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ORIGINAL ARTICLE *Andrology*

Glycoconjugates recognized by peanut agglutinin lectin in the inner acellular layer of the lamina propria of seminiferous tubules in human testes showing impaired spermatogenesis

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BACKGROUND: The aim of this study was to evaluate the histochemical characteristics of the thickened inner acellular layer (IL) of the lamina propria specifically present in the human seminiferous tubules of testes showing impaired spermatogenesis.

METHODS: Eighteen biopsies for the investigation of infertility and 10 orchiectomies for testicular cancer and cryptorchidism were used. Lectin staining [peanut agglutinin (PNA), *Maackia amurensis* (MAA), *Sambuccus nigra* (SNA)], PNA lectin staining with sialidase digestion, immunohistochemistry and binding assay of progesterone were performed and analysed quantitatively.

RESULTS: The IL of the thickened lamina propria of the seminiferous tubules in the testes showed PNA lectin affinity and binding affinity for progesterone. Both affinities of MAA and SNA were in the IL of only fairly thickened lamina propria. Furthermore, a positive correlation was present between the thickness of the lamina propria and the accumulation of glycoconjugates showing PNA lectin affinity ($r = 0.829$, $P < 0.001$) or progesterone ($r = 0.629$, $P < 0.001$) in the IL. However, ILs show no immunoreactivities of progesterone receptor, androgen receptor or human serum albumin. Progesterone inhibited the binding affinity of PNA lectin to the IL ($P < 0.001$), but not the affinity to the spermatogenic cells. In addition, sialidase digestion increased the PNA affinity not in the IL but in the spermatogenic cells ($P < 0.001$).

CONCLUSIONS: These results indicate that the IL of the thickened lamina propria always consists of glycoconjugates with PNA lectin affinity and possible binding affinity to progesterone. In addition, the glycoconjugates in the IL may be predictors of abnormal spermatogenesis in the testes of infertile patients.

Key words: glycoconjugates / peanut agglutinin lectin / lamina propria / progesterone / male infertility

Introduction

The lamina propria of human seminiferous tubules is composed of 5–7 incomplete, partly overlapping layers of flat, elongated cells (Bustos-Obregon and Holstein, 1973). These cellular laminae are separated by layers of connective tissue fibres and amorphous ground substances (Bustos-Obregon, 1976). In cases of disturbed spermatogenesis, the lamina propria is often considerably thickened and its inner acellular layer (IL) is situated between the basement membrane and the outer cellular layer (Bustos-Obregon, 1974, 1976 ; Kretser *et al*., 1975; Gulkesen *et al*., 2002). Such a phenomenon is not characteristic in animals other than humans because the construction of the lamina propria appears to be highly species specific (Christl, 1990).

Previously, we showed that the laminae propriae varies in thickness in the seminiferous tubules of testes with different diagnoses, and there

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is a correlation between the thickness of the lamina propria and spermatogenesis in the tubule (Sato et al., 2008). Furthermore, the seminiferous tubule with the thinnest lamina propria does not appear to have a clear IL and shows almost complete spermatogenesis when the patient has no Y chromosome deletion (Sato et al., 2008). There is a possibility that the increase in the lamina propria thickness precedes spermatogenetic failure (Sato et al., 2008). These observations suggest that this morphological characteristic may be an important predictive factor for spermatogenesis in the testes of infertile patients.

To our knowledge, there have been no reports showing a common substance specifically present in the IL of thickened laminae propriae in seminiferous tubules showing impaired spermatogenesis, although there are several reports concerning the substances present in the lamina propria (Paollanen et al., 1985; Christl, 1990; Davidoff et al., 1990; Santamaria et al., 1990; Takaba, 1990; Virtanen et al., 1997; Gulkesen et al., 2002; Sato et al., 2008). For example, laminin, collagen type IV, fibronectin and desmin are present in either or both the basement membrane and the outer cellular membrane of the lamina propria; however, these molecules are not in the IL of thickened laminae propriae (Paollanen et al., 1985; Christl, 1990; Davidoff et al., 1990; Virtanen et al., 1997). Glycoconjugates stained by periodic acid-Schiff, Masson's trichrome or collagens I and III are always observed, independent of thickness of the lamina propria (Paollanen et al., 1985; Christl, 1990; Santamaria et al., 1990; Takaba, 1990; Virtanen et al., 1997): in other words, these substances do not change secondary to the presence of the IL.

On the other hand, glycoconjugates showing affinity for peanut agglutinin (PNA) lectin, that binds preferentially to D-galactose (β-1,3)-*N*-acetyl-D-galactosamine, are observed in the thickened lamina propria of seminiferous tubules of testes in patients with Sertoli cell-only syndrome or Morris's syndrome (Wollina et al., 1989; Gheri et al., 2004). Glycoconjugates that show affinity for PNA lectin may be useful for analysis of the ILs of thickened laminae propriae because in these syndromes, the testes show thickened laminae propriae with obvious ILs (Wollina et al., 1989; Gheri et al., 2004). Furthermore, patients with Sertoli cell-only syndrome or hypospermatogenesis showing some thickening of the lamina propria in the seminiferous tubules, showed abnormal androgen synthetic pathways with predominant progesterone synthesis (Fredricsson et al., 1989a), although progesterone is not one of the predominant metabolites in normal human adult testes (Fredricsson et al., 1989b). Therefore, we suggest that progesterone may accumulate in the thickened lamina propria. There have been no reports showing the localization of progesterone or progesterone receptors (PRs) in the lamina propria. In addition, the relationship among abnormal metabolism of progesterone, thickening of the lamina propria and impaired spermatogenesis remains unclear.

In this study, we examined the substances present in the IL of the lamina propria by cytochemical and immunohistochemical techniques. We utilized PNA lectin, progesterone-related antibodies and fluorescent-conjugated progesterone for the detection of its principal components. Further analyses were performed to identify sialic acid linkage to the glycoconjugates using PNA lectin with enzymatic digestion, *Maackia amurensis* (MAA) lectin and *Sambucus nigra* (SNA) lectin. We also analysed the correlation between thickness of the lamina propria and the amounts of these substances to determine whether they may be used as markers of impaired spermatogenesis.

