Stromal Cells From Human Long-Term Marrow Cultures Are Mesenchymal Cells That Differentiate Following a Vascular Smooth Muscle Differentiation Pathway

By Marie C. Galmiche, Victor E. Koteliantsky, Josette Brière, Patrick Hervé, and Pierre Charbord

In human long-term marrow cultures connective tissue-forming stromal cells are an essential cellular component of the adherent layer where granulomonocytic progenitors are generated from week 2 onward. We have previously found that most stromal cells in confluent cultures were muscle-specific actin isoforms. The present study was carried out to evaluate the time course of α-SM-positive stromal cells and to search for other cytoskeletal proteins specific for smooth muscle cells. It was found that the expression of α-SM in stromal cells was time dependent. Most of the adherent spindle-shaped, vimentin-positive stromal cells observed during the first 2 weeks of culture were α-SM negative. On the contrary, from week 3 to week 7, most interdigitated stromal cells contained stress fibers whose backbone was made of α-SM-positive microfilaments. In addition, in confluent cultures, other proteins specific for smooth muscle were detected: metavinculin, h-caldesmon, smooth muscle myosin heavy chains, and calponin. This study confirms the similarity between stromal cells and smooth muscle cells. Moreover, our results reveal that cells in vivo with the phenotype closest to that of stromal cells are immature fetal smooth muscle cells and subendothelial intimal smooth muscle cells; a cell subset with limited development following birth but extensively recruited in atherosclerotic lesions. Stromal cells very probably derive from mesenchymal cells that differentiate along this distinctive vascular smooth muscle cell pathway. In humans, this differentiation seems crucial for the maintenance of granulopoiesis. These in vitro studies were performed to find out whether we could detect cytoskeletal proteins that may be more specific for smooth muscle cells than vimentin or α-SM actin. We looked for metavinculin, h-caldesmon, smooth muscle myosin heavy chains (SMMHCs), and calponin. In addition to the ubiquitous protein vinculin, smooth muscle cells express metavinculin that results from alternative splicing of the vinculin gene. This 150-Kd protein differs from the 130-Kd vinculin by an insert of 68 amino acid residues close to the C-terminal end of the vinculin molecule. Because metavinculin is observed as vinculin in focal contacts, it is likely to play an essential role by linking actin microfilaments to integrins and extracellular matrix molecules. In some of the smooth muscle thin filaments, a 150-Kd caldesmon variant is found. This protein results from an alternative splicing of the caldesmon gene, usually translated in non-smooth muscle cells by a 75-Kd protein. High (h) and low (l) molecular weight caldesmons are involved in the regulation of actomyosin complexes (in coordination with calmodulin and tropomyosin), in the bundling of actin filaments, and in the binding of thin filaments to the cell membrane. Myosin heavy chains differ because of their tissue origin. In smooth muscle cells, two smooth muscle–specific myosin heavy chains have been recently described; SM-1 (204 Kd) and SM-2 (200 Kd) are observed in addition to the ubiquitous nonmuscle myosin heavy chain of 196 Kd. Myosin heavy chains provide the force for cell locomotion in association with actin and under the regulation of calcium, ATP, and a set of actin-binding proteins. Among these, calponin, a 34-Kd protein, is found in smooth muscles. This protein interacts with calmodulin and pre-
Table 1. Antibodies Used for the Phenotypic Characterization of Stromal Cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope</th>
<th>Species Specificity</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-sm-1 (clone 1A4)</td>
<td>α-SM actin isoform</td>
<td>No</td>
<td>Sigma</td>
</tr>
<tr>
<td>Antipolyvalent actin</td>
<td>Actin</td>
<td>No</td>
<td>Sigma</td>
</tr>
<tr>
<td>Clone VIM 13.2</td>
<td>Vimentin</td>
<td>No</td>
<td>Sigma</td>
</tr>
<tr>
<td>Clone Vin 11.5</td>
<td>Vinculin and metavinculin</td>
<td>No</td>
<td>Sigma</td>
</tr>
<tr>
<td>Clone 1BZ</td>
<td>No</td>
<td>Levi and al^{28}</td>
<td></td>
</tr>
<tr>
<td>Clone Cald 8</td>
<td>h- and i-Caldesmon</td>
<td>No</td>
<td>Sigma</td>
</tr>
<tr>
<td>Clone h-CD</td>
<td>h-Caldesmon</td>
<td>Yes (human)</td>
<td>Frid and al^{30}</td>
</tr>
<tr>
<td>Clone CALP</td>
<td>Calponin</td>
<td>No</td>
<td>Frid and al^{30}</td>
</tr>
<tr>
<td>Clone SMMS-1</td>
<td>Smooth muscle</td>
<td>No</td>
<td>Frid and al^{30}</td>
</tr>
<tr>
<td></td>
<td>Myosin heavy chain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

sents a molecular region homologous to skeletal troponin T. In this report, it is shown that stromal cells synthesize metavinculin, the 150-Kd caldesmon isoform, smooth muscle myosin heavy chains, and calponin.

