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REGULAR ARTICLE

Kartiki V. Desai · Kathleen C. Flanders
Paturu Kondaiah

Expression of transforming growth factor-β isoforms in the rat male accessory sex organs and epididymis

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Abstract We studied the expression and distribution of transforming growth factor-β (TGF-β) isoforms in the rat male accessory sex glands and the epididymis. Our data demonstrate the expression of both TGF-β1 and -β3 isoforms in ventral prostate (VP), seminal vesicle (SV), coagulating gland (CG), and epididymis (E) by Northern blot analysis. In addition, there was differential expression of TGF-β3 in the three regions of epididymis, the corpus region being the highest. Immunostaining data showed intense staining for latent TGF-β1 in all the male accessory glands. In contrast, no staining using antibodies specific for active TGF-β1 was observed. No expression of TGF-β2 was evident either by immunohistochemistry or Northern blot analysis. The presence of mature TGF-β3 protein was observed in the secretory epithelium of VP, CG, and corpus E. There was no detectable staining of TGF-β3 in the seminal vesicle and caput and cauda regions of epididymis. These data suggest possible differential regulation of TGF-β isoform expression in the male reproductive system and predict unique roles for individual TGF-β isoforms in sperm maturation and maintenance.

Key words Coagulating gland · Epididymis · Gene expression · Prostate · Seminal vesicle · Rat (Wistar)

Introduction

Transforming growth factor beta (TGF-β) was first isolated by virtue of its ability to promote anchorage-independent growth of normal rat fibroblasts. Subsequently, TGF-β was shown to elicit profound effects on the cell growth, differentiation, and development (Roberts and Sporn 1990). Three highly conserved but distinct isoforms of TGF-β (β1, β2, and β3) that belong to a superfamily of growth modulators have been described in the mammals (Kingsley 1994). TGF-βs play a dominant role in immunesuppression, carcinogenesis and tumor suppression, angiogenesis, and tissue remodeling and repair. They are potent inducers of the extracellular matrix (ECM), mediate cell-to-cell communication and chemotaxis, and cause inhibition of epithelial cell growth. In vitro studies demonstrate a redundancy in TGF-β isoform action, as these isoforms can replace one another in many biological assays and signal via the same set of receptors designated as type I and type II (Massague et al. 1994). Despite the similarity in biological response, TGF-β isoforms exhibit variable affinity to the cognate receptors and, depending upon the cell type, TGF-βs differ in the extent of biological response in in vitro assays (Graycar et al. 1989). Although TGF-β1 has long been studied as a prototype of the three isoforms, several lines of evidence point out to their discrete actions. For example: (a) TGF-βs display complex and disparate control elements in their promoter regions, suggesting independent regulation leading to their differential expression in various cells and tissues (Roberts et al. 1991); (b) biological assays are capable of distinguishing these isoforms (Merwin et al. 1991; Roberts et al. 1990); and (c) although the targeted gene disruption of each isoform resulted in embryonic or perinatal mortality, the phenotypes were distinct. Null mutants of TGF-β1 display severe inflammation and impairment of heart development (Kulkarni et al. 1993; Letterio et al. 1994). TGF-β2 knockout mice display a wide variety of defects in the development of skeletal, cardiac, lung, eye, spinal, inner ear, and urinogenital tissues (Sangford et al. 1997). In contrast, null mutants of TGF-β3 resulted in abnormal lung development and cleft palate (Kaartinen et al. 1995; Proetzel et al. 1995). These phenotypes establish the importance of correct spatial and temporal expression of TGF-β isoforms during organogenesis and emphasize their functional individuality in vivo.