Materials and Methods

Tissue preparation

Eighteen testicular biopsy specimens from cases of obstructive azoospermia ($n = 3$), varicocele ($n = 11$) and non-obstructive azoospermia ($n = 4$) and orchiectomized specimens from patients with cryptorchidism ($n = 2$) or testicular tumour ($n = 8$) were obtained from St. Marianna University Hospital and Ofuna-Chuo Hospital, Japan. Patients were 32–54 years of age. No patient showed chromosomal abnormality or had a history of hormonal therapy or radiotherapy.

The samples were divided into three pieces soon after biopsy or orchiectomy. One part was frozen in liquid nitrogen immediately and kept at −80°C in a deep freezer for cryosections. The other portions were fixed immediately in Bouin's solution or 10% formalin at 4°C for 24 h and later embedded in paraffin. Cryosections or paraffin sections (5 μM thick) were prepared from each specimen. Samples prepared using different fixatives were analysed separately. The tissues from patients with testicular cancer did not contain any tumour regions, confirmed by haematoxylin and eosin staining. Seminiferous tubules were classified into five types according to the thickness of the lamina propria, from thinnest to thickest (types I–V) as previously described (Sato et al., 2008). Briefly, on transverse section of each seminiferous tubule, the thickness of three randomly selected positions of the lamina propria was measured and the mean thickness was used for classification of tubule type.

This study was approved by the Ethical Committee/Institutional Review Board of St. Marianna University School Hospital and Ofuna-Chuo Hospital. Every participant gave informed consent for the use of their samples in this study.

Lectin histochemistry

Deparaffinized and rehydrated paraffin tissue sections and cryosections fixed with 4% paraformaldehyde (PFA, Sigma, Tokyo, Japan) in phosphate-buffered saline, pH 7.2 (PBS, Wako, Saitama, Japan) were used. Sections were blocked in 3% H_2O_2/methanol and incubated with 40 μg/ml biotinylated PNA lectin that preferentially bound to D-galactose (β-1,3)-*N*-acetyl-D-galactosamine (Vector Laboratories, Burlingame, CA, USA), 20 μg/ml biotinylated MAA lectin that preferentially bound to *N*-acetylneuramic acid (α-2,3) galactose (Vector Laboratories) or 20 μg/ml biotinylated SNA lectin that preferentially bound to *N*-acetyl neuramic acid (α-2,6) galactose/*N*-acetyl-D-galactosamine (Vector Laboratories) for 30 min at 37°C (Maekawa and Nishimune, 1985). As a control, sections were incubated with 40 μg/ml biotinylated PNA lectin in the presence of 200 mM galactose, 20 μg/ml biotinylated MAA lectin in the presence of excess amount of fetuin (Sigma) and 20 μg/ml biotinylated SNA lectin in the presence of excess amount of fetuin (Rogerieux et al., 1993). Peroxidase-conjugated streptavidin (Nichirei Co., Tokyo, Japan) was applied and signals were developed with aminoethylcarbazole (AEC) (Nichirei Co.). Sections were counterstained with haematoxylin and mounted using aqueous permanent mounting solution (Nichirei Co.) with a cover glass. For lectin histochemistry, the samples fixed with Bouin's solution were used for analysis first and results were then confirmed using samples fixed with the other fixatives.

PNA lectin histochemistry with enzymatic digestion

In order to determine whether the glycoconjugates contain sialic acids, sialic acid was removed by pretreatment of the sections with 0.1 unit/ml sialidase; specificity for sialic acids linked to glycoconjugates at α-2,3, α-2,6 and α-2,8 (neuraminidase Type X from *Clostridium perfringens*, Sigma) in 0.25 M sodium acetate buffer, pH 5.5 containing 5.5 mM

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$CaCl_2$ and 154 mM NaCl for 18 h at 37°C before staining with biotinylated PNA lectin (Arenas *et al*., 1998; Gheri *et al*., 2009). To investigate the presence of acetylic groups by deacetylation of sialic acid residues, the sections were pre-incubated with 0.1% KOH in 70% ethanol for 30 min at room temperature before sialidase treatment (Gheri *et al*., 2009). Controls for sialidase digestion were prepared by incubation of sections with enzyme-free buffer before staining with biotinylated PNA (Plendl *et al*., 1989). The samples fixed with Bouin's solution were used for these analyses first and the results confirmed using samples fixed with different fixatives.

Immunohistochemistry

Deparaffinized and rehydrated paraffin sections fixed with 10% formalin were blocked in 3% H_2O_2/methanol or in levamisole (Roche, Tokyo, Japan) as appropriate. After incubation of the slides with 10% goat serum in PBS for 1 h, primary rabbit polyclonal antibodies against PR (pre-commercially diluted) (Nichirei Co.), [1 : 100, PR C-19; Santa Cruz Biotechnology (SCB), Santa Cruz, CA, USA] and androgen receptor (AR) (1 : 100, AR N-20; SCB) or alkaline-phosphatase-labelled anti-human serum albumin (HSA) goat polyclonal antibody (1 : 80; Bethyl Laboratories Inc., Montgomery, TX, USA) were applied to the slides and incubated for 1 h. All antibodies were diluted with 1% bovine serum albumin (BSA, Sigma) in PBS. Each antibody was pre-absorbed with excess antigen: progesterone-3-(*O*-carboxymethyl)oxime BSA (3-CMO-BSA progesterone) (Sigma), blocking peptides of PR(C-19)P or AR(N-20)P (SCB) and HSA (Sigma) adjusted to a working concentration for each primary antibody and used as negative controls. All sections except those that reacted with anti-HSA antibody were incubated with goat anti-rabbit immunoglobulin G (Fab') labelled with an amino acid polymer-peroxidase complex (Histofine Simple Stain MAX PO, Nichirei Co.) for 1 h. The sections were visualized using AEC or First Red (Histofine Simple Stain First Red solution, Nichirei Co.) as appropriate, counterstained and mounted as previously described. All reactions were performed at room temperature.

