Cell Tissue Res (2001) 304:271–278
DOI 10.1007/s004410000262

REGULAR ARTICLE

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Increased expression of non-muscle myosin heavy chain-B in connective tissue cells of hypertrophic rat urinary bladder

Received: 2 April 2000 / Accepted: 27 June 2000 / Published online: 23 March 2001

Abstract Expression of the non-muscle myosin heavy chain-B (NM-MHC-B, also denoted as the embryonic smooth muscle myosin heavy chain, SMemb) was examined in rat urinary bladder during growth in response to a partial urinary outflow obstruction. Following obstruction, the weight of the urinary bladder increased more than five-fold within 10 days. Immunohistochemistry with a polyclonal antiserum against the C-terminal sequence of NM-MHC-B revealed very few NM-MHC-B immunoreactive cells in the control urinary bladders. In hypertrophic bladders, the number of NM-MHC-B immunoreactive cells markedly increased. The majority of such cells were found in the interstitium surrounding smooth muscle bundles and also in the subserosal and submucosal layers. Western blot analysis showed that the NM-MHC-B expression was transient; the content of NM-MHC-B immunoreactive material had doubled 10 days after obstruction and then declined towards the control level after 6 weeks. Immunohistochemistry revealed co-localization of NM-MHC-B and vimentin within the same cells. NM-MHC-B did not co-localize with smooth muscle actin, suggesting that the source of NM-MHC-B is not a de-differentiated smooth muscle cell or myofibroblast but a non-muscle cell possibly reacting to tissue distension or stress. The NM-MHC-B-positive cells could have a role in the production of extracellular matrix and growth factors or be involved in modulation of spontaneous contractile activity.

Keywords Urinary bladder · Hypertrophy · Non-muscle myosin heavy chain-B (SMemb) · Myosin · Rat (Sprague Dawley, female)

This work was supported by grants from the Swedish Medical Research Council (04x-8268 and 04x-13406) and the Deutsche Forschungsgemeinschaft Mo 362/5–3 (I.M.)

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Introduction

Growth of smooth muscle tissue can be initiated in response to increased functional demands, e.g. an increase in tension and/or strain on the wall of hollow organs, such as arterial vessels, urinary bladder, intestine or pregnant uterus. In the urinary bladder, adaptive growth, in response to urinary outflow obstruction, is associated with alterations in cytoskeletal and contractile proteins in the smooth muscle cells and changes in contractile behaviour (Malmqvist et al. 1991; Sjuve et al. 1996). The growth of the bladder wall also involves alterations in its extracellular matrix and non-smooth muscle components (Gabella and Uvelius 1990), e.g. an increase in total tissue collagen (Uvelius and Mattiasson 1984). Few connective tissue cells are present in the normal bladder wall (Gabella and Uvelius 1990) but these cells may have a role in the formation of the bladder matrix. Other possible functions have also emerged. Mesenchymal cells in the subserosal layer have been suggested to be able to differentiate into mature smooth muscle (Buoro et al. 1993). Interstitial cells exhibiting an NO-induced increase in cGMP (Smet et al. 1996) and staining properties similar to that of the interstitial cells of Cajal (ICC), with tentative excitatory functions (Hussain et al. 2000), have been reported in human bladder. The role of connective tissue cells in the control of bladder function is not known in detail and possible adaptative changes in these cells during bladder growth are unresolved.

The myosin II heavy chain (MHC) includes several isoforms with specialized functions in muscle and connective tissue cells. In smooth muscle, two MHC isoforms (SM1, 204 kDa; SM2, 200 kDa) are produced by alterna-

tive mRNA splicing (Babij and Periasamy 1989). The expression of SM1 and SM2 is regulated differently during development; it seems that SM2 is expressed after birth (Kuro-o et al. 1991), whereas SM1 is expressed at the same level throughout development. Non-muscle MHCs (NM-MHC-A, 196 kDa; NM-MHC-B, which is also denoted as embryonic smooth muscle myosin heavy chain or SMemb, 200 kDa; Kawamoto and Adelstein 1991) are encoded by separate genes (Simons et al. 1991; Katsuragawa et al. 1989) and have been detected in non-muscle cells and in smooth muscle during development and in association with some pathophysiological conditions (Aikawa et al. 1993; Roelofs et al. 1995). Interestingly, the non-muscle myosins can be expressed in smooth muscle during proliferation (Kuro-o et al. 1991) and during phenotypic modulations between mesenchymal stem cells, myofibroblasts and smooth muscle cells (Buoro et al. 1993).

