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Transient tenascin enhancement is an early event after androgen ablation in rat prostate

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Abstract Tenascin (tenascin-C), a mesenchymal glycoprotein, is expressed in many tissue remodeling processes. We evaluated tenascin expression during androgen-deprivation-related involution of the rat prostate. At set intervals following castration and subsequent testosterone repletion, prostates were removed in 30 adult rats. Each prostate was immunostained with a polyclonal antiserum against rat tenascin and keratin antibodies specifically directed against exocrine basal cells and luminal cells in the prostate glandular structure. Morphologic impressions were semiquantatively evaluated using a computer-assisted image analysis system. Rat prostates showed a transient increase in the periglandular tenascin expression directly following castration that reached a maximum at day 3. At day 6, tenascin expression was similar to control prostates. This was accompanied by a decrease of cells in the luminal cell layer. The weakest tenascin immunoreactivity was noted on day 14 after androgen withdrawal. This process was reversed by androgen repletion. This study shows that in the rat prostate tenascin expression may be androgen dependent and that during androgen deprivation-related involution tenascin expression is probably associated with tissue remodeling by stromal-epithelial interactions.

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Introduction

Tenascin (tenascin-C) is a multidomain glycoprotein with a hexameric structure consisting of disulfide-linked subunits of 220–320 kDa, each of which comprises various domains with possibly independent functions [5, 21, 23, 31, 35]. Intense tenascin expression is observed during organogenesis [4]. In the mature individual tenascin is normally at a low level; however, its expression can be markedly upregulated in tissues undergoing remodeling as, for instance, during wound healing or in malignant tumors [16]. Studies suggest that tenascin may be involved in tissue remodeling via stromal-epithelial interactions [1, 2, 4]. Tenascin is conserved in all vertebrate species examined [7]. These findings indicate that tenascin has an important role in many biologic and pathologic processes such as cell differentiation, proliferation and tumor progression.

Under normal conditions, levels of tenascin expression in the prostate are low, whereas enhanced tenascin expression in the human prostate is associated with disturbances of the basement membrane and/or basal cell layers, for example, in prostatic intraepithelial neoplasia (PIN) and prostate carcinoma (PCa) [10, 33, 39, 40]. Studies indicate that tenascin expression in prostatic stroma correlates with androgen levels. For example, immunohistochemical studies in developing fetal prostate tissue show strong and diffuse tenascin expression around maturing glands. High levels of tenascin expression are also observed during postnatal development of human prostate, especially during puberty when androgen levels increase significantly [10, 25]. This also indicates the role androgens play in regulating tenascin expression. To date studies show that as soon as 1 week after androgen withdrawal tenascin expression in the prostate is upregulated [33]. However, studies have not

described the effect of androgen deprivation on tenascin expression during the first week following castration.

Using a computer-assisted image analysis system this study addresses the effect of androgen deprivation on tenascin expression starting directly postcastration. We also investigated tissue remodeling within the exocrine epithelial compartment of the prostate, which also results from androgen deprivation, as in this remodeling process tenascin probably plays an important role.

Materials and methods

Tissue specimen

Twenty-seven 6-month-old male Wistar rats were castrated and divided into nine groups of three rats each. At 1, 2, 3, 6, and 14 days postcastration, a group of three rats was killed, prostates were harvested, and immediately stored in liquid N_2 until required. In the remaining 12 rats on day 14, a testosterone-filled silastic capsule was implanted subcutaneously in the flank, this restored serum testosterone to control levels (approx. 3 ng/ml) [11]. At 1, 3, 8, and 12 days postimplantation, a group of three rats was sacrificed, their prostates were collected and processed as described. Prostates from three normal male Wistar rats served as controls.

