Phenotypic Modulation of Intima and Media Smooth Muscle Cells in Fatal Cases of Coronary Artery Lesion

Hiroyuki Hao, Giulio Gabbiani, Edoardo Camenzind, Marc Bacchetta, Renu Virmani, Marie-Luce Bochaton-Piallat

Objectives—Characterize the phenotypic features of media and intima coronary artery smooth muscle cells (SMCs) in mildly stenotic plaques, erosions, stable plaques, and in-stent restenosis.

Methods and Results—Expression of α-smooth muscle actin (α-SMA), smooth muscle myosin heavy chains (SMMHCs), and smoothelin was investigated by immunohistochemistry followed by morphometric quantification. The cross-sectional area and the expression of cytoskeletal proteins in the media were lower in restenotic lesions and, to a lesser extent, in stable plaques compared with mildly stenotic plaques and erosions. An important expression of α-SMA was detected in the intima of the different lesions; moreover, α-SMA staining was significantly larger in erosions compared with all other conditions. In the same location, a striking decrease of SMMHCs and a disappearance of smoothelin were observed in all situations.

Conclusions—Medial atrophy is prevalent in restenotic lesions and stable plaques compared with mildly stenotic plaques and erosions. Intimal SMCs of all situations exhibit a phenotypic profile, suggesting that they have modulated into myofibroblasts (MFs). The high accumulation of α-SMA–positive MFs in erosions compared with stable plaques correlates with the higher appearance of thrombotic complications in this situation. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: α-smooth muscle actin ■ erosion ■ smooth muscle myosin heavy chains ■ smoothelin ■ stable plaque

Thrombosis after plaque rupture is the most frequent cause of coronary mortality, followed by thrombosis over an erosion, whereas a smaller but significant proportion of deaths corresponds to the presence of stable plaques without evidence of thrombosis.1 Several factors have been suggested to participate in the mechanisms of plaque rupture, such as altered matrix turnover and blood rheology, increased coagulation, and recurrent infections.2 Macrophages and T lymphocytes are increased at site of plaque rupture, whereas erosions and stable plaques contain relatively few inflammatory cells.1,2 The causes of plaque rupture as well as those of thrombosis formation on nonruptured plaques are obviously crucial problems but remain at present controversial and probably depend on the concurrent action of several noxious events.

Smooth muscle cells (SMCs) are well-accepted players in the evolution of the atheromatous plaque3 and may also participate in the establishment of conditions facilitating plaque rupture and/or thrombus formation through several activities, including vasospasm4 and synthesis of extracellular matrix components5 or of proteolytic enzymes.5 Remodeling and deformation of the intima through tensile force generation by SMCs could also be instrumental in the process of plaque rupture. In this respect, no much is presently known concerning the phenotypic features of human SMCs in erosions and stable plaques; in particular, no systematic study has been made to establish whether SMCs have modulated into myofibroblasts (MFs)6 during these situations. This has become relevant in view of recent findings suggesting that force generated by MFs to produce tissue remodeling involves mechanisms different from those classically attributed to SMC contraction.6,7

To define the phenotypic changes of media SMCs and to test the hypothesis of a possible myofibroblastic modulation of intimal SMCs, we have taken advantage of well-characterized coronary mildly stenotic lesions (<50% cross-sectional luminal narrowing) compared with those observed in patients with sudden death and presenting erosions or stable plaques (>75% luminal narrowing).1 We have also included in our study coronary lesions of patients with restenosis after stent application.8 The phenotype of SMCs has been investigated by morphometric quantification of the expression of well-accepted SMC differentiation markers, such as α-smooth muscle actin (α-SMA),9 smooth muscle

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myosin heavy chains (SMMHCs), and smoothelin. Our results show that media atrophy is maximal in restenotic lesions and stable plaques, whereas erosions and mildly stenotic plaques exhibit similar features; moreover, intimal SMCs in all conditions exhibit remarkably dedifferentiated features, supporting the assumption that they have modulated into MFs. Finally, the intima of erosions shows a significantly higher accumulation of α-SMA–expressing cells compared with the other situations including stable plaques.