Mesenchymal cells have been detected in the subserosal layer by means of antibodies that recognize the 196-kDa NM-MHC-A and have been suggested to be able to transform into smooth muscle cells during hypertrophy and regeneration of the urinary bladder (Roelofs et al. 1995; Buoro et al. 1993; Faggian et al. 1998). The 200-kDa NM-MHC-B is expressed in embryonic rabbit aorta smooth muscle in addition to SM1 (Kuro-o et al. 1991). Interestingly, the expression of NM-MHC-B is reinduced in proliferating smooth muscle during atherosclerosis in rabbit (Kuro-o et al. 1991) and man (Aikawa et al. 1993), during post-stenotic dilation in rabbit carotid artery (Okamoto et al. 1992) and in culture (Kawamoto and Adelstein 1991). During cardiac hypertrophy, NM-MHC-B has been found to increase in activated stromal fibroblasts (Shiojima et al. 1999). Studies on NM-MHC-B expression in smooth muscle tissues have focused on the vasculature and, thus, knowledge regarding the expression of the NM-MHC-B isoform in other types of smooth muscle tissue and possible changes during growth is limited at present. The purpose of this study has been to investigate (1) cell proliferation by the incorporation of bromodeoxyuridine (BrdU) and (2) the possible presence of NM-MHC-B in the rat urinary bladder wall during adaptive growth in response to outlet obstruction.

Materials and methods

Surgical procedures and tissue preparations

Urinary bladder hypertrophy was induced by a partial ligature of the urethra in female Sprague-Dawley rats as described previously (Uvelius et al. 1984). The rats were killed 3 days, 10 days or 6 weeks after obstruction. Aged-matched rats served as controls. For experiments on the reversibility of urinary bladder growth, the obstructive ligature was removed after 10 days and the animals were killed 6 weeks after the obstruction was performed. The urinary bladders were removed, blotted between two filter papers and weighed. Pieces from the midsection of the bladder were cut out and fixed for immunohistochemistry (see below). For the biochemical experiments, the mucosa was removed with a pair of scissors, after which the remaining bladder tissue was frozen in liquid nitrogen and stored at –80°C. All animal experiments were carried out according to the guidelines of the local animal ethics committee.

Antibody production

A polyclonal antiserum against the synthetic carboxy terminal peptide sequence of NM-MHC-B/SMemb (Thr-Ser-Asp-Val-Asn-Glu-Thr-Gln-Pro-Pro-Gln-Ser-Glu) was raised in rabbits by immunization and affinity-purified essentially according to Calovini et al. (1995). The antiserum was used to detect the content of the NM-MHC-B isoform on Western blots and its cellular localization in immunohistochemistry (dilution 1:200).

Western blot analysis of NM-MHC-B expression

Bladder tissue samples (20–30 mg) were homogenized in SDS-sample buffer (5% SDS, 50 mM TRIS/HCl pH 7.5, 250 mM sucrose, 75 mM urea, 60 mM β-mercaptoethanol), boiled for 2 min and centrifuged. The protein content of the supernatant was determined by the method of Lowry et al. (1951). Aliquots from the supernatant were separated by SDS-polyacrylamide gel electrophoresis with a 5% separation gel in a Bio-Rad minigel system (Richmond, USA). The gels were run for 3 h (30 mA constant current at 3°C). Proteins from the SDS gels were transferred to nitrocellulose (Hybond-C, 45 µm; Amersham) in a Bio-Rad Mini-protean II electrophoresis system in a buffer containing 25 mM TRIS, 192 mM glycine, 0.1% SDS, 20% (v/v) methanol. The nitrocellulose was blocked with ovalbumin and then incubated with the NM-MHC-B antiserum for 2 h at room temperature at a concentration of 0.5 µg IgG/ml followed by the secondary peroxidase-conjugated antibody (anti-rabbit IgG; Sigma, St. Louis, USA) for 1 h at room temperature. The NM-MHC-B protein band was visualized by the enhanced chemiluminesence reaction (ECL, Amersham), recorded on an X-ray film (X-Omat, Kodak), and scanned densitometrically (ScanPack, Biometra, Germany).

Immunohistochemistry and BrdU measurements

Whole wall strips from the urinary bladder from obstructed (3 and 10 days) and control rats were pinned flat without stretching onto balsa wood and fixed overnight in a mixture of 2% formaldehyde and 0.2% picric acid in phosphate buffer (pH 7.2) followed by rinsing in Tyrode solution containing 10% sucrose. Specimens were frozen on dry ice and cut in a cryostat to a thickness of 10 µM. The NM-MHC-B antiserum described above or monoclonal antibodies to vimentin (code no. V4630, dilution 1:1280, Sigma) or smooth muscle actin (code no. A2547, dilution 1:400, Sigma) were used to demonstrate the cellular location of the proteins. The site of the antigen-antibody reaction was visualized by fluorescein isothiocyanate (FITC)-conjugated antibodies to rabbit IgG raised in pigs (DAKO, Copenhagen, Denmark) or affinity-purified FITC-conjugated antibodies to mouse IgG (Jackson Immuno Research Laboratories, USA). For double-staining, the primary monoclonal antibodies were visualized by using an affinity-purified anti-mouse IgG labeled with tetramethyl rhodamine isothiocyanate (Jackson Immuno Research Laboratories).