Immunohistochemistry

Five consecutive frozen sections (5 μm) from each prostate were cut and fixed in acetone for 10 min. After preincubation with normal rabbit serum, one section was incubated for 60 min at room temperature with a polyclonal antiserum against rat tenascin diluted 1:100 (kindly provided by Dr. E.J. Mackie, Addenbrookes Hospital, Cambridge, UK) [17], then rinsed in phosphate-buffered saline (PBS) and incubated with peroxidase-labeled sheep anti-rabbit secondary antibodies (1:100, 30 min.). Peroxidase activity was detected with 3,3 diaminobenzidine (0.5 g/l, Sigma, St Louis, Mo.). Sections were counterstained with hematoxylin. Bovine serum albumin/PBS (1%) was used instead of the primary antiserum as a negative control. The second section was stained with hematoxylin & eosin for histologic purposes.

To assess the influence of androgen manipulation on the epithelial compartments, the other sections were incubated with the keratin (K) antibodies RCK103, RGE53 and RCK107 (courtesy of Dr F Ramaekers, Maastricht, The Netherlands). Antibodies RCK103 and RCK107 both stain basal cells (keratin 5, 14), while RGE53 (keratin 18) stains luminal cells. Detailed characteristics of these antibodies and the protocol used for the immunostaining have been described previously [4, 30].

Evaluation of immunoreactivity

All slides were examined by two authors (Y.X., J.v.d.L.) and an overall assessment of immunostaining was formulated. Immunostaining was histologically assessed and scored. For tenascin the thickness of the rim around prostate glands was graded as narrow, medium or wide. Intensity of immunostaining was scored as weak, moderate or strong. Perivascular staining served as an internal control. Keratin immunostaining was semiquantitatively scored. The thickness of the basal and luminal cell layers was assessed as narrow, medium or broad. Furthermore, heterogeneity or homogeneity of staining was separately noted. The reference point from a narrow rim of tenascin staining was the control rat, which also served as a control for keratin staining.

Expression of tenascin was semiquantitatively assessed using the Vidas^{plus} image analysis system (Kontron, Eching, Germany). Microscopic images were digitized by a 3-chip CCD camera (DXC-325P, Sony) mounted on a conventional light microscope (Axioskop, Zeiss) using a 40× objective (NA = 0.75). The system was calibrated using a set of standardized grey filters. Based on morphology, the rat prostate can be divided into two distinct parts, i.e. ventral and dorsal/lateral lobes [13]. From each lobe, 20 different fields were selected containing prostate glands. Recorded images were shown on an image monitor and tenascin expression was indicated manually using a pointer. Shading correction was applied to the images to correct for unequal illumination of the microscopic field. Optical density of the DAB staining was calculated for every image point within the region indicated by the user. The mean optical density (MOD) of the tenascin immunostaining in the region indicated was calculated and used as a measure for the average amount of tenascin per area unit of prostate tissue. MOD data were evaluated by multiple variance analysis (ONEWAY, $P < 0.0001$). Selected pairs between different time points were analyzed by the Mann-Whitney U-Wilcoxon rank sum W-test at the 0.05 level (SPSS/PC software). This technique was also used to evaluate the effect of androgens on the different cell types stained by the keratin antibodies. The epithelial compartment positive for the respective keratin antibodies in the glandular structure was traced with the pointer and the image analysis system calculated the thickness of the cell layers.

Results

Tenascin expression in normal rat prostates

A narrow periglandular rim of focal and weak staining was noted in the control animals (Fig. 1A).

Tenascin expression in degenerating rat prostates

Prostatic glands showed enhanced tenascin expression directly after castration. One day after castration an increase in staining intensity and distribution within each animal was noticeable. This manifested itself by a broadening of the band of periglandular tenascin expression. The intensity of immunostaining was only slightly increased. Enhancement reached its highest level 3 days after castration. Image analysis supported the morphologic observations. The MOD had increased strikingly during the same period from 0.1101 in control prostates to MOD 0.1562 (Fig. 2A). After day 6, the distribution and intensity of tenascin expression had fallen to below the level of the control animals. During the following 8 days tenascin expression continued to fall, reaching its lowest level 14 days after castration

