Proliferation modulates intestinal smooth muscle phenotype in vitro and in colitis in vivo

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Nair DG, Han TY, Lourenssen S, Blennerhassett MG. Proliferation modulates intestinal smooth muscle phenotype in vitro and in colitis in vivo. Am J Physiol Gastrointest Liver Physiol 300: G903–G913, 2011. First published February 10, 2011; doi:10.1152/ajpgi.00528.2010.—Intestinal inflammation causes an increased intestinal wall thickness, in part, due to the proliferation of smooth muscle cells, which impairs the contractile phenotype elsewhere. To study this, cells from the circular muscle layer of the rat colon (CSMC) were isolated and studied, both in primary culture and after extended passage, using quantitative PCR, Western blot analysis, and immunocytochemistry. By 4 days in vitro, both mRNA and protein for the smooth muscle marker proteins α-smooth muscle actin, desmin, and SM22-α were reduced by >50%, and mRNA for cyclin D1 was increased threefold, evidence for modulation to a proliferative phenotype. Continued growth caused significant further decrease in expression, evidence that phenotypic loss in CSMC was proportional to the extent of proliferation. In CSMC isolated at day 2 of trinitrobenzene sulfonic acid-induced colitis, flow cytometry and Western blotting showed that these differentiated markers were reduced in mitotic CSMC, while similar to control in nonmitotic CSMC. By day 35 post-trinitrobenzene sulfonic acid, when inflammation has resolved, CSMC were hypertrophic, but, nonetheless, showed markedly decreased expression of smooth muscle protein markers per cell. In vitro, day 35 CSMC displayed an accelerated loss of phenotype and increased thymidine uptake in response to serum or PDGF-BB. Furthermore, carbachol-induced expression of phospho-AKT (a marker of cholinergic response) was lost from day 35 CSMC in vitro, while retained in control cells. Therefore, proliferation reduces the expression of smooth-muscle-specific markers in CSMC, possibly leading to altered contractility. However, inflammation-induced proliferation in vivo also causes lasting changes that include unexpected priming for an exaggerated response to proliferative stimuli. Identification of the molecular mechanisms of intestinal smooth muscle cell phenotypic modulation will be helpful in reducing the detrimental effects of inflammation.

intestinAl inflammation causes altered motility in both acute and chronic disease, such as inflammatory bowel disease, which is evidence of challenges to the regulatory mechanisms of smooth muscle cells (SMC) and the enteric nervous system that exceed their capacity for homeostasis. However, symptoms of dysmotility can persist after resolution of inflammation, raising the possibility of permanent alterations to the cellular structure of the intestine. Repeated or protracted inflammation may cause serious malfunction, such as stricture formation in Crohn’s disease, where surgical intervention is often the only recourse. However, recurrence is very common and often detectable soon after surgery (4, 27).

Analysis of the changes at the cellular level may be necessary to understand the altered smooth muscle function that is characteristic of the inflamed state. For example, intestinal wall thickening and proliferation of intestinal SMCs (ISMC) occurs in intestinal inflammation in both human disease, as well as in animal models, with poorly understood consequences (3, 6, 19, 24). Furthermore, in studies of isolated ISMC obtained from animal models, we and others have shown that inflammation causes substantial alterations to the contractile responses to neural stimuli, including altered regulation of intracellular calcium (30, 31), with evidence for persistence of these changes after inflammation has resolved (29).

Rodent models of intestinal inflammation have shown intestinal wall thickening due to hyperplasia of ISMC, with striking increases in ISMC number that are not reversed upon the resolution of inflammation (19). While increased tissue mass may compromise contractility at the tissue and organ level, very little is known of the effect of hyperplasia in vivo on ISMC phenotype. Elsewhere, this is recognized as an important component of disease processes, such as reactive airway disease, atherosclerosis, and the stenosis of coronary vasculature (22, 23).