**Methods**

**Selection of Cases**

Human coronary artery lesion slides were provided by the Armed Forces Institute of Pathology (Washington, DC). The clinical classification of the lesions was based on histological observations as previously described. A total of 6 mildly stenotic plaques, 12 stable plaques, and 20 erosions were examined. Mildly stenotic plaques had ≤50% cross-sectional luminal narrowing; these specimens came from patients who died of causes other than cardiac events. Coronary arteries of patients undergoing sudden death (stable plaques and erosions) had ≥75% cross-sectional luminal narrowing by an atherosclerotic plaque or a plaque with a superimposed thrombus. Stable plaque was defined as an intimal plaque in the absence of a luminal thrombus. Erosion consisted of an acute thrombus in direct contact with intimal plaque. In addition, a total of 13 stented coronary arteries with restenotic intimal hyperplasia not resulting in sudden cardiac death were studied.

**Antibodies and Immunoblotting**

The following primary antibodies were used: (1) mouse monoclonal Iggs recognizing α-SMA (clone 1A4); (2) affinity-purified rabbit polyclonal Iggs recognizing both SMMHCs, SMMHC 1 and SMMHC 2, one produced in our laboratory, and the other commercially available (bt-562, Biomedical Technologies Inc, Stoughton, Mass); (3) mouse monoclonal Igg1 (clone r4a) recognizing smoothelin; (4) mouse monoclonal Igg1 recognizing cd68 specifically by macrophages (clone kpl; Dako, Glostrup, Denmark); (5) mouse monoclonal Igg1-specific of cd34, typical of endothelial progenitor cells, and fibrocytes (clone qhend 10; Dako); or (6) mouse monoclonal Igg1 specific of desmin (clone d33; Dako). Specificity of the antibodies was investigated by immunoblotting on different muscle and nonmuscle tissues. Detailed methods are available online at http://atvb.ahajournals.org.

**Histology and Immunohistochemistry**

Paraffin-embedded sections (5-m-thick) were stained with hematoxylin and eosin. Immunostainings for (α-SMA, SMMHCs, smoothelin, and cd68 were performed on sections adjacent to those stained histologically. Anti-SMMHCs antibodies produced similar results; image analysis was performed on the specimens stained with the anti-SMMHCs purchased from Biomedical Technologies Inc. To standardize image analysis quantification, times of antibody incubation and chromogen development were scrupulously observed and, for a given antibody, all sections were processed simultaneously. Before using the first antibody, immunoreactivity was intensified by: 1 microwave treatment (750 w) for 5 minutes in citrate buffer (10 mm, pH 6.0) for (α-SMA, desmin, and cd34, 3 microwave treatments for cd68, or 1 pressure cooker treatment for 3 minutes in citrate buffer for SMMHCs and smoothelin. Goat anti-mouse-biotinylated or anti-rabbit-biotinylated antibodies (Dako) were used as secondary antibodies, and the presence of the specific proteins was revealed by means of the streptavidin-biotin-peroxidase complex method (Dako) followed by hemalum counterstaining.

**Image Analysis**

For quantitative immunohistochemistry, sets of images (795×596 pixels) covering the whole cross-sectional area of the specimens were acquired with a high-sensitivity color camera (Coollview; Carl Zeiss, Jena, Germany) mounted on an Axioskop (Carl Zeiss) equipped with a 3200-Kelvin tungsten light. An apochromat 4x/0.10 (1 pixel corresponding to 13.69 μm²) and a plan-neofluar 10x/0.30 (1 pixel corresponding to 2.1904 μm²) objectives were used. Images were subsequently analyzed using the software KS400 (Kontron System; Carl Zeiss). Pixels were selected according to hue (ie, dominant color tone), lightness (ie, color intensity), and saturation (ie, color purity) components. Therefore, immunostaining was automatically discriminated from the unstained portion of the specimen. The threshold of positive staining above the background was determined on the control in which only the second antibody was used. Cross-sectional area of media, intima, and in-stent intima were manually defined. In addition, intimal necrotic core was manually excluded from the image; the remaining cross-sectional area (intima minus core in Tables 1 and 3) corresponded to the area of interest. Results were calculated as area of immunostaining (mm²), area of immunostaining/corresponding cross-sectional area and area of SMMHC or smoothelin immunostaining/area of α-SMA immunostaining.

