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Alterations in expression of myosin and myosin light chain kinases in response to vascular injury

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Abstract

Histochemical analysis of balloon-injured rat carotid arteries revealed a coordinated expression of nonmuscle myosin heavy chain-A and -B (NM-A and NM-B) in response to injury. Expression of these nonmuscle myosin forms shifts from the media to the adventitia and intima. In contrast, expression of smooth muscle myosin heavy chain-1 (SM-1) within the media is not altered, whereas smooth muscle myosin heavy chain-2 (SM-2) expression declines. Western blotting shows a statistically significant increase in expression of NM-A that occurs within 6 h in response to carotid injury, suggesting this myosin form may be an appropriate experimental marker for proliferating, migrating cells in injured vessels. No overall change in the relative expression level of NM-B was detected, suggesting that compensatory declines in media expression are balanced by increases in the intima and adventitia. Expression of SM-1 did not change in response to injury, whereas the expression of SM-2 significantly declined between 24 h and 7 days. Expression of myosin light chain kinase is also negatively regulated, and the decline in its expression parallels downregulation of SM-2.

Keywords

smooth muscle; restenosis; vascular remodeling

Smooth muscle contractility and growth are important to normal vessel wall development, and abnormal proliferation of smooth muscle cells can contribute significantly to the pathophysiological changes associated with restenosis after angioplasty, hypertension, and atherosclerosis (29,30). Many studies have established that smooth muscle cells exhibit phenotypic changes during the process of vascular remodeling (28). Although the precise changes that occur are variable, depending on the species and pathology, common factors exemplifying the phenotypic plasticity exhibited by smooth muscle cells include alterations in cytoskeletal organization, composition, and distribution. These changes also include distinct alterations in the expression of myosin heavy chain (MHC) isoforms, an event that is likely to have major effects on smooth muscle contractility, migration, and proliferation.

Several isoforms of smooth muscle MHC have been identified and shown to arise via alternative splicing at both the 5' and 3' ends of the mRNAs (2,22). Alternative splicing within the 3' end of the smooth muscle MHC gene results in unique carboxyl termini that distinguish smooth muscle MHC-1 (SM-1) and smooth muscle MHC-2 (SM-2; see Ref. 16). The

physiological role of the alternatively spliced carboxyl termini that results in SM-1 and SM-2 isoforms is not understood, but this region may serve as a signal transduction site, as SM-1 is phosphorylated by at least one protein kinase (16). In addition to SM-1 and SM-2, the expression of additional distinct MHCs, the nonmuscle MHCs in vascular smooth muscle, has been demonstrated in human, porcine, and rabbit animal models (15,18,33,34). Two forms of nonmuscle MHC have been identified and shown to arise from separate genes (16). Previous studies have shown that the mRNA encoding non-muscle MHC-B (NM-B) is predominantly expressed in embryonic and postnatal aortas and is downregulated in adult tissues. Other studies have shown that the levels of the mRNA encoding NM-B are increased in intimal smooth muscle cells and adventitial myofibroblasts in experimental atherogenesis and human atherosclerotic lesions (1,2,7,19,20,24). However, it is still not clear if increases in mRNA levels occur in parallel with an increase in the NM-B protein or if the expression is equally distributed throughout restenotic lesions.

In all types of smooth muscle, including vascular smooth muscle, the contractile response involves activation of myosin to a force-generating state. Activation of myosin to result in contraction occurs when myosin regulatory light chains (RLC) are phosphorylated by myosin light chain kinase (MLCK; see Ref. 11). Phosphorylation of RLC by MLCK is also important for cellular migration and division (11,17). A 220-kDa MLCK (nonmuscle MLCK) and a 130-kDa MLCK (smooth muscle MLCK) have been identified and shown to be expressed in both smooth and nonmuscle tissues. These MLCKs are derived from the same gene and differ only by the presence of ~1,000 residues that extend the amino terminus of the larger form, suggesting that their biochemical activities are similar (10–12). Although some studies have examined the expression of MLCKs in normal cells and tissues, little information is available about changes in expression of MLCKs in pathological conditions (6,10,11). In view of the importance of understanding at the protein level how expression of contractile proteins in vascular smooth muscle changes temporally and spatially in response to injury, we have, in the current study, examined the expression patterns of MHCs and MLCKs in balloon-injured rat carotid vessels.

