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Acute Inflammation Promotes Early Cellular Stimulation of the Epithelial and Stromal Compartments of the Rat Prostate

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BACKGROUND. It has been proposed that prostatic inflammation plays a pivotal role in the pathophysiology of benign hyperplasia and prostate cancer. However, little information is available about the prostatic reaction to bacterial compounds in vivo. Our aim was therefore to evaluate the early effects of bacterial infection on rat ventral prostate compartments.
METHODS. Using a rat model of acute bacterial prostatitis by *Escherichia coli*, we analyzed the histological and ultrastructural changes in the prostate at 24, 48, and 72 hr postinfection. Prostatic tissues were immunostained for prostatic binding protein (PBP), ACTA2, ErbB1, and ErbB2 receptors, TUNEL, and markers of cell proliferation. Dot and Western blots for PBP, ACTA2, ErbB1, ErbB2, and TGFβ1 were also performed.
RESULTS. The prostatic epithelium became hypertrophied, with increases in PBP and ErbB1 expression at 24 hr postinfection. Moreover, inflammation induced the expression of ErbB2, a receptor strongly involved in carcinogenesis. These alterations were more pronounced at 48 hr, but the epithelium also showed apoptosis and finally atrophy at 72 hr postinfection, with a decrease in PBP and ErbB receptors. Interestingly, the epithelial cells exhibited a high level of proliferation in response to the bacteria. The stromal reaction to acute inflammation was initially characterized by smooth muscle hypertrophy. Afterwards, muscle cells acquired a secretory phenotype, with a reduction in ACTA2 at 72 hr postinfection.
CONCLUSIONS. Prostatic inflammation, even at the early stages, promotes atrophic and proliferative changes, and the upregulation of ErbB receptors together with dedifferentiation of smooth muscle cells. These data suggest that repetitive reinfections could lead to uncontrolled growth in the prostate gland. *Prostate 70: 1153–1165, 2010.* © 2010 Wiley-Liss, Inc.

KEY WORDS: **prostate inflammation; bacterial infection; cell stimulation; proliferation; smooth muscle cells**

INTRODUCTION

The prostate gland is the most disease-prone organ in men. Illnesses affecting the human prostate such as prostatitis, benign hyperplasia, and prostate cancer are major causes of morbidity and death in developed countries. However, the etiology and pathophysiology of these diseases remain poorly understood. In recent years, many studies have led to hypothesize a strong association among the three conditions.

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Moreover, it has been proposed that prostatic inflammation plays a pivotal role in the pathophysiology of benign prostatic hyperplasia [1] as well as in the development of prostate cancer [2].

Escherichia coli is a pathogen frequently associated with both acute and chronic bacterial prostatitis in humans [3,4]. It has been speculated that chronic low-grade colonization with *E. coli* can cause prostate pathology via the release of endotoxin over a long period of time, which, in concert with dihydrotestosterone, might lead to hyperplasia and cancer of the prostate [5]. There has also been a renewed interest in the pathogenesis, diagnosis, and treatment of the prostatitis syndromes since the latter half of the 1990s (6). With new basic research activity being instigated, including animal models [7] and immunological analysis [8,9]. However, these investigations have been mainly focused on the nature and extent of inflammatory cell infiltrates, thus omitting the role played by both resident stromal and epithelial cells in the prostatic reaction to infection. In this sense, the study of the interactions between pathogens and resident cells and their early consequences on prostatic tissues deserves priority treatment, since it is well established that this initial host response strongly dictates the development of chronic pathologies in other organs [10,11].

Among the multiple parameters involved in tissue-specific proliferation/differentiation homeostasis, the epidermal growth factor (EGF) family comprises several mediators that act in an autocrine and paracrine manner on their corresponding specific cell membrane receptors (EGFR or ErbB) and mounts an effective reparative response to any attack on the biophysical integrity of the epithelium [12]. In addition, ErbB can be activated by transactivation from a variety of G-protein-coupled receptors, integrins, and cytokine receptors [13,14]. In this way, ErbB family acts as the major transducer of disparate cell functions, including changes in the proliferation rate and cellular shape, and the regulation of proinflammatory activation. Typically, the ErbB family of receptors comprises four closely related receptor tyrosine kinases: ErbB1 (EGFR), ErbB2 (HER2/*neu*), ErbB3 (Her3), and ErbB4 (Her4), with all of these being crucially involved in proliferation/survival of epithelial cells [12]. Moreover, mutations affecting their expression or activity can result in cancer. Indeed, this has been well established for the ErbB2 molecule, which is widely considered to be an important oncoprotein in many types of human cancer [13,15,16]. The expression of ErbB1 ligands is known to increase during the injury and inflammation of different organs and tissues. For instance, the levels of ligands such as TGFα and HB-EGF rise markedly in the inflammatory reaction of the skin during wound healing, and the targeted disruption of ErbB1 has been shown to impair reepithelialization after injury [17]. However, most studies regarding the ErbB system in the prostate gland have focused mainly on prostate cancer [18–20], with little knowledge being acquired about the effects of inflammatory conditions on the expression of these receptors in this gland.

Epithelial cells lining the reproductive tract are responsible for other homeostatic mechanisms in the host defense, as they are equipped with various antimicrobial, proinflammatory, and immunomodulatory substances which can deal with continual exposition to external injures [21]. In the prostate, many of these molecules have been identified [21–24], but there is still a lack of information about their regulation by inflammatory stimuli. The major secretory protein from the epithelium of the rat ventral prostate is prostatic binding protein (PBP) [25], also called prostatein, which belongs to the secretoglobin family of proteins. The parent member is uteroglobin (UG), a multifunctional cytokine-like and anti-inflammatory protein with potent phospholipase A_2-inhibitory activity [26]. We have previously described lung UG expression being modulated by allergic inflammation [27], and by exposure to an organo-phosphorated insecticide [28]. This protein is a principal component of the female reproductive tract and UG is overexpressed in inflammatory conditions [29]. Although PBP has been described to be an essential immunosuppressive factor of prostatic secretions [30,31], its regulation by infection and the physiological role of PBP in the prostate are not completely understood.

The interstitial smooth muscle cells and fibroblasts are dynamic elements of the prostatic stroma and assume the role of adapting this compartment to the reduced epithelial activity following castration [32]. Also, Tuxhorn et al. [33] have demonstrated an important reactive stroma against cancer, which is a mixture of fibroblast, myofibroblast, and endothelial cells, thus highlighting the responsiveness of interstitial tissue in the prostate gland. Furthermore, it has been recently shown that prostate smooth muscle cells are able to secrete inflammatory mediators in response to bacterial compounds [34] and cytokines [35] in vitro. Nevertheless, the contribution in vivo of stromal components in response against microorganisms has not been elucidated.

