Contribution of Intimal Smooth Muscle Cells to Cholesterol Accumulation and Macrophage-Like Cells in Human Atherosclerosis

Running title: Allahverdian et al.; Smooth muscle foam cells in human atherosclerosis

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Abstract

Background—Intimal smooth muscle cells (SMCs) contribute to the foam cell population in arterial plaque, and express lower levels of the cholesterol exporter ATP-binding cassette transporter AI (ABCA1) when compared to medial arterial SMCs. The relative contribution of SMCs to the total foam cell population and their expression of ABCA1 when compared to intimal monocyte-derived macrophages, however, are unknown. While expression of macrophage markers by SMCs following lipid loading has been described, the relevance of this phenotypic switch by SMCs in human coronary atherosclerosis has not been determined.

Methods and Results—Human coronary artery sections from hearts explanted at the time of transplantation were processed to clearly delineate intracellular and extracellular lipids and allow co-staining for cell-specific markers. Co-staining for Oil Red O and the SMC-specific marker SM α-actin of foam cell rich lesions revealed that 50±7% (avg ± SEM, n=14 subjects) of total foam cells were SMC-derived. ABCA1 expression by intimal SMCs was significantly reduced between early and advanced atherosclerotic lesions, with no loss in ABCA1 expression by myeloid-lineage cells. Co-staining with the macrophage marker CD68 and SM α-actin revealed that 40±6% (n=15) of CD68-positive cells originated as SMCs in advanced human coronary atherosclerosis.

Conclusions—These findings suggest SMCs contain a much larger burden of the excess cholesterol in human coronary atherosclerosis than previously known, due in part to their relative inability to release excess cholesterol via ABCA1, when compared to myeloid-lineage cells. Our results also indicate that many cells identified as monocyte-derived macrophages in human atherosclerosis are in fact SMC-derived.

Key words: smooth muscle cell, foam cells, atherosclerosis, macrophage, cholesterol homeostasis
Introduction

Smooth muscle cells (SMCs) are the main cell type in early arterial intimal thickenings, and a major component of most stages of human atherosclerosis. Like monocyte-derived macrophages, SMCs express scavenger receptors and become foam cells upon exposure to lipoproteins. In spite of these findings, and the many descriptions of SMC-derived foam cells in human atherosclerotic lesions, the relative contribution of SMCs to total foam cell formation and macrophage-like cells in human atherosclerosis has not been studied. The apparent scarceness of intimal SMCs in mouse models of atherosclerosis has contributed to the de-emphasis of these cells as contributors to foam cell formation, although a recent study suggests SMCs lacking classic SMC markers are prevalent in mouse arterial intima. When compared to monocyte-derived macrophages, there is a striking lack of literature regarding the mechanisms that control cholesterol metabolism in arterial SMCs.

In addition to cholesterol uptake, foam cell formation depends on the cell’s ability to release excess cholesterol, the rate-limiting component of which is mediated by the membrane lipid transporter ATP-binding cassette transporter A1 (ABCA1). We previously reported ABCA1 mRNA as well as SMC-specific ABCA1 protein levels are diminished in the intimal layer of human coronary arteries. This suggested formation of SMC foam cells is due in part to their reduced ability to efflux excess cholesterol via ABCA1. To determine the relative importance of SMC cholesterol accumulation in the intima, in the current studies we also compared intimal SMC- and myeloid cell-specific expression of ABCA1 in early and late stages of human atherosclerosis.

Previous studies have indicated lipid loading induces expression of macrophage markers and a gain of phagocytic activity by arterial SMCs. The presence of cells expressing both
SMC and macrophage markers has been demonstrated in *en face* preparations of human aortic intima. These findings raise the possibility that some atherosclerotic lesion cells expressing macrophage markers, and assumed to be of monocyte origin, may be in fact be SMCs that have an altered phenotype following lipid loading. The extent of this phenotypic switch has not been previously investigated in human coronary atherosclerosis.