Binding assay of progesterone

After washing the cryosections with PBS, the sections were incubated with progesterone 3-(*O*-carboxymethyl)oxime: BSA-fluorescein isothiocyanate conjugate (FITC-3-CMO-BSA progesterone, Sigma) at 5 μg/ml in PBS, or with FITC-BSA (Sigma) at 5 μg/ml in PBS as a control, for 30 min at 4°C. Furthermore, pre-incubation with progesterone (Sigma) at 5 μg/ml in PBS was performed for 30 min at 4°C to evaluate the blocking effect for binding sites of progesterone. The stock solution of progesterone was dissolved in ethanol and diluted 200 times with PBS before use. The sections were then washed and mounted using anti-fade fluorescence aqueous permanent mounting solution with a cover glass, and images were recorded using an Olympus fluorescent microscope and a digital camera (Pixera Penguin 600CL; Diractor™, CA, USA). The paraffin sections were also used for these binding experiments to confirm the data using cryosections.

Inhibition assay of lectin binding by progesterone

For this experiment, the samples fixed with Bouin's solution were used for analysis of the data first and then the results were confirmed using samples fixed with formalin or the cryosamples. Deparaffinized and rehydrated paraffin sections or cryosections fixed with 4% PFA/PBS were washed in PBS. The slides were then pre-incubated with progesterone (5 μg/ml in PBS, Sigma) for 30 min at 4°C and after washing with PBS they were incubated with biotinylated PNA lectin. Lectin binding to the sections was detected and the slides were mounted as described previously.

Statistical analysis

The surface areas occupied by immunostaining, lectin affinity or progesterone-binding affinity portions of the IL or spermatogenic cells

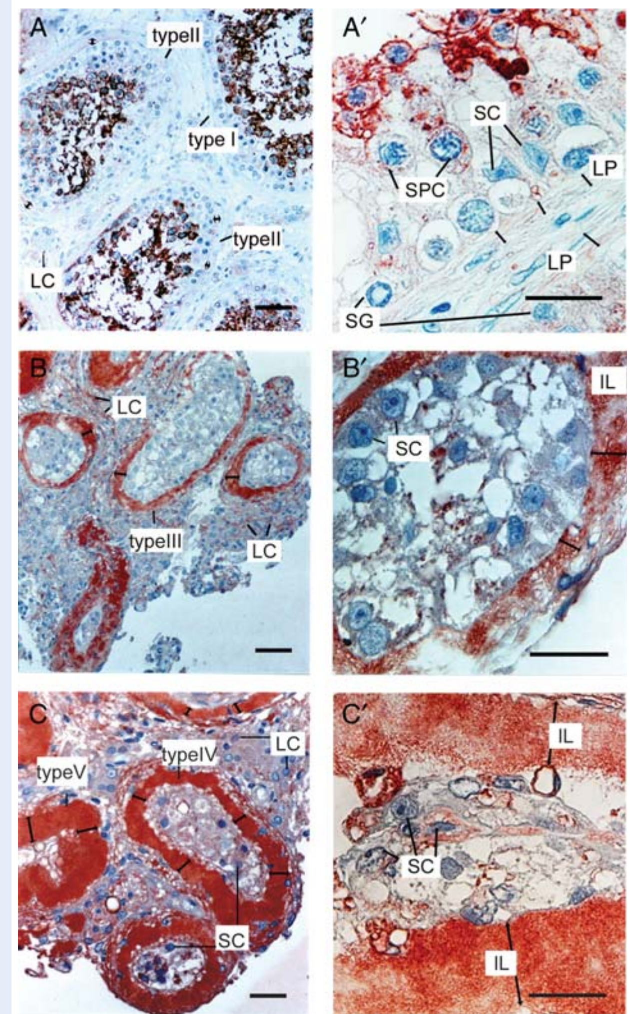

Figure 1 PNA lectin affinity of human testes. (**A**) Types I and II tubules showing strong PNA lectin affinity to spermatogenetic cells. IL in type II tubules showed weak PNA lectin affinity. (**A′**) Magnification of type I tubules showing negative PNA lectin affinity on lamina propria and Sertoli cells (SCs). (**B**) Type III tubule showing strong PNA lectin affinity to the IL of the lamina propria and weak affinity to SC. (**B′**) Magnification of (B). (**C**) Types IV and V tubules showing strong PNA lectin affinity to SC and IL of the lamina propria. (**C′**) Magnification of type V tubule. Leydig cells (LCs) between the tubules (types I–III) with thinner laminae propriae showed weak PNA lectin affinity (A and B); however, the cells between the tubules (types IV and V) with thicker laminae propriae showed strong affinity (C). The signal of PNA lectin affinity was visualized by the red coloration. SG, spermatogonia; SPC, spermatocytes; double arrowheads, lamina propria (LP) (A′), IL of the lamina propria (A, B, B′, C, C′); scale bars, 50 μm (A–C) and 20 μm (A′–C′).

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were measured as optical density or luminance intensity using an automatic image analyser (WinRoof ver. 6; Mitani Co., Ltd., Tokyo, Japan). After the automatic binalization was applied to the images, optical density data were reversed for adjustment to luminance intensity and finally the staining intensity was measured and expressed as arbitrary units. The average arbitrary units of layers or cells labelled with each reagent per experiment was determined from an average of five fields in each sample. The fields examined were chosen randomly by examiners blinded to the tissue treatments. The number of immunopositive Sertoli cells in each type of seminiferous tubules was counted by eye on the histological section. The data were represented as mean value of % immunopositive Sertoli cells/total Sertoli cells in each tubule. All values are expressed as mean ± SD. Student's *t*-test was used to assess all differences between pairs using Windows Excel 2004 (Microsoft Corp., Redmond, WA, USA). The correlations between the data were analysed with Spearman's rank correlation test using StatView 5.0 (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered significant.

