERIAL AND METHODS

Specimens

Eleven BPH samples were collected at the time of surgery. The protocol had previously received ethical approval, and informed consent was obtained from all patients. Finasteride (MK 906) was generously supplied by Merck, Sharp and Dohme (Rahway, NJ).

Explant cultures

Explant cultures were performed as described previously (Geller *et al.,* 1993) with some modifications. BPH samples were placed on ice in RPMI-1640 supplemented with 7.5% $NaHCO_3$, L-glutamine (200 mM) and 5% charcoal-treated (steroid-free) FCS (FCS-SF) (GIBCO-BRL, Gaithersburg, MD) and minced with a scalpel into 2 × 2 mm cubes. Explants were next placed in culture dishes with an adequate volume of RPMI-1640 supplemented with 5% FCS-SF and 10 nM testosterone (T) and incubated for 3 days at 37°C in 95% O_2, 5% CO_2. From day 4, explant cultures were transferred into each of the following media: 1) medium C: basal RPMI with 5% FCS-SF (control group); 2) medium T: basal RPMI with 5% FCS-SF and T (10 nM); and 3) medium FT: basal RPMI with 5% FCS-SF and finasteride (F) (10 mM) plus T (10 nM). Cultures were maintained under the above conditions for an additional period of 15 days. Media were replaced every other day, and explant samples for RNA extraction and immunohistochemistry were obtained on days 3, 10 and 18 of culture.

Testosterone and DHT hormonal levels in culture media

Testosterone and DHT levels were measured in the culture media of all experimental culture conditions on days 3, 10 and 18 by a radioimmunoassay with the testosterone [^{125}I]-coated tube radioimmunoassay kit (Spectria, Orion Diagnostica, Espoo, Finland) and DSL-9600 ACTIVE dihydrotestosterone [^{125}I]-coated tube radioimmunoassay kit (Diagnostic Systems, Webster, TX), using suitable standards and controls. DHT determination required a prior extraction procedure with n-hexane:ethanol (98:2). Ninety percent of the DHT was recovered from the media with this procedure. The interassay variation of the DHT determination was 7%.

Immunohistochemical staining

Formalin-fixed paraffin-embedded 5 μm sections were stained with haematoxylin-eosin. Additional sections were used for immunohistochemical staining of epithelial basal cell high m.w. keratin

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TABLE I – SPECIFIC PRIMERS FOR THE SEMI-QUANTITATIVE RT-PCR[1]

EGFr^{f}	5′-ACA GCC ATG CCC GCA TTA GCT CTA A-3′
EGFr^{r}	5′-GGA ATG CAA CTT CCC AAA ATG TGC C-3′
c-*erb*B-2^{f}	5′-CCT CTG ACG TCC ATC ATC TC-3′
c-*erb*B-2^{r}	5′-CGG ATC TTC TGC TGC CGT CG-3′
gapdh^{f}	5′-TGG TAT CGT GGA AGG ACT CAT GAC-3′
gapdh^{r}	5′-ATG CCA GTG AGC TTC CCG TTC AGC-3′

[1]Superscript f, forward primer; superscript r, reverse primer.

903, using the purified mouse monoclonal IgG_1 antibody (MAb) 34βE12, specific for basal cells, (Biogenex, San Ramon, CA) as well as streptavidin-biotin complex (LSAB Kit, Dako, Copenhagen, Denmark) and diaminobenzidine (DAB; Sigma, St Louis, MO) as follows: slides were pre-digested in pronase (Sigma) and 0.5 mg/ml phosphate buffered saline (Life Technologies, Gaithersburg, MD) for 10 min. Primary incubation with the 34βE12 MAb was performed at 1:20 dilution overnight at room temperature. Secondary incubation with a biotinylated horse antibody anti-mouse antibody at 1:500 dilution (Vector, Burlingame, CA) was carried out for 30 min at room temperature. Streptavidin-biotin amplification was carried out for 30 min. The slides were developed with DAB for 10 min as a chromogen and counterstained.

