

Estrogen receptor β -deficient female mice develop a bladder phenotype resembling human interstitial cystitis

Otabek Imamov*, Konstantin Yakimchuk*, Andrea Morani*, Thomas Schwend*, Osamu Wada-Hiraike*, Sergei Razumov†, Margaret Warner*, and Jan-Åke Gustafsson**

*Department of Biosciences and Nutrition, Karolinska Institute, Novum, SE-141 86 Stockholm, Sweden; and †Moscow Institute of Urology, 3-Parkovaya 51, Moscow 105425, Russia

Contributed by Jan-Åke Gustafsson, April 18, 2007 (sent for review March 18, 2007)

Interstitial cystitis/painful bladder syndrome is a disease seen mostly in women, and symptoms tend to be worse premenopausally or during ovulation. The four cardinal symptoms of interstitial cystitis/painful bladder syndrome are bladder pain, urgency, frequency, and nocturia. Estrogen has been implicated in the etiology of this disease, but the role of the two estrogen receptors (ER), ER α and ER β , has not been investigated. We found that, in the bladders of WT mice, ER β is expressed in the basal cell layer of the urothelium. Bladders of male ER $\beta^{-/-}$ mice were intact and morphologically indistinguishable from those of their WT littermates. However, in female ER $\beta^{-/-}$ mice, there was ulceration and atrophy of bladder urothelium concomitant with infiltration of $\gamma\delta$ T cells concentrated in the areas of atrophy and shedding of urothelium. The data support the idea that activated $\gamma\delta$ T cells are causing the damage to the urothelium. The hyperactivity of T cells may be because of an imbalance between ER α and ER β signaling in female ER $\beta^{-/-}$ mice. Our data suggest that reduced ER β signaling might have a role in the pathogenesis of interstitial cystitis, and ER β could be a candidate for a target of medical therapy.

$\gamma\delta$ T cells | urothelium | painful bladder syndrome

The etiology of interstitial cystitis (IC) is unknown, and available treatment options are limited and mostly palliative. There is a clear role for the immune system in this disease: increased numbers of T lymphocytes, particularly $\gamma\delta$ T cells, abnormal urothelial HLA, and overexpression of HLA class II (1) in the urothelium and lamina propria in patients with IC strongly suggest a role for autoimmunity in development of IC (2). Because IC is almost exclusively a disease of young women, and symptoms tend to worsen premenopausally or during ovulation, the role of estrogen receptors (ERs) is likely to be important.

The bladder is one of those tissues that were long thought to be nonestrogen-responsive on the basis of the lack of ER α expression. The discovery of the second ER, ER β , provided an explanation for the effects of estrogen in several ER α -negative tissues such as the prostate, intestine, and lung, where ER β serves as a modulator of terminal differentiation of epithelium (3–7). ER β and ER α knockout mice (ER $\beta^{-/-}$ and ER $\alpha^{-/-}$) have been invaluable in analyzing and dissecting the functions of ER β and ER α (8, 9).

The predominant ER in the bladder is ER β , with very little ER α expression (10, 11). Reduced ER β expression levels have been reported in the bladders of rats with chemically induced cystitis (12). In the present study, with the use of ER $\beta^{-/-}$ mice, we have investigated the role of ER β in the bladder urothelium. We report that, in female ER $\beta^{-/-}$ but not in ER $\alpha^{-/-}$ mice, there is autoimmune destruction of urothelium resembling IC.

Results

Immunohistochemical staining of WT mouse bladder sections showed that ER β is expressed in bladder urothelium and localized in the basal cell layer (Fig. 1). In the bladders of female

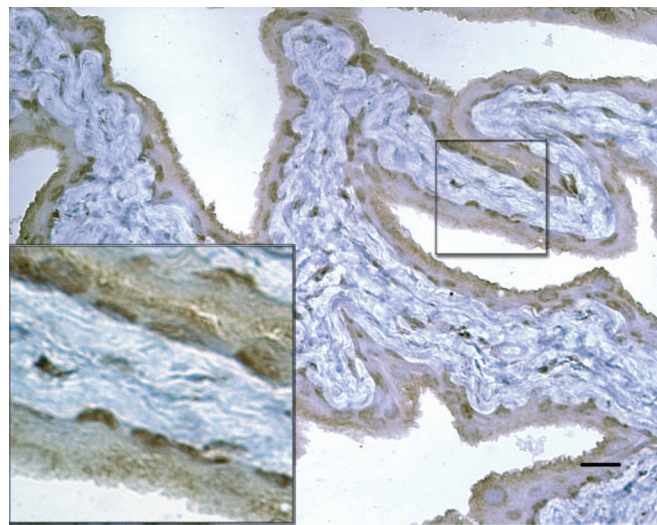


Fig. 1. ER β expression in female bladder section. Note positive nuclei in basal layer of cells. (Magnifications: main image, $\times 20$; Inset, $\times 60$.)