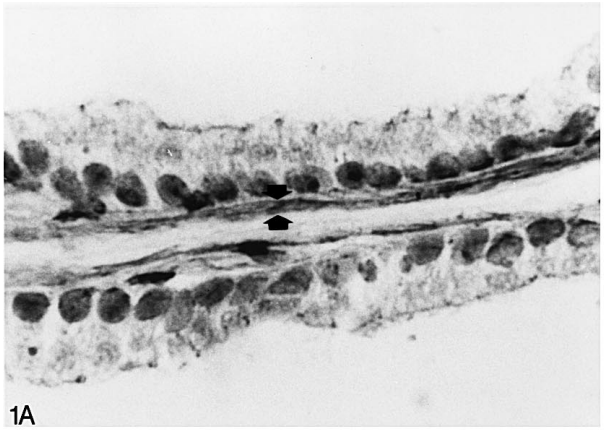
►

Fig. 1 Normal rat prostatic tissue, (**A**) stained for tenascin, (**B**) stained for K18 luminal epithelial cells

Fig. 2 Rat prostate 3 days after castration, showing enhanced tenascin expression (**A**), and a large decrease of luminal epithelial cells stained for keratin 18 (**B**)

Fig. 3 Rat prostate 14 days after castration, tenascin immunoreactivity (**A**), and (**B**) luminal epithelial cells stained for K18