Images for illustrations were acquired with an Axioskop (Carl Zeiss) using a 3200-Kelvin tungsten light with a high-sensitive, high-resolution color camera (AxioCam; Carl Zeiss) using an apochromat 4x/0.10 and an oil-immersion plan-neofluar 40x/1.4 objectives (Carl Zeiss).

**Statistical Analysis**

Results are shown as mean±SEM. For statistical evaluation, results were analyzed by means of the Student t test. Differences were considered statistically significant at values of P<0.05.

**Results**

The histological features of mildly stenotic plaques, erosions, stable plaques, and restenotic lesions have been reported previously. We limit our description to some general morphological changes and to new morphometric data, concerning essentially the accumulation of SMC differentiation markers.
TABLE 2. Cytoskeletal Protein Expression in Media

<table>
<thead>
<tr>
<th></th>
<th>α-SMA (mm²)</th>
<th>α-SMA/media (%)</th>
<th>SMMHC (mm²)</th>
<th>SMMHC/media (%)</th>
<th>SMMHC/α-SMA</th>
<th>Smoothelin (mm²)</th>
<th>Smoothelin/media (%)</th>
<th>Smoothelin/α-SMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mildly stenotic plaques</td>
<td>1.155 ± 0.364§</td>
<td>52.2 ± 3.7†§</td>
<td>0.727 ± 0.327§</td>
<td>31.6 ± 6.0†§</td>
<td>0.628 ± 0.120§</td>
<td>0.313 ± 0.140§</td>
<td>12.3 ± 3.5†§</td>
<td>0.245 ± 0.075§</td>
</tr>
<tr>
<td>Stable plaques</td>
<td>0.775 ± 0.068§</td>
<td>68.5 ± 2.9†§</td>
<td>0.544 ± 0.056§</td>
<td>50.8 ± 5.5†§</td>
<td>0.745 ± 0.067§</td>
<td>0.270 ± 0.057§</td>
<td>22.5 ± 3.0†§</td>
<td>0.331 ± 0.046§</td>
</tr>
<tr>
<td>Erosions</td>
<td>1.474 ± 0.143§</td>
<td>80.1 ± 2.9†§</td>
<td>0.837 ± 0.104§</td>
<td>46.6 ± 3.1†§</td>
<td>0.608 ± 0.059§</td>
<td>0.360 ± 0.069§</td>
<td>19.4 ± 3.4§</td>
<td>0.232 ± 0.039§</td>
</tr>
<tr>
<td>Stented plaques</td>
<td>0.521 ± 0.088†‡</td>
<td>35.8 ± 4.4†‡</td>
<td>0.182 ± 0.050†‡</td>
<td>14.7 ± 4.1†‡</td>
<td>0.346 ± 0.066†‡</td>
<td>0.040 ± 0.012†‡</td>
<td>4.0 ± 1.2†‡</td>
<td>0.081 ± 0.022†‡</td>
</tr>
</tbody>
</table>

Results are given as mean ± SEM.
Significant difference from *mildly stenotic plaques, †stable plaques, ‡erosions, and §stented plaques.

General Features
The cross-sectional area of the media (Table 1) was similar in mildly stenotic plaques and erosions; in both cases it was significantly larger compared with stable plaques (P < 0.05 and P < 0.01, respectively) and restenotic lesions (P < 0.01 and P < 0.001, respectively). No difference was present between the last 2 situations. Thus media atrophy is higher in stable plaques and restenotic lesions compared with the other situations.

The cross-sectional area of the intima was smaller in mildly stenotic plaques compared with the other situations (P < 0.05 always) (Table 1). It remained significantly smaller in mildly stenotic plaques versus restenotic lesions (P < 0.05) after eliminating the area of the necrotic core. Intima/media was lower in mildly stenotic plaques compared with the other situations (P < 0.01 versus stable plaques and P < 0.05 versus erosions and restenotic lesions). It was also significantly lower in erosions versus stable plaques (P < 0.01) and restenotic lesions (P < 0.001). When intima/media was calculated after elimination of the necrotic core area, no difference was present between mildly stenotic plaques and erosions; both were significantly lower compared with stable plaques (P < 0.05 in both cases) and restenotic lesions (P < 0.05 and P < 0.001, respectively); stable plaques were lower compared with restenotic lesions (P < 0.05). These results confirm that mildly stenotic plaques show less advanced lesions compared with the other situations.