To visualize proliferating cells, 25 mg/kg BrdU (Sigma) was injected intraperitoneally at 12 h; this was repeated 2 h before sacrifice, after which the urinary bladders were fixed and cut as described above. The cryosections were subjected to DNA hydrolysis by incubation in 1 M HCl at 56°C for 30 min before being processed for immunohistochemistry with monoclonal anti-BrdU antibodies (code no. M0744, dilution 1:20, Dako, Copenhagen, Denmark) as described above.

Statistics

Values are given as means ± SEM with the number of animals investigated within parentheses. Comparisons were made by using Student's *t*-test for unpaired values.

Results

Table 1 shows the summarized data for bladder weight. The bladders increased about fivefold in weight during the 10-day-obstruction period. Removal of the obstruction after 10 days caused the bladder weight to return to values about twice the normal value.

Immunohistochemical analysis of NM-MHC-B expression

Figure 1 shows sections of urinary bladders from a control and from animals at 3 and 10 days after obstruction stained with the NM-MHC-B antibodies. A small number of immunoreactive cells occurred in the serosal and submucosal layers of the control sections. After 3 days of obstruction, an increased number of immunoreactive cells was noted in the subserosal and submucosal layers. At this time, immunoreactive cells also appeared in the interstitium between the muscle bundles. After 10 days, numerous cells displaying intense staining were noted in the connective tissue throughout the bladder wall. Such cells were particularly abundant in the submucosal and subserosal layers. The NM-MHC-B-immunoreactive cells were fibroblast-like in that they were elongated, slender and fusiform. No NM-MHC-B immunoreactivity was noted in the smooth muscle cells.

Figure 2 shows micrographs of control and 10-day-obstructed bladders double-immunostained for smooth muscle actin and NM-MHC-B. Antibodies to smooth muscle actin stained the cells in the smooth muscle bundles and did not co-localize with NM-MHC-B reactivity. NM-MHC-B immunoreactivity co-localized to a large extent with vimentin immunoreactivity in fibroblast-like cells in the hypertrophic bladder preparations (Fig. 3). Very few cells were vimentin-positive but NM-MHC-B-negative. In the control bladder, vimentin-immunoreactive cells were also frequent but the vast majority did not harbour any NM-MHC-B-immunoreactive material.

Detection of cell proliferation with BrdU staining

In order to detect whether the cells expressing NM-MHC-B also revealed an increased DNA turnover, animals were injected with BrdU before sacrifice. Immunocytochemical detection of BrdU incorporation in sections of urinary bladders from controls and from animals 3 and 10 days after obstruction is shown in Fig. 4. In