In general, the onset of cell division in SMCs compromises contractile function in favor of an increased emphasis on metabolic activity. Thus, agents, such as growth factors, inflammatory cytokines, and altered extracellular matrix proteins, drive vascular SMC change from the contractile phenotype to a pathophysiological synthetic phenotype with ensuing replication, increased secretion of extracellular matrix proteins, and migration. Similar changes in airway disease are implicated in the outcome of increased muscle mass and hyperactivity (12).

In these systems, PDGF-BB is recognized as a potent growth factor, causing proliferation, as well as the strong inhibition of expression of smooth muscle-specific genes, such as smooth muscle α-actin (α-SM actin), myosin heavy chain and desmin, among others (32). Recently, our laboratory has also identified PDGF-BB as the key factor responsible for ISMC growth in vitro, as well as in the trinitrobenzene sulfonic acid (TNBS)-induced model of rat colitis (25). In this animal model, ISMC proliferation is apparent by day 2 of inflammation and leads to a threefold increase in ISMC number by day 6 of colitis (19). Furthermore, we described sporadic stricture formation in this model, which correlated with locally severe damage to the enteric nervous system (20). This suggested that proliferation both could impair ISMC phenotype in the acute phase of inflammation, and also, if unchecked, could contribute to stricture formation and the essentially permanent compromise of intestinal function.

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Therefore, we examined the phenotype of the circular SMC (CSMC), both in the TNBS model, as well as in vitro using established approaches, proposing that proliferation could cause characteristic patterns of alteration to the expression of smooth muscle-specific proteins. This identified profound changes to phenotype, both in the onset of primary culture in vitro and in the acute phase of colitis in vivo. Surprisingly, this also uncovered the presence of a lasting impact of prior proliferation on CSMC phenotype, apparent long after recovery from inflammation. This has the potential to aid in studies in assessment of molecular approaches to the control of ISMC proliferation, which seek to maintain the contractile phenotype in vivo.

METHODS

Animals. Adult Sprague-Dawley rats were obtained from Charles River Laboratories (Montreal, PQ, Canada) and housed in pairs in microfilter-isolated cages for 7 days before use, with free access to food and water. To induce inflammation, 500 μl of 200 mM TNBS (Sigma) dissolved in 50% ethanol were infused into the colon (8 cm proximal to the anus) of rats lightly anesthetized with inhaled isoﬂurane, as previously described (25, 31). All experimental procedures were approved by the Queen’s University Animal Care Committee.

Isolation of primary CSMC. ISMC were isolated as previously described (25). Briefly, the middescending colon was removed, opened, and placed in HEPES-buffered Hank’s saline at pH 7.35. The mucosa and submucosa were discarded, and small strips of circular smooth muscle (CSM) were removed with fine forceps, verified for orientation under a microscope, and incubated in a digestive solution (papain, 0.5 mg/ml; BSA, 1 mg/ml; Dl-dithiothreitol, 1 μM; and collagenase type F, 0.5 mg/ml; all from Sigma) for 2 h at 4°C, 1 h at room temperature, then 1 h at 37°C. Isolated cells were seeded into 24-well plates on plastic or on collagen-coated glass cover slips and maintained in DMEM with 5% fetal calf serum (FCS) (Invitrogen). The absence of glia, vascular SMCs, and interstitial cells of Cajal was confirmed by immunocytochemistry using relevant antibodies, as described previously (25). The appearance of these cultures was photographed using phase contrast microscopy (Olympus IMT-2). Some cohort cultures were passaged twice (P2) or 10 times (P10) to determine the effect of continued proliferation on phenotypic characteristics.

Quantitative RT-PCR. Total RNA was isolated from freshly isolated CSMC or cultured using RNeasy kits (Qiagen), according to the manufacturer’s protocol. Total RNA (~1 μg) after DNase I treatment was converted into cDNA using iScript RT kit (Bio-Rad). cDNA (~150–200 ng) was taken for real-time PCR using iTaq Fast SYBR Green supermix with ROX (Bio-Rad) in an Applied Biosystems 7500 System Sequence Detection Software, version 1.3, using the ΔΔCt comparative method of relative quantification. Results were expressed as fold change relative to the D0 control cells for α-SM actin and desmin and to D4 control cells for cyc- lin D1.