METHODS

Antibodies and cells

The anti-chicken gizzard smooth muscle MLCK monoclonal (K36, 1:10,000 dilution; Sigma) antibody was used to detect both 130- and 220-kDa MLCKs. Antibodies to 204-kDa SM-1 (1:5,000 dilution), 200-kDa SM-2 (1:5,000 dilution), 200-kDa nonmuscle myosin-A (NM-A; 1:30,000 dilution), and 196-kDa NM-B (1:30,000 dilution) were generated using peptides representing unique regions at the carboxyl terminus of these myosin isoforms. The peptides used were SM-1 (CDADFNGTKSSE), SM-2 (CSKLRGPPPQETSQ), NM-A (CDGAEKPAE), and NM-B, (CDVNETQPPS; see Refs. 5 and 22). The peptides were coupled to keyhole limpet hemocyanin and were injected in rabbits to produce antibody. All anti-peptide antibodies were affinity purified and tested by Western blotting to determine their specificity. COS-7 and A10 rat embryonic thoracic aorta cell lines were obtained from the American Type Culture Collection (Manassas, VA). Human platelet extracts were generously provided by Dr. Robert Wysolmersky (St. Louis University School of Medicine, St. Louis, MO).

Balloon-injured rat carotid artery model

Male Sprague-Dawley rats weighing 200–250 g were anesthetized by intra-peritoneal injection of ketamine (80 mg/kg) and acepromazine (0.1 mg/kg). A midline incision was made, and the left carotid artery was isolated. The distal external carotid artery was ligated, and a small arteriotomy was made. A no. 2 French Fogarty balloon catheter (Baxter Edwards LIS, Irvine, CA) was inserted and advanced ~2 cm below the carotid bifurcation. The balloon was distended with saline to 2 atmospheres using an ACS Inflator Plus (Advanced Cardiovascular Systems,

Temecula, CA) and was gently withdrawn to the level of the bifurcation. The balloon was deflated, and the procedure was repeated for a total of three times. The external carotid artery was ligated, and the incision was closed. At varying intervals after balloon injury the rats were killed by an intraperitoneal injection of pentobarbital sodium, and the left and right carotid arteries were harvested.

Temporal expression of myosin and MLCKs in the rat carotid artery after vascular injury

At 1 h, 3 h, 6 h, 24 h, 3 days, or 7 days, the animals were euthanized, and the injured left and right control carotid arteries were removed. The arteries were cleaned, snap-frozen in liquid N₂, and pulverized to a fine powder. Proteins were extracted by homogenization of the frozen tissue powder in lysis buffer (1% Nonidet P-40, 10 mM MOPS, pH 7.0, 1.25 mM EGTA, 10 mM dithiothreitol, 50 mM MgCl₂, 300 mM NaCl, 100 µg/ml phenyl-methylsulfonyl fluoride, 4 µg/ml leupeptin, and 10 µg/ml aprotinin). Total protein concentration in each sample was determined using the Bradford protein assay. To control for variations in protein determination, each sample was quantitated at least three times, and the average value was used for analysis. To control for animal variations in response to injury at least four to six animals were examined at each time point.