Fig. 4 Rat prostate 8 days after testosterone implantation, (**A**) tenascin expression, (**B**) regenerated luminal epithelial cell layers stained for K18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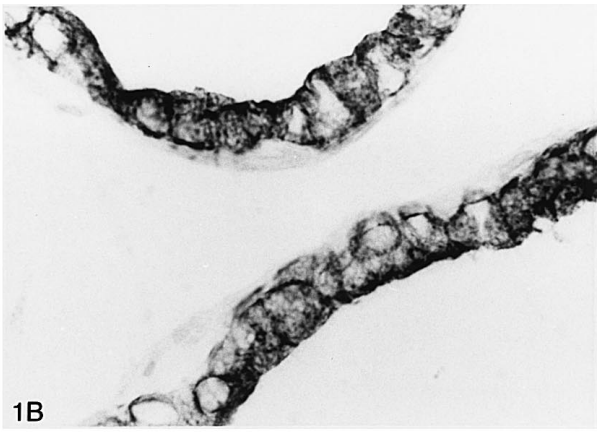
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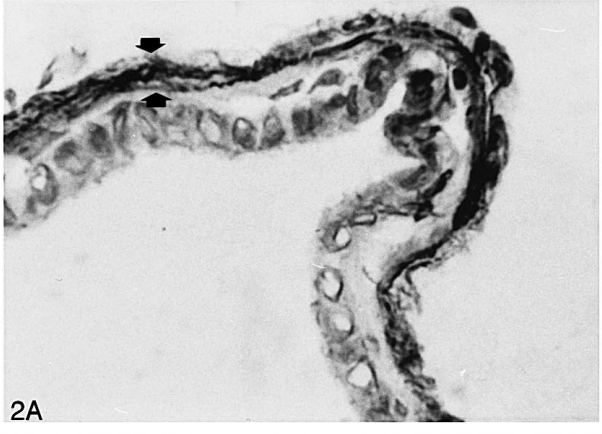
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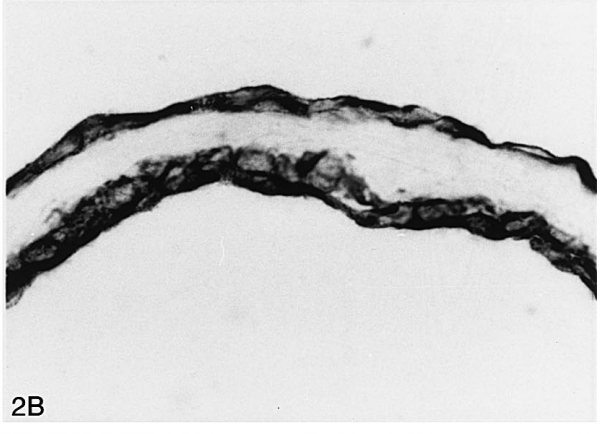
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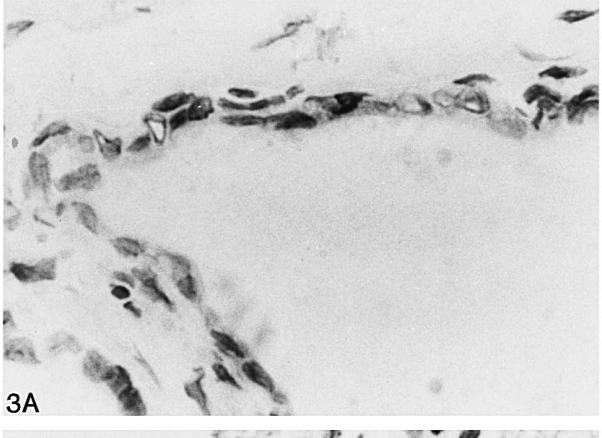
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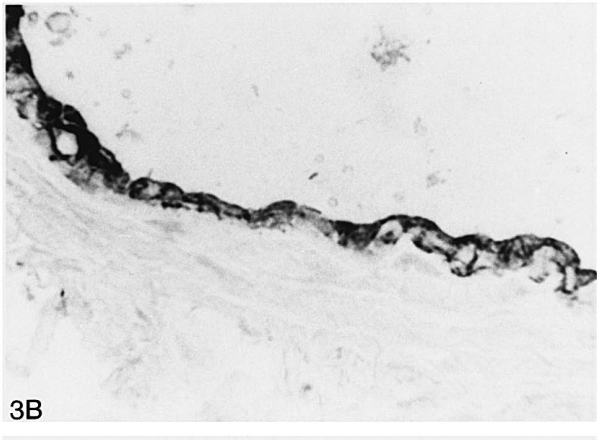
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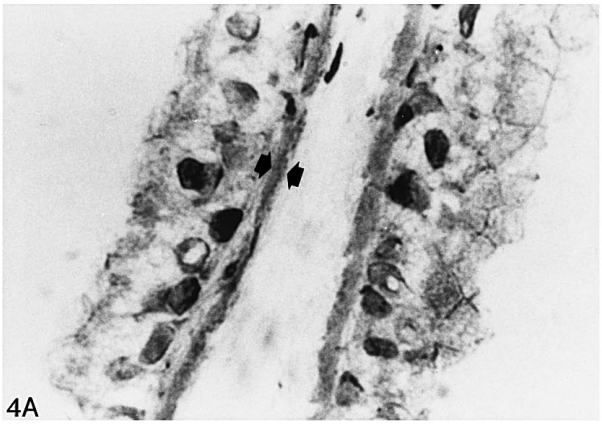
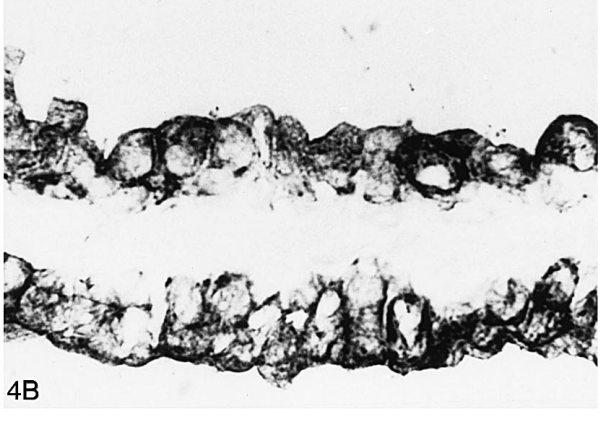

4A


4B

(MOD 0.0481), which was considerably lower than control levels (Fig. 3A).