Immunocytochemistry. Immunocytochemistry was used to detect the expression of proliferating cell nuclear antigen (PCNA; 1:1,000; DAKO), or smooth muscle phenotype markers (α-SM actin, 1:2,000; DAKO; desmin, 1:1,000; Chemicon; smoothelin, 1:500; Santa Cruz; or SM22-α, 1:2,000, ABCAM) in primary ISMC. Cells were fixed in 4% neutral buffered formalin for 10 min to detect PCNA or in 70% ethanol for 10 min to detect α-SM actin, desmin, or SM22-α, followed by incubation in primary antibodies overnight at 4°C. Cells were then incubated in the appropriate Alexa-linked secondary antibodies for 1 h at room temperature, and stained cells were visualized with fluorescence microscopy (BX51; Olympus). Nuclei were labeled with Hoechst 33342 (1 μg/ml; Sigma). Images were acquired using Image Pro Plus (version 6; Media Cybernetics) with standard camera settings.

Proliferation assay. [3H]thymidine incorporation assays were performed to evaluate the growth responses to PDGF, as described previously (18). Cultures were maintained in serum-free media for 3 days, then replaced with either culture media alone (control), or with media containing FCS or PDGF-BB (PeproTech; Cedarlane). After 17 h, [3H]thymidine was added for 5 h, and the cells were then processed for scintillation counting. Counts were normalized to cohort negative controls and expressed as fold increase over control.

Flow cytometry. CSMC were isolated from the colons of animals that received TNBS 2 days previously, as described above. These were fixed with 70% ethanol before labeling with antibodies to α-SM actin, and nuclei were labeled with propidium iodide (PI; 10−7 M) to detect nuclear DNA content. These were subjected to standard fluorescence-activated cell sorting (FACS) analysis (EPICS ALTRA HSS) to remove immune cells (α-SM actin negative) and sort the α-SM actin-positive CSMC into dividing and nondividing populations, based on the intensity of nuclear PI. Cells were then processed for Western blot analysis, as described below.

Western blot analysis. Since changes in cell size and volume may accompany alteration to CSMC phenotype, with unknown consequences to the expression of housekeeping proteins, experiments were performed on a per-cell basis. An equal number of cells in each condition were lysed in buffer (in mM: 10 Tris-HCl, 150 NaCl, 5 EDTA, 0.2% Triton X-100, 0.5% sodium deoxycholate; pH 8.0, with 10 μM leupeptin, 10 μM aprotinin, and 1 mM phenylmethylsulfonyl fluoride), resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane using a wet transfer apparatus (Bio-Rad). Blots were incubated in blocking buffer (5% non-fat milk in Tris-buffered saline–0.2% Tween 20 or 5% BSA in Tris-buffered saline–0.2% Tween 20) for 1 h at room temperature, following incubation in primary antibodies overnight at 4°C. These included α-SM actin (1:5,000; DAKO), desmin (1:1,000; Chemicon), SM22-α (1:5,000; Abcam), and Act and phospho-AKT (pAKT) (both 1:1,000; Cell Signaling). This was following by incubation with appropriate horse-radish peroxidase-linked secondary antibodies for 1 h at room temperature before visualizing bands using a chemiluminescent substrate (Super Signal, Pierce). Exposed films were converted to gray scale, and the band intensity determined using Image Pro Plus.

Statistics. Quantitative data are expressed as means ± SE of n animals. Differences are considered significant for P values ≤ 0.05 using ANOVA.