This study confirms that stromal cells differentiate following a smooth muscle differentiation pathway. Moreover, it reveals a phenotypic similarity between stromal cells and a subset of vascular smooth muscle cells: immature fetal smooth muscle cells and aortic subendothelial intimal cells with limited development following birth but extensive recruitment in atherosclerotic lesions.^{21-23}

Eventually, we have examined what could be the in vivo BM counterpart for the vascular smooth muscle--like stromal cells. We found on trephine marrow biopsies of adults without hematologic abnormalities a subset of α-SM-positive cells lining sinuses or located within the hematopoietic logettes. These cells, previously called myoid cells by Smitt-Griff et al.,^{24} presented long and thin cytoplasmic extensions in intimate contact with cells from the granulocytic series. Myoid cells may therefore be the likely in vivo counterpart for stromal cells.

MATERIALS AND METHODS

Dexter-type cultures. After obtaining informed consent, BM samples were collected from patients undergoing thoracic surgery.

Long-term marrow cultures were established by a modification of the method of Gartner and Kaplan.^{2} Twenty million unseparated nucleated BM cells were suspended in 25 cm² culture flasks in McCoy's medium with 12.5% horse serum, 12.5% fetal calf serum, and 10⁻⁶ mol/L cortisol and incubated at 33°C. Half the medium was removed weekly and replaced by fresh medium. Each week, a flask was sacrificed and granulomonocytic progenitors were cultured from nonadherent and adherent cells as previously described.^{3}

For immunofluorescence studies, Ficoll-Hypaque-separated cells were cultured on plastic lab-tek chamber slides (Miles, Naperville, IL). Each well was seeded with 3 × 10⁵ nucleated cells.

Depletion of hematopoietic cells. When a confluent layer was obtained, the supernatant was removed and adherent cells were treated with collagenase (250 U/mL; Sigma, St Louis, MO) for 2 hours at 37°C. The cell suspension was incubated for 30 minutes at room temperature, with a mixture of antibodies (Dakopatts, Denmark) directed against CD14, CD33, CD45, and CD68 epitopes. One microliter (0.3 μg) of each antibody per 10⁶ cells was used. After incubation, the cells were collected by centrifugation, washed two times with Hank's balanced salt solution, and then treated with dynabeads M-450 coated with a goat antimouse IgG (Dynal SA, Oslo, Norway) at a ratio of 3 beads per cell. The hematopoietic cells were then removed by means of a magnet and the remaining stromal cells resuspended in phosphate-buffered saline (PBS)–EDTA (10 mmol/L), benzamidine (10 mmol/L), and phenylmethylsulfonyl fluoride (PMSF) (10 μmol/L) for protein extraction.

Cell line culture. The rat smooth muscle line A7r5 from embryonic thoracic aorta was obtained from the American Type Culture Collection (ATCC CRL 1444). The L2ori- marrow stromal cell line was generated from long-term marrow culture stromal cells transfected by electroporation with an SV-40 vector lacking the origin of replication.^{25} It was a generous gift from Dr A. Keating (Toronto General Hospital, Ontario, Canada). These two cell lines were grown in the medium described above. The flasks were incubated at 37°C in a fully humidified atmosphere with 5% CO₂. When the adherent layers reached confluence, the cells were trypsinized and passaged.

Preparation of uterus samples. Fragments of uterus were obtained from patients undergoing hysterectomy. Fragments were frozen in liquid nitrogen. Frozen fragments were pulverized. The tissue powder was then boiled in buffer as described for other cell extracts.