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K.V. Desai · P. Kondaiah (✉)
Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore 560 012, India
Fax: 91–80–3345999; e-mail: paturu@serc.iisc.ernet.in

K.C. Flanders
Laboratory of Cell Regulation and Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

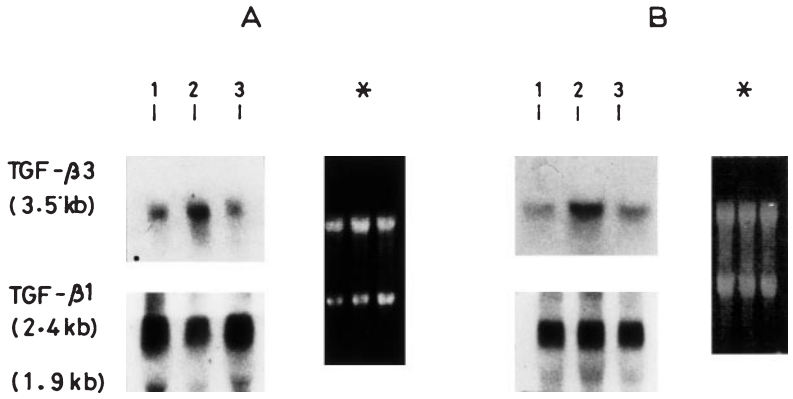


Fig. 1A, B Northern blot analysis of total RNA from rat accessory sex organs and epididymis. Twenty micrograms of total RNA isolated from the rat accessory sex glands (n=5) was analyzed on a 1.2% agarose gel. Northern hybridizations were carried out using P^{32}-labeled mouse transforming growth factor-β3 (TGF-β3) and rat TGF-β1 cDNAs. Representative autoradiograms are shown. **A** *Lanes 1*, *2*, and *3* represent RNA isolated from the coagulating gland, seminal vesicle, and ventral prostate. **B** *Lanes 1*, *2*, and *3* represent the caput, corpus, and cauda epididymis, respectively. (*Asterisks* ethidium bromide staining of the respective gel)

TGF-β1 has been implicated in the morphogenesis of the rat neonatal seminal vesicle and ventral prostate (Tanji et al. 1994; Timme et al. 1994). Also, castration-induced regression of the ventral prostate was associated with increased levels of TGF-β1 isoform (Kyprianou and Isaacs 1989). Several lines of evidence suggest a specific role for TGF-βs during prostate carcinogenesis (reviewed by Barrack, 1997). However, there are no data on the expression of TGF-β1 in other male accessory organs. Further, TGF-β3 expression and regulation has not been documented in these organs. It is important to assess the expression and regulation of these closely related isoforms to analyze the distinct roles played by TGF-βs in the male reproductive system. In this report we present data on the expression and tissue distribution of TGFβs in the rat ventral prostate (VP), seminal vesicle (SV), coagulating gland (CG), and epididymis (E) by northern blot analysis and immunohistochemistry.

Materials and methods

The caput, corpus, and caudal regions of the E, VP, SV and CG of 35- to 45-day-old male Wistar rats (IISc strain; n=5) were dissected and snap-frozen in liquid nitrogen for RNA isolation or fixed in Bouin's fixative after fluid expression for immunohistochemistry.

RNA extraction and hybridization

Frozen samples were used for extraction of total RNA using one-step purification by guanidium isothiocyanate (Chomczynski and Sacchi 1987) and RNA concentration was determined by measuring absorbance at 260 nm. Twenty micrograms of total RNA was analyzed on a 1.2% agarose-formaldehyde gel and blotted onto Hybond-N nylon membrane (Amersham, UK). The prehybridization and hybridizations were carried out at 65° C in a buffer containing 1% bovine serum albumin, 7% SDS, 1 mM EDTA, and 0.5 M disodium hydrogen phosphate (pH 7.0; Church and Gilbert 1984). The probes were labeled with [^{32}P]dCTP (NEN, Du Pont, USA) by random primed method using the Megaprime labeling kit (Amersham, UK). The hybridizations were carried out for approx. 16 h and the blots were washed with 2×SSC at room temperature for 30 min and for another 30 min with 0.2×SSC at 65°C and exposed to Hyperfilm (Amersham, UK) at –70°C. These blots were sequentially hybridized to mouse TGF-β3 cDNA (Denhez et al. 1990), mouse TGF-β2 cDNA (Millan et al. 1991), and subsequently to rat TGF-β1 cDNA probe (Qian et al. 1990).