Considering the importance of the stromal elements in the development and progression of prostate pathologies together with the critical role of epithelial secretions in the maintenance of glandular homeostasis, the aim of the present work was to characterize the early response of both compartments to *E. coli* in a rat model of bacterial prostatitis.

MATERIALS AND METHODS

Animals

Wistar strain male rats, 12 weeks old, and weighing 250–350 g, were housed in air-conditioned quarters, under a controlled photoperiod (14 hr light/10 hr darkness) and free access to rodent food and tap water. Animal care and experiments were conducted following the recommendations of the International Guiding Principles for Biomedical Research Involving Animals, as issued by the Council for International Organizations of Medical Sciences (CIOMS).

Bacterial Prostatitis Model

A strain of *Uropathogenic E. coli*, isolated from patients with complicated urinary tract infection, was stored at −20°C and grown overnight in tryptic soy broth at 37°C when required for inoculations. The cells were spun, washed three times and resuspended in PBS to give 10^8 cells per ml.

Surgical procedures and bacterial inoculation were performed following a previously published protocol from our lab [23,24]. Briefly, animals were anesthetized with intraperitoneal 1.25% sodium pentobarbital (45 mg/kg), and the lower abdomen and back were swabbed with 70% alcohol. A longitudinal incision of the lower abdomen of about 15–20 mm in length was made (medial laparotomy) to expose the ventral prostate. Prostatitis was induced via an injection of 200 μl of *E. coli* diluted in sterile PBS (10^8 CFU per ml). This bacterial solution was injected via a 30-gauge needle directly beneath the capsule of both ventral lobes. Sham injected controls received 200 μl of PBS. The peritoneum, muscle, and skin were then closed with a 5–0 surgical suture. Animals were sacrificed at 24, 48, or 72 hr after bacterial inoculation, with these protocols being performed three times as independent experiments and a total of 18 rats being used for each time point.

Collection of Tissues and Samples

In order to evaluate the extension of the infectious process, at the end of the protocols, the peritoneal cavity of all of the animals was opened and examined macroscopically. Indeed, samples of the prostate complex, as well as the seminal vesicles, bladder, and epididymis were harvested and studied by using routine histological techniques and microbiological cultures as described below. In addition, the ventral prostate lobes from control and infected animals were excised and divided into two segments. The left lobule was dissected and its intermediate region was processed for light and electron microscopy, while the right lobule was used to do tissue homogenates or to extract prostatic secretion.

For Western blot, the prostate tissue was lysed on ice and 200 μl of cold PBS containing 1.25% Igepal CA-630, 1 mM EDTA, 2 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin was added. The lysate was then centrifuged at 14,000*g* for 30 min at 4°C to pellet the Igepal CA-630-insoluble material, and the supernatant was withdrawn and stored in aliquots frozen at −70°C until required.

To obtain prostate fluid, intact lobes from the ventral prostate were washed in ice-cold buffer, blotted onto filter paper, nicked with a sharp blade, and then centrifuged at 14,000*g* for 20 min to collect the prostate supernatant. This fraction was then used to carry out dot blot analysis.

Microbiological Study

Pieces of ventral, anterior, and dorsolateral prostates, bladder, seminal vesicles, and epididymis from control and infected rats were cultured on agar McConkey (Sigma–Aldrich, St. Louis, MO) for 24 hr at 37°C to assess qualitatively the development of colonies. The bacteria were isolated and identified using conventional Gram staining. The samples were considered sterile if no microorganisms were detected 48 hr after culture.

Histopathological Analysis

Tissue samples of ventral prostate and other organs of the urogenital tract were formalin-fixed and processed for routine histological examination, hematoxylin–eosin (H&E) staining and immunohistochemistry (IHC). Other prostate blocks were fixed in Karnovsky mixture, treated with 1% osmium tetroxide, and embedded in Araldite. For light microscopy, 1 μm thick sections were cut serially and stained using a silver technique according to Sutter and Roulet [36], making identification easier of the tissue structures such as collagen fibers, basement membranes, and glycoproteic components [27].

Electron Microscopy

Tissue for electron microscopy was diced into 1 mm^3 fragments and fixed in Karnovsky's mixture containing 2% (v/v) glutaraldehyde and 4% (w/v) formaldehyde in 0.1 M cacodylate buffer, pH 7.3, at 4°C for 24 hr. These samples were postfixed in 1% osmium tetroxide for 2 hr and washed in 0.1 M cacodylate buffer, before being dehydrated through a graded series of cold acetone and embedded in Araldite epoxy resin. Ultrathin sections were cut in a JEOL JUM-7 ultramicrotome and examined in a Zeiss LEO 906E electron microscope with digital acquisition of images.

For ultrastructural immunocytochemistry, prostate tissue blocks were embedded in acrylic resin (LR-White, London Resin Corporation, Berkshire, England), but omitting osmium fixation.

Immunocytochemistry

Slides from paraffin-embedded prostates were cleared with xylene and rehydrated in a series of descending concentrations of ethanol solutions. Then, a microwave pretreatment (antigen retrieval method) was performed. To block endogenous peroxidase activity, slides were treated with H_2O_2 in methanol for 15 min. Sections were incubated for 30 min in 5% normal goat serum (Sigma–Aldrich) to block non-specific binding, followed by overnight incubation with 1/2,000 diluted polyclonal rabbit antibody to prostatein (developed by Dr. Maccioni [30]) at 4°C in a humidified chamber. Then, the sections were incubated with a biotinylated secondary antibody (Jackson Immunoresearch, West Grove, PA) and ABC complex (VECTASTAIN Vector Labs, Southfield, MI). Diaminobenzidine (DAB; Sigma–Aldrich) was used as a chromogen substrate for 10 min at RT, and sections were rinsed in running water. Meyer hematoxylin was used as a counterstaining solution. The expression of two markers was also evaluated by IHC to characterize the stromal cellular phenotype, with the procedure used being similar to the above-mentioned for PBP. Monoclonal antibodies to vimentin (diluted 1/100) and smooth muscle α-actin (ACTA2) (Novocastra, Newcastle, UK) were used diluted 1/50, and a goat anti-mouse biotinylated IgG (Amersham Pharmacia, Freiburg, Germany) was applied as a secondary antibody.

For ErbB2, we utilized a monoclonal antibody against HER2/*neu* (A0485, Dako, Glostrup, Denmark), diluted 1/350. The immunostaining was semi-automated and performed on a TechMate 500 plus (Dako) by using the DAKO Envision+ Detection Kit and following the manufacturer's instructions. The deparaffinized tissue sections were treated with heat prior to the staining procedure to enable epitope retrieval by immersion of tissue sections in citrate buffer pH 6.0 in an autoclave at 121°C for 1 min.

To assess primary antibody specificity, additional slides were incubated in parallel by replacing primary antibodies with normal rabbit or mouse serum. For controlling nonspecific binding of the secondary antibody, the primary antibody was replaced with PBS–bovine serum albumin.