RNA extraction and semi-quantitative RT-PCR

The RNA extraction and the reverse transcription were performed as described by Chomczynski and Sacchi (1987) and Sambrook *et al.* (1989). All RNA samples were treated with DNAase I (Boehringer Mannheim, Germany) at 37°C for 1 hr, and reverse transcribed at 37°C for another 60 min with MMuLV-reverse transcriptase (Boehringer Mannheim) to obtain cDNAs. The following semi-quantitative RT-PCR procedure was performed as previously described (Frye *et al.,* 1989), using specific primers for EGFr (amplified fragment of 109 bp) (Lönn *et al.,* 1993), c-*erb*B-2 (98 bp) (Yamamoto *et al.,* 1986) and glyceraldehyde-3-phosphate-dehydrogenase as control (gapdh) (190 bp) (Nobori *et al.,* 1994) (see Table I). A double and simultaneous PCR amplification of c-*erb*B-2 or EGFr and gapdh cDNAs was performed respectively for each sample. RT-PCR assays were performed with 0.4 mM of gapdh primers and 1 mM of EGFr or c-*erb*B-2 primers. PCRs were carried out as follows: 94°C × 10 min, and 94°C × 1 min, 55°C × 1 min, 72°C × 2 min for 30 cycles. The amplification reaction was completed with a final extension run at 72°C × 7 min. All reactions were stopped at the midpoint of the exponential phase of the amplifications (data not shown). All samples were amplified at the same time and with the same stock solutions of Mg^{2+}, Taq polymerase (Boehringer Mannheim) and primers, and using 100 ng of cDNAs as templates, 1.5 mM magnesium and 5 units of Taq polymerase. The reaction mixture was electrophoresed in ethidium bromide-stained 3% agarose gel and the integrated optical density (IOD) of the resolved bands quantified using a Cue-2 Image Analyzer (Olympus, Tokyo, Japan). The reported relative contents of c-*erb*B-2 and EGFr cDNAs are obtained by expressing their values on the basis of gapdh cDNA content for each sample and normalized to their respective controls at 10 and 18 days of each treatment group. Statistical analysis was performed using the mean ± SEM of the results.

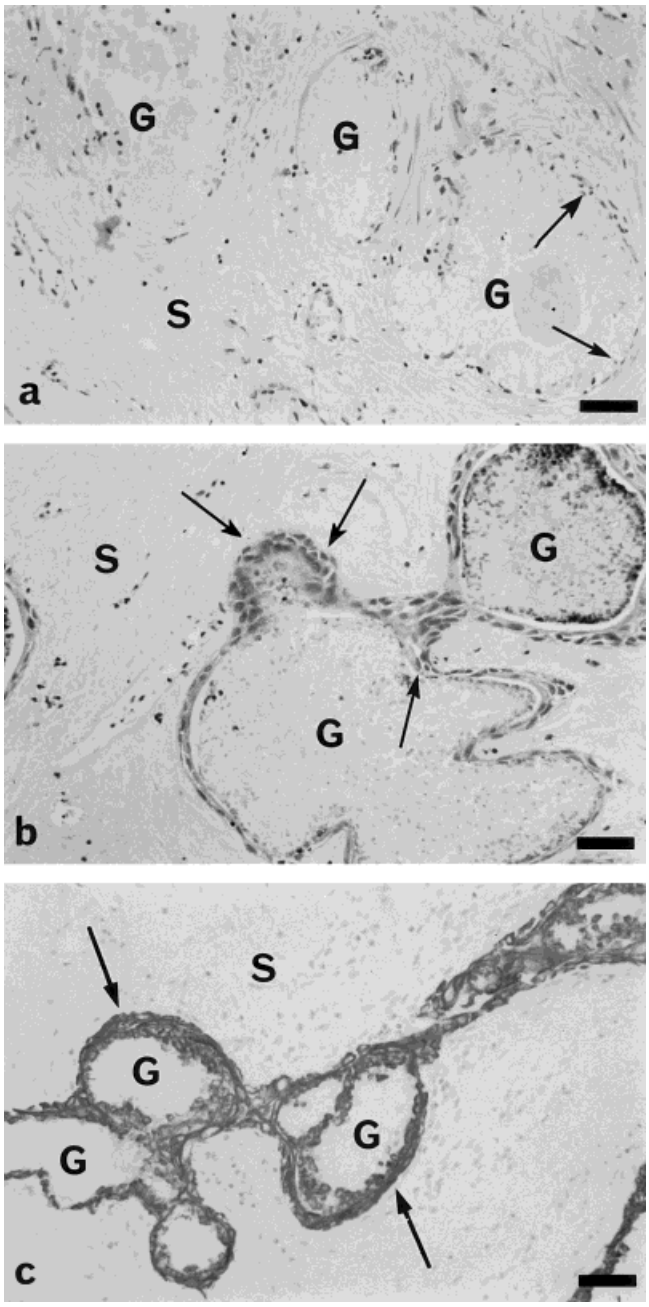

FIGURE 1 – Re-epithelialization in explant cultures. *(a)* Hematoxylin/eosin staining of an explant at day 3. Only epithelial basal cells are present in the glands (arrows). *(b)* Hematoxylin/eosin staining of an explant at day 10 of culture with cell re-epithelialization (arrows). *(c)* Basal cell keratin 903 immunohistochemical staining of an explant at day 10 of culture. Re-epithelialized cells are stained in brown (arrows). Glandular (G) and stromal (S) components are indicated. Scale bars = 40 μm.