Specificity of Antibodies
Detailed Results and Figure I are available online at http://atvb.ahajournals.org.

Cytoskeletal Protein Expression in Media
Immunostaining of the media for SMC differentiation markers confirmed and extended the previous observations (Table 2). The area of α-SMA expression (Figures 1a, 2a, 3a) was smaller in restenotic lesions compared with all other situations (P < 0.01 versus mildly stenotic, P < 0.05 versus stable plaques, and P < 0.001 versus erosions). Stable plaques showed an α-SMA-positive area similar to that of mildly stenotic plaques and smaller than that of erosions (P < 0.001). Remarkably, erosions presented the largest α-SMA-positive area. Similar results were obtained when α-SMA expression was calculated as α-SMA/media (Table 2). These results were essentially reflected by the distribution of SMMHCs (Figures 1c, 2c, 3c) and smoothelin (Figures 1e, 2e, 3e) with, however, some differences. The highest degree of dediffentiation, ie, the lowest expression of these markers was seen, as expected, in the media of restenotic lesions. In particular, the area of SMMHC and smoothelin expression was significantly lower in restenotic lesions versus mildly stenotic plaques (P < 0.05 for SMMHCs and P < 0.01 for smoothelin), stable plaques (P < 0.001 for both proteins), and erosions (P < 0.001 for both proteins). The area of SMMHC expression was significantly different between stable plaques and erosions (P < 0.05) but not the area of smoothelin expression. When SMMHC and smoothelin expressions were calculated as area of immunostaining/media, they were lower in restenotic lesions compared with mildly stenotic plaques (P < 0.05 for SMMHCs and P < 0.01 for smoothelin), stable plaques (P < 0.001 for both proteins), and erosions (P < 0.001 for both proteins). No difference was observed between stable plaques and erosions. SMMHC and smoothelin expression, calculated as SMMHC/α-SMA or smoothelin/α-SMA, ie, as values

Figure 1. Cytoskeletal protein expression in stable plaques. Immunostaining for α-SMA (a, b), SMMHCs (c, d), and smoothelin (e, f) at low (a, c, e) and high (b, d, f) magnification. High-magnification images were taken in the intimal thickening region. Bar=500 μm in a, c, e; bar=50 μm in b, d, f.
representative of SMC differentiation level, was lower in restenotic lesions versus mildly stenotic plaques (P < 0.05 for SMMHCs and P < 0.01 for smoothelin), stable plaques (P < 0.001 for both proteins), and erosions (P < 0.05 for SMMHCs and P < 0.01 for smoothelin). Our results demonstrate that restenotic lesions induce the highest degree of media atrophy followed by stable plaques, whereas mildly stenotic plaques and erosions are the less effective in this respect. Staining for desmin gave general results similar but weaker than those obtained for SMMHCs indicating that morphometric analysis would not be contributory to result evaluation; hence, we decided not to complete the quantification.

**Cytoskeletal Protein, CD68, and CD34 Expression in Intima**

When the intima of the different lesions was examined after α-SMA staining, the accumulation of positive cells appeared clearly prevalent in erosions (Figure 2a, 2b) and in-stent restenotic lesions (Figure 3a, 3b) compared with mildly stenotic plaques and stable plaques (Figure 1a, 1b). In the first 2 situations, positive cells were located close one to another, whereas in stable plaques they appeared surrounded by extracellular matrix deposits; moreover, positive cells of stable plaques were more regularly aligned compared with the 2 other situations. The most striking observation was, however, the dramatic decrease of SMMHC expression and the almost complete disappearance of smoothelin from the intima in all situations, with the positivity of media for the same proteins serving as internal control (Figure 1c to 1f; Figure 2c to 2f; Figure 3c to 3f).