RESULTS

Adaptation of CSMC to tissue culture decreases markers of the differentiated phenotype. To study the characteristics of CSMC on adaptation to tissue culture, we used an established primary culture model described previously (25). Freshly isolated CSMC show an elongated, spindle shape (Fig. 1A), becoming flattened with extended processes by day 4 in vitro (Fig. 1B). By day 6 in culture, cell proliferation was apparent,
resulting in the appearance of overlapping cells and the typical hill-and-valley appearance of cultured SMCs (Fig. 1C).

CSMC cultured for 4 days showed an overall decrease in expression of markers of the contractile phenotype over freshly isolated cells. Quantitative real-time PCR analysis showed a substantial decrease in mRNA for α-SM actin, desmin, and SM22-α by −65, 80, and 60% relative to control, respectively. In contrast, expression of cyclin D1, a key regulator of cell proliferation, was increased by >250% in cultured CSMC relative to control (Fig. 1D). Western blot analysis of contractile marker expression showed that changes in protein levels occurred that paralleled the changes to mRNA levels, with expression of α-SM actin, desmin, and SM22-α decreased by 60–80% relative to control (Fig. 1E).

Immunocytochemistry to detect α-SM actin, desmin, and SM22-α showed strong but characteristically variable expression by day 4 in culture (Fig. 2, A–C), along with the onset of vimentin expression that was coexpressed with these SMC markers (Fig. 2, D–F). While many cells expressed significant levels of these phenotypic markers, others showed little or no expression. To determine whether the intensity of contractile marker expression corresponded to mitotic status, we colabeled these cultures with antibodies to PCNA. Double labeling of day 4 cultured cells using SM22-α and PCNA antibodies suggested that the expression of PCNA and SM22-α in a given cell was inversely proportional. Cells with strong PCNA expression that are entering S phase of the cell cycle expressed much lower levels of SM22-α than quiescent cells with little or no PCNA expression (Fig. 3, A–C). Analysis of cultured CSM double labeled using α-SM actin and PCNA showed a similar result (Fig. 3D). Therefore, there appeared to be two distinct cell populations in cultured CSM, with strong expression of phenotypic markers indicative of a quiescent cell, and weaker expression correlating with the initiation of the cell cycle.

The phenotypic changes are reversible at early passage. CSMC in the inflamed colon undergo proliferation early in colitis, then revert to a contractile, nonproliferating phenotype by day 6 post-TNBS (13, 19). We tested whether proliferating CSMC are able to undergo a similar reversal in phenotype in vitro by culturing cells in 5% serum for 4 days, then in 0.25% serum for a further 7 days to remove the stimulus to undergo further proliferation, but to maintain viability. Primary, passage 1 (P1) or passage 2 (P2) cells cultured in low-serum conditions for 7 days showed enhanced expression of α-SM actin compared with control cells exposed to 5% serum (Fig. 3). In addition, cells maintained in 5% serum showed an increased expression of PCNA relative to cells incubated in low-serum conditions (compare Fig. 3, E and F). In cells exposed to low-serum conditions, quantitative PCR analysis showed that mRNA levels for α-SM actin were nearly 14-fold