Immunohistochemistry

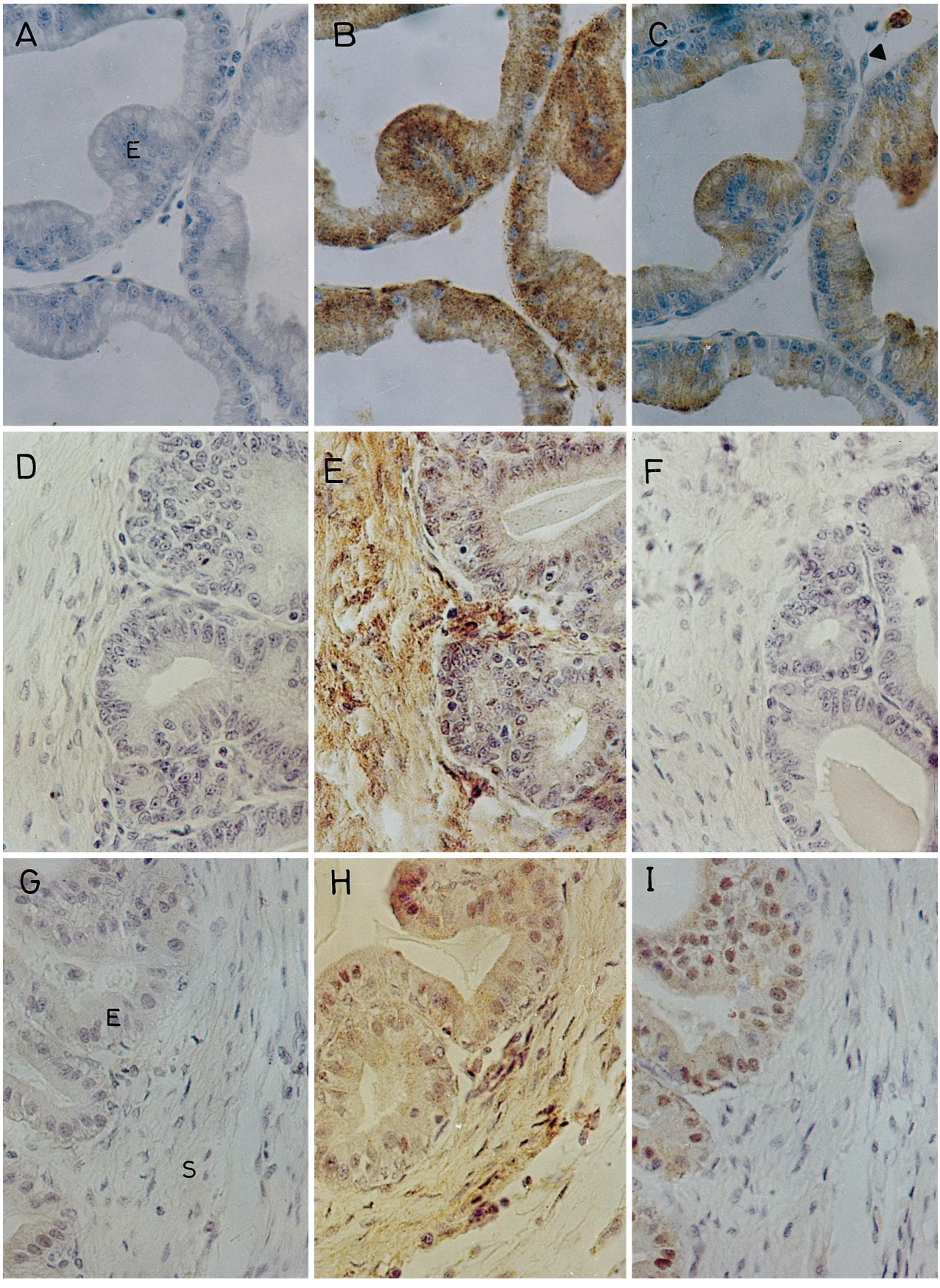
For immunohistochemistry, tissues were embedded in Paraplast (Sigma) after processing and fixation. Serial sections of 5–6 μm thickness were used to immunolocalize TGF-β isoforms. The antibodies used in the present study are polyclonal antisera raised in rabbit against synthetic peptides: (1) β1-pre (amino acids 266–278 of β1-protein in the precursor region); (2) LC and CC (amino acids 1–30 of the mature β1-protein); (3) TGF-β2 (amino acids 50–75 in the mature region); and (4) TGFβ-3 (amino acids 50–60 in the mature region). These antibodies have been extensively characterised for their specificity in immunohistochemical studies (Flanders et al. 1989, 1991). In brief, serial sections were dehydrated in grades of alcohol, and cellular peroxidase was inactivated by treatment with H_2O_2 in methanol. After treatment with testicular hyaluronidase, the nonspecific sites were blocked by 5% normal goat serum. Sections were exposed to either 5–10 μg/ml of the purified IgG fraction of primary antibodies or preimmune IgG at the same concentration at 4°C overnight. The bound primary antibody was detected using the Vectastain ABC system (Vector Labs; Flanders 1989). Color reaction was developed using diaminobenzidine (Sigma Chemical, USA) as substrate. Sections were counterstained with hematoxylin and mounted in Permount. Mouse embryo sections were used as a positive control for antibody detection.