Our results suggest that in human coronary intima, a very large proportion of foam cells are SMC- rather than myeloid cell-derived, the intimal SMCs in advanced lesions have a selective reduction in ABCA1 expression when compared to myeloid-origin cells, and a large percentage of cells expressing macrophage markers in advanced atherosclerosis are SMC- rather than monocyte-derived.

Methods

Tissue sections

Explanted hearts donated by consenting patients undergoing heart transplantation were obtained from the Cardiovascular Registry at the Centre for Heart Lung Innovation, University of British Columbia following approval by the UBC Clinical Research Ethics Board. Coronary artery tissues from 31 hearts were used for lipid fixation and staining in order to obtain samples from 14 subjects containing sufficient foam cells for cell identification studies (online-only Supplemental Table 1). Coronary artery sections from 24 patients across the range of atherosclerosis stages were used for immunohistochemical studies (online-only Supplemental Table 2).

Lipid fixation

Formalin-fixed human coronary artery tissues were processed to carefully preserve lipid content
in paraffin sections.\textsuperscript{16,17} Briefly, formalin-fixed tissues (1-2 mm thick) were kept in an emulsion of linoleic acid (60% practical grade, MP Biomedicals) and lecithin (Jamieson Natural Sources) in 70% ethylene glycol (Sigma) at 56 °C for 3-5 days. Tissues were rinsed for at least 8 h in several changes of 70% ethanol followed by several changes of distilled water, immersed in 2% chromic acid for 24 h at 4°C, then rinsed in several changes of distilled water for 24 h. Finally, tissues were placed in a 5% sodium bicarbonate solution for 24 h followed by rinsing in water for at least 8 h. Tissues were then embedded and cut in paraffin sections and stained with Oil Red O. This method preserves neutral lipids in tissues for subsequent lipid staining. Comparison of tissues fixed in this way and by frozen section showed a similar extent, intensity and distribution of lipid staining in types I-IV human coronary atherosclerosis using the two methods.\textsuperscript{14} OCT embedded frozen sections were analyzed for comparison as previously described.\textsuperscript{12}

**Immunostaining**

To determine the cell type of origin of foam cells, formalin-fixed paraffin-embedded tissues with lipids preserved were cut in 5 \( \mu \)m thick sections and stained histochemically with Oil Red O and counterstained with hematoxylin and eosin to ensure the presence of lipids in coronary artery sections. Once the presence of foam cells or extracellular lipid were confirmed in intimas, serial sections were double-stained with SM \( \alpha \)-actin (Abcam) or CD45 (Dako) antibodies and Oil Red O. Briefly, sections were de-waxed, rehydrated, and incubated in Tris/EDTA PH 9.0 buffer for 30 minutes for antigen retrieval. Slides were then blocked with Dako universal block for 30 minutes followed by incubation with SM \( \alpha \)-actin or CD45 antibodies overnight. AlexaFluor® 488-conjugated donkey anti-mouse IgG was used to detect SM \( \alpha \)-actin and CD45. Sections were then processed for Oil Red O staining with subsequent staining of nuclei using Hoechst 33342.
To determine the origin of cells expressing ABCA1 and macrophage markers, formalin-fixed, paraffin-embedded tissues were cut in sections and double stained with SM α-actin, CD68, CD45, or ABCA1 antibodies. Sections were dewaxed and antigen retrieved as described above and then co-incubated with polyclonal rabbit anti-human SM α-actin (Abcam), mouse monoclonal anti-human SM α-actin (Abcam), monoclonal mouse anti-CD68 antibody (Dako), rabbit polyclonal anti-CD45 antibody (Abcam), mouse anti-CD45 (Dako), or rabbit polyclonal anti-ABCA1 antibody (Novus Biologicals) overnight. AlexaFluor® 488-conjugated donkey anti-mouse IgG and AlexaFluor® 594-conjugated donkey anti-rabbit IgG were used for detection. Nuclei were stained using Hoechst 33342.