RESULTS

Testosterone and DHT hormonal levels in the culture media

We assessed the levels of testosterone and DHT in the media of each cultured explant at the time of RNA extraction. No detectable levels of DHT were found in the media of the control groups (in which no testosterone was added) or in the media of the finasteride plus testosterone-treated explants, in which DHT synthesis is inhibited. DHT concentration in the testosterone-treated groups was 135 ± 18 pmol/L at day 10 and 140 ± 20 pmol/L at day 18. No testosterone was detected in the culture media of the testosterone-treated explants, in which DHT synthesis is not inhibited.

Epithelial cell content in BPH explant cultures

To assess the content and integrity of epithelial cells in our cultures, tissue samples were collected on days 3, 10 and 18 of culture, fixed, paraffin-embedded and hematoxylin/eosin-stained as described above. Densitometric analysis of those samples showed that epithelial cell content was very low on day 3 in all culture

TABLE II – MEAN ± SEM OF THE EGFr/gapdh AND c-*erb*B-2/gapdh NORMALIZED RATIOS[1]

	10 days			18 days		
	C	T	FT	C	T	FT
EGFr	1.0 ± 0.15	0.9 ± 0.15	0.9 ± 0.15	1.0 ± 0.15	1.6 ± 0.15[2]	0.4 ± 0.15[2,3]
c-*erb*B-2	1.0 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	2.5 ± 0.2[2]	2.7 ± 0.2[2]

[1]C, control group; T, testosterone group; FT, finasteride plus testosterone group.–[2]Statistical significance compared with day 10.–[3]Statistical significance compared with testosterone group.

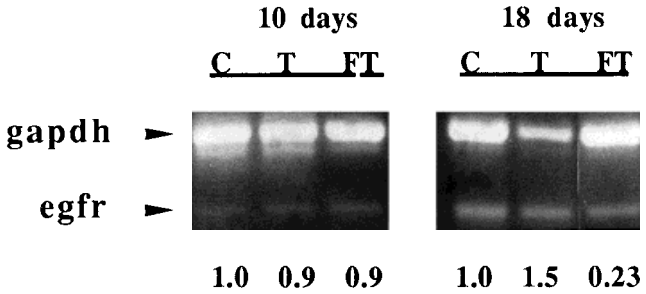

FIGURE 2 – Semi-quantitative RT-PCR of EGFr/gapdh. Amplified bands of control (C) and testosterone (T)- and finasteride plus testosterone (FT)-treated groups on days 10 and 18 of culture of one representative explant culture. The EGFr/gapdh IOD normalized ratios are listed below each lane, taking the ratio of control group as one arbitrary unit.

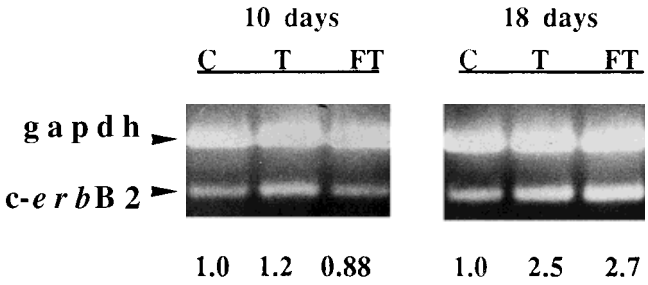

FIGURE 3 – c-*erb*B-2/gapdh semi-quantitative RT-PCR. Amplified bands of control (C) and testosterone (T)- and finasteride plus testosterone (FT)-treated groups on days 10 and 18 of culture of one representative explant culture. c-*erb*B-2/gapdh IOD normalized ratios are listed below each lane, taking the ratio of control group as one arbitrary unit.

conditions and only basal cells could be seen in the glands of the explants (Fig. 1*a*). When cultures of days 3 and 10 were compared with day 18, we detected an increase in the basal epithelial cell content and a progressive re-epithelialization process (Fig. 1b). Indeed, re-epithelialization was progressive from day 3 to day 10 of culture, with no differences observed among the various treatments. This androgen-independent basal cell re-epithelialization was confirmed in all culture groups by immunohistochemistry of basal epithelial cell high m.w. keratin 903 (Fig. 1c). Densitometric analysis showed no differences in the basal epithelial cell content among treatment groups between days 10 and 18 of culture.