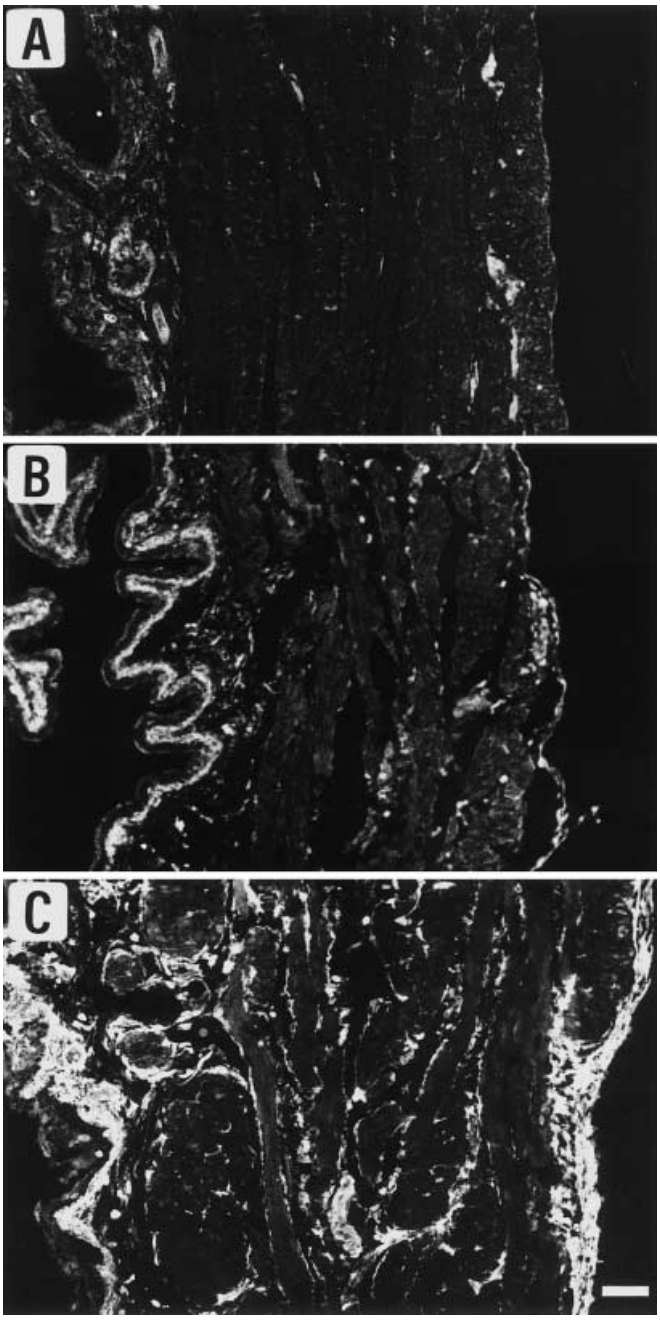

Fig. 1 Cryosections of rat urinary bladder from (**A**) control, (**B**) 3 days and (**C**) 10 days after partial obstruction of the urethra. Immunoreactivity to NM-MHC-B. *Bar* 50 µm

Table 1 Urinary bladder weight in control bladders, bladders subjected to partial outflow obstruction for 3 days, 10 days and 6 weeks, and bladders subjected to a 10-day-obstruction period followed by removal of the ligature and sacrifice 6 weeks after the initial operation

	Controls	3 days	10 days	6 weeks	Ligature removed
Urinary bladder weight (mg)	61±3 (*n*=6)	104±9 (*n*=6)	322±44 (*n*=6)	490±104 (*n*=6)	126±7 (*n*=6)