Fig. 1. Adaptation of circular smooth muscle cells (CSMC) to tissue culture sharply decreases mRNA and protein levels for markers of the differentiated smooth muscle (SM) phenotype. A–C: representative phase-contrast images showing adaptation of CSMC to primary culture. A: phase-contrast appearance of live, freshly isolated CSMC at 30 min postisolation. Some cells have begun to shorten and become more round (e.g., arrow). Scale bar, 100 μm. B: by 4 days in vitro, CSMC are attached, and many are flattened with extended processes. Scale bar, 40 μm. C: by 6 days in vitro, CSMC are well established, and cell growth has occurred to form a typical SM culture with regions of high density and overlapping cells, as well as lower density or open areas. Inset: image of a single SM at 6 days in culture. Scale bars, 40 μm. D: quantitative PCR (qPCR) analysis of SM markers showing a significant decrease in mRNA levels for α-SM actin (SMA), desmin, and SM22-α in cells cultured for 4 days relative to freshly isolated cells. In contrast, cyclin D1 mRNA in these cells was increased almost threefold. E: protein expression of SM markers in cells cultured for 4 days showed decreases that paralleled the changes in mRNA levels. Top: typical outcome of Western blots showing reduced expression of all 3 markers in day 4 cultured vs. freshly isolated CSMC. Each lane represents protein expression per 5,000 cells. Bottom: histogram of quantified immunoblotting analysis of cultured CSM. Each point represents the mean ± SE of integrated optical density (IOD) values relative to freshly isolated control (n = 3 animals per time point; P < 0.05).
higher than control CSMC in 5% FCS (Fig. 3G). Similarly, Western blot analysis to detect expression of α-SMA showed a significantly higher level of protein in cells maintained in low-serum relative to high-serum conditions (Fig. 3H). Therefore, the reduction in contractile protein expression in CSMC cultured in 5% FCS can be reversed under conditions of growth inhibition with exposure to minimal serum.

Since proliferation caused a reduction of phenotypic markers in early passage cultures of CSMC, we determined the effect of continued proliferation on CSMC phenotype, with cells maintained until P10 (late passage) and compared with early passage cells (P2). As shown in Fig. 4, cells in late-passage cultures appeared to have a reduced size relative to cells at P2. Labeling of cultures with SM22-α antibodies indicated a dif-

Fig. 2. Immunocytochemistry of primary cultures of CSMC showing altered expression of markers of the differentiated phenotype with adaptation to culture. A: appearance of α-SMA labeling in CSMC cultured for 4 days. A low-magnification view (inset) shows strong labeling, but higher magnification reveals a nonuniform appearance, with strong cytoplasmic labeling among some cells adjacent to cells with little or no α-SMA expression (e.g., arrow). Nuclei are labeled with Hoechst (scale bar, 20 μm). B and C: a similar labeling pattern is observed in CSMC labeled with antibodies to desmin and SM22-α, respectively. Cells with strong labeling are interspersed with cells with little or no marker expression (e.g., arrows) (scale bars, 50 μm). D–F: images of dual-label immunocytochemistry of primary cultures of CSMC showing that adaptation to culture includes the onset of expression of vimentin. D: merged image showing expression of vimentin (red) in SM22-α-positive (green) CSMC at 4 days in vitro. E and F: lower magnification of field in D showing the individual appearances of SM22-α expression (E) and vimentin (F), with strong cytoplasmic expression of each marker that overlapped in cultured cells. The inset shows the field in E after refocusing, photographed with reduced exposure, to show the strong SM22-α staining of rounded CSMC still in the process of culture adaptation. These cells are SM22-α positive, but vimentin negative (e.g., arrow in inset, E, and D). Scale bars: 30 μm (D) and 50 μm (E and F).
ference in phenotype, with expression in cells at P2 much stronger than in cells at P10 (Fig. 4, C and D). Quantitative PCR analyses of expression of α-SM actin, desmin, and SM22-α showed a substantial decrease in mRNA levels in cells at P10 relative to P2 by 55, 85, and 80%, respectively. However, expression of cyclin D1 increased by nearly 60% in P10 cells relative to P2 (158.6 ± 21.0%; n = 3), suggesting a higher proportion of proliferating cells in later passage CSMC. The result of Western blot analysis for protein expression showed a similar outcome, with reduced expression of phenotypic markers in late-passage cells relative to cells at P2 (Fig. 4, F and G).

Proliferation of CSMC in vivo causes phenotypic alterations. Proliferation of CSMC is significant by day 2 of TNBS colitis in the rat (19). To assess the effect of proliferation on CSMC phenotype in vivo, we developed the methods needed for cell isolation and subsequent FACS. First, we isolated CSMC from the inflamed rat colon at day 2 of colitis, with phase-contrast microscopy showing characteristic CSMC, as well as the persistent presence of immune cells (Fig. 5A). Within this mixed population, we used dual-label immunocytochemistry for the smooth muscle marker smoothelin (25) and PCNA, to identify a subpopulation of PCNA-positive CSMC (Fig. 5, B and C) among a majority of PCNA-negative CSMC, as well as double-negative immune cells (Fig. 5C).