Immunofluorescence studies. They were performed as previously described.^{5} When a confluent layer was obtained, the supernatant was removed. The adherent layer was washed three times with PBS, fixed with formaldehyde (3.7% [vol/vol] in PBS) for 5

Table 2. Generation of α-SM Actin- and Vimentin-Positive Adherent Cells in Long-Term Bone Marrow Cultures: Effect of Culture Age

<table>
<thead>
<tr>
<th>Days of Culture</th>
<th>Degree of Confluence</th>
<th>No. of Experiments</th>
<th>Vimentin Positive Fields (%)</th>
<th>α-SM Actin-Positive Fields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Range</td>
<td>Range</td>
</tr>
<tr>
<td>&lt;14</td>
<td>C1</td>
<td>6</td>
<td>96-100</td>
<td>98.5 ± 1.0</td>
</tr>
<tr>
<td>14-21</td>
<td>C2</td>
<td>9</td>
<td>96-100</td>
<td>98.5 ± 0.5</td>
</tr>
<tr>
<td>21-42</td>
<td>C3</td>
<td>9</td>
<td>100</td>
<td>100 ± 0.0</td>
</tr>
<tr>
<td>&gt;42</td>
<td>C2/C3</td>
<td>10</td>
<td>96-100</td>
<td>99.0 ± 1.0</td>
</tr>
</tbody>
</table>

The degree of confluence of the adherent layer was graded C1 to C3. C1: isolated adherent cells only. C2: some contacts between adherent cells are present. C3: adherent cells constitute a confluent layer. C2/C3: part of the adherent cells that formed previously a confluent layer detached. At least 50 microscope fields (0.009 mm²) were scored for each experiment. A field was considered positive when at least one cell with bright stress fibers or intermediate filaments was observed. Statistical analysis was performed using the Mann and Whitney U test.

* P < .01 compared with the value for <14 days of culture.
† P < .01 compared with the value for 14-21.
‡ P < .01 compared with the value for days 21-42.
Fig 1. Immunofluorescence with the MoAb α-SM-1 on adherent cells after 21 (A and B) or 35 (C and D) days of culture. (A,C) Immunofluorescence. (B,D) Corresponding phase contrast photomicrographs (bar = 10 μm). At day 21, the layer is not confluent: In the 0.015-mm² field shown in this figure, there are 7 α-SM-positive stromal cells out of 24 cells (29%). At day 35, the layer is confluent; there are approximately 33 α-SM-positive cells out of 48 (68%). At this stage, the numeration of cells and especially of α-SM-positive cells is very difficult owing to the intertwining of stromal cell pseudopods making up a syncytial meshwork.

Fig 2. Detection by Western blot of α-SM actin and vimentin in adherent cells from Dexter-type cultures. Cells were harvested from week 2 (lane 1) or week 5 (lane 2) adherent layers. (A) Immunoblot with α-sm-1. (B) Immunoblot with VIM 13.2. (a) Corresponds to actin in panel A and (v) corresponds to vimentin in panel B. For both time points, proteins were extracted from identical number of cells, as shown by identical vimentin bands in panel B.
MARROW STROMAL CELL CYTOSKELETON

A

1 2 3 4 5

α SM

B

1 2 3 4 5

Actin

Fig 3. Detection by Western blot of total actin and α-SM actin in extracts from uterus (lane 1), cultured smooth muscle cells from the A7r5 line (lane 2), cells from the transformed L2ori- stromal cell line (lane 3), confluent adherent layer devoid of hematopoietic cells (lane 4), and fresh uncultured BM cells (lane 5). (A) Immunoblot with α-sm-1. (B) Immunoblot with antiaxin polyclonal antibody.

The protein loads were adjusted so that each sample contained equivalent amounts of actin, as shown by similar intensity of bands when using an antipolyclonal actin antibody (panel B).

fluorescence microscopy using a Leitz Aristoplan (Wild Leitz, Wetzlar, Germany).

Since stromal cells were interdigitated, it was not possible to determine the precise number of positive or negative cells with α-SM-

1 or Vim 13.2 MoAbs. For a given MoAb, we counted the percentage of fields with positive cells, as reported previously. The percentage of positive fields was determined by counting 50 fields. A field was considered positive when at least one cell with bright stress fibers or intermediate filaments was observed. Each field measured 0.009 mm². When layers were confluent, there were 15.5 ± 0.5 (mean ± SEM) cells per field. The relatively high value of the SEM (measurements were made on 95 fields) was related to the difficulty of counting cells at confluency, there were several layers of stromal cells with intertwined pseudopods.

The significance of the results obtained for different degrees of confluence was analyzed using Mann and Whitney's nonparametric U test.