Results

Northern hybridization

Hybridization of the total cellular RNA from various tissues using TGF-β-specific probes demonstrated the ex-

Fig. 2A–I Immunohistochemistry of TGF-β isoforms in ventral prostate (VP), seminal vesicle (SV), and coagulating gland (CG). Representative photomicrographs of sections (×132) stained with isoform-specific antiserum are shown. **A–C** VP; **D–F** SV; **G–I** CG. The isoforms are indicated at the *top*. VP displayed the highest amount of staining for latent TGF-β1 and active TGF-β3 (**B, C**), whereas SV was completely devoid of β3-specific staining. *Arrowhead* (**C**) indicates the β3-positive stromal cell in the VP. (*E* Epithelium, *S* stroma)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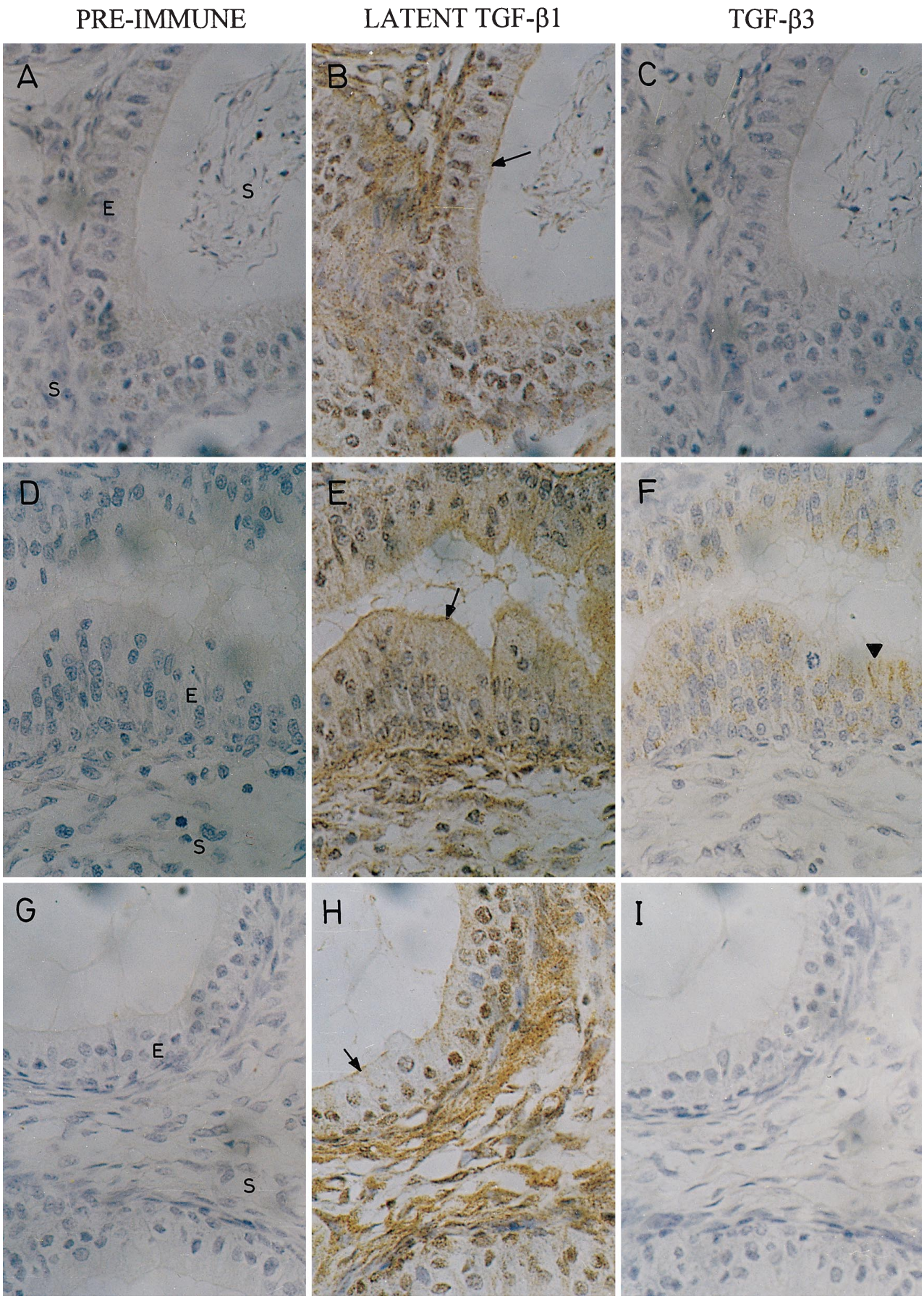
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pression of β1 and β3 isoforms in all the tissues studied (Fig. 1). However, no β2-specific mRNA was observed in any of these organs. Overexposure of the blots resulted in the detection of 4.8-kb and 3.5-kb transcripts with very high lane backgrounds (data not shown), indicating that there is very low level of expression in these organs. Between the tissues, the level of TGF-β1 expression appeared to be higher in the CG and that of TGF-β3 was higher in the SV in comparison with the other organs (Fig. 1A). Interestingly, in the epididymis (Fig. 1B), the corpus region (E2) showed sixfold higher expression of β3 than the caput (E1) and cauda (E3) regions as quantitated by densitometric scanning (data not shown). However, there was no difference in the expression of the TGF-β1 between epididymal sub-regions.