Confocal microscopy

Confocal images were acquired with a Leica AOBS SP2 laser scanning confocal microscope (Leica, Heidelberg, Germany) using a high-resolution Leica 63X/1.4 Plan-Apochromat oil immersion objective. The acquisition software was Leica Confocal Software TCS SP2. All images and spectral data were captured using PMT detectors (R6357; Hamamatsu, Shizuoka-ken, Japan) located inside the scan head. A total of 50 Z-stacks were acquired from the whole section thickness (5 μm) to reconstruct 3-D images. Spectral scanning was performed on each fluorescent dye to confirm its specificity as well as to ensure that there was no overlap in the emission signal range collected for each fluorescent dye used in this study (data not shown).

Image analysis

Slides were viewed using a Nikon Eclipse TE300 inverted microscope, illuminated by a Nikon Super High Pressure Mercury Lamp, filtered using DAPI, FITC, or rhodamine filter sets, and captured using a Spot digital camera (Diagnostic Instruments).

*ImagePro Plus®* 5.1 image analysis software was used to combine three obtained images.
Quantification of SMC foam cells

The relative contribution of SMC foam cells to total intimal foam cells in human atherosclerotic lesions was determined by two independent observers counting foam cells that were strongly positive for or lacking SM $\alpha$-actin. Quantitation of foam cells was performed by comparing images of the same field of view showing DAPI merged with Oil Red O or SM $\alpha$-actin immunofluorescence, and applying a grid on each image to reference specific cells between the two images. Foam cells were first identified as cells expressing strong Oil Red O fluorescence, and the expression of SM $\alpha$-actin in each foam cell was determined by locating it in the corresponding SM $\alpha$-actin micrograph using the grid. Using this method, SM $\alpha$-actin positive foam cells were counted for each field and the entire tissue section and expressed as a percent of total foam cells. Foam cells staining weakly for SM $\alpha$-actin were not included in the SMC foam cell estimate, but were counted as part of the non-SMC foam cell fraction. There was no statistically significant difference between percent of SMC foam cells counted by the first and second observers.

ABCA1 co-localization

ABCA1 expression in intimal SMCs and myeloid cells was determined by measuring the degree of co-localization between ABCA1 and SM $\alpha$-actin or CD45, respectively, using ImagePro Plus® 5.1 image analysis software. Briefly, areas of interest were selected, separating the intima and media of vessels using the internal elastic lamina as identified from adjacent sections stained with Movat’s pentachrome. Color segmentation was used to determine ABCA1, SM $\alpha$-actin, and CD45 positivity as previously described.12 Pixels were pseudo-colored and merged so that the ABCA1 co-localization with either SM $\alpha$-actin or CD45 could be determined by summing the
total area of co-localization pixels for each section and expressing it as a percentage of either total SM \( \alpha \)-actin or CD45 area. This measurement was done by one observer blinded to identify the protein markers and lesion stage.

**Quantification of SM \( \alpha \)-actin+/CD68+ cells in intima**

To determine the proportion of intimal CD68 positive cells expressing SM \( \alpha \)-actin, a quantitative immunofluorescence microscopy technique was used. Cells positive for CD68 and positive or negative for SM \( \alpha \)-actin immunoreactivity were counted by two independent observers using the methods described above, with slight modification. Briefly, a grid was applied to immunofluorescence micrographs showing CD68 counterstained with DAPI. The expression of SM \( \alpha \)-actin in CD68 positive cells was assessed by locating each CD68 positive cell in the matching SM \( \alpha \)-actin micrograph. Nucleated cells expressing CD68 only or CD68 and SM \( \alpha \)-actin were each totaled and the percentage of total CD68 positive cells expressing SM \( \alpha \)-actin was determined for each section. This measurement was done by two observers with one blinded to identity of protein markers and lesion stage. Only cells staining strongly positive for both SM \( \alpha \)-actin and CD68 were counted as SM \( \alpha \)-actin+/CD68+ cells. There was no statistically significant difference between percent of SM \( \alpha \)-actin+/CD68+ cells counted by the first and second observers.