Results

Glycoconjugates detected by PNA lectin in testes

First, we examined the PNA lectin affinity in the lamina propria. The affinity of PNA lectin was observed in the clear IL of thickened laminae propriae in types II–V seminiferous tubules (Fig. 1B and C), but not in the thin lamina propria without a clear IL in type I tubules (Fig. 1A) in the testes with various diagnoses. The thickness of the lamina propria showed a positive correlation with PNA lectin affinity in the IL ($r = 0.829$, $P < 0.001$) (Table I). The affinity of PNA lectin to IL showed the same tendency among the different fixatives. Hapten sugar inhibited all PNA lectin binding (data not shown). These results first suggest that there were glycoconjugates showing PNA lectin affinity specifically on the IL of the lamina propria in the testes from patients with various diagnoses.

PNA lectin affinity was also detected in spermatogenic cells and in some Leydig and Sertoli cells (Fig. 1). Some spermatogonia and many spermatocytes showed weak and strong staining of PNA lectin at the cellular membrane, respectively (Fig. 1A′). Sertoli cells showed no affinity to PNA lectin in type I seminiferous tubules with thin laminae propriae (Fig. 1A′), but showed weak affinity to PNA lectin in types II–V seminiferous tubules with thickened laminae propriae (Fig. 1B′ and C′). Leydig cells showed weak affinity to PNA lectin when surrounded by types I–III seminiferous tubules (Fig. 1A and B) but showed strong affinity to PNA lectin when surrounded by types IV and V seminiferous tubules (Fig. 1C). These staining patterns were observed in all categories of diagnosis.

PNA lectin histochemistry with enzymatic digestion

Both ILs of thickened laminae propriae and spermatogenic cells showed PNA lectin reactivity, regardless of whether the samples were subjected to sialidase or deacetylation-sialidase treatment (Fig. 2A–F and Table II). The intensity of PNA lectin affinity on spermatogenic cells after sialidase treatment was higher than that in samples not subjected to sialidase treatment ($P < 0.001$) (Fig. 2A and C; Table II). Furthermore, the intensity of PNA lectin affinity after deacetylation-sialidase treatment was slightly higher than that after sialidase treatment in spermatogenic cells ($P < 0.001$) (Fig. 2A and E; Table II). In contrast to the PNA lectin affinity to spermatogenic cells, the intensity of PNA lectin affinity on ILs of thickened laminae propriae after sialidase and deacetylation-sialidase treatment was not increased compared with that in samples that were not treated ($P < 0.001$) (Fig. 2B, D and F; Table II). These staining patterns were observed in all categories of diagnosis and did not differ among the different fixatives.

MAA lectin and SNA lectin histochemistry in human testes

Furthermore, ILs of thickened laminae propriae in types III –V tubules showed weak and non-homogeneous MAA lectin reactivity and faint SNA reactivity (Fig. 2G and H) although IL in types I–II did not show any staining. Compared with the lectin affinity in ILs of thickened laminae propriae, strong MAA affinity was observed in both inner and outer membranes of laminae propriae (Fig. 2G) and in Sertoli cells, and strong SNA affinity was in Leydig cells, Sertoli cells and myoid cells (Fig. 2H). Spermatogenic cells did not show any staining of SNA and weak staining of MAA as same as the previous report by Arenas *et al*. (1998) (data not shown). Hapten sugar inhibited both MAA and SNA binding (data not shown). These staining patterns were observed in all categories of diagnosis and did not differ among the different fixatives.

Immunolocalization of progesterone in human testes

The immunohistochemical study revealed that progesterone was present in the clear IL of the thickened lamina propria in types II–V

Table I PNA lectin affinity and immunolocalization of progesterone in lamina propria of the human seminiferous tubules.

	Seminiferous tubules classified by thickness of lamina propria				
	Type I	Type II	Type III	Type IV	Type V
PNA	0.71 ± 1.39^a	6.27 ± 3.70^b	23.94 ± 9.00^c	44.28 ± 8.40^d	43.45 ± 5.53^d
Progesterone	0^a	2.94 ± 0.81^b	8.33 ± 1.34^c	17.57 ± 1.09^d	20.16 ± 1.09^e

Values are mean ± SD. Data (arbitrary units) with a different superscript within each line are significantly different (Student's *t*-test, $P < 0.005$).

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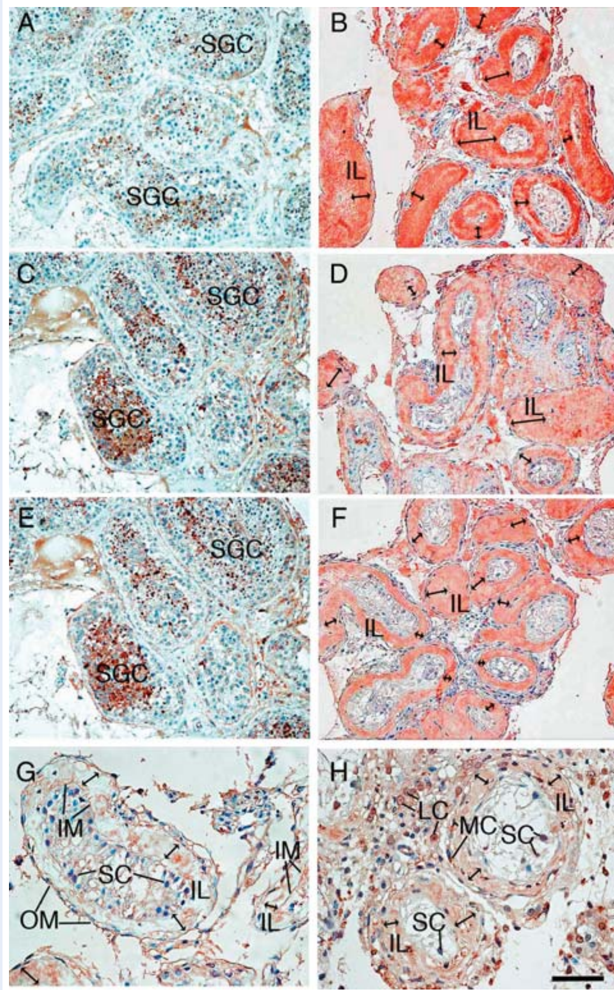