ER $\beta^{-/-}$ mice, there were specific morphological changes, i.e., ulceration and atrophy of bladder urothelium. A comparison of the differences in morphology between ER $\beta^{-/-}$ mice and their WT littermates is shown in Fig. 2. At 8 months of age, there are epithelial atrophy, massive ulcerations of the bladder, and invasion of immune cells in the stroma and epithelium in ER $\beta^{-/-}$ mice. Bladders of male ER $\beta^{-/-}$ mice were intact and morphologically indistinguishable from their WT littermates. The morphological changes became evident when mice were 6 weeks of age, which corresponds to the time of puberty in mice. There was progressive destruction of the urothelium with age.

Bladder permeability assessed by measuring the serum/urine concentration coefficient of sodium fluorescein after it had been noninvasively instilled into the bladder revealed a significantly higher bladder permeability in female ER $\beta^{-/-}$ mice (0.12 ± 0.009) than in WT littermates (0.07 ± 0.03 ; $P = 0.01$). Bladder permeability in ER $\alpha^{-/-}$ female mice was not different from that of their WT littermates (Fig. 3).

There was no difference between ER $\beta^{-/-}$ and WT male mice in the total glucosaminoglycan (GAG) content of urine (male

Author contributions: S.R., M.W., and J.-Å.G. designed research; O.I., K.Y., A.M., and O.W.-H. performed research; O.I., K.Y., T.S., and M.W. analyzed data; and O.I., M.W., and J.-Å.G. wrote the paper.

Conflict of interest: J.-Å.G. is a shareholder and consultant of KaroBio AB.

Abbreviations: ER, estrogen receptor; GAG, glucosaminoglycan; IC, interstitial cystitis.

†To whom correspondence should be addressed. E-mail: jan-ake.gustafsson@mednub.ki.se.

© 2007 by The National Academy of Sciences of the USA

develop at ≈ 6 weeks of age, which corresponds to mouse puberty.

In 8-month-old female $ER\beta^{-/-}$ mice with manifested urothelial atrophy, bladder permeability is almost twice higher than in their WT littermates (Fig. 3). Bladder permeability was not affected by loss of $ER\alpha$ (in $ER\alpha^{-/-}$ mice). No bladder permeability tests were done in male mice because of technical difficulties in performing bladder catheterization of males.

To evaluate the differentiation process of bladder urothelium, we used Ck20 as an established marker protein for terminally differentiated urothelium. In 5-week-old female mice, bladder expression of Ck20 was similar in $ER\beta^{-/-}$ and WT mice. Thus, the differentiation of mouse urothelium, unlike prostatic epithelium, is not affected by the absence of $ER\beta$ (6). In contrast to the prostate, where $ER\beta$ is expressed in the differentiating epithelium, in the bladder, $ER\beta$ is predominantly expressed in the basal epitheliocytes. Its role in the bladder may not be regulation of differentiation. Because differentiation was not affected in $ER\beta^{-/-}$ urothelium, GAG production was probably not reduced and could not account for the primary mechanism of urothelial destruction. The difference of GAG content in urine was perhaps attributed to the high amount of cellular debris in urine of $ER\beta^{-/-}$ female mice.

In the bladders of 3.5-month-old mice, i.e., before the phenotype appeared, electron microscopic studies showed that the layer of GAGs on the apical side of urotheliocytes was even and equally well seen in $ER\beta^{-/-}$ and WT mice. The number and distribution of high resistance tight junctions on the cell–cell membranes were similar in both genotypes. Thus the bladder epithelium in $ER\beta^{-/-}$ female mice before the manifestation of phenotype appears to be well differentiated.

Because $ER\beta$ plays a role in the immune system, and one of the possible pathogenetic mechanisms of IC involves autoimmunity, we analyzed infiltrating immune cells in $ER\beta^{-/-}$ and WT female bladders. The role of mast cells in IC has previously been investigated. Increased numbers of mast cells have been found in mucosa and lamina propria of the bladders of patients with IC, and mast cells have been suggested to contribute to the pathogenesis of IC (15, 16). However, using an experimental rat model of autoimmune IC, Luber-Narod *et al.* (17) found no statistical differences in the bladders between experimental animals and controls.