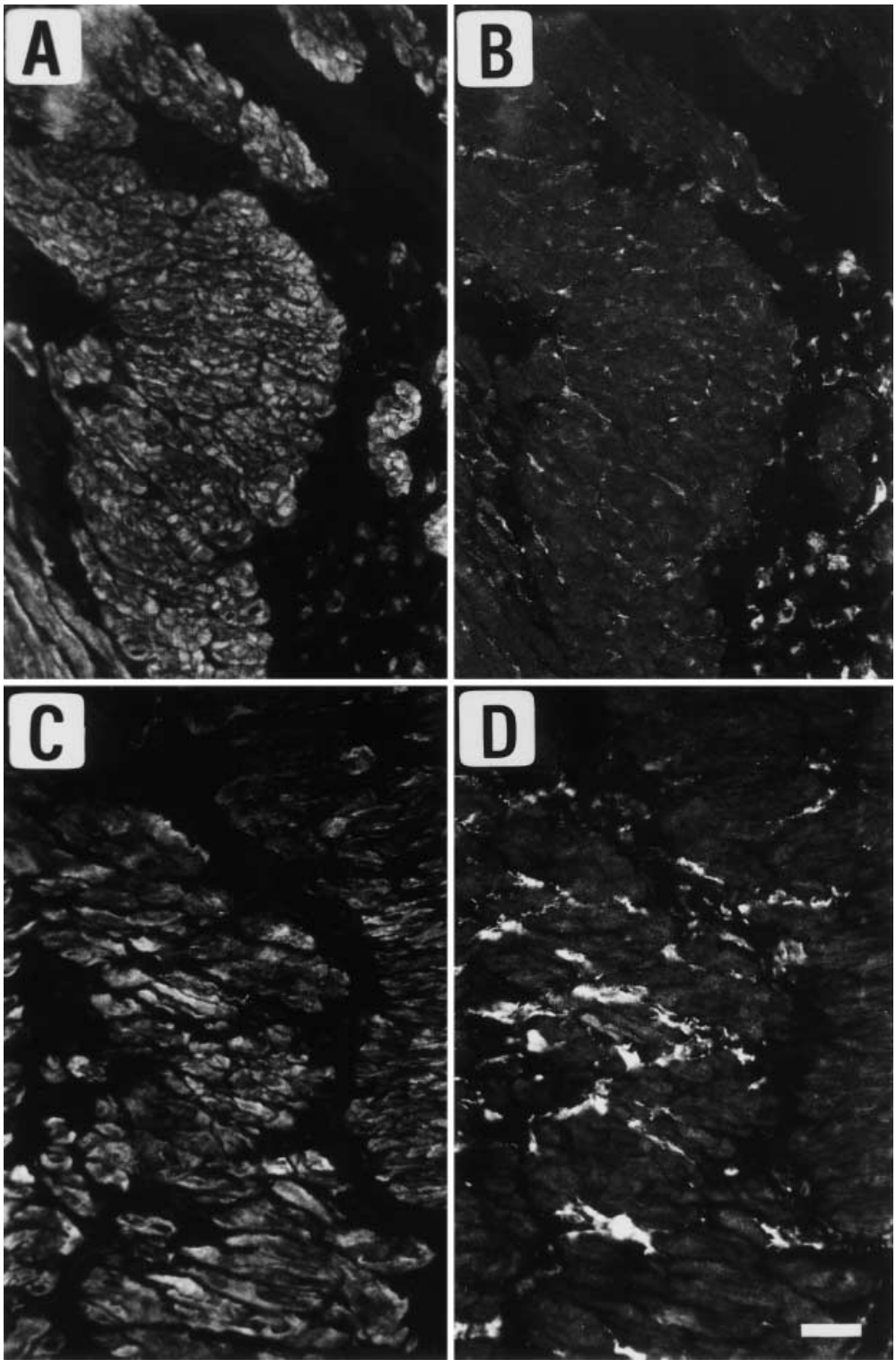

Fig. 2 Cryosections double-stained for smooth muscle actin (**A, C**) and NM-MHC-B (**B, D**) for control (**A, B**) and 10-day-obstructed (**C, D**) urinary bladder. Note the lack of NM-MHC-B staining in the smooth muscle cells. *Bar* 30 µm

control tissue, labelling was mainly detected in the urothelium. After 3 days of obstruction, the incorporation of BrdU increased particularly in the subserosal layer and also at the periphery of the muscle bundles. A similar pattern was observed after 10 days of obstruction.

Tissue content of NM-MHC-B

Gel electrophoretic separation and Western blot analysis were performed in order to follow the time-course of the expression of NM-MHC-B in the urinary bladder tissue. Figure 5A shows the intensity of the immunoreaction to the antibody against NM-MHC-B (ECL signal) plotted