Western blotting and quantitation

Western blotting was performed as described previously (9). Equivalent amounts of total cellular protein (25 µg, prepared as described above) were fractionated by electrophoresis through a 4% SDS poly-acrylamide gel and transferred to nitrocellulose. Immunoreactive proteins on Western blots were visualized using the Pierce Supersignal detection system according to the manufacturer's directions. Densitometry was performed on each sample after Western blotting using National Institutes of Health Image (version 1.62) and appropriate macros. Short exposures of the Western blots that were not saturating were used for quantitation. To analyze the densitometric data, the ratio of the raw densitometric units from the left (injured) and contralateral right (control) vessel for each individual animal in the sample group was determined (L/R ratio). The mean of these L/R ratios for each time point was calculated using a standard statistical program in Excel. Bars in Figs. 1–8 represent the calculated mean value of the ratios $\pm$ SE. ANOVA was used to determine if a statistically significant difference existed between the mean of the expression in the left (injured) vessels and the right (control) vessels at each time point. Means were considered statistically different at $P \leq 0.01$. To control for variations in extraction, loading, and transfer of the proteins in the tissue samples, the relative level of expression of α -smooth muscle actin (Sigma) was determined to examine the potential level of experimental variation in the sample groups at each time point. These results (data not shown) confirmed that α -smooth muscle actin signal for all samples within either the injured or control groups did not vary by more than ± 0.2 densitometry units at each time point. In addition, to control for variations in transfer efficiency, data were used for quantitation of the means at each time point only when the densitometry signal from the A10 control lane was within ± 0.2 densitometry units of that obtained with the same antibody for all time points.

Immunohistochemistry

At the indicated times after injury, carotid arteries were fixed by perfusion fixation under physiological pressure with phosphate-buffered paraformaldehyde (4%, 0.1 mol/l) as described previously (21). The carotid arteries were excised and embedded in paraffin, and sections were prepared (>200 µM). Immunocytochemical staining for MHC isoforms was carried out using paraffin sections of carotid arteries. Each section was incubated with affinity-purified anti-peptide antibodies. Dilutions of antibodies were 1:3,000 for NM-A and NM-B and 1:50 for SM-1 and SM-2. Subsequent incubations with fluorescence-conjugated anti-rabbit IgG were

performed as previously described (21). Control sections were reacted with secondary antibody or nonimmune serum (nonimmune IgG). At least four sections from three different animals were examined for each antibody.

RESULTS

Characterization of antibodies to smooth muscle and nonmuscle MHCs

Western blotting was used to determine the specificity of the affinity-purified antibodies to MHCs. Figure 1A shows that the SM-1 and SM-2 affinity-purified antibodies each react with a single high-molecular-weight band in a smooth muscle tissue (bovine trachea). These high-molecular-weight bands are absent when the affinity-purified antibodies are reacted against cell extracts prepared from fibroblast cell lines, which lack expression of smooth muscle myosin isoforms (data not shown). Reaction of both antibodies on a single blot produces two closely migrating bands of 204 and 200 kDa. Reaction of affinity-purified NM-A and NM-B against extracts from cells that express both NM-A and NM-B (A10), only NM-A (platelets), or only NM-B (COS) shows that these antibodies react appropriately with a single high-molecular-weight band of ~200 kDa. These data together with previously published data (13) show that these antibodies react appropriately with a single specific isoform of smooth or nonmuscle MHCs.

Expression of NM-A is rapidly upregulated and SM-2 is rapidly downregulated after vascular injury

Several previous studies have shown that transcriptional alterations that lead to increased or decreased levels of the mRNAs encoding myosin isoforms occur in response to injury. To learn whether parallel changes in the protein expression levels of the corresponding MHCs occur, isoform-specific antibodies to SM-1, SM-2, NM-A, and NM-B were used to detect these MHC isoforms in control (right) and injured (left) rat carotid arteries. At various times after balloon injury, tissues were harvested and analyzed by Western blotting. Figure 2 shows Western blots analyzing the expression of MHCs for four representative animals for each time point. At each time point, left (injured) and paired right (control) carotid arteries from at four to six animals were analyzed. Figure 3 shows the mean of the ratios of the expression in left compared with right control vessels ($\pm$ SE) for each time point. Sufficient numbers of animals were used to achieve statistical significance ($P < 0.01$) to control for animal-to-animal variation in the response to injury as well as for variations in sample extraction, quantitation, transfer, and loading. Statistical significance ($P < 0.01$) was determined by comparing the mean of the left sample group with the mean of the right sample group at each time point. These results show that a rapid, statistically significant increase in the expression of NM-A occurs in the injured, left carotid arteries between 3 and 6 h. Overall, expression of NM-A increases in the left (injured) carotid vessels three- to fourfold after injury, although between 6 and 24 h a small relative decrease in expression occurs. In contrast to earlier studies examining the mRNA levels for NM-B, no significant increase in the overall expression of NM-B protein was detected in injured vessels (2,3,7,18,20,24). Similarly, no significant change in expression of SM-1 occurred in the injured carotid vessels during this time course, whereas expression of 200 kDa SM-2 was significantly downregulated (10-fold) beginning by 24 h and continuing to 7 days.