**Statistical analysis**

All data are expressed as average \( \pm \) SEM. Significant differences between two independent experimental groups were determined using two tailed Student’s \( t \)-tests, with a value of \( P<0.05 \) considered significant. Paired \( t \)-tests were performed to determine statistical differences between measurements obtained by the first and second observers and ABCA1 immunoreactivity between SMCs and myeloid-origin cells in advanced lesions.
Results

Detection of Extra- and Intracellular Lipid in Arterial Wall Sections

To identify the origin of foam cells using cell-specific markers, we first adapted a method to stain lipids using Oil Red O in formalin-fixed rather than frozen tissues.\textsuperscript{16,17} This method allows a much clearer distinction between extracellular and intracellular neutral lipids than can be achieved using standard Oil Red O staining of frozen tissues. Figure 1B demonstrates extracellular lipids in proximity to smooth muscle cells (SMCs), stained brown using anti-SM $\alpha$-actin antibody, much more clearly than can be seen in a similarly-stained section using standard methods for frozen samples (Figure 1A). Figure 1D demonstrates staining of intracellular lipids in foam cells of a more advanced lesion, also with a high degree of clarity compared to a frozen section (Figure 1C).

Origin of Foam Cells in Human Coronary Artery Atherosclerotic Intima

Of coronary artery sections from 31 subjects treated using this enhanced lipid fixation technique, 7 showed extracellular lipid staining only with no foam cells (type I atherosclerosis, Table 1).\textsuperscript{18} The extracellular lipid in these sections was localized to the deep intima, as indicated in Figure 1B. In 6 out of 7 of these subjects the intima was also rich in SMCs, with myeloid cells only observed in the superficial intima close to the lumen, as previously reported.\textsuperscript{19,20} Lesions from 10 subjects showed advanced atheromas as indicated by a large necrotic core and fewer intact foam cells (type IV atherosclerosis). Specimens from 14 subjects showed foam cells clearly visible in the intima (types II and III atherosclerosis). These 14 specimens were therefore used to determine the relative contribution of SMCs to total foam cell formation. The expression patterns of actin isoforms including SM $\alpha$-actin in 9783 samples from normal and diseased human specimens by Affymetrix gene expression array found no evidence of SM $\alpha$-actin expression by
myeloid lineage cells. SM α-actin was therefore used to identify SMC- from myeloid lineage cell-derived foam cells. Sections treated first to preserve lipids were stained with SM α-actin followed by Oil Red O to identify SMC-derived foam cells (Figure 2). Foam cells either positive or negative for SM α-actin staining were quantitated in multiple images of these sections (subject characteristics, number of images studied and percent SMC foam cells per section are shown in Online-only Supplemental Table 1). The results showed that 50±7% (avg±SEM, n=14 subjects) of foam cells also co-stained strongly with SM α-actin. This likely represents an underestimate of SMC contribution to total foam cells, due to a report of very low or no SM α-actin expression by some intimal SMCs. Overall, these results suggest the majority of foam cells in human coronary atherosclerosis are SMC- rather than myeloid cell-derived.

Expression of ABCA1 is Lower in Intimal Smooth Muscle Cells from Advanced Compared to Early Native Atherosclerosis

We previously reported that ABCA1 expression is reduced in SMCs in the intimal when compared to the medial layer of human coronary arteries, providing a potential reason for increased SMC foam cell formation in the intima. To determine whether the reduction in intimal SMC ABCA1 expression is dependent on lesion stage, we determined ABCA1 protein levels in the SMCs of early (types I and II) and advanced (types III and IV) atherosclerotic lesions. As shown in Figures 3A and 3B, intimal SMCs in advanced lesions express significantly less ABCA1 as compared to intimal SMCs in early atherosclerosis when normalized to SM α-actin staining.