Morphometric data (Table 3) demonstrated that the α-SMA-positive area was significantly higher in erosions compared with mildly stenotic plaques (P < 0.05), stable plaques (P < 0.01), and in-stent restenotic lesions (P < 0.01). In the 3 last situations, the α-SMA-positive area was similar. The α-SMA expression, calculated as α-SMA/intima minus core (or α-SMA/in-stent intima for restenotic lesions), was significantly higher in erosions compared with mildly stenotic plaques (P < 0.05) and stable plaques (P < 0.001), and in-stent restenotic lesions compared with stable plaques (P < 0.05). SMMHC expression was the highest in mildly stenotic plaques when calculated as SMMHC-positive area (P < 0.05 versus stable plaques and erosions, and P < 0.01 versus restenotic lesions) or as SMMHC/intima minus core (P < 0.05 versus stable plaques, P < 0.001 versus erosions and P < 0.01 versus restenotic lesions). SMMHC expression calculated as SMMHC/α-SMA was higher in mildly stenotic plaques compared with the other situations (P < 0.01 versus stable plaques, and P < 0.001 versus erosions and restenotic lesions); it was significantly higher in stable plaques versus erosions (P < 0.001) and restenotic lesions (P < 0.01). In all situations, SMMHC-positive areas were located mainly in the proximity of the media. Smoothelin was practically absent in all situations. CD34 was present, as expected, in endothelial cells; it was expressed in a negligible number of intimal cells in all lesions (data not shown). CD68-positive area was
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**TABLE 3. Cytoskeletal Protein Expression in Intima**

<table>
<thead>
<tr>
<th></th>
<th>α-SMA (mm²)</th>
<th>α-SMA/intima (%)</th>
<th>α-SMA/intima minus core (%)</th>
<th>SMMHC (mm²)</th>
<th>SMMHC/intima minus core (%)</th>
<th>SMMHC/α-SMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mildly stenotic plaques</td>
<td>0.628±0.067§</td>
<td>20.1±4.5</td>
<td>22.6±2.6§</td>
<td>0.214±0.071</td>
<td></td>
<td>7.5±0.28§</td>
</tr>
<tr>
<td>Stable plaques</td>
<td>0.806±0.104§</td>
<td>15.3±1.5§</td>
<td>23.3±3.29§</td>
<td>0.092±0.019†</td>
<td>2.8±0.8†</td>
<td>0.113±0.017†</td>
</tr>
<tr>
<td>Erosions</td>
<td>1.804±0.201¶</td>
<td>30.5±2.7†</td>
<td>39.6±3.3†</td>
<td>0.100±0.022†</td>
<td>2.1±0.4†</td>
<td>0.050±0.008†</td>
</tr>
</tbody>
</table>

| Stented plaques*         | 0.867±0.129§ | 25.4±3.4‡        | 31.6±2.4§                   | 0.038±0.020† | 1.3±0.7†                   | 0.041±0.020† |

Results are given as mean±SEM.
ND indicates not done.
*Values of in-stent intima except for α-SMA/intima.
Significant difference from †mildly stenotic plaques, ‡stable plaques, §erosions, and ¶stented plaques.

Discussion

Our work brings new quantitative data on the distribution of well-established SMC differentiation markers in the media and intima of human coronary arteries undergoing lesions such as mildly stenotic plaques, erosions, stable plaques, and restenosis after stent implantation. The specificity of the antibodies we have used to identify α-SMA, SMMHCs, and smoothelin has been established by several laboratories9–11 and has been confirmed here by immunoblotting using human smooth muscle, myofibroblastic, and nonmuscle tissues (see information online at http://atvb.ahajournals.org). We are conscious that, because of the characteristics of the material we had at our disposal, we were limited to the use of morphological techniques; however, we tried to quantify the morphometric results as precisely as possible.

Our results confirm and extend previous observations suggesting that atheromatous plaque formation results in atrophy of the underlying media14–16 and show that stable plaques are more effective, in this respect, than mildly stenotic plaques and erosions; the most important atrophy of the media is produced, not unexpectedly, by stent implantation. The smaller degree of atrophy in the media below the erosion compared with the other situations is compatible with the possibility that vasospasm is more easily generated in this condition. It also suggests that lesions resulting in erosions develop relatively rapidly compared with those resulting in stable plaques. When the distribution of SMC differentiation markers was studied morphometrically in the media, the occurrence of atrophy was confirmed by a decrease of the areas occupied by SMMHCs and smoothelin compared with the area occupied by α-SMA; however, it should be noted that these SMC differentiation markers remained largely present in medial SMCs during all pathological situations studied.