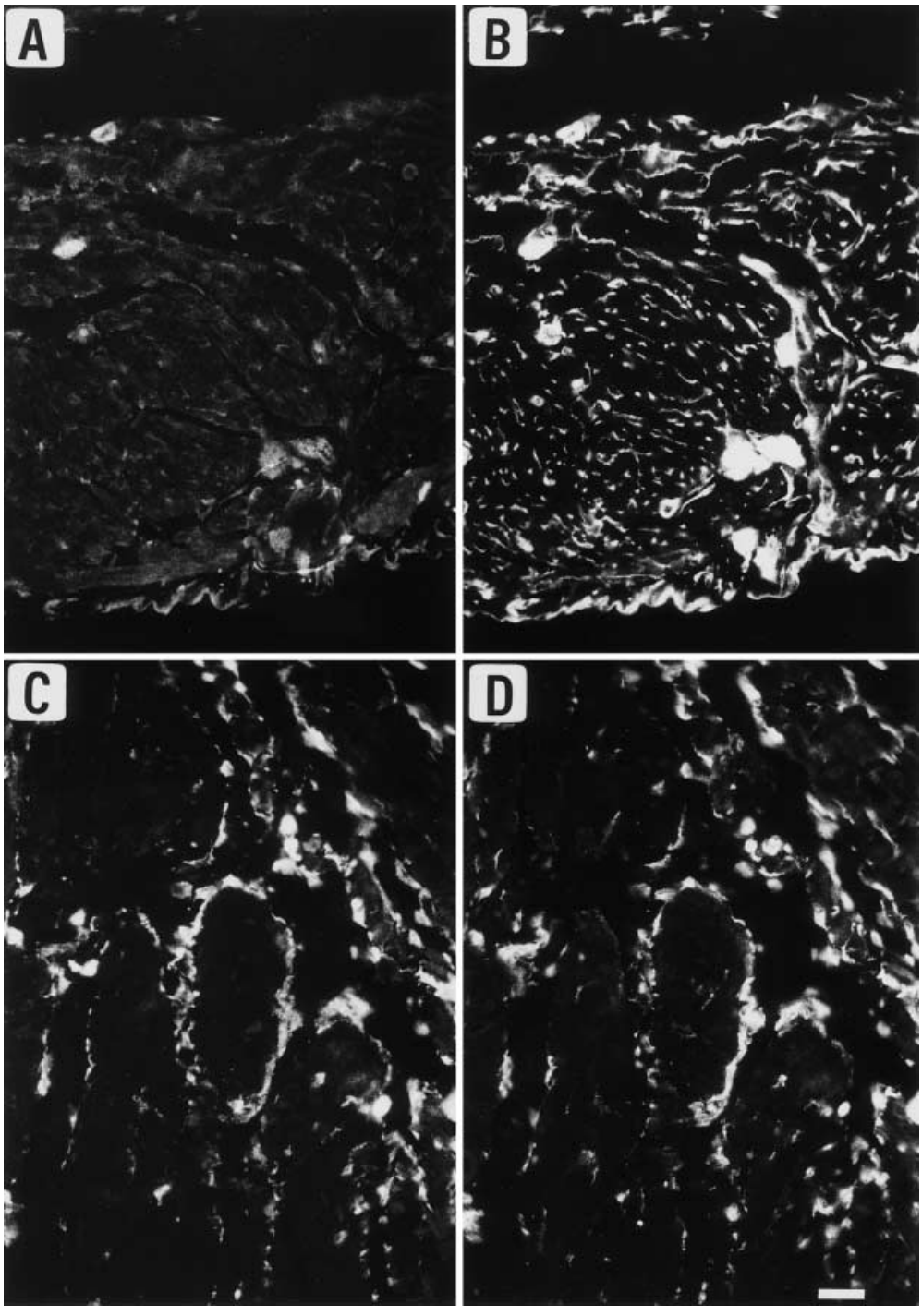

Fig. 3 Cryosections double-stained for NM-MHC-B (**A, C**) and vimentin (**B, D**) from control (**A, B**) and 10-day-obstructed (**C, D**) urinary bladder. All NM-MHC-B-immunoreactive cells also contain vimentin. In addition, vimentin-immunoreactive cells without NM-MHC-B can be detected. *Bar* 50 μm

against the amount of protein on the gel. The ratio of NM-MHC-B to tissue protein increased more than two-fold in the bladder 10 days after obstruction. An increased NM-MHC-B content was observed even at 3 days, although the value was not statistically significant. After 6 weeks of obstruction, immunoreactivity to NM-MHC-B was lower than after 10 days and resembled control values. Removal of the obstruction 10 days post-operatively resulted in an immunoreactivity at 6 weeks comparable with that of control values. The binding of the antibodies could be blocked by the addition of excess synthetic peptide antigen used for immunization prior to the antibody incubation, thus indicating specific antigen recognition.

The extraction of proteins for electrophoresis was performed quantitatively and, when we expressed the NM-MHC-B ECL intensity normalized to tissue wet weight, the pattern was similar to that shown in Fig. 5, i.e. an initial increase at 3 and 10 days and a return towards control values at 6 weeks.