Expression of ABCA1 is Lower in Smooth Muscle Cells Compared to Myeloid-origin Cells in Advanced Atherosclerotic Lesion Intima

To investigate whether the reduction in ABCA1 expression in advanced lesions is unique to
SMCs, we also determined ABCA1 expression by myeloid-lineage cells in early and later stage atherosclerosis. CD45 is expressed in all leukocytes including monocyte-derived macrophages, but is not expressed by SMCs. We therefore compared ABCA1 expression in SM α-actin- and CD45-expressing cells in early (types I and II) and advanced (types III and IV) lesions. Compared to the reduction of ABCA1 expression in intimal SMCs (Figure 4A upper panels), there was no reduction in ABCA1 expression by myeloid-lineage cells in advanced lesions (Figure 4A, lower panels). Normalization of ABCA1 and SM α-actin co-staining to SM α-actin levels in SMCs, or ABCA1 and CD45 co-staining to CD45 levels in myeloid-lineage cells from multiple early and advanced lesions is shown in Figure 4B. The results show significantly lower ABCA1 expression by SMCs as compared to myeloid-origin cells, with no loss of ABCA1 expression by myeloid cells in advanced lesions.

**Presence of CD68- and SM α-actin-positive Smooth Muscle Cells in the Intima of Human Coronary Artery Atherosclerosis**

Lipid-loaded human and murine SMCs have been shown to express macrophage markers in vitro, and the presence of intimal cells expressing both SMC and macrophage markers has been reported in human aorta. In the current studies early (types I and II) and advanced stage (types III and IV) human coronary lesions were stained with SM α-actin (SMC-specific) and CD68, typically considered to be a macrophage lineage marker, to determine the extent of this phenotypic switch in vivo. Our results show the presence of cells expressing both SM α-actin and CD68, especially in advanced lesions (Figure 5A). Cells co-staining strongly for both SM α-actin and CD68 were expressed as a percentage of total CD68+ cells. The data indicate that 18±3% (n=9) and 40±6% (n=15) of CD68+ cells express SM α-actin in early and advanced atherosclerotic lesions, respectively (Figure 5B). Co-localization of SM α-actin and CD68 was
also examined using confocal microscopy. Immunoreactivity against SM α-actin and CD68 showed relative colocalization within cells (Figure 5C). Spectral scanning of the cells expressing both SM α-actin and CD68 also confirmed presence of the emission signal range for Alexa 488 (SM α-actin) and Alexa 594 (CD68) in single cells (data not shown).

Not all CD68-positive Cells in Human Atherosclerotic lesions are of Myeloid Origin

To further investigate whether CD68-expressing cells in human atheromas are all of myeloid origin, we performed co-staining with CD68 and the specific myeloid cell lineage-marker CD45. Double staining of CD45 and CD68 showed that not all of the CD68 positive cells express CD45. The white boxes in Figure 6 panels A-C and confocal image panel D indicate a myeloid-origin cell expressing both CD68 and CD45. The orange boxes in panels A-C and confocal image panel E indicate a spindle-shaped cell expressing CD68, but not CD45. We measured the fraction of CD68+CD45- cells in total CD68+ cells in advanced atherosclerosis. The analysis showed that 34±8% (n=11) of CD68+ cells do not express CD45, suggesting they are not of myeloid origin. These findings further support the expression of CD68 by non-myeloid origin cells in human atherosclerosis, and are consistent with our findings of co-expression of CD68 by intimal SMCs.

Discussion

The existence of smooth muscle foam cells in human atherosclerosis has been known for decades, but the contribution of SMCs to total foam cells and cholesterol accumulation in the plaque has been unknown. In the current study we present data suggesting a large proportion, at least 50%, of total foam cells in human coronary intimas are smooth muscle cell- rather than monocyte-derived. Consistent with this major contribution of SMCs to foam cell formation, we
also found a specific reduction in expression of the cholesterol-efflux promoting protein ABCA1 by SMCs between early and advanced stage atherosclerotic lesions, which was not observed in myeloid lineage cells. Also consistent with previous reports showing the expression of macrophage proteins by SMCs following lipid loading,\textsuperscript{13,14} we report for the first time that a high percentage of macrophage-marker expressing cells in human coronary intima are SMC rather than myeloid in origin. These combined findings suggest a much larger role than previously realized for intimal SMCs as a site of excess cholesterol accumulation and as a source of macrophage-like cells in human atherosclerotic plaque.