Determination of Cell Proliferation and Apoptosis

In order to label proliferating cells in the prostate gland, rats infected for 24, 48, or 72 hr, and sham control animals were injected with BrdU (50 mg/kg, intraperitoneally, Sigma, St. Louis, MO) 1 hr prior to sacrifice. The rats were then anesthetized, and the ventral prostate harvested and processed for paraffin-embedding. Four-micron thick paraffin sections were immunostained with a monoclonal antibody against BrdU (Amersham Pharmacia), using a biotinylated goat anti-mouse IgG and ABC reagent (Vector Labs) as described above. The number of BrdU-labeled epithelial cells was measured in 500 epithelial cells and expressed as the percentage of BrdU-labeled epithelial cells [37]. This index was calculated for at least three different areas per rat and repeated in three animals for each time point of analysis.

The apoptotic nature of cell death was confirmed in TUNEL stained sections by using an In Situ Cell Death Detection Kit (POD; Roche Molecular Biochemicals, Mannheim, Germany) following the steps recommended in the manufacturer's instructions. Paraffin-embedded sections were dewaxed, rehydrated, and pretreated with 20 μg of proteinase K per ml for 15 min at room temperature. After rinsing, the sections were incubated for 2 hr in a reaction buffer containing TdT, dATP, and fluorescein-11-dUTP. At the end of this incubation, the sections were rinsed with a stop-wash buffer for 30 min at 37°C, and the mixture was then replaced by peroxidase-conjugated antifluorescein antibody. Immunocomplexes were visualized by exposure to H_2O_2 and DAB. The percentage of TUNEL-positive cells was calculated counting at least 5,000 epithelial cells by rat and repeated at least in three animals per period analyzed.

As a positive control, we used prostate sections from 48 hr postcastrated rats and observed the appearance of a large number of immunostained nuclei. Negative controls were also run by omitting the addition of TdT in the reaction buffer, which resulted in no color staining.

Ultrastructural Immunocytochemistry

For immunoelectron microscopy, Karnovsky-fixed prostates were dehydrated in increasing concentrations of ethanol solutions, up to 90%, and embedded in LR-White (London Resin Company, Hampshire, UK). Then, LR-White thin sections mounted on 250 mesh nickel grids were incubated overnight on a drop of rabbit anti-prostatein diluted 1/2,000, 1/100 mouse anti-vimentin, or 1/100 mouse anti-ACTA2, and immunoreactive sites were labeled with 15 nm colloidal gold/anti-rabbit IgG complex (Ted Pella, Inc., Redding, CA) or 16 nm colloidal gold/anti-mouse IgG complex (Electron Microscopy Sciences, Hatfield). Controls were performed by replacing the primary antibody with normal rabbit or mouse serum, or using PBS–bovine serum albumin.

Western Blot

Prostate homogenates (50 μg proteins) were run in a 15% acrylamide gel for 2 hr at 30 mA, using a mini-protean II electrophoresis system (Bio-Rad Laboratories). To assess the corresponding molecular weight, a full range rainbow molecular weight marker was used (Amersham Pharmacia). Proteins were transferred to a nitrocellulose membrane, and nonspecific binding was blocked at room temperature with PBS containing 5% dried skim milk and 0.1% Tween-20. Membranes were rinsed and incubated for 3 hr with a rabbit polyclonal antibody diluted 1/400, which recognizes TGFβ (sc-146, Santa Cruz Biotech, CA) 1/200 mouse anti-ACTA2 (Novocastra), 1/250 rabbit anti-HER2 (Dako), or 1/200 goat anti-EGFR (Santa Cruz Biotech). After washing, the blots were incubated with a peroxidase-conjugated (HRP) goat anti-rabbit or anti-mouse secondary antibody (Jackson Immunoresearch), and exposed with ECL Western blot detection reagents (Amersham Pharmacia) following the manufacturer's instructions. Emitted light was captured on Hyperfilm (Amersham Pharmacia) and densitometry was performed by applying the Scion Image software (V. beta 4.0.2, Scion Image Corp., Frederick, MD). The relative expression was compared among different treatments by taking the control group value as a reference.

The expression of ACTB (1/5,000; monoclonal anti-β-actin; Sigma–Aldrich) was used as an internal control to confirm the equivalent total protein loading.

Dot Blot Analysis

Prostatic secretion was diluted in PBS buffer, pH 7.4, and protein measurement was performed using the Bio-Rad kit as described above. Samples were then matched at a concentration of 100 μg/ml, and 5 μl of each sample was spotted onto a Hybond C Super membrane (Amersham Pharmacia). The membrane was then blocked with 5% fat-free milk in PBS buffer for 1 hr, before being incubated with the rabbit anti-prostatein serum diluted 1/4,000 in blocking buffer overnight at 4°C. After washing with PBS–Tween-20 buffer, the membrane was treated with an alkaline phosphatase conjugated purified anti-rabbit IgG (Sigma–Aldrich) and revealed with BCIP/NBT tablets (SigmaFast, Sigma). The labeling obtained was evaluated using the Scion Image software as described above for Western blot.

Statistical Analysis

The characterization of data was accomplished by comparing their mean values ± standard error of the mean from three independent experiments. Data from more than two groups were analyzed using analysis of variance (ANOVA) with Tukey as the posttest to compare all pairs of columns. Statistical analyses were performed using the InStat V2.05 program from GraphPad, Inc. (La Jolla, CA).

RESULTS

Analysis of Inflammatory Progression

In control animals inoculated with PBS, the ventral prostate gland consisted of a simple columnar epithelium, with a homogeneous secretory content and scarce elements in the stroma (Fig. 1A,D). After 24 hr of infection, the interstitial compartment exhibited a notable inflammatory infiltrate throughout the gland, which mainly comprised numerous neutrophils and macrophages. At the site of the inoculation, focal infiltrates of lymphocytes and macrophages were frequently observed and, more rarely, small abscesses in some rats. These inflammatory changes were more pronounced at 48 hr after *E. coli* inoculation, with intense edema, reduction of the glandular lumen, and loss of the prostatic secretion occurring (Fig. 1B). At 72 hr after infection, there was an alteration of the prostatic histoarchitecture, with a great number of inflammatory cells invading the glandular compartment, leading to the obliteration of the prostatic acini and epithelial atrophy (Fig. 1C,F). All animals from the infected group showed similar gradual histological findings, consisting in acute bacterial prostatitis. In addition, bacterial cultures of the ventral prostates were positive for *E. coli* in these animals (data not shown).

In order to determine the extension of the infection to other sites of the male reproductive tract, we analyzed the seminal vesicles, bladder, and epididymis, all of which exhibited just a few signs of inflammation such as slight edema and vasodilatation. On the other hand, dorsolateral prostates showed moderate inflammatory infiltrates and positive cultures for *E. coli*. However, the inflammatory changes in this lobe did not display the signs of progression seen in the ventral lobe.