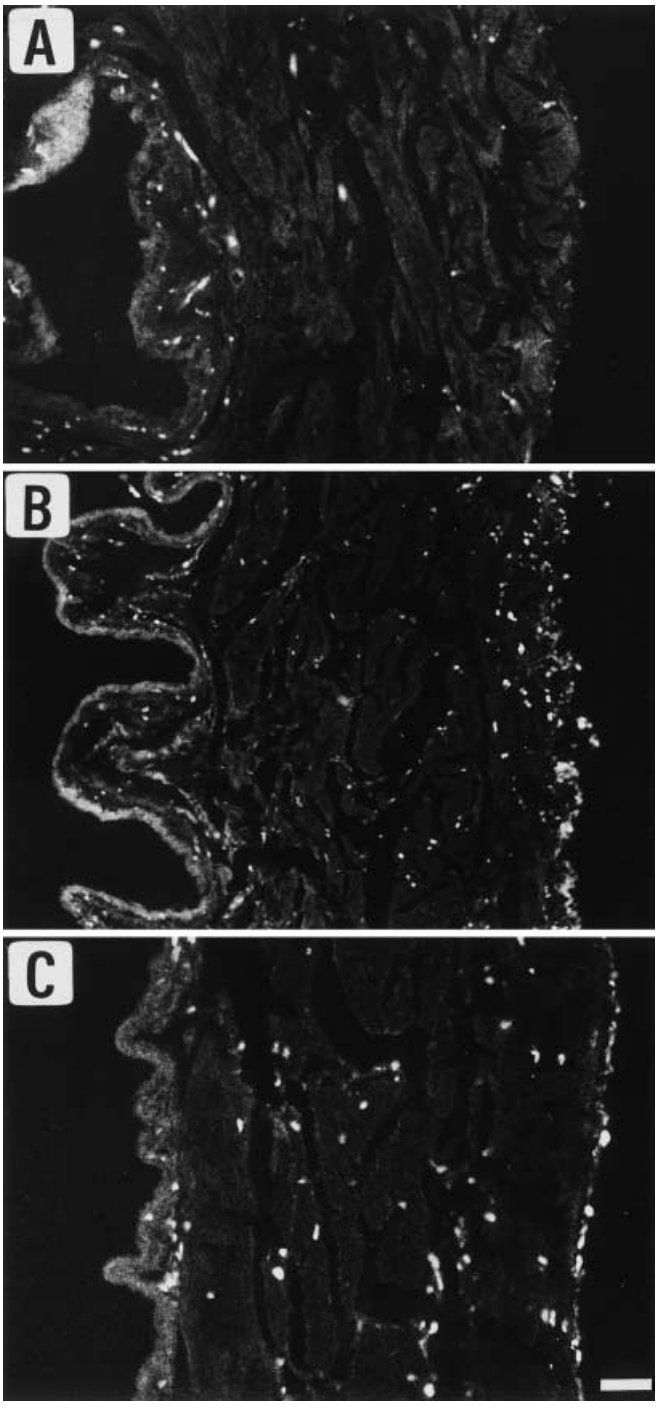

Fig. 4 BrdU-labelling visualized by immunostaining in rat urinary bladder in control (**A**), 3 days (**B**) and 10 days (**C**) after obstruction of the urethra. In control preparations, BrdU immunoreactivity is located in the epithelium and this reactivity persists in the obstructed preparations. The number of BrdU-positive cells within the subserosa and submucosa is makedly increased after 3 days of obstruction compared with control. After 10 days, the BrdU labelled cells are mainly located at the periphery of the smooth muscle bundles and in the subserosa. *Bar* 60 μm

Discussion

In this study, we have found that the expression of NM-MHC-B (also denoted as SMemb) dramatically increases in the rat urinary bladder during adaptive growth. The increased levels of NM-MHC-B are transient, returning to near control level within 6 weeks. NM-MHC-B may have a contractile function, since it is expressed in neonatal smooth muscle cells and supports sustained contractions in a smooth-muscle-MHC knockout mouse model (Morano et al. 2000). Interestingly, in the present study, NM-MHC-B has not been found to be localized to the smooth muscle cells but is mainly present in fibroblast-like cells within the connective tissue surrounding the smooth muscle bundles and in the subserosa and submucosa.

Previous studies have suggested a phenotypic transition from fibroblasts in the subserosa via myofibroblasts to mature smooth muscle cells in obstructed rabbit bladder (Buoro et al. 1993; Roelofs et al. 1995). The cells described in these studies were visualized by a platelet non-muscle myosin (196 kDa, NM-MHC-A) antibody that did not react to brain-derived myosin (SMemb/NM-MHC-B; Frid et al. 1993). In addition, these cells also contained α-actin. This suggests that the NM-MHC-B-positive and α-actin-negative cells observed in the present investigation represent a different cell population or another transitional state.

The relative content of NM-MHC-B in hypertrophic smooth muscle declines from its peak value at 10 days to a level similar to that of the control at 6 weeks. Whether this decline is attributable to the total phenotypic transition of activated cells to fully differentiated smooth muscle cells or whether the activated cells revert back to a non-activated "non-muscle" cell type is a matter for speculation. Injury to the serosa or urinary outlet obstruction have been found to activate the proliferation of a population of subserosal mesenchymal cells (Roelofs et al. 1995; Faggian et al. 1998). It is noteworthy that the staining properties of these cells differ from the NM-MHC-B-positive cells in the present study in that they contain α-actin and NM-MHC-A. A transition in smooth muscle phenotype, including a stage with NM-MHC-B expression, could be important for the migration of cells into the injured or growing tissue; indeed, NM-MHC-B has been reported to increase in proliferating smooth muscle cells (Kawamoto and Adelstein 1991; Aikawa et al. 1993; Okamoto et al. 1996). However, in our investigation, no evidence of this change in phenotype has been found. In general, the growth of rat urinary bladder is mainly attributable to cell hypertrophy and not hyperplasia, since few mitoses have been noted (Gabella and Uvelius 1990). This suggests that proliferation of the smooth muscle cells is not a major phenomenon in hypertrophy and, consequently, that the NM-MHC-B-positive cells are not recruited to increase smooth muscle mass.