Immunohistochemistry

The localization of TGF-β1 protein in the male accessory sex glands was studied using two types of antibodies: β1-pre that detects TGF-β1 protein in association with the latency-associated peptide, and LC and CC that recognize biologically active protein. Our results indicate that copious amounts of latent TGF-β1 are present in all the organs studied, with VP being the highest (Fig. 2). In the VP, all columnar epithelial as well as the stromal cells and the interductal fibrous connective tissue stained intensely for β1-pre (Fig. 2). Cytosolic localization of staining may indicate active synthesis of the protein in the epithelial cells. On the other hand, in SV staining was restricted to the stroma and was intense in the connective tissue. No staining was detectable in the epithelium (Fig. 2). Diffused distribution of latent β1 protein was observed in the coagulating gland (Fig. 2). In all the three regions of E, the extracellular matrix surrounding the stromal cells showed intense localization of this isoform, although the epithelium dispalyed a comparitively weaker reactivity lining the luminal surface of the cells (Fig. 3). Interestingly, both LC and CC antibodies could not detect the presence of active TGF-β1 protein in any of the organs studied (data not shown). Hence, to substantiate the β1-pre staining, we have employed a monoclonal antibody (mAb VB3A9) raised against overexpressed latent precursor protein (Gleveis et al. 1996). An identical staining pattern was observed, confirming the presence of latent TGF-β1 in the tissues studied.