Human $\gamma\delta$ T cells have also been demonstrated to be associated with IC (2). $\gamma\delta$ T cells participate in the elimination of stressed or damaged cells. Killing of damaged cells by $\gamma\delta$ T cells involves perforin/granzyme and Fas/Fas ligand-dependent pathways (18, 19). Evidence for a pathogenic role of $\gamma\delta$ T cells in autoimmunity has been reported (20). Other studies suggest a regulatory role of $\gamma\delta$ T cells in autoimmune responses. It has been shown that E2 administration enhances Ag-specific Th1 cell responses through $ER\alpha$ expression in hematopoietic cells (21). Most $\gamma\delta$ T cells are biased toward Th1 responses (22). In our study, we observed that in $ER\beta^{-/-}$ mice $\gamma\delta$ T cells were concentrated in the areas of atrophy and shedding of urothelium (Fig. 5A). Our data suggest that $\gamma\delta$ T cell are activated in $ER\beta^{-/-}$ mice, and this response may be the cause of the damage to the urothelium. Our previous results indicated that $ER\beta$ is the predominant type of ER in lymphocytes (23), and negative effects of estrogen on the immune system may be mediated by $ER\beta$ (24). The disruption of the $ER\beta$ gene in $ER\beta^{-/-}$ mice may cause abnormal activation of systemic and local regulatory immune response. Previous studies demonstrated that 28% of IC patients develop autoimmune exocrinopathy, Sjogren's syndrome (SS) (25). Our studies show that ArKO mice, which have complete loss of estrogen, spontaneously develop signs of lymphoproliferative autoimmunity, which particularly resembles SS (7).

The accumulation of activated macrophages is an important event in pathogenesis of autoimmune diseases and chronic infections. $\gamma\delta$ T cells engage in regulatory interactions with

macrophages and dendritic cells (26) and play a significant role in macrophage homeostasis. Up-regulation of macrophage cytokine production by $\gamma\delta$ T cells during bacterial infection was recently demonstrated (27). In $ER\beta^{-/-}$ mice, the increased recruitment of macrophages to lamina propria may be caused by activated $\gamma\delta$ T cells. In our study, we have found high infiltration with both $\gamma\delta$ T cells and macrophages in bladder of 3.5-month-old $ER\beta^{-/-}$ female mice. It is possible that at this stage, $\gamma\delta$ T cells and macrophages may act synergistically, damaging urothelium and lamina propria.

Several pieces of evidence indicate that IC, chronic pelvic pain, and bacterial prostatitis might share the same pathogenetic mechanism. It has been reported that up to 70% of men with chronic abacterial prostatitis have cystoscopic signs of IC (28, 29). There is a published hypothesis that links together prostatitis, IC, chronic pelvic pain, and urethral syndrome. In this report, the author postulates that these diseases share “dysfunctional urinary epithelium and potassium recycling” impairment (30). Furthermore, all these diseases have been reported to respond to phytoestrogens, such as sitosterols, quercetin, and genistein (31–33). Many phytoestrogens, like genistein, are better ligands for $ER\beta$ than $ER\alpha$ (34, 35). Because the morphological changes in female $ER\beta^{-/-}$ bladders, described in the present study, resemble those seen in IC, one could speculate that $ER\beta$ might be a valuable target for pharmacological intervention in IC, chronic pelvic pain, and chronic abacterial prostatitis.

Materials and Methods

Animals were used in accordance with the guidelines for care and use of experimental animals issued by Stockholm's Södra Djurförsöksetiska Nämnd. Mice were fed a standard diet with ad libitum access to water. Mice were bred from heterozygous mice. Genotyping using PCR was performed on DNA isolated from the tails of 2-week-old mice, as described elsewhere. Mice were killed by cervical dislocation. For immunohistochemical studies, bladders were removed, rinsed in ice-cold phosphate-buffered saline (PBS), and fixed overnight in 4% buffered paraformaldehyde. The bladders were routinely embedded in paraffin, cut in 4- μ m sections, and subsequently mounted on organosilane-coated slides.

Detection of GAG Content in Urine. Animals were killed as described above, and the urine was collected by puncturing the bladder wall. The total GAG content of urine was obtained by using the Di Ferrante (36) method, modified by Goldberg and Cotlier (37) and Pennock *et al.* (38). The analysis procedure was done as described by Panin *et al.* (39).