Tenascin protein expression in regenerating rat prostate

The decreased tenascin expression after androgen depletion appeared to be reversible. Three days after androgen reconstitution tenascin staining had not increased significantly compared with day 14 postcastration. By day 8, however, the staining intensity had increased significantly from MOD 0.0622 on the first day after androgen repletion to MOD 0.0998, this was similar to the noncastrated controls (Fig. 4A). The distribution of staining was also similar to the controls and had become considerably broader in comparison with day 14 post castration.

In Fig. 5 and Table 1 the results are summarized, the mean optical density of tenascin expression in the ventral lobes at different time points is shown. There was a significant difference in tenascin immunostaining between several selected pairs of time points. In the dorsal/lateral lobes the pattern and intensity of tenascin expression during androgen manipulations were identical to the ventral lobes.

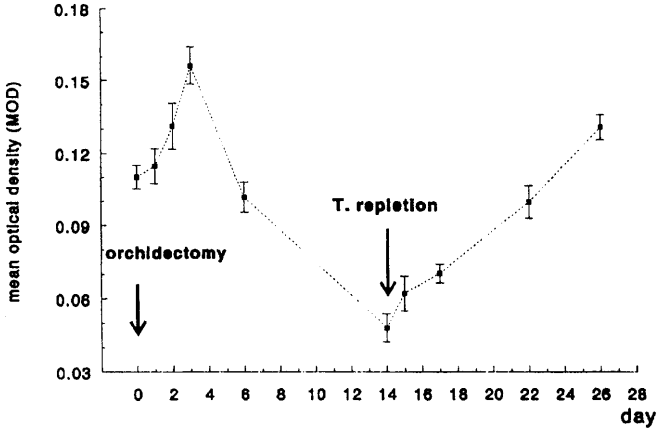


Fig. 5 Mean optical density (MOD) of tenascin (*T*) at multiple time points during androgen ablation and repletion in ventral lobes of rat prostate

Table 1 Mean optical density (MOD) of tenascin immunoreactivity in ventral lobes of rat prostrates ($n = 30$). *S* significant difference, *NS* no significant difference, *SE* standard error

Day	Day 0	Day 6	Day 15	MOD ± SE
0				0.1101 ± 0.0048
1				0.1146 ± 0.0073
2	NS			0.1312 ± 0.0095
3	S	S		0.1562 ± 0.0078
6	NS		S	0.1017 ± 0.0063
14	S			0.0481 ± 0.0058
15	S	S		0.0622 ± 0.0071
17				0.0704 ± 0.0039
22	NS		S	0.0998 ± 0.0068
26	S		S	0.1309 ± 0.0052

Tissue remodeling processes in rat prostate epithelial structure during androgen depletion and repletion

In normal prostate: RCK103 and RCK107 showed a wide rim of staining in the basal cell compartment, RGE53 stained the luminal cells. Following androgen ablation in the H&E stained slides we observed a rapid and dramatic loss of epithelial cells. This decrease was restricted to the K18 positive cells representing the luminal cell compartment. Additionally, we observed a less striking increase of cells in the basal compartment positive for K5 + K14. The decrease in luminal cells became apparent between days 3 and 6 postcastration, after which no further reduction was observed (Figs. 2B, 3B). Testosterone administration to the castrates on day 14 induced a striking regeneration of the luminal epithelial cells. A clear increase in the thickness of luminal-cell layer was apparent on day 8 after androgen repletion reaching the range of the control animals (Fig. 4B). Using the computer-assisted image analysis system, however, we could not objectify the above morphologic impression statistically because of the enormous variation within each animal and within each group.