The TGF-β3-specific antiserum used in our study detects only the active protein. Expression of TGF-β3 was localized exclusively to the secretory epithelium of all the male accessory glands except the SV. In the VP, specific staining was localized to the glandular epithelial lining of the distal tubules and it decreased marginally in the intermediate tubules (not shown). Whereas the basal epithelial cells lining the tubules and the stromal compartment of the VP were negative for TGF-β3 reactivity, occasionally cells in the stroma did stain for this isoform (Fig. 2). In addition, the epithelial lining in the CG was also positive for β3 (Fig. 2). Interestingly, the staining specific to mature TGF-β3 protein was very low and restricted only to the epithelial cells of the corpus E (Fig. 3). No detectable color reaction was observed in the caput and the cauda E (Fig. 3). The color reaction encompassed the cytoplasm of the glandular cells. Despite the presence of β3 mRNA in SV, no immunostaining was detected either in the epithelium or the interstitial cells of this tissue using β3 antibodies (Fig. 2). We observed nuclear staining using β1-pre in all the organs and β3 displayed nuclear localization in the CG. The significance of this pattern is not known.

Fig. 3A–I Immunolocalization of TGF-β isoforms in the three regions of epididymis. **A–C** Caput epididymis; **D–F** corpus epididymis; **G–I** cauda epididymis (×132). The isoforms are indicated at the *top* of the panel. Note the presence of β3 only in the epithelium of the corpus region (*arrowhead in* **F**). *Arrows* indicate latent β1-specific staining on the surface of the glandular cells in all the three regions. (*s* Stroma, *E* epithelium, *S* sperm present in the epididymal lumen)

Discussion

The ability of various growth factors to serve as autocrine and/or paracrine mediators to restructure and fine-tune the instructive signals between the mesenchyme and the epithelium has been well documented in different organ systems. The importance of growth factor requirement in the differentiation of the male reproductive system has been established by experiments carried out on developing rat neonatal VP. In these organ cultures, keratinocyte growth factor (KGF) alone was capable of eliciting complete development of the tissue, a process thought to be completely androgen-dependent. On the other hand, the presence of testosterone is essential for complete differentiation and for the epithelial branching morphogenesis of the SV, although KGF can induce the growth of SV in culture (Cunha et al. 1994). The androgen effects on the stromal cells result in the production of soluble growth factors, which in turn mediate the arrest of epithelial cell growth. Previously, this effect was thought to be mediated by TGF-β1 (Goldstein et al. 1991; McKeehan and Adams 1988; Wilding et al. 1989) but it was later shown to be due to the "prostate derived epithelium inhibiting factor" (p-EIF; Kooistra et al. 1995). TGF-β1, -β2, and -β3 have unique roles and are autocrine/paracrine modulators of the mesenchymal-epithelial interactions (Millan et al. 1991). The proper maintenance and function of male accessory glands necessitates such an intimate interaction between the cells. Hence, in the present study the distribution of the TGF-β isoforms has been evaluated.

Our Northern blot data clearly demonstrated the expression of both TGF-β1 and TGF-β3 in the rat male accessory sex organs. The TGF-β mRNA presence seldom reflects the status of the TGF-β protein levels, and hence assessment of the cellular distribution of these isoforms would give novel insights into their biological action. In accordance with the RNA profile, intense staining for the latent β1 isoform was observed in all the organs stud-

ied. Depending upon the antiserum employed to detect this isoform, conflicting data has been generated. However, immunostaining using antibodies LC and CC which recognize the intracellular and the extracellular TGF-β1, respectively, failed to reveal immunoreactive TGF-β1 protein in any of the organs used in our study. Recently, TGF-β1 was localized to the prostatic smooth muscle cells, whereas the epithelium of the VP was devoid of such immunoreactivity (Nemeth et al. 1997). We could not detect β1 presumably because our peptide antisera were not sensitive enough. In the same study, RT-PCR using total RNA isolated from separated stromal and epithelial cells revealed the presence of β1 mRNA predominantly in the stromal cells. In another report, polyclonal goat anti-human LAP antibody, AB-246-PB (which detects the latent β1), localized the inactive isoform to the epithelium as well as the stroma in the normal human prostate (Perry et al. 1997). This pattern is similar to that revealed by the β1-pre antiserum in the rat VP and other organs. Taken together, our data suggest that TGF-β1 protein under normal physiological conditions is essentially inactive, as it is incapable of interacting with its cognate receptors in association with the precursor. Hence, the plausibility of activation of β1 under certain conditions, for example, under androgen ablation, exists. Indeed, the upregulation of TGF-β1 has been reported in the VP during castration-induced regression (Kyprianou and Isaacs 1989), which may accompany and or result in a concomitant activation of the latent isoform.