The morphometric analysis of the intima revealed that α-SMA–positive cells were quite numerous; however, differently from those of the media, they had lost SMMHC expression very importantly and smoothelin expression almost completely. These features support the assumption that in all the situations examined intimal SMC have acquired the myofibroblastic phenotype.17 The largest α-SMA–positive cross-sectional area (ie, highest quantity of MFs) was present in erosions, whereas SMMHCs/α-SMA was highest in stable plaques compared with erosions suggesting that in this situation the transition toward the myofibroblastic phenotype is less developed. Intimal SMC were practically negative for CD34, suggesting a local rather than a bone marrow-derived cell origin.13 A low percentage of CD34-positive cells has been reported in in-stent restenosis compared with primary lesions of atherectomy specimens;18 we cannot confirm this observation but our results could be caused by the high degree of lesions severity in our specimens.

The changes observed in human lesions appear different from the phenotypic features of SMCs present in the intima of pig coronary arteries after chronic administration (up to 18 months) of a cholesterol-rich diet19 or after stent implantation.20 In these situations, pig intimal SMCs express always SMC markers, including smoothelin, although in experimental chronic plaques, SMMHCs and smoothelin expression decreased significantly compared with control coronary arteries after 1 year of cholesterol-rich diet; it did also increase again during plaque recovery because of the interruption of the diet.21 Our findings underline once more the problems connected with the interpretation of results obtained in experimental models and their extrapolation to the human situation.

A systematic study of SMC differentiation marker expression in human plaques in relation to the location or to the age of the lesion is presently not available; however, there are indications that well-developed atheromatous plaques of the aorta express advanced SMC differentiation markers such as desmin,22 SMMHCs, and smoothelin.23 We do not know why coronary plaques behave differently than aortic plaques as far as SMC differentiation marker expression is concerned, but it should be noted that the coronary plaques we have studied represent a subset of very advanced lesions that have been responsible of a fatal event.1

The possibility that human coronary intimal SMCs are modulated into MFs may suggest an alternative contractile mechanism for these cells. MFs have been described in granulation tissue during wound healing and in practically all fibrocontractive diseases, in which they participate in the generation of isometric tension and in the synthesis of extracellular matrix components; these phenomena are in turn responsible for granulation tissue remodeling and retraction.6,24 The main marker of the myofibroblastic phenotype is the expression of α-SMA.17 MFs may also express other SMC markers such as SMMHCs, but have not been yet...
shown to express smoothelin,20,22 a very late marker of SMC differentiation.11 MFs have been shown to derive mainly from the modulation of pre-existing fibroblasts, but evidence has also been presented indicating their derivation from several sources including: epithelial cells in locations such as the kidney interstitium,23 blood-borne stem cells,26 local pericytes,27 and vascular SMCs.27 Obviously modulation of SMCs into MFs implies the loss of specific differentiation markers such as SMMHCs and smoothelin.

Recent work indicates that the mechanisms regulating force production by MFs are different from those classically described for SMC contraction. In SMCs the elevation of intracellular Ca++ is the main process through which an immediate and reversible force is created.28 Moreover phosphorylation of the myosin light chains leading to contraction is regulated by myosin light chain kinase.29 On the contrary, MFs generate isometric tension through stress fiber contraction that is regulated by a Rho/kinase--depending mechanism.30 Activated Rho/kinase has been shown to promote the contraction of isolated stress fibers,31 and the maintenance of a long-lasting tensile activity probably depends on the concurrent inhibition of myosin phosphatase.6,31 These mechanisms explain why granulation tissue contraction is characterized by a slow and continuous retractive activity that is not reversible as in the case of SMC contractile activity. Moreover, deposition of extracellular matrix stabilizes the tissue remodeling and deformation produced through the force generated by MFs.32,33 The role of α-SMA in inducing stress fiber contraction and force generation by MFs is now well-established both in vitro and in vivo.34 This force is then transmitted to the surrounding tissue through specialized focal adhesions.35 It is noteworthy that α-SMA and, hence, MF accumulation is prevalent in erosions compared with stable plaques. Further work is needed to understand the mechanisms inducing SMCs modulation into MFs in coronary plaques, but it is noteworthy that the large accumulation of these cells in the intima of erosion correlates with the high incidence of thrombosis.