Early Activation of the Prostatic Epithelium After Bacterial Inoculation

As early as 24 hr after bacterial inoculation, the prostatic epithelial cells displayed several morphological signs of cellular activation that were corroborated by electron microscopy. Highly hypertrophied cells exhibited nucleus with big nucleolus and numerous secretory granules in the apical compartment, characteristics that were maintained and frequently more pronounced at 48 hr postinfection (Fig. 1E). Furthermore, this early cell activation was accompanied by a high expression of PBP (Figs. 1H and 5), a key marker of

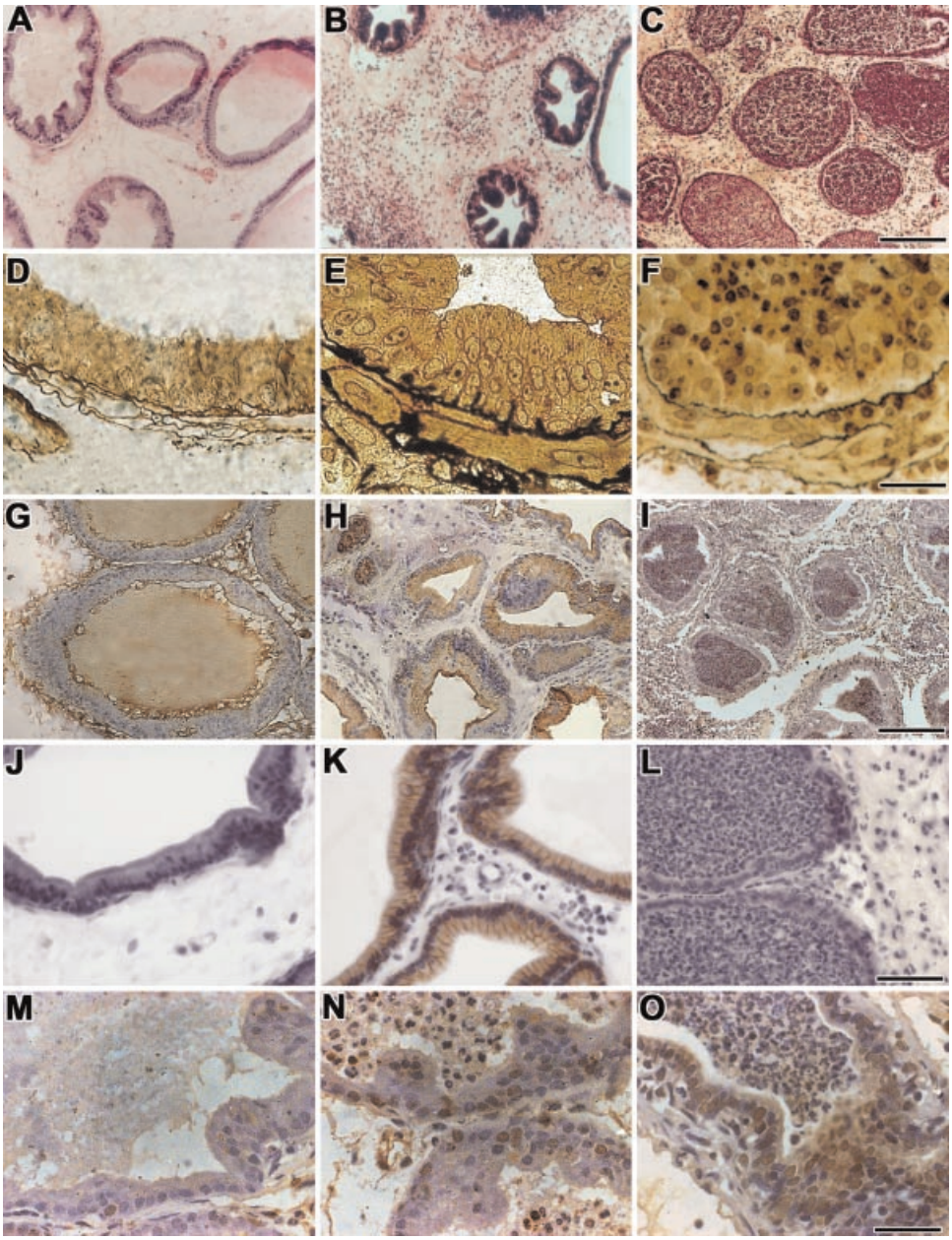

Fig. 1. Morphological and immunocytochemical evaluation of ventral prostate sections from control animals (**A**,**D**,**G**,**J**,**M**), and at 48 hr (**B**,**E**,**H**,**K**,**N**) or 72 hr postinfection (**C**,**F**,**I**,**L**,**O**). The hematoxylin–eosin staining (A–C) shows a progressive interstitial inflammatory infiltrate which invaded the prostatic acini at 72 hr postinfection. The prostatic reaction to inflammation included early epithelial hypertrophy and an increase in the thickness of the periacinar smooth muscle layer, as revealed by the methenamine–silver technique on araldite sections (D–F). In addition, infected animals show increased epithelial expression of PBP (G–I) and the occurrence of the receptor ErbB2 (J–L), with both proteins declining at 72 hr postinfection. Prostate sections were also immunostained to visualize BrdU-labeled cells (M–O), with the epithelial cell proliferation being clearly increased in infected animals. Bars indicate 120 μm (A–C), 20 μm (D–F), 105 μm (G–I), and 30 μm (J–O). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

prostatic function [25], that localized in the secretory granules (Fig. 2C,inset). In correlation, cells exhibited a well-developed Golgi complex (Fig. 2A) and abundant rough endoplasmic reticulum (appearing mainly as concentric lamelar membranes, Fig. 2B). Finally, microvilli at the cell surface were also pronounced in these animals (Fig. 2C).