These cells were then sorted using FACS to obtain two populations of SMCs, according to their mitotic status, either nonmitotic (G1) or mitotic (S/G2/M). For this, experiments first determined that ethanol fixation of isolated CSMC did not interfere with subsequent Western blotting for smooth muscle marker proteins (not shown), thus allowing the use of immunocytochemistry for α-SM actin to identify SMCs, followed by routine staining of DNA with PI. FACS analysis identified a subset of mitotic CSMC (7.7 ± 1.6%; n = 5) within the total CSMC population, while no mitotic cells were identified in CSMC from control rats. Western blotting of the CSMC that were recovered after sorting showed no significant differences in expression of desmin or SM22-α between control CSMC and nonmitotic CSMC from day 2 animals (Fig. 5). However, this approach did show a large and consistent decrease in the mitotic population, with mean expression of desmin and SM22-α reduced by >50% (P < 0.05 vs. control or nonmitotic CSMC on day 2; Fig. 5F). From this, we conclude that the early stage of TNBS colitis causes the onset of proliferation in a subset of CSMC that selectively and profoundly decreases their expression of markers of smooth muscle phenotype.

Fig. 3. Growth inhibition reverses phenotypic modulation in CSMC in vitro. A–C: images of CSMC labeled with antibodies to SM22-α (red; A) and proliferating cell nuclear antigen (PCNA; green; B), with an overlap of the two images shown in C. Cells with strong SM22-α labeling showed little expression of nuclear PCNA (e.g., arrows), and, conversely, CSMC with low SM22-α expression showed strong nuclear labeling with PCNA (arrowhead). D: dual-labeled image of α-SMA and PCNA showing similarly reduced α-SMA expression in CSMC with strong nuclear PCNA expression (e.g., arrows), and, conversely, CSMC with low SM22-α expression showed strong nuclear labeling with PCNA (arrowhead). 

E and F: comparison of outcomes of immunocytochemistry for α-SMA in primary cultures of CSMC that were cultured for 4 days and then either maintained in 5% serum or switched to 0.25% serum for 7 days. α-SMA expression remained low in cells incubated in high serum (E), but increased in low serum (F). Nuclei are labeled with Hoechst. G and H: confirmation of increased α-SMA expression in cohort cultures of CSMC showing increased mRNA levels in 0.25% serum by qPCR (G) and Western blotting (H), with inset (H) showing a representative Western blot, with each lane representing 5,000 cells. n = 3 Separate isolations per condition. * P < 0.05 vs. 5% fetal calf serum (FCS). Scale bars, 40 μm.
Persistence of an altered phenotype is evident in CSMC from the recovered colon. In previous studies of the contractility of freshly isolated CSMC, we showed that altered responses to neurotransmitters were present among CSMC isolated from the colon of rats administered TNBS 35 days previously, compared with control CSMC (29). Since this might correlate with persistence of alterations to the expression of phenotypic markers, we assessed this in CSMC from the colons of rats at 35 days post-TNBS colitis. First, we found that these cells were characteristically enlarged compared with control cells, with quantification of phase-contrast images showing that mean cell area was nearly 30% greater in day 35 CSMC (Fig. 6A). However, Western blotting showed significant reduction of α-SM actin, desmin, and SM22-α protein expression per cell (Fig. 6B).