Although Timme et al. (1994) have previously failed to detect the TGF-β3 transcript, we could demonstrate the presence of a specific 3.5-kb mRNA in the VP. We observed only epithelial staining for β3 in the VP, CG, and corpus E, similar to that observed for the β2 and β3 isoform in the human prostate (Perry et al. 1997). Although, β1 was shown to inhibit the proliferation of rat prostatic cells (Martikainen et al. 1990), and as we could not detect any active β1 in the male accessory sex organs, we propose that TGF-β3 most probably acts as the autocrine inhibitor of epithelial cell growth to check uncontrolled proliferation. Subsequently, such an inhibition may benefit the process of differentiation of these cells. Action of β3 in these epithelial cells can be feasible only in the presence of TGF-β receptors type I and type II. Indeed our personal observations (K.V. Desai and P. Kondaiah, unpublished work) show the colocalization of the both these receptors in most of the organs studied. The absence of β3 immunoreactivity in SV and the pattern of TGF-β1 and -β3 staining in the E was intriguing, since there were detectable levels of mRNA for both the isoforms in these tissues. This differential staining suggests stringent regulation of these factors, which is essential considering their multifunctional nature. Moreover, in addition to transcriptional and translational control of TGF-β expression (Arrick et al. 1991; Kim et al. 1992), activation is a key regulatory step in TGF-β action. This postsecretion process results in the release of latency-associated peptide (LAP), which otherwise remains noncovalently attached to the secreted mature peptide (Roberts et al. 1990). As the antibodies used in our localization studies could detect only the active proteins, it is possible that β2 and β3 in these tissues exist in a latent form and a specific signal could trigger the activation step under certain physiological conditions. Another reason for the lack of staining could be the presence of very low levels of active proteins in these tissues, which are undetectable by the antiserum employed. The differential staining pattern of TGF-β3 in the E points to a unique role for β3 in the maturation of sperm. Several genes including growth factors such as nerve growth factor (NGF) are known to exhibit region-specific expression in the subdivisions of epididymis (Cornwall and Hans 1996), although no corpus-specific protein or mRNA has yet been reported. Both TGF-β1 and -β2 have been specifically localized to the germ cell compartment of the rat testis and were shown to display an age- and stage-specific distribution in the cycle of the seminiferous epithelium. This suggests important isoform-specific roles during germ cell maturation (Teerds and Dorrington 1993). Similarly, in boar testis, stage-specific isoform staining for all the TGF-β isoforms and type I and type II TGF-β receptors has been demonstrated (Caussanel et al. 1997). The physiological significance of this differential expression is not known. We could not detect the presence of β3 isoform on the surface of the sperm present in the epididymal lumen. Hence, this isoform, unlike β1 and β2, may exert its effect on sperm maturation indirectly by influencing the secretions from the corpus E. The maturation of the sperm involves epididymal processing of the sperm surface proteins. As TGF-βs are involved in regulating the activity of the matrix proteases by inducing specific inhibitors, a similar corpus-specific function of TGF-β3 would limit the proteolysis initiated in the caput region. More studies are required to elucidate the regulation and the function of β3 in the corpus region. Our attempt to study TGF-β2 distribution in these organs failed to detect any staining or mRNA expression for this isoform.

Preliminary data from our laboratory suggest that TGF-β3 is modulated upon castration in the VP. It would be of interest to see whether testosterone has a similar effect in the modulation of TGF-β1 and -β3 in other male accessory glands, revealing either a common or differential regulation of these two isoforms. In conclusion, in this report we have demonstrated the differential expression and distribution of TGF-β isoforms in the rat male accessory glands and E and suggest that tight regulation of these factors exists.

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