We then analyzed the expression of two members of the ErbB family, bearing in mind the importance of these receptors in normal and malignant prostate epithelial growth [12,13,15,16]. Strikingly, there was an induction of the oncoprotein ErbB2 in the infected prostate (Fig. 3). This expression of ErbB2 was localized on the plasma membrane of epithelial cells in some

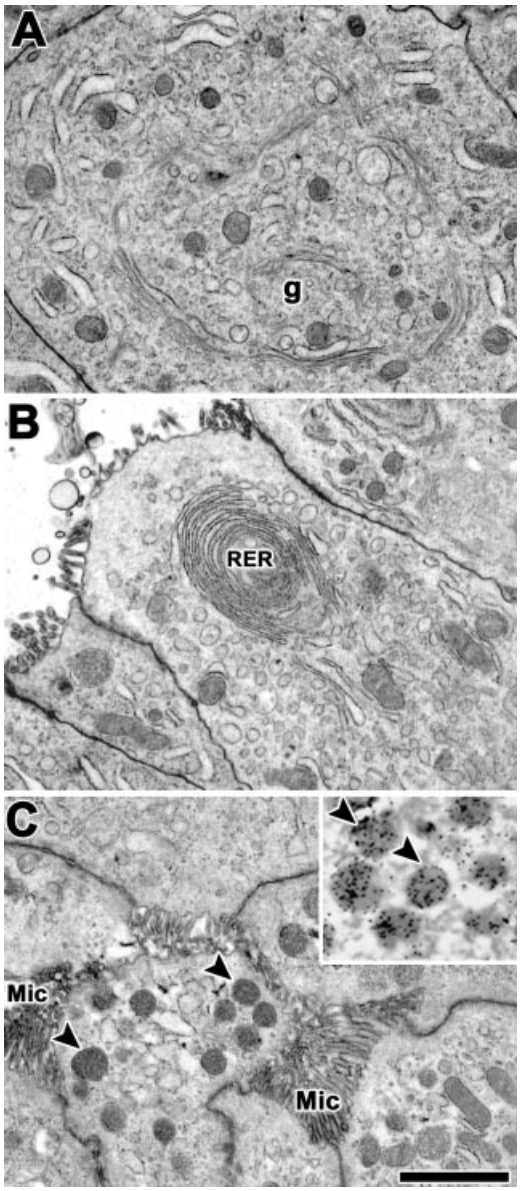

Fig. 2. Ultrastructural evidence of prostatic epithelial stimulation at 24 hr postinfection. Using electron microscopy, araldite-embedded ultrathin sections exhibited several signs of cell activation, such as a well-developed Golgi complex (g in **A**), rough endoplasmic reticulum (RER in **B**), and microvilli (Mic in **C**). Arrowheads denote the presence of numerous mature granules, which were immunopositive for PBP (inset in **C**). Bar: 1 μm.

acini at 24 hr postinfection, but this was more pronounced at 48 hr (Fig. 1K). Although the immunostaining of this oncoprotein was absent at 72 hr after bacterial inoculation (Fig. 1L), it was still detected by Western blot at this time (Fig. 3). In agreement with this finding, the expression of ErbB1 was more intense at 48 hr postinfection compared to control animals (Fig. 3).

In order to determine whether the epithelial activation was also associated with cell proliferation, we

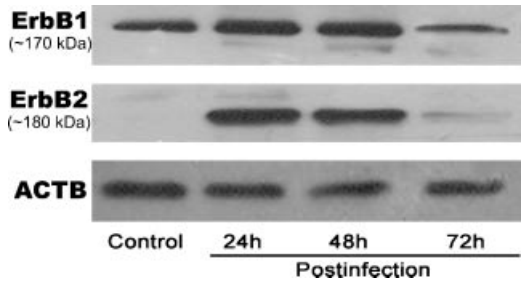

Fig. 3. Western blot results of the ErbBI and ErbB2 receptors in rat ventral prostate after bacterial infection. Representative bands corresponding to control or infected rats. Note the induction of ErbB2 at 24 and 48 hr postinfection. Actin was used as a control for equal loading. The experiment was repeated three times.

evaluated the incorporation of BrdU by control and infected rats and performed IHC for BrdU-labeled nuclei within the ventral prostate. Although control animals had a very low labeling index, BrdU staining was clearly increased in the prostate tissue of rats with bacterial prostatitis (Table I). Interestingly, labeling for BrdU showed a clear increase in epithelial cell proliferation, especially in the acini near foci of intense inflammatory infiltrates (Fig. 1N,O). In fact, the BrdU labeling index reached its highest value at 72 hr postinfection, at which point, the epithelial compartment was completely invaded by inflammatory cells.

As shown in Figure 1C,F, after 72 hr of bacterial inoculation, the acinar invasion of neutrophils correlated with signs of epithelial atrophy. At this time, the prostatic epithelial cells lost their typical cylindrical appearance, turning cuboidal or round in shape (Fig. 1F), with a corresponding poor development of their proteinopoietic organelles correlated with a very weak or absent immunostaining for PBP (Fig. 1I).

E. coli Infection Provoked Apoptosis of Prostatic Epithelial Cells

Following the initial hypertrophy of epithelial cells, a large number of apoptotic figures were observed in the methenamine–silver-stained semi-thin sections of prostates from infected animals within the epithelial compartment (Fig. 4A). This apoptotic death mechanism was corroborated by TUNEL, being apparent at 24 hr after infection, although it was even more evident at 48 hr postinfection (Fig. 4B and Table I). At the electron microscopy level, the classical nuclear chromatin condensation in epithelial cells was sometimes associated with macrophages containing remains of apoptotic cells (Fig. 4C).

Effects of *E. coli* on PBP Expression

The PBP expression in bacterial prostatitis was studied not only as a parameter of epithelial cell

TABLE I. Epithelial Proliferation (Expressed as Percentage of BrdU-Positive Prostatic Epithelial Cells) and Epithelial Apoptosis (Expressed as Percentage of TUNEL-Positive Cells) Induced by Bacterial Prostatitis (Values Are Means ± SD, Six Animals per Group)

		Postinfection		
	Control	24 hr	48 hr	72 hr
Epithelial proliferation	5.88 ± 3.21	9.06 ± 3.84	17.76 ± 2.16**	23.91 ± 5.36***
Epithelial apoptosis	0.03 ± 0.02	1.18 ± 0.16**	2.64 ± 1.01***	0.70 ± 0.39**

**Significantly different than corresponding value in control, $P < 0.01$.
***Significantly different than corresponding value in control animals, $P < 0.001$.

activity but also to investigate whether infection could modulate the levels of this immunomodulatory protein. By applying immunocytochemistry, it was observed that PBP immunoreactivity accumulated mainly in the luminal secretion, while epithelial cells exhibited a weak staining around the nuclei (Fig. 1G). After bacterial inoculation, the whole apical cytoplasm appeared strongly labeled, with the nuclei being completely negative (Fig. 1H). Increased PBP immunostaining was localized within secretory granules (inset in Fig. 2C) as well as in the cytosolic compartment. However, and somewhat surprisingly, the luminal content was washed out (Fig. 1H), which may have been due to a contractile effect of bacterial infection on the muscular periacinar layer. This intense cytoplasmatic PBP expression was maintained up to 48 hr postinfection. Then, as shown in Figure 1I, a few positive epithelial cells were seen, whereas the luminal content showed immunoreactivity for PBP interspersed among the inflammatory cells invading the compartment at 72 hr after *E. coli* inoculation. The dot blot analysis confirmed the rise in PBP, with a peak value being found at 24 hr postinfection. However, this was followed by a decrease in its levels, until reaching similar values to controls at 72 hr after bacterial infection (Fig. 5).