One percent hexadecyltrimethylammonium bromide (CTAB) (Sigma, St. Louis, MO; no. H9151) was mixed with an equal volume of urine, incubated at room temperature for 2 h, and centrifuged at $20,000 \times g$ for 20 min at room temperature. Supernatant was discarded and tubes drained upside down on a filter paper. A thin layer of CTAB-GAG was dissolved by 1 ml of 60% isopropanol. The remaining CTAB-GAG undissolved in isopropanol was taken by rinsing with 8 ml of ethanol, containing 1% sodium acetate. Two washings were pulled together, mixed thoroughly, and kept at 4°C overnight. The precipitate was centrifuged at $25,000 \times g$, 10 min at 0°C. Supernatant was discarded and the pellet washed with ethanol. The tubes were dried and pellets resuspended with 0.5 ml of distilled water. Reagent dye solution [dimethyl methylene-blue-chloride (DMB)] was prepared as follows: 16 mg of DMB (Serva, Heidelberg, Germany) 5 ml of 95% ethanol, 2 g of sodium formate, and 2 ml of formic acid were mixed and diluted to 1.5 l with deionized water. As standards, samples of chondroitin-4-sulfate A sodium salt (Sigma–Aldrich, Stockholm, Sweden; no. 27042) were used in sequential concentrations. The DMB solu-

tion was added to each sample, and the absorbance was read at 535 nm. The results were calculated as mg/ml of urine.

Bladder Permeability Measurement. Mice were deeply anesthetized with avertin, bladders were catheterized by using modified soft ureteral catheter Ch. 3, the residual urine was drained out and 110 μ l of sodium fluorescein (Chauvin Pharmaceuticals, Ltd., Kingston-upon-Thames, Surrey, U.K.) saline solution (0.8 mg/ml) was instilled into bladders. After 15-min exposure, animals were killed; blood was obtained from heart and centrifuged at $1,000 \times g$ for 5 min to get clean serum. Bladder content was also collected. Fifty microliters of serum was mixed with 100 μ l of 0.2 M acetate buffer (pH 5.1) containing glucurase (Sigma, no. G4882) (5,000 units/ml), and incubated for 30 min. Ten microliters of urine was mixed with 20 μ l of acetate buffer with glucurase and incubated for 30 min. To serum sample 1.15 ml and to urine sample 220 μ l of carbonate buffer 0.2 M, pH 9.15, was added. The sodium fluorescein concentration was determined by using a fluorometer set at 494-nm excitation and 516-nm emission wavelengths. The permeability index was calculated as relation of urine concentration to serum concentration multiplied by 100.

Immunohistochemistry. Antigen retrieval was performed by microwave sections in 0.01 M citrate buffer, pH 6.0. The sections

were permeabilized with 0.1% Nonidet P-40 in PBS for 15 min at room temperature. Endogenous peroxidase was blocked by incubation for 30 min with a solution of 0.5% hydrogen peroxide. Tissue sections were incubated for 2 h at 4°C with 1% BSA in PBS. Primary antibodies were diluted in PBS containing 1% BSA; 1:50 dilution was used for both biotin-conjugated antibodies to $\gamma\delta$ TCR (GL-3; BD PharMingen, San Jose, CA) and antibodies to F4/80 (BD PharMingen); 1:75 dilution was used in staining for ER β (chicken ER β 503 antibody, produced in our laboratory). Bladder sections were incubated with primary antibodies overnight at 4°C. The ABC method was used to visualize the signal according to the manual provided by the manufacturer (Vector Laboratories, Burlingame, CA). Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (Dako, Carpinteria, CA), counterstained with Mayer's hematoxylin, and dehydrated through ethanol series, followed by exposure to xylene and mounting.

We thank Prof. Gerd Fricker (Institut für Pharmazie und Biotechnologie, Heidelberg, Germany) for valuable advice in interpreting permeabilization data. We also thank Dr. Jose Inzunza and Annemarie Witte for helping with laboratory animals. This study was supported by grants from the Swedish Cancer Fund and KaroBio AB.