Figure 2 PNA lectin affinity after sialidase digestion, *Maackia amurensis* II (MAA) lectin affinity and *Sambuccus nigra* (SNA) lectin affinity in human testis. (**A**) PNA lectin affinity in the spermatogenic cells (SGCs). (**B**) PNA lectin affinity in the inner acellular layers (IL) of thickened laminae propriae. (**C**) Increased PNA lectin affinity after sialidase treatment in the spermatogenic cells. (**D**) Decreased PNA lectin affinity after sialidase treatment in the ILs of thickened laminae propriae. (**E**) Increased PNA lectin affinity after deacetylation-sialidase treatment in the spermatogenic cells. (**F**) Decreased PNA lectin affinity after deaetylation-sialidase treatment in the ILs of thickened laminae propriae. All the PNA lectin histochemistry were performed after pre-incubation in the solution of sodium acetate buffer with (C–F) or without sialidase (A and B). (**G**) MAA lectin affinity (red) in IL of thickened lamina propria and high affinities in inner and outer membranes (IM, OM) of laminae propriae and SC, (**H**) Faint SNA reactivity (red) in the IL of thickened lamina propria and strong reactivity of SNA in myoid cells (MCs), some SCs and LCs. The signal of lectin affinity was visualized by the red coloration. Scale bars, 100 µm (A–F) and 50 µm (G and H).

seminiferous tubules (Fig. 3B and C), but not in the thin lamina propria in type I tubules (Fig. 3A) in the testes with various diagnoses. There was a positive correlation between the thickness of the lamina propria in seminiferous tubules and progesterone immunoreactivity in the IL ($r = 0.629$, $P < 0.001$) (Table I).

Table II PNA lectin affinity after enzyme digestion in laminae propriae of human seminiferous tubules and spermatogenic cells.

	PNA	Neu-PNA	KOH-Neu-PNA
IL	46.75 ± 8.17^{a}	30.44 ± 8.29^{b}	28.59 ± 6.67^{b}
SPC	56.47 ± 14.24^{a}	75.39 ± 18.36^{b}	89.44 ± 11.54^{c}

Values are mean ± SD. Data (arbitrary units) with a different superscript within each line are significantly different (Student's *t*-test, $P < 0.001$).
IL, inner acellular layer of thickened lamina propria; SPC, spermatocytes; Neu, neuraminidase (sialidase) treatment; KOH, 0.1% KOH in 70% EtOH (deacetylation) treatment.

Spermatogenic cells (spermatocytes, round spermatids and acrosomes of elongated spermatids) showed weak immunostaining of progesterone in type I seminiferous tubules (Fig. 3A). Immunoreactivity in both nuclear and cytoplasmic regions was observed in Leydig cells (Fig. 3A–C) and in most nuclei of Sertoli cells (Fig. 3A and B). There was a weak positive correlation between the thickness of the lamina propria in seminiferous tubules and the progesterone immunoreactivity in Sertoli cells ($r = 0.479$, $P < 0.0001$) (Table III).

These staining patterns were observed in all categories of diagnosis. The antigen-absorbed control did not show any immunostaining (Fig. 3A′–C′).

Binding affinity of progesterone to human testes

Next, we examined the binding affinity of progesterone to the ILs of thickened laminae propriae. FITC-3-CMO-BSA progesterone showed stronger binding affinity to the ILs of thickened laminae propriae (19.69 ± 2.29) in testes showing impaired spermatogenesis than FITC-BSA (2.03 ± 0.23, $P < 0.0001$) (Fig. 4A and B). Furthermore, pre-incubation of the sections with excess progesterone significantly inhibited the binding affinity of FITC-3-CMO-BSA progesterone to the ILs of thickened laminae propriae (3.20 ± 0.47, $P < 0.0001$) (Fig. 4C). These results indicate that ILs of laminae propriae had sufficient binding affinity for the preservation of progesterone.

Sertoli cells, Leydig cells and spermatogenic cells showed strong affinity to FITC-3-CMO-BSA progesterone in testes showing impaired spermatogenesis (Fig. 4A), although they showed weak affinity to FITC-BSA (Fig. 4B). In addition, pre-incubation of the sections with excess progesterone inhibited the binding affinity of FITC-3-CMO-BSA progesterone to Sertoli cells, Leydig cells and spermatogenic cells (data not shown). These results indicate that Sertoli cells, Leydig cells and spermatogenic cells also have binding affinities to progesterone.

These affinities were also observed in the paraffin sections although they were slightly weak. These binding patterns were observed in all categories of diagnosis in which the testes possessed seminiferous tubules with ILs of thickened laminae propriae.

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Immunolocalization of AR, PR and HSA in testes showing impaired spermatogenesis

To determine the progesterone-binding partner in the IL, we examined the occurrence of PR, AR and HSA, which were progesterone-binding candidates. PR, AR and HSA were not detected in the ILs of thickened laminae propriae by immunohistochemistry in any case (Fig. 5A–C). Furthermore, AR, PR and HSA were not detected in the any thickness of laminae propriae (data not shown). These results show that the binding of progesterone to the IL of the lamina propria was not dependent on the occurrence of PR, AR or HSA.

PR was localized in the spermatogenic cells as previously shown (Shah et al., 2005). PR and AR were expressed in both Leydig and Sertoli cells (Fig. 5A and B). The thickness of the lamina propria showed a weak negative correlation with the AR expression in Sertoli cells ($r = -0.263$, $P = 0.002$) but not with the PR expression in Sertoli cells ($r = -0.040$, $P = 0.495$) (Table III).

The antigen pre-absorbed control did not show any immunostaining (data not shown). These staining patterns were observed in all samples.