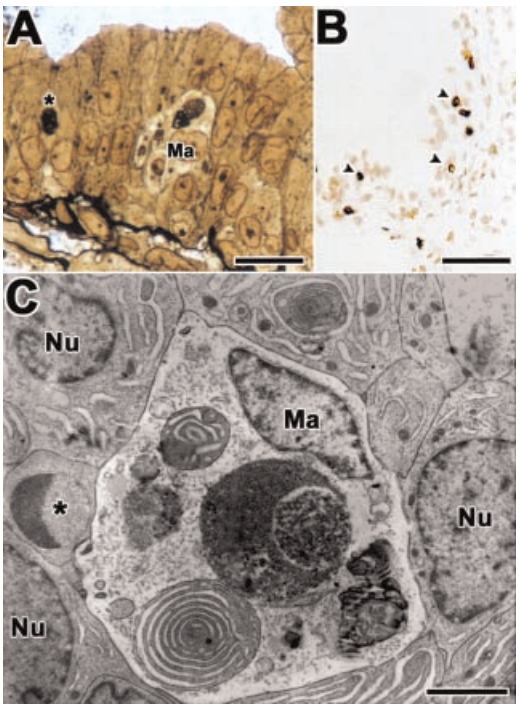

Fig. 4. Morphological analysis of epithelial apoptosis following bacterial infection. **A**: Methenamine–silver staining on araldite sections from rats infected for 48 hr revealing an apoptotic nucleus (*) as well as macrophagic cells (Ma) engulfing the apoptotic remains; bar: 15 μm. **B**: TUNEL-positive nuclei confirming apoptotic cell death (arrowheads). **C**: A representative electron microscope micrograph showing a macrophage with apoptotic debris. An apoptotic cell nucleus with typical chromatin condensation (*) surrounded by nuclei with normal appearances (Nu) is also seen. Bars indicate 15 μm (A), 45 μm (B), and 4 μm (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Stromal Reaction to Bacterial Infection

In control specimens, the ACTA2-positive smooth muscle cells formed narrow sheaths surrounding the acini and were separated by thin layers of loose connective tissue. Vimentin-positive fibroblasts were observed between the epithelium and muscle cells as well as outside the muscle layer. As shown in Figure 1D, the methenamine–silver technique strongly labeled the reticular basal membrane, with this method allowing easy delineation of the periacinar smooth muscle cells.

After bacterial inoculation, a marked hypertrophy of the periacinar layer was observed, associated with a strong methenamine–silver-stained extracellular matrix surrounding the muscle layer (Fig. 1E). The vimentin immunoreactivity formed two interrupted layers that enclosed the smooth muscle cells. Using electron microscopy, smooth muscle cells were seen to display stimulated nuclei with abundant mitochondria in the paranuclear region, while the myofibrillar apparatus was highly developed and ACTA2 positive (Fig. 6C and D). Moreover, the total amount of ACTA2,

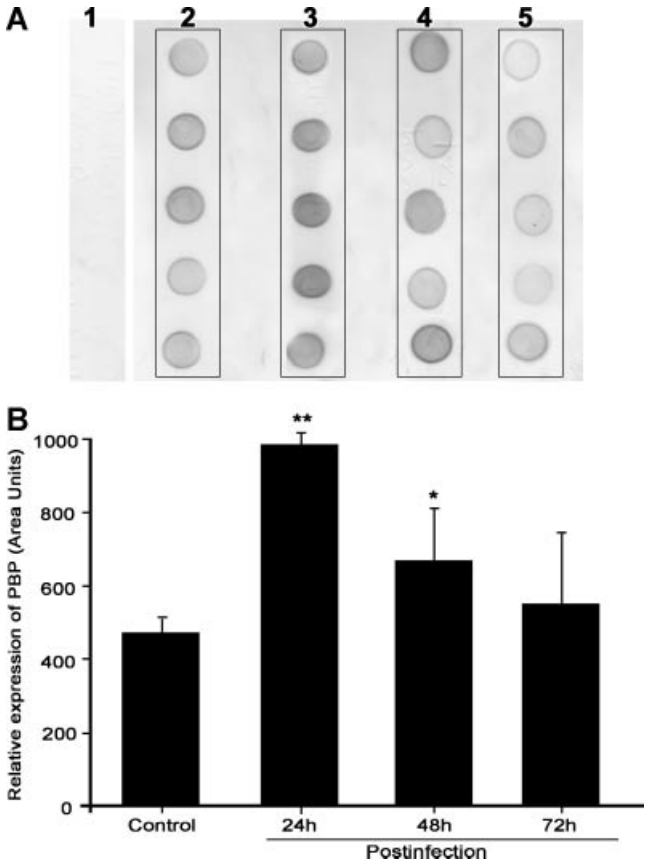

Fig. 5. Expression of PBP in ventral prostate samples. **A**: Dot blot of PBP showing five representative prostatic samples from control rats (in **2**), and infected animals for 24 hr (**3**), 48 hr (**4**), and 72 hr (**5**). In **1**, the same samples blotted in 2 were incubated with a normal rabbit serum to check antibody specificity. **B**: Densitometric analysis of dot blot exhibiting a significant increase in PBP expression after bacterial infection at 24 and 48 hr postinoculation. The experiments were repeated three time, using a total of 15 animals per time point. ** versus control, $P < 0.01$, ANOVA-Tukey. * versus control $P < 0.05$, ANOVA-Tukey.

observed by Western blot was significantly increased in these inflamed prostates (Fig. 7).

It was also evident that smooth muscle cells started progressively losing their normal electron density by 48 hr after infection, which was even more pronounced after 72 hr. This was correlated to a decrease in the ACTA2 immunoreactivity (Fig. 7) and well-developed proteinopoietic organelles, with the extracellular matrix exhibiting numerous collagen fibers (Fig. 6E). The density of the contractile filaments was also reduced with the ACTA2 reactivity being confined to narrow areas near the plasmalemma (Fig. 6F). In addition, vimentin-positive fibroblast showed signs of cellular stimulation, such as a prominent nucleolus and the increased development of intracellular membranes.