- Christmas TJ, Bottazzo GF (1992) *Clin Exp Immunol* 87:450–454.
- Christmas TJ (1994) *Br J Urol* 73:508–515.
- Forster C, Makela S, Warri A, Kietz S, Becker D, Hultenby K, Warner M, Gustafsson J-Å (2002) *Proc Natl Acad Sci USA* 99:15578–15583.
- Wada-Hiraike O, Imamov O, Hiraike H, Hultenby K, Schwend T, Omoto Y, Warner M, Gustafsson J-Å (2006) *Proc Natl Acad Sci USA* 103:2959–2964.
- Morani A, Barros RP, Imamov O, Hultenby K, Arner A, Warner M, Gustafsson J-Å (2006) *Proc Natl Acad Sci USA* 103:7165–7169.
- Imamov O, Morani A, Shim GJ, Omoto Y, Thulin-Andersson C, Warner M, Gustafsson J-Å (2004) *Proc Natl Acad Sci USA* 101:9375–9380.
- Shim GJ, Warner M, Kim HJ, Andersson S, Liu L, Ekman J, Imamov O, Jones ME, Simpson ER, Gustafsson J-Å (2004) *Proc Natl Acad Sci USA* 101:12628–12633.
- Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O (1993) *Proc Natl Acad Sci USA* 90:11162–11166.
- Krege JH, Hodgins JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson J-Å, Smithies O (1998) *Proc Natl Acad Sci USA* 95:15677–15682.
- Saunders PT, Maguire SM, Gaughan J, Millar MR (1997) *J Endocrinol* 154:R13–6.
- Taylor AH, Al-Azzawi F (2000) *J Mol Endocrinol* 24:145–155.
- Acar D, Cayan S, Aktas S, Tek M, Akbay E (2006) *NeuroUrol Urodyn* 26:309–316.
- Parsons CL, Lilly JD, Stein P (1991) *J Urol* 145:732–735.
- Hurst RE, Roy JB, Min KW, Veltri RW, Marley G, Patton K, Shackelford DL, Stein P, Parsons CL (1996) *Urology* 48:817–821.
- Pang X, Sant G, Theoharides TC (1998) *Urology* 51:939–944.
- Yamada T, Murayama T, Mita H, Akiyama K (2000) *Int J Urol* 7:292–297.
- Luber-Narod J, Austin-Ritchie T, Banner B, Hollins C, III, Maramag C, Price H, Menon M (1996) *Urol Res* 24:367–373.
- Nakata M, Kawasaki A, Azuma M, Tsuji K, Matsuda H, Shinkai Y, Yagita H, Okumura K (1992) *Int Immunol* 4:1049–1054.
- Huber S, Shi C, Budd RC (2002) *J Virol* 76:6487–6494.
- Olive C (1997) *Immunol Cell Biol* 75:102–106.
- Maret A, Coudert JD, Garidou L, Foucras G, Gourdy P, Krust A, Dupont S, Chambon P, Druet P, Bayard F, Guery JC (2003) *Eur J Immunol* 33:512–521.
- Yin Z, Chen C, Szabo SJ, Glimcher LH, Ray A, Craft J (2002) *J Immunol* 168:1566–1571.
- Shim GJ, Gherman D, Kim HJ, Omoto Y, Iwase H, Bouton D, Kis LL, Andersson CT, Warner M, Gustafsson J-Å (2006) *J Pathol* 208:408–414.
- Shim GJ, Wang L, Andersson S, Nagy N, Kis LL, Zhang Q, Makela S, Warner M, Gustafsson J-Å (2003) *Proc Natl Acad Sci USA* 100:6694–6699.
- van de Merwe JP, Yamada T, Sakamoto Y (2003) *Int J Urol* 10 (Suppl):S35–S38.
- Born WK, Reardon CL, O'Brien RL (2006) *Curr Opin Immunol* 18:31–38.
- Tramonti D, Andrew EM, Rhodes K, Newton DJ, Carding SR (2006) *Eur J Immunol* 36:1729–1738.
- Miller JL, Rothman I, Bavendam TG, Berger RE (1995) *Urology* 45:587–590.
- Berger RE, Miller JE, Rothman I, Krieger JN, Muller CH (1998) *J Urol* 159:83–85.
- Parsons CL, Greene RA, Chung M, Stanford EJ, Singh G (2005) *J Urol* 173:1182–1185.
- Bouic PJ, Etsebeth S, Liebenberg RW, Albrecht CF, Pegel K, Van Jaarsveld PP (1996) *Int J Immunopharmacol* 18:693–700.
- Shoskes DA, Zeitlin SI, Shahed A, Rajfer J (1999) *Urology* 54:960–963.
- Sun Y, Chen M, Lowentritt BH, Van Zijl PS, Koch KR, Keay S, Simard JM, Chai TC (2007) *Am J Physiol* 292:C106–C114.
- Miller CP, Collini MD, Harris HA (2003) *Bioorg Med Chem Lett* 13:2399–2403.
- Kuiper GG, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson J-Å (1996) *Proc Natl Acad Sci USA* 93:5925–5930.
- Di Ferrante NM (1967) *Anal Biochem* 21:98–106.
- Goldberg JM, Cotlier E (1972) *Clin Chim Acta* 41:19–27.
- Pennock CA, White F, Murphy D, Charles RG, Kerr H (1973) *Acta Paediatr Scand* 62:481–491.
- Panin G, Naia S, Dall'Amico R, Chiandetti L, Zachello F, Catassi C, Felici L, Coppa GV (1986) *Clin Chem* 32:2073–2076.