Discussion

In this study we investigated alterations in tenascin expression starting immediately following androgen withdrawal and immediately after androgen repletion in the rat prostate. We also attempted to quantify previously described effects of androgen withdrawal on the epithelial compartments of the prostate using semiquantitative morphologic methods [30]. By examining tissues at different time points during the androgen manipulation process we were able to demonstrate that in the rat prostate enhanced tenascin expression occurs quickly after androgen withdrawal and parallels the tissue remodeling process observed in the glandular compartment (androgen deprivation-related apoptosis and subsequent glandular involution). By using computer-based image analysis, we were able to semiquantify tenascin immunoreactivity, resulting in a more objective characterization of the process of involution and regression.

Many reports on various tissues under different conditions demonstrate that tenascin may play an important role during development and cell differentiation. This is mainly based on the fact that tenascin distribution is mainly spatially restricted in the periepithelial ECM, during early urogenital development [26], postnatal development of human prostate [25], uterine embryogenesis [14], and in neoplastic breast and colon tissues, and human prostate [9, 24, 40]. Because of its restricted location to areas where S-E interactions occur, differential periepithelial tenascin expression implies an active stromal role during tissue remodeling via S-E interactions. Therefore, we feel that the spatially restricted periglandular differential expression pattern of tenascin

may provide crucial information on tenascin biological functions in the rat prostate during tissue remodeling, subsequent to androgen manipulation. Thus, instead of evaluating the level of tenascin expression in the entire prostate by means of measuring the integrated optical density (IOD), we focused on the spatially restricted periglandular tenascin immunoreactivity in terms of MOD. Together with the descriptive results subjectively obtained, these data can provide information concerning the distributional pattern of tenascin immunoreactivity, and can largely prevent the possibility of misinterpreting the influence of the changes in epithelium/stroma ratio induced by androgen manipulation. Another reason for not using IOD in this study is that this parameter can be easily influenced by many factors, such as the thickness of the tissue sections which is not easily controlled in frozen sections. Obviously, the true level of tenascin expression in the entire prostate is an important parameter, but for this purpose, Western blotting would be more appropriate. Indeed, we attempted to include this in the pilot study; however, because we failed to extract enough tenascin from tissues, application of Western blot or ELISA techniques was not possible in this study.

Consistent with previous studies in human and rat tissues [10, 33, 40], tenascin immunoreactivity was observed in normal rat prostate tissue, albeit at low level. Obviously the swift activation in tenascin expression is closely associated with the effect of androgen withdrawal. Isaacs et al. [12] showed that within 2 h following castration, the serum testosterone level drops to less than 10% of its initial value. By 12–24 h after castration, the intracellular dihydrotestosterone (DHT) level is only 5% of the initial value. The related androgen receptor function changes reach their maximum by 24 h after castration. They result in major epigenetic reprogramming within the glandular cells in which the apoptosis pathway is reactivated [28]. Consistent with this are the indices of in situ DNA end-labeling which reach a maximum level 3 to 4 days after castration [38]. The subsequent fall in tenascin expression between day 4 and day 14 following androgen ablation probably reflects the fact that there has been extensive tissue involution in both epithelial and stromal compartments which results in a decrease in tenascin synthesis and secretion. Therefore, it seems that tissue involution induced by androgen ablation is the effect of an early alteration in the homeostasis of microenvironment in prostates of castrated rats. Interestingly, tenascin immunoreactivity at day 26, i.e. 12 days after androgen repletion, was even higher than that in the control (Table 1). Considering the limited content of testosterone installed in the implanted capsules, it may be possible that 12 days after implantation, the androgen level started to decrease again resulting in another androgen withdrawal and subsequent transient tenascin enhancement.