Finally, since TGFβ1 has been considered to be a major factor in prostatic stromal modifications, we

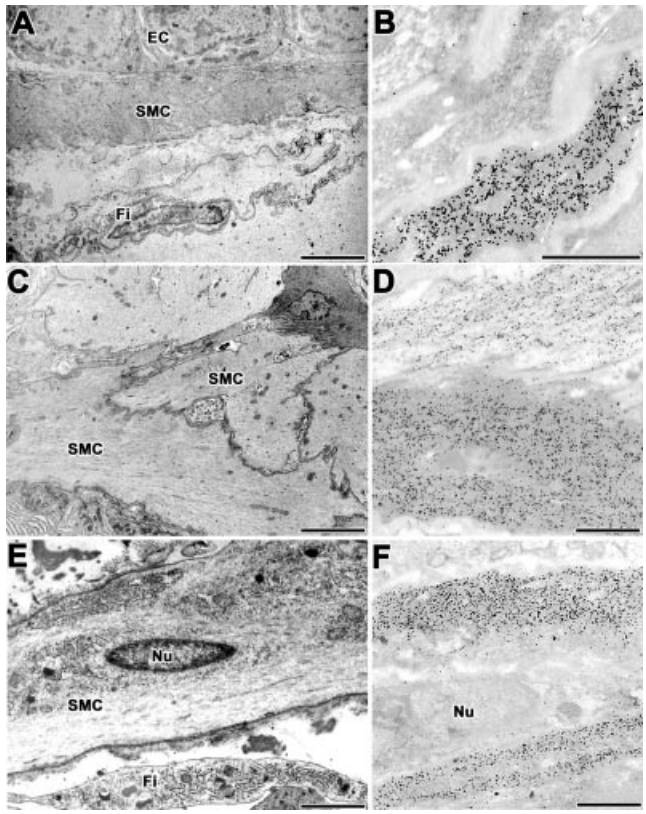

Fig. 6. Effects of inflammation on periacinar smooth muscle cells at ultrastructural level. Araldite sections from control animals (**A**), and at 24 hr (**C**), or 72 hr postinfection (**E**). Matching LR-White-embedded prostate ultrathin sections were immunostained with anti-ACTA2 (**B**,**D**,**F**). Note that smooth muscle cells (SMC) appear hypertrophic (C) with a rich content of ACTA2 (D) at 24 hr postinoculation. Later, this large development of organelles is accompanied by a loss of contractile filaments (E), with ACTA2 being restricted to peripheral zones (F). Fi, fibroblast; Nu, nucleus. Bar: 1 μm.

analyzed its levels in prostatic homogenates by Western blot, with a progressive increase in this growth factor being found in infected animals compared to controls (Fig. 8).

DISCUSSION

Bacterial compounds have been reported to cause a wide range of cellular responses in prostatic cells, including hypersecretion of epithelial proteins [38] and the increase of host defense molecules [22,24,39–41]. In the present work, we demonstrated that acute bacterial infection also produced an early important stimulation in both the epithelial and stromal compartments, which led to a subsequent loss of normal epithelial function and the dedifferentiation of smooth muscle cells in the prostate gland.

Traditionally, the study of the immune response against microorganisms has been confined to professional cells such as macrophages and lymphocytes. However, in recent years, an important role has been

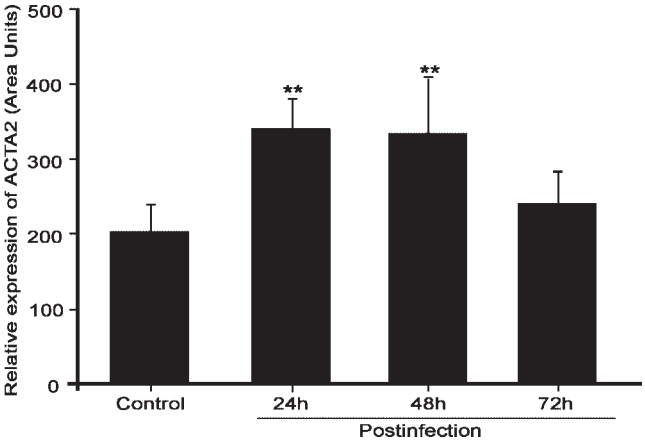

Fig. 7. Relative amounts of ACTA2, measured by densitometry of Western blots, in ventral prostate homogenates. The ACTA2 expression is significantly increased at 24 and 48 hr after bacterial inoculation in comparison with the control (**$P < 0.01$, ANOVA-Tukey). Note that ACTA2 expression decreases at 72 hr after bacterial infection. The values represent the mean of ACTA2 expression after normalization with ACTB expression of nine animals per group obtained from three independent experiments.

revealed for resident cells, especially in respiratory [42] and digestive infections [11]. In the prostate gland, epithelial cells in vitro were found to be able to secrete inflammatory mediators in response to LPS [39,43], *Chlamydia trachomatis* [44], and CpG [45]. Although few studies in vivo have been published, we previously reported that both rat [24] and human [41] prostatic epithelium constitutively expresses Toll-like receptor 4 (TLR4) to recognize Gram-negative bacteria. Furthermore, TLR4 and the anti-microbial protein SP-D increase after bacterial challenge [23,24], suggesting

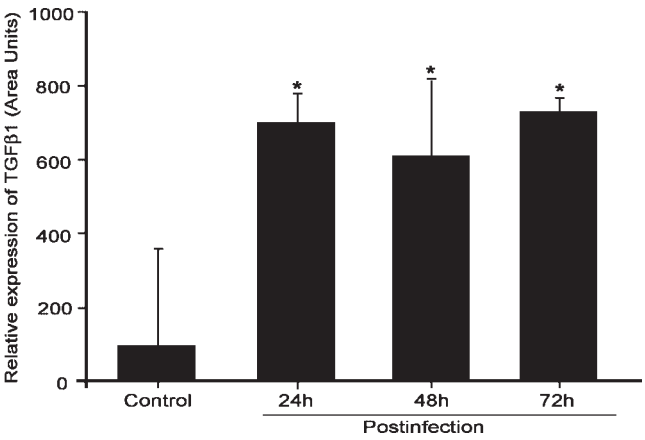

Fig. 8. Western blot analysis of TGFβI levels in prostatic homogenates. Densitometric evaluation of TGFβI expression showing a clear increase of this growth factor in the ventral prostate after bacterial infection. The values were normalized with ACTB expression and represent the mean of nine rats per group obtained from three independent protocols. * versus control, $P < 0.05$, ANOVA-Tukey.

that epithelial cells are central players in the early host–pathogen interactions in the prostate gland. This suggestion is now supported by our present ultrastructural findings, which indicate a strong cellular activation, as reflected in the increase in organelles engaged in protein synthesis and secretion.

PBP is the main epithelial secretory product of the rat ventral prostate [25], which belongs to the anti-inflammatory secretoglobin family of proteins [26]. High levels of these molecules have been described in association with inflammatory processes in the airways [27,28,46], as well as in the female reproductive tract [29]. In the current work, we found an early increase in PBP levels to be concomitant with epithelial cell activation, which was followed by a posterior decrease in this protein that was correlated with atrophic epithelium. Although the specific biological function of PBP remains still uncertain, it has been reported that it exerts a potent immunosuppressive activity by inhibiting the macrophage function [30], reducing the production of IL-2, and dampening the mitogen-induced proliferation of lymphocytes [31]. In this context, the initial overexpression of PBP after bacterial infection might act as a central strategy in modulating the early immune response by prostatic cells, with the posterior decrease of PBP being associated with the presence of large numbers of inflammatory cells and tissue destruction within the prostate gland. Finally, the concomitant upregulation of pro- and anti-inflammatory proteins in the genital tracts could constitute a mechanism for immunosuppressive status, characteristic of these sites, since an uncontrolled inflammatory reaction would be more dangerous for gametes than inflammatory stimuli.