Changes in expression of MLCKs in injured vascular tissue

To learn if in vivo temporal alterations in the expression of the 220- and 130-kDa isoforms of MLCK occur in response to injury, Western blots representing the expression levels of these MLCK were examined. Figure 4 shows the Western blotting results of this temporal expression study, and Fig. 5 shows the changes in expression of 220- and 130-kDa MLCKs in the left, injured vessels compared with the right control vessels that were determined as described in METHODS. These results reveal that a significant decrease in expression of 130-kDa MLCK

occurs in the left, injured carotid vessels by 24 h and continues until at least 7 days. The expression of 220-kDa MLCK is below the level of detection in control vessels, and its expression does not increase in response to injury.

In situ changes in expression of NM-A and NM-B

Examination of histological sections reacted with anti-NM-A antibodies revealed that, before injury, expression of NM-A occurs in the medial and endothelial layers of the vessel. After injury, the pattern of expression began to change and resulted in an increase in expression of NM-A. Consistent with the Western blotting experiments, the increase in expression of NM-A could be detected visually by immunohistochemical staining of tissue sections from injured vessels at 4 h postinjury. As the intimal layer developed, localization of the cells that were most intensely stained for NM-A shifted from the medial to the adventitial as well as the intimal layer, and the overall level of expression continued to increase until at least 2 wk postinjury (Fig. 6). In contrast, expression of NM-B was higher relative to NM-A in the medial and endothelial layers of the uninjured vessel. After injury, expression of NM-B had a similar pattern of expression to NM-A, being initially localized to the medial smooth muscle layer, where it then decreased as expression in the adventitial and neointimal layer increased (Fig. 7). These results suggest that expression of NM-A becomes rapidly upregulated in the medial layer, and then its expression becomes coordinated with NM-B in the newly forming intimal layer. This pattern of expression may represent the population of dedifferentiating smooth muscle cells that migrate from the media to form the neointima. These results may also suggest that adventitial cells can become activated in response to injury and either proliferate and/or become migratory to contribute to the negative and positive remodeling of the vessel in response to injury.

In situ changes in expression of SM-1 and SM-2

Figure 8 shows the results of staining histological sections from control (uninjured) and injured carotid arteries with antibodies to SM-1 and SM-2. Examination of these sections showed that expression of SM-1 does not appear to change in response to injury. Before injury, SM-1 was expressed in smooth muscle cells in the media, and after injury this MHC continued to be predominantly expressed in the media and at relatively low levels in the forming neointima. In contrast, expression of SM-2 appeared to be restricted to the media of uninjured vessels, and after injury its expression in the media visibly declined by 24 h consistent with Western blotting. In addition, SM-2 did not appear to be expressed in the newly forming intima although it is possible that some of the migrating smooth muscle cells that form the intima may have had levels of expression below that detectable by immunohistochemical techniques.

DISCUSSION

In this study, Western blotting and immunohistochemistry with specific antibodies was used to comprehensively examine the changes in the temporal and spatial expression of MHCs and MLCKs that occur in response to vascular injury. The results show a significant, rapid upregulation of the expression of NM-A but no overall change in the relative level of expression of NM-B. Histological studies show that, although the relative expression level of NM-B does not change, a significant change occurs in its spatial expression that leads to a coordinated expression of NM-A and NM-B in the adventitial and intimal regions of the injured vessel. Of the smooth muscle MHCs, only SM-2 exhibited a significant decline in expression that paralleled the temporal decline in expression of 130-kDa MLCK.