The ability to delineate foam cells for co-staining with SMC or myeloid lineage cell markers depended on adapting a prior method\textsuperscript{16,17} that allowed us to distinguish intracellular from extracellular lipid and maintain arterial wall architecture in formalin-fixed tissues for subsequent paraffin embedding and immunohistochemistry. Using this method we were able to clearly identify co-localization or absence of SM $\alpha$-actin with Oil Red O staining of intracytoplasmic lipids in foam cells. The conclusion that at least 50\% of total foam cells are SMC-derived due to SM $\alpha$-actin staining is based on the assumption that these cells originated as SMCs, rather than being the product of phagocytosis of apoptotic SMCs or SMC debris by myeloid-lineage foam cells. While staining of phagocytosed SM $\alpha$-actin in monocyte-derived macrophages is a potential limitation of our conclusions, to our knowledge this has not been reported in the literature, and staining of SM $\alpha$-actin in early and late endosomes would be expected to be quite weak. To reduce the chance of counting non-SMCs in the SMC foam cell pool, we only included foam cells staining strongly for SM $\alpha$-actin in this estimate. Another potential confounder is expression of SM $\alpha$-actin by myeloid lineage cells. Although it has been shown that cultured macrophages can express SMC markers including SM $\alpha$-actin after
stimulation with transforming growth factor-β or thrombin, to our knowledge there is no evidence for SM α-actin expression by myeloid lineage cells in human or mouse tissues in vivo. 

There is, however, the likelihood that we have not identified all of the SMCs involved in intimal foam cell formation. Gomez et al. have recently shown that an epigenetic mark of SMC lineage cells identifies a percentage of SMCs in advanced human coronary atherosclerosis that do not express typical SMC marker genes including SM α-actin. We did not perform this assay in our studies as the efficiency of finding this epigenetic mark in human intimal SMCs has not been determined, nor whether this discreet mark could be seen in cells first processed to stain intracellular lipids. While it will be important to attempt this method to further define the percentage of intimal foam cells originating as SMCs, the findings of the Owens group suggest our conclusion that at least 50% of intimal foam cells are SMC-derived is a conservative estimate, and the actual contribution of SMCs to the foam cell population may be much larger.

We previously reported a reduction in ABCA1 expression by intimal compared to medial coronary artery SMCs. The current finding of reduced ABCA1 protein in late-stage compared to early-stage atherosclerotic lesion SMCs, but not myeloid-lineage cells, suggests there is a SMC-specific defect in ABCA1 expression in advancing lesions. Reduced intimal SMC ABCA1 expression also provides a plausible explanation for the high percentage of foam cells originating from SMCs. We have also found that addition of exogenous oxysterols can partially correct ABCA1 expression in cultured intima-type SMCs, suggesting a potential defect in cholesterol trafficking and generation of endogenous oxysterols necessary to activate liver X-receptor-dependent expression of ABCA1 in these cells. While the exact nature and in vivo correlation of this defect remain to be confirmed, our results showing no loss of ABCA1 expression in
myeloid-lineage cells across lesion stages suggest specific defects in SMC cholesterol metabolism are a reason for the high amount of cholesterol accumulation in human atherosclerotic plaque.