Further differences appeared when day 35 CSMC were maintained in vitro under the same culture conditions as used for control CSMC. In primary cultures of day 35 CSMC, immunocytochemistry for α-SM actin showed a general decrease in expression compared with control cells (Fig. 6C; compare with Fig. 2A). This was reinforced by the outcome of examination of mRNA levels in primary cultures on day 4 in vitro, where day 35 CSMC showed surprisingly large decreases in mRNA for all markers, reaching only 10–20% of that in freshly isolated day 35 CSMC (Fig. 6D). These levels were significantly lower than those seen in CSMC from control
animals also cultured for 4 days ($P < 0.05$; $n = 5$). Furthermore, the expression of mRNA for cyclin D1 increased far more greatly than in control cells at the same time point (Fig. 6D), suggesting that the onset of proliferation and modulation of phenotype was more rapid in cells isolated from day 35 post-TNBS relative to control. In airway SMCs, PDGF activates the cyclin D1 promoter (26), and PDGF has been shown to be the dominant mitogen for ISMC proliferation (25). Therefore, we examined the possibility of a greater propensity for growth by determining $[^3H]$thymidine uptake in day 35 cells stimulated with either FCS or PDGF-BB and compared this to the control response. As shown in Fig. 6E, $[^3H]$thymidine incorporation into day 35 cells following FCS exposure was more than double that of control CSMC. However, the response to PDGF was even more striking, where the response was six- to eightfold greater relative to control for each dose of PDGF tested (0.5–50 ng/ml; $n = 3$ isolations, $P < 0.05$).

Earlier, we showed that acetylcholine caused contraction of freshly isolated CSMC and the characteristic phosphorylation of AKT, which was similar among day 35 and control CSMC (29). This approach detects early events in signal transduction, and, since the effect of proliferation on this response is unknown, we tested this in vitro, evaluating the expression of mRNA for muscarinic receptor 3 at 4 days in culture. While this decreased significantly in control cells, by 50% (31), day 35 CSMC showed approximately a 90% reduction in mRNA ($P < 0.05$ vs. control; Fig. 7A). To determine the outcome of cholinergic stimulation, we analyzed carbamyl-induced expression of p-AKT in control or day 35 CSMC at P2. This showed that the addition of $10^{-4}$ M carbamyl to control CSMC for 15 min resulted in an increase in p-AKT to 158% of unstimulated control. However, similar treatment of day 35 cells showed no significant change in expression of p-AKT with cholinergic stimulation (Fig. 7, B and C). This shows that proliferation of day 35 CSMC in vitro has a differentially severe impact on their cholinergic response compared with control cells.

Overall, we conclude that phenotypic alterations in day 35 CSMC are detectable in both freshly isolated cells and in their

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**Fig. 5.** CSMC isolated from the inflamed intestine show that proliferation causes modulation of SM phenotype in vivo. A: phase-contrast image of freshly isolated CSMC from the inflamed colon of rats at day 2 of trinitrobenzene sulfonic acid (TNBS) colitis, showing typical CSMC in the presence of immune cells (arrows). B and C: detection of the mitotic marker PCNA in freshly isolated CSMC by dual-label immunocytochemistry for the SM marker smoothelin (red) and PCNA (green), with merged labeling appearing yellow (arrow) in both an elongated and a rounded SM cell (arrows); contrast with the rounded, PCNA-negative CSMC (*). D: No-marks view of the field in B combined with detection of nonspecific nuclear fluorescence with Hoechst; note the double-negative putative immune cell (arrowhead). E: representative image of CSMC obtained by selection for α-SMA expression (green) using fluorescence-activated cell sorting (FACS), showing positive labeling of all cells (e.g., arrow); nuclei are labeled with propidium iodide (red). F: representative Western blot for desmin and SM22α expression in CSMC after sorting by FACS, showing decreased expression in mitotic vs. nonmitotic cells (2,500 cells/lane). D2, day 2. F: quantification of protein expression in mitotic vs. nonmitotic cells showing significant decrease in expression per cell in the mitotic population ($n = 3$ per condition; $^{*}P < 0.05$).
response to proliferation in vitro. While this decreases the expression of characteristic smooth muscle proteins in both cases, prior proliferation in vivo appears to prime these cells to respond to further mitogen exposure with enhanced proliferation.