Using the rat carotid injury model, we have shown that temporal expression of one MHC form, NM-A, rapidly increases after injury. This finding suggests that NM-A may be a useful marker to identify proliferating, migrating cells that can contribute to both the negative and positive

remodeling of injured vessels (14,25,31). Although the overall protein expression level of NM-B does not significantly change in response to injury, its spatial localization does. One explanation consistent with this observation is that, after injury, the declining expression of NM-B in the media is balanced by increased expression in the adventitia and neointima. This suggestion is consistent with previous *in situ* hybridization studies that have shown an increase in the level of mRNA for NM-B in both adventitial and intimal cells. The coexpression of NM-A and NM-B in both the adventitial and intimal layers correlates with the regions of injured vessels where proliferating, dedifferentiating vascular smooth muscle cells appear (1,7,18, 20,24). Although the physiological roles of the two nonmuscle MHC isoforms in smooth muscle tissues are not yet understood, it is becoming clear that NM-A and NM-B may regulate distinct motile processes like extension and retraction of cells (16). Thus the coexpression of these MHCs within the same regions of injured vessels may indicate that proliferating adventitial myofibroblasts and proliferating, dedifferentiating vascular smooth muscle cells may require both NM-A and NM-B forms to become competent for migration and proliferation.

Although no significant change in the relative level of expression of SM-1 was detectable by Western blotting or immunohistochemistry, expression of SM-2 protein becomes significantly downregulated within 24 h, and its expression continues to decline to an undetectable level by 7 days postinjury. This is consistent with other previous studies that have all shown that a decline in expression in SM-2 mRNA occurs in response to injury (1–3,26,27). The mRNAs encoding SM-1 and SM-2 arise from alternative splicing at the 3' end of the smooth muscle MHC gene. This together with the results of these studies suggests that the mRNA and/or the protein for SM-2 turns over more rapidly than the mRNA and/or the protein encoding SM-1 (4,22,32). Alternatively, regulation of the differential splicing to favor the mRNA encoding SM-1 might account for the static levels of SM-1 expression and decreasing levels of expression of SM-2. Together these results suggest that an unusual and distinct mechanism for regulating the expression of SM-1 and SM-2 occurs in the smooth muscle cells in injured tissues.

Paralleling the decline in expression of SM-2 MHC isoforms, we also find a statistically significant decrease in the relative level of expression of the 130-kDa MLCK. This decrease in expression begins at 6 h and continues until at least 7 days postinjury. The loss of a contractile smooth muscle MHC isoform (SM-2) and the protein kinase regulating its activity are consistent with the dedifferentiation of smooth muscle cells to a more embryonic phenotype and could potentially contribute to the impaired contractility of injured vessels. Neither SM-2 nor 130-kDa MLCK is highly expressed in developing embryos and are considered to be adult MHC and MLCK forms (2,10,11,23,28). Expression of the 220-kDa MLCK is not detected in either control or injured vessels, suggesting that this MLCK is not highly expressed in either endothelial or smooth muscle cells of arterial vessels, nor is it upregulated in response to injury in the rat. This observation is consistent with the suggestion that the 220-kDa MLCK may be an earlier developmental MLCK (8,9).

In summary, the experiments in this report show that balloon injury of the rat carotid artery results in a coordinated expression of NM-A and NM-B initially in the medial and adventitial regions within 24 h postinjury. Expression of both of these MHC isoforms is not confined to the adventitial region of injured vessels, as both are detected in the developing neointima. This positive change in expression in the adventitia occurs coupled with a decrease in expression of both NM-A and NM-B in the media of injured vessels. The positive increase in expression of NM-A is very rapid, being detectable within 3–6 h after injury and suggests that NM-A may be useful as an early experimental marker to identify proliferating, migrating cells in injured vessels. Expression of the major protein kinase regulating smooth muscle contractility, the 130-kDa MLCK, is negatively regulated, and this decline in expression parallels the downregulation of expression of one of the contractile MHC isoforms, SM-2. Together these

results highlight new data showing that the phenotypic modulation of protein expression levels of MHC and MLCKs in smooth muscle cells as a result of vascular injury can occur very rapidly in response to vascular injury. These studies also stress the complexity of the molecular regulation at both the transcriptional and translational level of the contractile proteins that are important for the differentiated contractile phenotype of smooth muscle tissues.

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Fig. 1.