Previously Völlmer et al. [33] observed an inductive effect on tenascin expression in rat prostates 1 or 2 weeks after androgen withdrawal. However, they did not examine prostate tissues in the first week after androgen withdrawal. In fact, it is in the first week that the rapid and transient enhancement in tenascin immunoreactivity is seen, as we reported, during the first 3 days following androgen ablation. In the aforementioned report, however, the procedure of scoring tenascin expression was not very clearly addressed; for example, we do not know how many animals were included in the study, and we do not know how many areas in one tissue section were scored. Furthermore, only the percentages of cases were available in tables I and II in their paper, and no formula was given for the calculation of scoring index. Finally, comparing the average scoring index from tables I and II in their paper, 1–2 weeks after castration, the standard deviation is up to 0.94 for the castration group (mean was 2.6) while the control group was 1.6 ± 0.5; obviously overlap in tenascin immunoreactivity between these two groups can not be ignored. We feel that it is difficult to intensively evaluate the results in the above-mentioned report and to compare them with ours. Besides differences in materials and methods applied, it is possible that the heterogeneity of ductal glands and the surrounding stroma in the rat prostate may contribute to the inconsistency between these results [8, 18, 19]. Furthermore, it is also possible that tenascin differential expression in response to androgen regulation not only manifests itself in distribution pattern, but also in time-related sensitivity of the various prostate glandular segments. Use of computer-assisted image analysis is expected to reduce the chance of arbitary interpretation of immunostaining, and may give a more objective result [36].

Research indicates that androgens do not affect tenascin expression directly. It is thought that transforming growth factor-β_1 (TGF-β_1) is important in regulating tenascin expression [21, 27, 29]. Furthermore, in rat prostate it has been demonstrated that TGF-β_1 secretion responds quickly to androgen level in a negative fashion [15, 20]. Therefore, TGF-β_1 probably plays a regulatory role in enhancing tenascin expression during castration by the following mechanism: the androgen level has an inverse relationship with the expression levels of TGF-β_1, while this molecule itself positively regulates the tenascin expression. This explains the enhancement of tenascin expression after castration. Furthermore, in this report we also studied the effect of androgen repletion on tenascin expression. Because androgen repletion in the rat prostate correlates with downregulation of the levels of mRNAs for TGF-β_1 and TGF-β_1 type II receptor [15, 20], the enhancement of tenascin expression after androgen supplementation is not likely caused by TGF-β_1. Studies using rat endometrial and mammary tumor models demonstrate that, irrelevant to hormonal control, proliferative activity and a low degree of differentiation of the epithelium can induce tenascin expression, implying that tenascin expression may be a consequence of a hormonally altered differentiation status of a hormone-dependent tissue [34]. In line with these observations, tenascin expression in the prostate is

raised following puberty and gradually decreases as the gland fully matures [25]. This probably accounts for the increased level of tenascin immunoreactivity during androgen repletion-induced tissue regeneration. In addition, many factors besides TGF-β_1 have been described regulating tenascin expression in different cell types [3, 32]. The androgen impact of these factors awaits further study.

In the second part of this study we evaluated whether the increase in tenascin expression following castration was associated with glandular tissue remodeling. The image analysis system could not confirm statistically an increase in the number of basal cells (cells positive for K5 + K14) or a decrease in luminal cells (positive for K18); however, we found this evident morphologically. Furthermore, previous studies have shown that prostate wet weight decreases following castration, and in a quantitative study a decrease in epithelial cells was observed [6, 30, 37]. The reason for failure could be intragroup variation in the rate of epithelial atrophy and/or heterogeneity in the prostate glandular structure itself, because certain cell types and their response to androgens differ with regards to their location in the prostate [22]. However, we feel that even though reliable quantification of the epithelial compartment thickness was not achieved with the image analysis method, the observed changes could represent tissue remodeling following androgen withdrawal when tenascin expression is enhanced.

One can only speculate toward the functional role of tenascin during this process which in vivo is largely unknown. The most prevalent theory is that the anti-adhesive properties of tenascin inhibit cell attachment and may play an essential role in maintaining structural integrity within tissues [23]. Furthermore, tenascin may prevent tissue regression by inducing the formation of a dense hyaline barrier around epithelial structures [16]. The outlined experiment may support this assumption.

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