DISCUSSION

The severe transmural inflammation of the intestine that occurs in TNBS-induced colitis in the rat models the effect of Crohn’s disease in humans. This has shown that significant
changes occur in the smooth muscle/myenteric plexus region (e.g., Ref. 8), contributing to the characteristically altered motility. Proceeding from earlier work showing inflammation-induced hyperplasia of ISMC that contributed to thickening of the intestinal wall; the present work examined the effect of proliferation among the ISMC is curtailed by day 6, and ISMC numbers are stable thereafter (19). This could reflect the reversal of a transient modulation of phenotype, as suggested to occur when mitogenic stimuli were removed in vitro, and indeed, by day 35 post-TNBS colitis, freshly isolated ISMC were similar to control cells in their contractile response to ACh (29). However, there were still altered responses to other stimuli, suggesting that restoration of the normal phenotype might not have occurred, and examination of phenotype by Western blotting showed uniform, significant decreases in the cellular content of all three markers. Since these day 35 ISMC rats were also hypertrophic, this indicates that the density of contractile filaments within each cell was greatly decreased. While not well understood, both smooth muscle hypertrophy and hyperplasia contribute to increased muscle mass in airway disease (1). A recent study of an airway sensitization and challenge model in mice found specific evidence for smooth muscle hypertrophy, measuring increases in SMC size that were comparable to those recorded here (2). Similar studies in rats showed decreased expression of key smooth muscle markers (21), implying that hypertrophy is associated with decreased density of expression in multiple systems.
The decreased expression of α-SM actin and desmin in proliferating CSMC in vitro and in freshly isolated day 35 CSMC indicates a decreased contractile ability, but the significance of the decreased SM22-α in these cells may extend beyond that. SM22-α (or transgelin) is an actin-binding protein expressed in mature SMCs that is downregulated in vascular disease (e.g., Refs. 17, 28). Recent evidence shows that SM22-α normally inhibits cell cycle progression through segregation of Ras and downregulation of the Raf-1-MEK-ERK signaling cascade (7), which implies that the downregulation of expression of SM22-α in ISMC has strong predictive value for increased proliferation and a modulated phenotype in vitro and in vivo.

Decreased expression of SM22-α in freshly isolated CSMC from rats on day 35 postcolitis was correlated with an increased potential for cell division that became apparent with tissue culture. These cells showed a much more extensive decrease of mRNA for smooth muscle markers than control CSMC under similar conditions, i.e., at day 4 of primary culture. The disproportionate increase in cyclin D1 message indicated a more rapid onset of cell proliferation, which was emphatically confirmed through their exaggerated S-phase response to FCS or PDGF-BB. Since this growth response is evidence of increased proliferation and taken with the evidence for more rapid dedifferentiation, it appears that day 35 CSMC will grow more readily and lose phenotype more rapidly than control cells.

Furthermore, the evidence that cholinergic responsiveness is essentially lost in these cells in vitro, while retained by control cells, indicates that the outcome of a repeated episode of proliferation in vivo might strongly compromise organ function. Here, suppressed pAKT levels in response to a cholinergic stimulus indicated altered signal transduction, but other major regulatory events, such as the phosphorylation of regulatory myosin light chain [e.g., 20-kDa myosin light chains (11, 14, 16)] could also contribute to changes in smooth muscle contractility. These, along with the expression of contractile proteins, may underlie the changes seen in the mouse model of intestinal fibrosis that results from repeated application of TNBS, where the histology shows an altered cellular composition with exaggerated extracellular matrix production (9, 10, 15). While yet unproven, it may be that a combination of inflammation-induced smooth muscle proliferation with regionally severe neural damage and the subsequent potentiation of the growth response might result in the dedifferentiated structure-like region observed in a subset of rats after recovery from TNBS colitis (20).

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No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