Epidermal growth factor receptor mRNA expression in BPH explant cultures

The mean ± SEM EGFr/gapdh IOD ratio at day 3 was 0.37 ± 0.2 and under-expressed when compared with fresh tissue (9.0 ± 2.0; not shown; Table II). A representative EGFr/gapdh RT-PCR assay at days 10 and 18 of culture is presented in Figure 2. The IOD ratios were normalized considering the ratio of control group as one arbitrary unit. The mean ± SEM of the normalized ratios of each treated group is shown in Table II. No differences in EGFr expression were found among groups at day 10, but at day 18 a significant under-expression was detected in the finasteride-treated groups, whereas an increased expression was found in the testosterone groups, compared with controls.

C-erbB-2 mRNA expression in BPH explant cultures

The mean ± SEM IOD ratios of c-*erb*B-2/gapdh obtained on day 3 was 0.35 ± 0.2. No statistical differences were found when compared with fresh samples 0.43 ± 0.3 (not shown). Expression levels of c-*erb*B-2 were measured on days 10 and 18 of culture and the ratios normalized. A representative c-*erb*B-2/gapdh assay of one explant is shown in Figure 3. No significance differences were found between groups treated with finasteride and groups treated with testosterone, at either day 10 or 18 of culture. Nevertheless, increased levels of c-*erb*B-2 were found when comparing day 18 with day 10 for both groups of treatment. Mean ± SEM values are summarized in Table II.

DISCUSSION

We have observed a marked decrease of epithelial cell numbers of explanted prostate tissue during the first 3 days of culture. This loss appears to be specific for epithelial cells; basal cells appear to remain intact throughout the culture period. Since 10 nM testosterone was applied to explants throughout this period, the loss of epithelial cells cannot be considered as being a consequence of androgen deprivation but rather due to an adaptation period. After the 3rd day of culture and during the following 7 days, epithelial cell content increased progressively in an androgen-independent manner. This re-epithelialization process has also been reported by others who used prostate epithelial explant cultures as an *in vitro* study model (Nevalainen *et al.,* 1993). The cells of the newly formed epithelium were positive for basal cell keratin 903, suggesting the possibility that basal cells can act as stem cells. Further studies are necessary to investigate this possibility, as suggested by other authors (Bonkhoff and Remberger, 1996).

Since EGFr and c-*erb*B-2 genes are physiologically expressed in epithelial cells of the prostate (Frydenberg *et al.*, 1991; Kuhn *et al.,* 1993; Montone and Tomaszewski, 1993; Sadasivan *et al.,* 1993), we assumed that the remaining basal cells are the source of EGFr and c-*erb*B-2 in our explants, although further studies must be conducted to confirm this point.

Although it must be take in consideration that mRNA levels may not reflect what it is happening at the protein level, our results suggest that EGFr expression is regulated in an androgen-dependent manner in BPH. Our results are in agreement with others showing increased EGFr expression in tumor cell lines treated with testosterone or DHT (Liu *et al.,* 1993; Sehgal *et al.,* 1994; Brass *et al.,* 1995), although EGFr expression may be relatively androgen independent in BPH (Sherwood and Lee, 1995) as it is in the androgen-independent tumor cell lines PC3 and DU145. Thus, it is possible that other androgen-independent regulatory pathways may regulate expression of EGFr. This question awaits further investigation.

On the other hand, c-*erb*B-2 expression increased in the testosterone-treated explants, suggesting a stimulatory effect of DHT. However, no effects of finasteride were found when comparing expression levels of the testosterone-treated groups with the finasteride plus testosterone groups, in which c-*erb*B-2 levels were

also found to be increased. This suggests that testosterone could also have a stimulatory effect on the transcription of c-*erb*B-2, although its overexpression could also be explained by the existence of other non-androgen-regulatory pathways. The possible role of such androgen-independent pathways in the regulation of EGFr and c-erbB-2 expression as well as the role of the basal cells in BPH needs further investigations.

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