In conclusion, our findings suggest that, in the situations described here, intimal SMCs acquire a myofibroblastic phenotype and open the possibility that tension production and tissue remodeling by these cells induce, in conjunction with other established factors, such as vasoaspsam and proteolytic enzyme expression,5 coronary plaque remodeling, thus facilitating plaque fissure formation and ultimately thrombus development. Further studies are needed to better characterize the features of intimal MFs as well as the factors influencing their contractile activity. In any event, our observations suggest that a hitherto unexplored additional mechanism plays a role in the establishment of atheromatous plaque and restenosis complications.

Acknowledgments

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References


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Methods

Antibodies and Immunoblotting
The specificity of the following primary antibodies was tested by immunoblotting: 1) mouse monoclonal IgG2a recognizing α-smooth muscle actin (α-SMA, clone 1A4); 2) 2 affinity-purified rabbit polyclonal IgGs recognizing both smooth muscle myosin heavy chains (SMMHCs), SMMHC 1 and SMMHC 2, one produced in our laboratory and the other commercially available (BT-562, Biomedical Technologies Inc., Stoughton, MA); both antibodies do not recognize non-muscle isoforms A and B; and 3) mouse monoclonal IgG1 (clone R4A) recognizing smoothelin isoforms A (59 kD, mainly expressed in visceral smooth muscle cells (SMCs)) and B (110 kD; predominantly expressed in vascular SMCs).

In order to verify on our material that the antibodies can discriminate SMCs from MFs, the following specimens were processed for SDS-PAGE and immunoblotting: human and porcine coronary artery media, human platelets (known to contain non-muscle [NM] MHC-A), rat brain (known to contain NMMHC-B), Dupuytren’s nodule (selected as a tissue representative of a myofibroblastic lesion), human colon muscular layer, SMCs cultured from porcine coronary artery media and human lung fibroblasts MRC-5. In three separate experiments, filters were incubated with either anti-α-SMA, anti-SMMHCs or anti-smoothelin antibodies. In order to detect small amount of NMMHCs, we loaded the following amounts of total proteins: 30 µg for non-muscle specimens, 5 µg for muscle tissues and 10 µg for cultured SMCs.

Results

Specificity of antibodies
Immunoblots using the commercially available anti-SMMHC antibodies revealed type 1 and 2 SMMHCs at 200 kDa and 204 kDa, respectively, in both human and porcine coronary artery media and in cultured porcine SMCs whereas these isoforms were not present in human platelets, rat brain, MRC-5 fibroblasts and Dupuytren’s nodule (Figure I). Similar results were obtained with the anti-SMMHC antibody produced in our laboratory (data not shown). The anti-smoothelin antibody demonstrated a band at 110 kDa, corresponding to the smoothelin isoform B, in human and porcine coronary artery media, cultured porcine SMCs and, to a smaller extent, in human colon. The smoothelin isoform A, detected at 59 kDa, was highly expressed in human colon and to a lesser extent in human coronary artery media. No signal was detected in human platelets, rat brain, MRC-5 fibroblasts and Dupuytren’s nodule. As expected, α-SMA was present in all SMC samples and in Dupuytren’s nodule and absent in human platelets, rat brain and MRC-5 fibroblasts. We can thus conclude that the combined use of our antibodies discriminates between SMCs and MFs.

References


**Figure legends**

**Figure I:** Immunoblots showing the expression of SMMHCs, smoothelin and α-SMA in porcine coronary artery media (a), cultured porcine coronary artery SMCs (b), human coronary artery media (c), human colon muscular layer (d), human platelets (e), rat brain (f), MRC-5 fibroblasts (g) and Dupuytren’s nodule (h). Note that: SMMHC 1 and 2 and smoothelin are present in smooth muscle specimens, α-SMA is present additionally in Dupuytren’s nodule, and human platelets, rat brain and MRC-5 fibroblasts are negative for the tested antibodies. NL: not loaded.