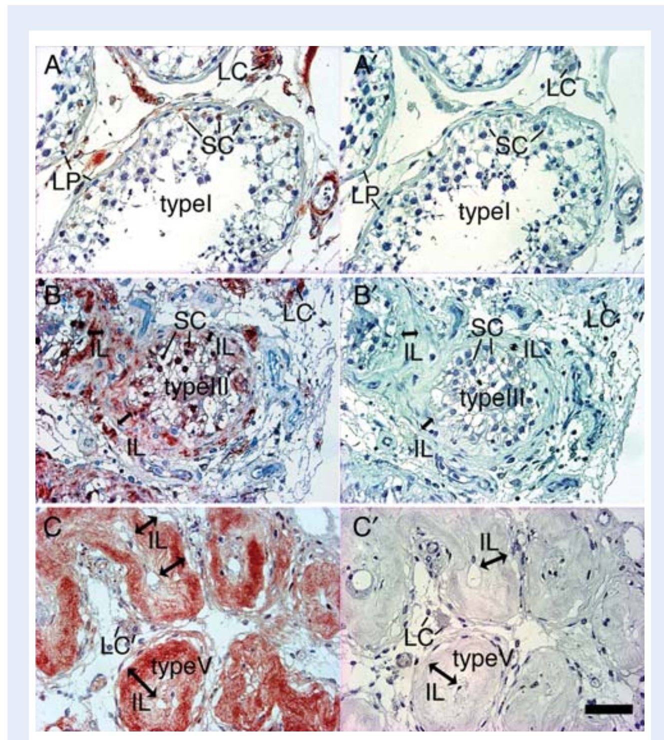

Figure 3 Immunoreactivity of anti-progesterone antibody in human testes. (**A**) A few progesterone-immunoreactive SC were observed in type I tubules. The lamina propria of type I tubules did not show any immunoreactivity. LC showed strong immunoreactivity; however, spermatogenic cells (spermatocytes, round spermatids and elongated spermatids) and some MC showed weak immunoreactivity. (**A′**) Negative control of (A). (**B**) Progesterone immunoreactivity was detected in SC, MC, LC and the IL of the thickening lamina propria (LP) in type III tubules. (**B′**) Negative control of (B). (**C**) Strong progesterone immunoreactivities were detected in MC and ILs of the very thick lamina propria in type V tubules. (**C′**) Negative control of (C). As negative control, progesterone antibody pre-absorbed with excess antigen was used for staining. Positive immunoreactivity produced a red coloration. Double arrowheads, IL of the lamina propria; scale bar, 50 µm.

Inhibition of binding of PNA lectin by progesterone

Furthermore, we examined whether progesterone inhibited the affinity of PNA lectin to IL because of their coincident localization. Pre-incubation of the slides with progesterone strongly inhibited PNA lectin from binding to the ILs of thickened laminae propriae (PNA, 46.78 ± 6.59; progesterone + PNA, 9.40 ± 1.07; $P < 0.001$) (Fig. 6).

Progesterone did not inhibit PNA lectin binding to germ cells in the testes in the same manner as for IL (PNA, 77.94 ± 11.92; progesterone + PNA, 77.83 ± 6.89; $P = 0.496$) (Fig. 6). Pre-incubation of the slides with progesterone slightly inhibited the binding to both Sertoli and Leydig cells (Fig. 6A–D).

These results indicate that glycoconjugates on spermatogenic cells detected using PNA lectin differed from those of the IL because the binding affinity to the cells was not inhibited by progesterone (Fig. 6E). These staining patterns were observed in all categories of diagnosis and did not differ among the different fixatives.

Discussion

The present report shows that there are glycoconjugates demonstrating PNA lectin affinity specifically to the IL of the lamina propria in the human testes, and that the amount of the glycoconjugates increased in accordance with the thickness of the lamina propria. Furthermore, the glycoconjugate was not observed in type I tubules without an obvious IL showing complete spermatogenesis. We previously demonstrated that the increase in the lamina propria thickness proceeded

Table III Immunolocalization of progesterone, PR and AR of Sertoli cells in the human seminiferous tubules.

	Seminiferous tubules classified by thickness of lamina propria				
	Type I	Type II	Type III	Type IV	Type V
Progesterone	54.53 ± 14.92^{a}	55.59 ± 26.4^{a}	65.39 ± 28.42^{b}	82.01 ± 19.48^{c}	86.58 ± 16.81^{c}
PR	96.09 ± 5.51^{a}	$98.50 \pm 2.73^{b,c}$	$97.55 \pm 5.19^{a,c}$	96.03 ± 7.40^{a}	$98.97 \pm 2.83^{b,c}$
AR	97.43 ± 3.04^{a}	81.98 ± 25.69^{b}	$77.15 \pm 38.82^{b,d}$	$74.06 \pm 32.52^{b,d}$	$67.60 \pm 39.45^{c,d}$

Values are mean ± SD%. Data (% immunopositive Sertoli cells/total Sertoli cells) with a different superscript within each line are significantly different (Student's t-test, $P < 0.05$).

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spermatogenetic disturbance (Sato *et al.*, 2008). These findings suggest that the glycoconjugates showing PNA lectin affinity to the lamina propria may also precede impaired spermatogenesis.