Previous observations that human aortic intimal SMCs13 as well as cholesterol-loaded human and mouse arterial SMCs in culture express macrophage markers13,14 led us to examine whether SMCs in human coronary lesions also express macrophage markers. We confirmed the presence of SM α-actin+/CD68+ cells in human coronary atherosclerosis, with approximately 40% of CD68+ cells in advanced lesions being of SMC origin (Figure 5). Again, our inclusion only of cells staining strongly for SM α-actin reduces the chance that we have included myeloid lineage cells staining for phagocytosed SMC fragments; the likely absence of SM α-actin expression by some intimal SMCs suggests this could also be an underestimate. Consistent with this estimate is our finding that approximately 34% of CD68+ cells in advanced lesions do not express CD45, a myeloid cell lineage marker not found to be expressed by SMCs.24-27 This further indicates that cells expressing both SM α-actin and CD68 but not CD45 are of SMC rather than myeloid origin. While the functional significance of SMCs assuming a macrophage phenotype in vivo is not yet known, cultured mouse aortic SMCs converted to a macrophage gene expression pattern following lipid loading also showed an increase in phagocytic activity.14 Our results along with previous studies13 indicate that many intimal cells, identified as CD68+ in tissue sections and assumed to be of monocyte or other myeloid cell origin, are in fact SMCs that have converted to a macrophage-like phenotype.

The most striking aspect of these findings is the heightened importance they suggest should be placed on understanding cholesterol metabolism and its dysregulation in intimal SMCs, in addition to intimal monocyte-derived macrophages and other myeloid lineage cells.
The perceived lack of importance of intimal SMCs to the composition of plaque in mouse models of atherosclerosis, which the recent Gomez et al. studies suggest has been unrecognized due to loss of classic SMC markers, is a likely reason there has been so little attention paid to arterial SMC cholesterol metabolism and the role of SMC foam cells in plaque biology. Whether mouse models of atherosclerosis exhibit the same defects in intimal SMC cholesterol metabolism, to support their use in studying this aspect of atherogenesis, remains to be seen.

In conclusion, the current studies suggest a much larger role for intimal SMCs in foam cell formation and over-accumulation of cholesterol in human atherosclerosis than previously assumed. While myeloid-lineage cells express abundant ABCA1 in both early and late-stage atherosclerosis, the selective decline in ABCA1 expression by later stage intimal SMCs provides a likely reason for this contribution of SMCs to total foam cell formation, and as a repository for much of the excess cholesterol in the artery wall. The expression of a macrophage marker by intimal SMCs also suggests many of the intimal macrophages previously assumed to be of monocyte origin are in fact SMCs assuming a macrophage-like state. Further understanding of the mechanisms of intimal SMC cholesterol loading and unloading will open up new targets for the prevention of cholesterol accumulation in the artery wall and its’ clinical outcomes.

Acknowledgments: We thank the members of the Cardiovascular Registry in the Centre for Heart Lung Innovation for their excellent assistance and technical skill that allowed us to complete these studies.

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Conflict of Interest Disclosures: None.
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Table 1. Classification of Atherosclerotic Lesions According to Stary et al.\(^{18}\)

<table>
<thead>
<tr>
<th>AHA classification</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Type I (initial)</td>
<td>Accumulation of smooth muscle cells in the intima with isolated macrophage foam cells</td>
</tr>
<tr>
<td>Type II (fatty streak)</td>
<td>Intracellular lipid accumulation/foam cells</td>
</tr>
<tr>
<td>Type III (intermediate)</td>
<td>Type II changes with extracellular lipid pools or small core</td>
</tr>
<tr>
<td>Type IV (atheroma)</td>
<td>Type II changes with obvious core and overlying cap</td>
</tr>
</tbody>
</table>

Figure Legends:

Figure 1. Detection of lipids in the intimal layer of human coronary arteries in frozen sections and after lipid fixation in formalin-fixed tissues. Frozen human coronary artery sections were fixed with formalin and stained with Oil Red O (panels A and C). In another series of experiments non-frozen formalin-fixed human coronary arteries were processed to preserve lipids for staining in paraffin sections as described in Methods (B and D). For a better demonstration of extracellular lipid panels A and B have been stained with Oil Red O and SM\(\alpha\)-actin to identify SMCs (brown staining). Inset images show cross sections of the arteries from which images were obtained. Lipids accumulated intracellularly are indicated by arrows in panel D. Original magnification X40. I: intima; L: lumen; M: media.