Characterization of myosin heavy chain (MHC) antibodies (AB). Western blotting of bovine tracheal tissues (Tr; A) or A10 cells, platelets, or COS cells (B) was used to confirm the specificity of affinity-purified antibodies to 204-kDa smooth muscle myosin-1 (SM-1), 200-kDa smooth muscle myosin-2 (SM-2), and the 200-kDa nonmuscle myosins-A (NM-A) and -B (NM-B). Bovine tracheal tissue expresses both SM-1 and SM-2. Platelets express only NM-A, and COS cells express only NM-B. Each lane represents 25 μ g total protein. C.E., cell extract.

Fig. 2.

Expression of nonmuscle and smooth muscle MHCs in control and injured rat carotid arteries. *A* and *B*: Western blots examining the expression of NM-A (*A*) and NM-B (*B*). *C* and *D*: Western blots of expression of SM-1 (*C*) and SM-2 (*D*). Each lane represents 25 μ g total protein from an injured left carotid and the control uninjured right carotid (indicated by nos. above each lane). A10 represents cell extract from A10 rat embryonic thoracic aorta cell line, which is included as a positive control. Expression of NM-A rapidly increases in injured left carotid arteries between 3 and 6 h. Expression of 200-kDa SM-2 becomes downregulated by 24 h. No change in expression of NM-B or SM-1 is detected.

Fig. 3.

Densitometry of Western blotting to detect expression of smooth and nonmuscle MHC isoforms. Western blots reacted with antibodies to detect either NM-A (A), NM-B (B), SM-1 (C), and SM-2 (D) were analyzed as described in METHODS. Bars depict means $\pm$ SE ($n = 4-6$) of the ratios of expression in the left (L) compared with right (R) carotid arteries for each time point. *Mean of the expression in the left (injured) vessel group is statistically ($P < 0.01$) different compared with right (control) vessel group at the same time point.

Fig. 4.

Western blotting to detect expression of myosin light chain kinases (MLCKs) in control and injured rat carotid arteries. Western blots to examine the expression of 220-kDa nonmuscle MLCK (220-kDa MLCK; A) and 130-kDa smooth muscle (130-kDa MLCK; B). Each lane represents 25 μ g total protein extracted from an injured left carotid and the contralateral uninjured right carotid (indicated by nos. above each lane). The 130-kDa and 220-kDa MLCK were detected with monoclonal antibody that recognizes a sequence common to both MLCKs. A10 represents cell extract from A10 rat embryonic thoracic aorta cells that is included as a positive control. Expression of 220-kDa MLCK is undetectable either before or after injury, whereas expression of 130-kDa MLCK declines by 24 h.

Fig. 5.

Densitometry of Western blotting to detect expression of 130-kDa (A) and 220-kDa (B) MLCKs. Western blots reacted with a monoclonal antibody to detect the 130- and 220-kDa MLCK were analyzed as described in METHODS. Bars depict the means $\pm$ SE ($n = 4-6$) of the ratios of expression in the left compared with right carotid arteries for each time point. *Mean of the expression in the left (injured) vessel group is statistically ($P < 0.01$) different compared with right (control) vessel group at the same time point.

Fig. 6. Immunohistochemical localization of NM-A in rat carotid arteries. Representative sections showing immunohistochemical localization of NM-A in the right (uninjured) and left (injured) rat carotid artery at 4 h, 4 days, 7 days, and 2 wk after injury. Control section shows a 7-day postinjury section reacted with normal IgG in place of primary antibody. Regions of the injured and uninjured vessels are indicated as follows: A, adventitia; I, intima; M, tunica media; L, lumen.

Fig. 7. Immunohistochemical localization of NM-B in rat carotid arteries. Representative sections showing immunohistochemical localization of NM-B in the right (uninjured) and left (injured) rat carotid artery at 4 h, 4 days, 7 days, and 2 wk after injury. Control section shows a 7-day postinjury section reacted with IgG in place of primary antibody.

Fig. 8.

Immunohistochemical localization of SM-1 and SM-2 in rat carotid arteries. Representative sections showing immunohistochemical localization of NM-A in the right (uninjured) and left (injured) rat carotid artery at 24 h, 3 days, and 7 days after injury. Control section shows a 7-day postinjury section reacted with normal IgG in place of primary antibody.