As indicated by the BrdU incorporation in the hypertrophic bladder, it seems that cell proliferation occurs

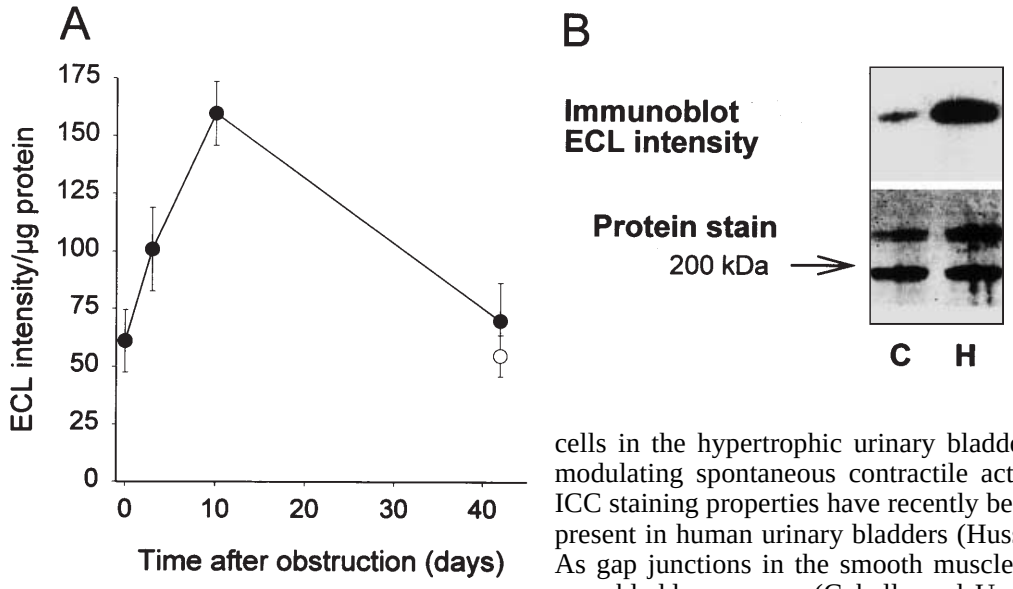

Fig. 5 **A** Change over time in the expression of non-muscle myosin heavy chain B (NM-MHC-B) in control and hypertrophic rat urinary bladder. Western blot analysis with the enhanced chemiluminescence (ECL) signal of the myosin heavy chain band following use of the NM-MHC-B antiserum. ECL signals were related to the tissue protein content. *Open circle* indicates expression of NM-MHC-B in bladders subjected to a 10-day obstruction period, wherafter the ligature was removed and the animals were killed 6 weeks after the initial operation. Values are means ± SEM. (n=6 in each group). **B** Analysis of the in vivo expression of the NM-MHC-B in control (*C*) and 10-day-obstructed rat urinary bladder (*H*). *Top* Western blot analysis (ECL staining) with the NM-MHC-B antiserum, *bottom* myosin heavy chain as detected by protein staining (Coomassie-blue)

preferentially in the vicinity of the muscle bundles. This coincides with the location of cells expressing NM-MHC-B. Other localizations of NM-MHC-B-containing cells include the submucosal and subserosal layers, which also show a high degree of proliferation. No NM-MHC-B expression or BrdU incorporation has been found in the bladder smooth muscle component. The NM-MHC-B-positive cells also express vimentin but not smooth muscle actin indicating that they are probably not myofibroblasts, but rather a fibroblast-like cell type. The function of these cells could be to contribute to the synthesis of extracellular matrix components. Hypertrophy is accompanied by alterations in the passive compliance of the bladder wall (Damaser et al. 1996) and in the extracellular matrix, e.g. with regard to the amount of collagen (Uvelius and Mattiasson 1984). Earlier studies have suggested that a small population of activated fibroblasts/fibrocytes in the interstitium of the bladder wall are involved in collagen synthesis (Gabella and Uvelius 1990; Uvelius and Mattiasson 1984). It is possible that this cell population corresponds to the NM-MHC-B-positive cells found in the present study.

Recently, NM-MHC-B has been detected in the ICC and gastrointestinal stromal tumors (Sakurai et al. 1999). Since intestinal ICC have been ascribed a pacemaker function, it is possible that the NM-MHC-B-positive cells in the hypertrophic urinary bladder play a role in modulating spontaneous contractile activity. Cells with ICC staining properties have recently been reported to be present in human urinary bladders (Hussein et al. 2000). As gap junctions in the smooth muscle cells in the urinary bladder are rare (Gabella and Uvelius 1990), it is tempting to speculate that the propagation of activating impulses is mediated by cells outside the smooth muscle cell bundles.

In conclusion, hypertrophy of the rat urinary bladder is accompanied by a transient increase in NM-MHC-B-expressing cells in the connective tissue between smooth muscle bundles. The physiological function of these cells remains to be elucidated but they might (1) develop into mature smooth muscle cells, (2) produce extracellular matrix components, (3) release growth factors or (4) generate or modify pacemaker activity in the hypertrophying urinary bladder. NM-MHC-B could be a valuable marker for strain-induced bladder-tissue growth.

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