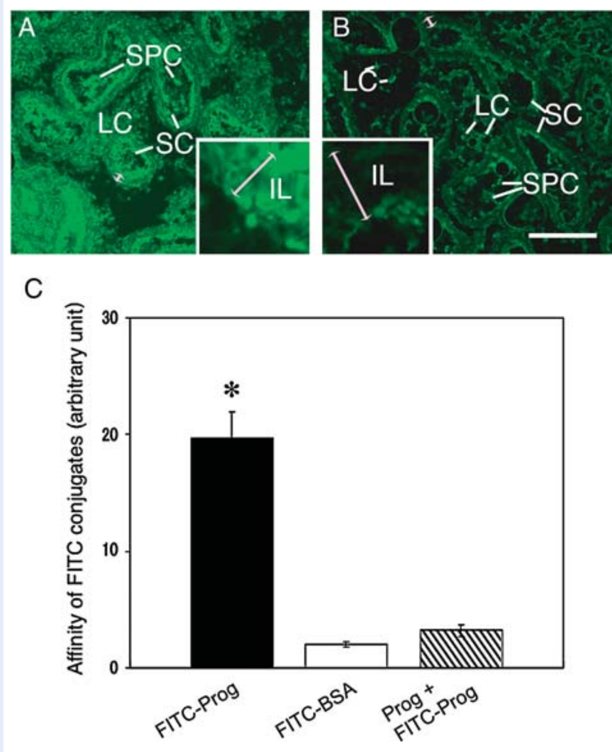

Figure 4 Binding of progesterone to the testes showing impaired spermatogenesis. (**A**) Fluorescein isothiocyanate (FITC)-3-CMO-BSA progesterone showed strong binding (green) to the IL of thickened laminae propriae, LC, germ cells [spermatocytes (SPC)] and SC. Inset: The IL (pink double arrowheads) was stained with FITC-3-CMO-BSA progesterone. (**B**) FITC-BSA (green) showed quite low affinity to IL and germ cells, but still showed binding to SC and LC. Inset: The IL (pink double arrowheads) was not stained with FITC-BSA. (**C**) Affinity of FITC conjugates to IL. Asterisk indicates the significant difference among all pairs of the groups (Student's *t*-test, $P < 0.0001$). FITC-Prog, FITC-3-CMO-BSA progesterone; Prog, pre-incubation with progesterone; scale bar, 100 μm (each inset is an ×8 magnification of each original).

Sialic acids are frequently found as terminal units of glycoconjugate oligosaccharides. PNA lectin affinity after sialidase digestion showed that at least the sialic acids linked α-3, α-6 or α-8 to galactose or *N*-acetylgalactosamine interfered with the binding affinity of PNA lectin to glycoconjugates not in IL but in spermatogenic cells, even if there was sialic acid binding. These results of spermatogenic cells using different fixatives confirmed the previous reports (Arenas *et al*., 1998; Gheri *et al*., 2009). Furthermore, the results of both affinities of SNA lectin and MAA lectin showed that the ILs of thickened laminae propriae contained the sialic acids linked α-3 or α-6 to galactose or *N*-acetylgalactosamine only when the lamina propria was fairly thickened. In other words, the amounts of the sialic acids linked α-3 or α-6 to galactose or *N*-acetylgalactosamine did not increase coincident with increase of the glycoconjugates showing affinity to PNA lectin.

The results of the present study with SNA lectin affinities to lamina propria confirmed both of the positive staining results in aged testes and the almost negative staining results in testes from young men fixed with Carnoy's solution (Gheri *et al*., 2009). The seminiferous tubules of aged testes are expected to have thickened lamina propria because of the increase in the thickness of lamina propria with aging (Johnsen *et al*., 1986). On the other hand, the formalin-fixed autopsy samples showing complete spermatogenesis which were used for SNA lectin staining by Arenas *et al*. (1998) might not contain fairly thickened lamina propria in seminiferous tubules, leading to no reactivity of SNA lectin in lamina propria as well as our present results in thinner lamina propria, because there is a correlation between the thickness of lamina propria and the spermatogenesis disturbance (Sato *et al*., 2008). Contradictory reports of SNA lectin affinity in lamina propria of seminiferous tubules (Arenas *et al*., 1998, Gheri *et al*., 2009) may not only result from use of different fixatives but also may be caused by differences in thickness of the lamina propria among samples.

Furthermore, the MAA lectin specificity also revealed the weak affinity to IL of fairly thickened lamina propria, as in the previous study of Gheri *et al*. (2009) using aged men, who are expected to have seminiferous tubules with thickened lamina propria. However, compared with the SNA lectin staining, MAA lectin also showed the higher affinities on both inner and outer membrane of the thinner lamina propria. This staining of the membranes of the lamina propria may explain the results by Arenas *et al*. (1998) who showed the MAA lectin affinity in all seminiferous

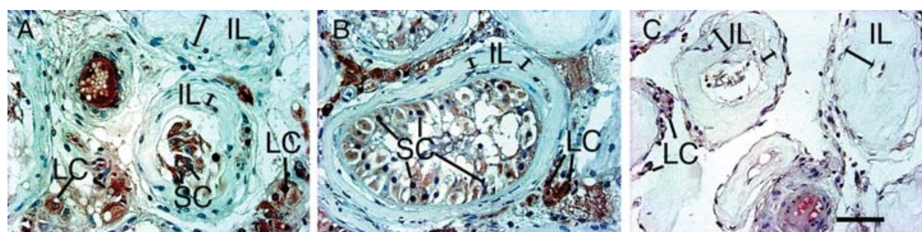

Figure 5 Immunohistochemistry of PR, AR and HSA on the IL of the thickened lamina propria. The IL of the thickened lamina propria in the seminiferous tubules showed negative staining with anti-PR antibody (**A**), anti-AR antibody (**B**) and anti-HSA antibody (**C**). Positive immunoreactivity produced a red coloration. Antigen pre-absorbed antibody did not show any staining on any sample. Double arrowheads, IL of the lamina propria; scale bar, 50 μm.

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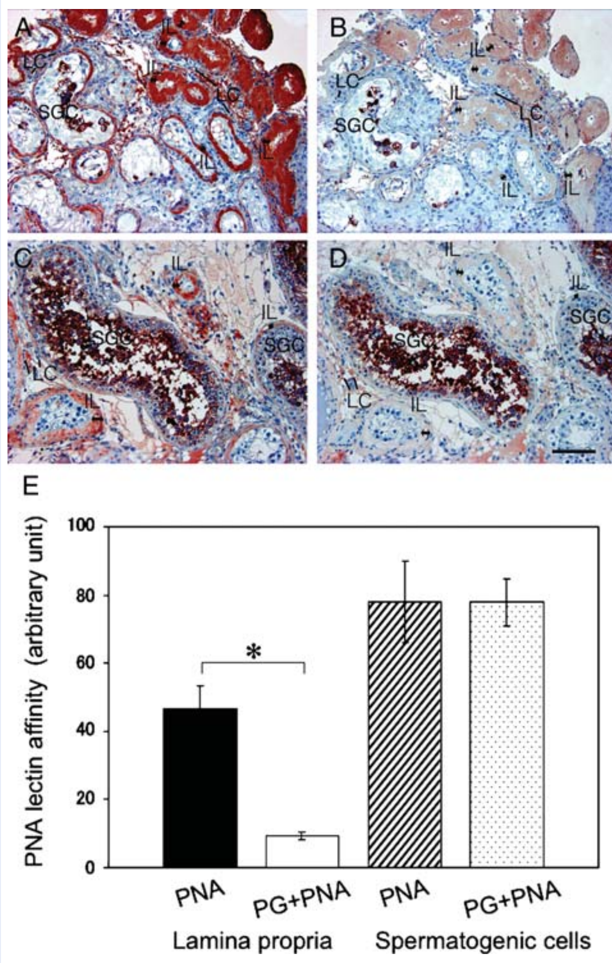