Figure 2. Identification of SMC foam cells in the intima of human coronary artery atherosclerosis. Immunohistochemical studies were carried out with co-staining of SM\(\alpha\)-actin with horseradish peroxidase- (arterial cross-section, brown) or Alexa 488-tagged (green) secondary antibodies and Oil Red O (red). Nuclei were stained with DAPI (blue). The Merge panel indicates SMC-derived foam cells containing SM\(\alpha\)-actin and Oil Red O. Scale bar 200 \(\mu\)m.
Figure 3. ABCA1 and SM α-actin immunoreactivity in the intima of coronary arteries in early and advanced atherosclerosis. A. Coronary artery sections from patients with early (types I and II lesions) and advanced (types III and IV) native atherosclerosis were double stained with SM α-actin (green) and ABCA1 (red) antibodies. Nuclei were stained with DAPI (blue). B. The extent of co-localization between ABCA1 and SM α-actin in multiple specimens was determined by color segmentation, and normalized to SM α-actin staining area. Scale bar 500 μm. M: media; I: intima; L: lumen.

Figure 4. ABCA1 immunoreactivity in SMCs and myeloid-origin cells in the intima of human coronary artery atherosclerosis. A. Coronary artery sections from patients with advanced native atherosclerosis were double stained with SM α-actin or CD45 (green) and ABCA1 (red) antibodies, and colocalization determined in the merged images. Nuclei were stained with DAPI (blue). Scale bar 500 μm. I: intima; L: lumen. B. The extent of co-localization between ABCA1 and SM α-actin normalized to SM α-actin, or ABCA1 and CD45 normalized to CD45 staining in early and advanced lesions were determined by color segmentation.

Figure 5. Presence of CD68- and smooth muscle α-actin-positive SMCs in the intima of human coronary artery atherosclerosis. A. Coronary artery sections from patients with early and advanced native atherosclerosis were stained with hematoxylin and eosin. Adjacent sections were double stained with SM α-actin (green) and CD68 (red) antibodies. Nuclei were stained with DAPI (blue). Arrows show cells that co-express SM α-actin and CD68. I:intima; L:lumen. Scale bar 23 μm. B. ImagePro Plus was used to co-localize green (SM α-actin), red (CD68), and blue (nuclei) as indicated in panel A, and intact cells expressing both SM α-actin and CD68 were
normalized to total CD68-positive cells in early and advanced lesions. C. 3-dimensional confocal image of a cell expressing both SM α-actin and CD68. X, Y, and Z axes of co-staining in a single cell are as indicated. Scale bar 5.7 μm.

**Figure 6.** Presence of CD68-positive cells without myeloid-specific marker in human atherosclerotic intima. Coronary artery sections were double stained with CD45 (red, A) and CD68 (green, B) antibodies, and co-staining determined in the merged image (C). Nuclei were stained with DAPI (blue). Scale bar 47 μm. Confocal microscopy indicating co-staining of CD68 and CD45 in the white-boxed cell (D) and CD68 only in the orange-boxed cell (E). Scale bar 2.9 μm.
Figure 1
Figure 2
Figure 3A
Co-localization of ABCA1 and SM-Î± actin / SM-Î± actin area

Early (n=8)            Advanced (n=9)

p<0.05

p=0.040

Figure 3B
Figure 4A
Co-localization of ABCA1 and SM-actin or CD45 / SM-actin or Cd45 area

\[ p = 0.011 \]

Figure 4B
Figure 5A
Figure 5B-C
Figure 6
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Supplemental Material
**Supplemental Table 1. Subject characteristics, number of images studied and percent SMC foam cells per section**

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<tr>
<th>Subject no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Primary diagnosis</th>
<th>Number of images studied</th>
<th>Percent of SMC foam cell</th>
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<td>M</td>
<td>Not available</td>
<td>3</td>
<td>75.6</td>
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Supplemental Table 2. Clinical characteristics of patients

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