Apoptosis of host epithelial cells appears to be an evolutionarily conserved response to microorganisms across the animal and plant kingdoms [47], with rapid induction of apoptosis in pathogen-infected host cells being critical to restoring the normal cell growth and function. For instance, it has been demonstrated that intestinal cells undergo apoptosis after *E. coli* or *Salmonella* infection [48]. Moreover, Klumpp et al. [49] have reported that *Uropathogenic E. coli* induces extrinsic and intrinsic cascades to initiate urothelial programmed cell death. Accordingly, apoptosis is a host defense mechanism whereby the bladder lining is shed and adherent bacteria are purged during voiding [50]. In the present work, the occurrence of apoptotic figures induced by *E. coli* was more evident at 48 hr postinfection and preceded the atrophy of the prostatic epithelium. In this regard, it is reasonable to hypothesize that the early postinfection hours would be sufficient for prostatic epithelial cells to initiate antimicrobial programs and inflammatory signals, necessary for removing pathogens. Subsequently,

epithelial cell death could be triggered not only by *E. coli* itself but also by the cytokines present in the inflammatory microenvironment, such as TNFα or TGFβ1, which had elevated levels after bacterial infection.

The fact that prostatic resident cells can be activated in response to bacterial infection might represent a beneficial mechanism for eliminating microorganisms at first glance. However, the consequence of chronic inflammatory signals on epithelial cells could also constitute a pivotal component in the pathophysiology of many human diseases. In fact, prostatic inflammation has recently been considered a key factor in the development and maintenance of hyperplasia [1] and prostate cancer [2], with an inflammatory environment possibly modifying the balance between cellular growth and turnover, thus leading to an uncontrolled proliferation. Related to this, Elkahwaji et al. [51] have reported that chronic inflammation induces reactive hyperplasia associated with oxidative stress injury, thereby supporting this proposed link among inflammation, oxidative DNA damage, and prostate carcinogenesis. In the present study, we demonstrated that epithelial proliferative changes in the rat prostate are early events following bacterial invasion. Interestingly, the highest proliferative index was observed in areas closed to intense inflammatory infiltrates, suggesting that proinflammatory signals, even at acute stages of infection, are a strong proliferative stimulus for prostatic epithelial cells.

Several lines of evidence indicate that the ErbB family of receptors greatly contributes to epithelial proliferation, differentiation, and inhibition of apoptosis [37]. Furthermore, ErbB1 and ErbB2 are overexpressed or abnormally activated in several epithelial malignancies. Consequently, this finding eventually led to the United States Food and Drug Administration's (FDA) approval of several agents specifically targeting these receptors [52]. We report here an initial overexpression of ErbB1, indicating the occurrence of activation and upregulation in this system after bacterial infection. Furthermore, the expression of ErbB2 was transiently expressed in infected prostates. This molecule is a co-receptor for many different ligands and is often transactivated by EGF-like ligands, thereby resulting in the formation of ErbB1-ErbB2 heterodimers [13]. As this pattern generates more potent signals than homodimers [53], ErbB2 has been implicated in the neoplastic transformation of prostate carcinoma [15,16].

An increased activity of ErbB1 in conditions that seriously affect the prostate such as castration [37] and cancer [54] has been previously demonstrated. However, to our understanding, this is the first evidence of changes in ErbB proteins in the rat ventral prostate induced by bacterial infection. Owing to the importance of the ErbB family in cancer, the increase in the expression of these receptors, along with the epithelial proliferation associated with bacterial prostatitis, appear to support the hypothesis of inflammation inciting carcinogenesis. Nevertheless, the reduction in ErbB1 and the absence of ErbB2 observed at 72 hr postinfection suggest a strict regulation over these molecules, at least in the acute phase of prostatitis. In addition, this finding indicates a complex homeostatic balance, wherein more factors are necessary to break the normal control avoiding malignant growth in the prostate gland.

It has been reported that the stromal compartment critically influences the initiation and/or maintenance of proliferative pathologies in the prostate gland [32–34]. Indeed, in the present work we describe a rapid stromal response to bacterial infection, characterized mainly by hypertrophy and the acquisition of a secretory phenotype in smooth muscle cells. Related to this, a lot of evidence suggests that smooth muscle cells are metabolically dynamic cells with the potential to express and secrete numerous highly active signaling proteins [55]. In addition, these cells can originate myofibroblasts with potent secretory activities, which are considered to be an important component of the stroma accompanying prostate cancer [33]. In a previous work, we showed that prostatic smooth muscle cells respond in vivo to bacterial infection by increasing their expression of TLR4 [24]. Recently, Penna et al. [34] further confirmed that prostatic stromal cells from patients express all of the TLRs, with their ligation leading to the secretion of IL-8, CXCL10, and IL-6. In addition, these authors propose that stromal cells represent nonprofessional antigen-presenting cells, being able to induce and sustain inflammatory processes within the prostate [34]. Together, these data indicate that smooth muscle cells may play a role in defending the prostate gland against microorganisms.

It is well known that TGFβ1 is critical for prostatic smooth muscle regulation, with its increase after castration being related to hypertrophy of the periacinar layer [56]. Moreover, it is believed that this factor is important in the development of the stromal changes accompanying proliferative diseases, such as hyperplasia and prostate cancer [33,57]. Therefore, we decided to evaluate the levels of TGFβ1, which were shown to increase in a time-dependent manner after infection. This early increase of TGFβ1 might have been responsible for the hypertrophy of smooth muscle observed at 24 hr postinfection. Afterwards, the presence of potent proinflammatory signals may have acted as dedifferentiator factors on the prostatic smooth muscle cells. In agreement with this hypothesis, it has

been previously shown that IL-8 induces phenotypical changes on prostatic stromal cells in vitro, leading to the development of myofibroblastic cells [58].

CONCLUSIONS

The present results demonstrate an early activation of unspecific cell mechanisms triggered by bacterial infection in the ventral prostate. Among them, hypertrophied epithelial cells increase PBP probably to control the inflammatory process, a strategy that is abruptly interrupted by epithelial atrophy. In addition, acute inflammation promotes proliferative changes and the upregulation of ErbB receptors, thus suggesting that repetitive reinfections could lead to uncontrolled growth. Finally, prostatic smooth muscle cells become secretory cells in response to *E. coli*, which allow them to cooperate actively with epithelial and immune cells in host defense against pathogens. However, these infection-induced cellular alterations might also represent key factors in the pathophysiology of many prostate diseases.

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