Figure 6 Inhibition of binding of PNA lectin by progesterone. (**A**) High affinities of PNA lectin to the IL of the thickened lamina propria, SGC and LC in the testes with impaired spermatogenesis. (**B**) PNA lectin affinity to the SGC and faint affinity to the IL of the thickened lamina propria and LC in the testes showing impaired spermatogenesis after pre-incubation with progesterone. (**C**) High affinities of PNA lectin to various stages of SGC, IL of the thickened lamina propria and LC in the testes showing spermatogenesis. (**D**) PNA lectin affinity to SGC and faint affinity to the IL of the thickened lamina propria and LC in the testes showing spermatogenesis after pre-incubation with progesterone. The signal of PNA lectin affinity was visualized by the red coloration. Double arrowheads, IL of the thickened lamina propria; scale bar, 50 µm. (**E**) PNA lectin affinity to IL of the thickened lamina propria and SGC with or without pre-incubation of progesterone. Asterisk indicates the significant difference in PNA lectin affinities between with (PG + PNA) and without pre-incubation of progesterone (PNA) (Student's *t*-test, $P < 0.001$).

tubular components, including lamina propria, independent of patient age.

Further examination showed that the IL of the thickened lamina propria demonstrated binding affinity to progesterone. The amount of progesterone in the lamina propria increased synchronously with the increasing thickness of the lamina propria. One of the reasons for the higher concentration of progesterone in testes of patients with Sertoli cell–only syndrome or hypospermatogenesis (Fredricsson *et al.*, 1989a,b) may be related to the fact that their testes mainly contained seminiferous tubules with thickened laminae propriae.

The binding of progesterone to the IL of the lamina propria was not dependent on the presence of progesterone-binding candidates (AR, PR and HSA). The amount of both progesterone and glycoconjugates showing affinity to PNA lectin in the IL increased in parallel to the increase in the thickness of the lamina propria. There has been no report that the galactosyl (β-1,3)-*N*-acetylgalactosamine structure can directly bind to progesterone. However, we can consider that the progesterone-binding site in the IL may be in, or near, the glycoconjugates of the PNA lectin binding site in the IL, because PNA lectin affinity to the IL was specifically inhibited after pre-incubation with progesterone.

Glycoconjugates on the IL detected by PNA lectin were thought to differ from those of spermatogenic cells because the binding affinity to the cells was not inhibited by progesterone. In addition, there was a difference in the linkage of sialic acids and glycoconjugates showing PNA lectin affinities between the IL and the spermatogenic cells. In future studies, we need to fully characterize the glycoconjugates specifically in IL that show affinity to PNA lectin and progesterone-binding.

The thickening lamina propria in the seminiferous tubules of testes of humans with pathological conditions is thought to prevent reciprocal regulatory effects between the cells inside and outside of the lamina propria (Kretser *et al.*, 1975). Progesterone has the binding affinity to AR (Freyberger *et al.*, 2010), which is thought to be important for spermatogenesis. The increased level of glycoconjugates in the IL may have a protective role for spermatogenic cells in the seminiferous tubules by blocking the influx of an excess amount of the progesterone from the stroma.

Sertoli cells express several proteins that are related to the signals essential for the development and differentiation of germ cells (Sharpe *et al.*, 2003). Some of these expression levels differ between testes showing normal spermatogenesis and those with impaired spermatogenesis (de Kretser *et al.*, 1998; Silva *et al.*, 2002; Sharpe *et al.*, 2003). Ours is the first study to show that the characteristics of Sertoli cells tend to change according to the thickness of the lamina propria. These cellular changes may be caused by the inhibition of the reciprocal regulation between the cells by the thickened lamina propria. The thickness of the lamina propria showed a weak correlation with Sertoli cell possession of progesterone, and a weak negative correlation with the AR, but not PR, expression in Sertoli cells. AR expression in Sertoli cells is mainly observed in mature Sertoli cells after the pre-pubertal period, suggesting a role in spermatogenesis (Sharpe *et al.*, 2003). A decrease in the expression of AR on Sertoli cells in seminiferous tubules with thickened laminae propriae may induce impaired spermatogenesis. Furthermore, in addition to reducing number of AR, the AR may not work as effectively because progesterone interferes with testosterone binding to AR (Tindal *et al.*, 1984).

The affinity to PNA lectin in Sertoli cells increased in the seminiferous tubules with thickened ILs. There are reports concerning an increase in PNA lectin binding sites in cells in culture in the presence of estradiol and progesterone (Daxenbichler *et al.*, 1986). The

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presence of progesterone in both Sertoli cells and thickened ILs may increase PNA-binding sites in both cells and the IL.

In conclusion, this study provides new and important information about the substances (such as progesterone and glycoconjugates recognized by PNA lectin) in the IL of the lamina propria. In future studies, we need to characterize the glycoconjugates and investigate the role of these substances in the changes of the IL of the lamina propria because the IL is a specific feature of seminiferous tubules showing spermatogenetic disturbances and may be a predictor of abnormal spermatogenesis.

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Authors' roles

Y.S. designed and performed the experiments, interpreted the results and wrote the manuscript. S.N. performed the interpretation of the results. M.Y. performed histological examination. T.O. performed statistical analysis of the data. T.I. organized collecting samples and supervised this study.

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Conflict of interest

None declared.

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