Western blots. The technique was carried out as previously described. Cells were washed in PBS with EDTA (10 mmol/L), sonicated, and the amount of proteins for each sample measured using the bicinchoninic acid protein assay reagent from Pierce Chemical Co (Rockford, Ill.). The proteins were solubilized in 2% (wt/vol) sodium dodecyl sulfate (SDS), 100 mmol/L tris (pH 6.8), and 2% (vol/vol) β-mercaptoethanol and heated at 100°C for 5 minutes. For Western blot studies using antibodies directed against actin, vimentin, vinculin, caldesmon, and calponin, samples were run on 7.5% or 10% polyacrylamide minigel (8.3 cm × 10.2 cm, thickness 0.75 mm) (Bio-Rad laboratories, Richmond, CA) at 40 mA current per slab for 30 minutes and transferred for 1 hour at 100 V to nitrocellulose paper. For Western blot studies using antibodies directed against myosin isoforms, samples were run overnight in 7% gel (7% acrylamide, 0.091% N,N-methylenebisacrylamide) at 7 mA current per slab using larger gels (16 cm × 18 cm, thickness 1.5 mm). The transfer was performed for 30 minutes at 16 V using a semidry electrophoretic transfer cell (Bio-Rad).

After transfer, the free binding sites were saturated by incubating the nitrocellulose paper with 5% (wt/vol) dried milk, 5% (wt/vol) bovine serum albumin (BSA) in TBS (tris buffer saline)-Tween (0.1% vol/vol) at room temperature for 1 hour. The paper was then incubated with the appropriate dilution of monoclonal or polyclonal antibody (Table 1) for 2 hours at room temperature and washed in TBS with 0.1% Tween three times for 5 minutes. To reveal actin, vimentin, vinculin, and calponin, the paper was incubated with peroxidase-linked goat antimouse IgG (Amersham, UK) or goat antirabbit IgG (Sigma), diluted at 1/300 and 1/3,000, respectively, for 1 hour at room temperature. Positive bands were revealed with diaminobenzidine peroxidase tablets from Sigma. To increase the sensitivity to detect caldesmon and myosin heavy chains, we used a chemiluminescence method (Kit ECL, Amersham). Peroxidase-linked goat antimouse IgG(1/3,000) was incubated with the nitro-
cellulose paper as previously described before applying the enhancement mixture.

For caldesmon and vinculin, respective amounts of isoforms were measured by densitometry. Photographs were taken from the blots and negatives were scanned at 360 nm using a densitometer (Sebia, France).

**Bone marrow biopsies.** BM biopsies were performed, after informed consent, in adults (age: 28 to 43 years) staged for Hodgkin’s disease. Biopsies from patients with stage I or IIA disease were selected.

BM biopsies were taken from the posterior iliac crest using a Jamshidi's needle. Biopsies were fixed in Bouin's solution and decalcified in a solution of RDO (Labonord, Lille, France). Specimens were included in paraffin and 5-μm thick sections were cut. Some sections were stained with hematoxylin-eosin; others were processed for immunohistochemistry.

Sections were incubated in toluene for 5 minutes (four times) and in ethanol for 5 minutes (four times). They were then incubated for 20 minutes in methanol with 0.2% hydrogen peroxide to allow the extinction of endogeneous peroxidases. They were then washed

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**Fig 5.** Metavinculin to vinculin ratio in extracts from uterus, cultured smooth muscle cells (A7r5), stromal cell line (L2ori-), confluent adherent layer prior (Adh.) and after (strom.) depletion of hematopoietic cells and fresh uncultured bone marrow cells (m.). Western blots were performed, as described in Fig 4, using the Vin 11.5 MoAb. On photographs of blots, amounts of each protein were measured by densitometry and the ratio of metavinculin/vinculin was then calculated and normalized to 1 (1 = vinculin + metavinculin). For each sample, several experiments were performed: Each bar gives the mean value and the different experiments are indicated by dots. For fresh BM, six experiments gave a value of zero demonstrating the absence of metavinculin.

**Fig 6.** Immunofluorescence with the MoAb 1B2 (that recognizes an epitope present on vinculin and metavinculin) on adherent cells after 21 days of culture (bar = 10 μm). Focal contacts are visible as distinct elongated dots in stromal cells.
Fig 7. h-Caldesmon to l-caldesmon ratios in extracts from uterus, cultured smooth muscle cells (A7r5), confluent adherent layer prior (Adh.) and after (strom.) depletion of hematopoietic cells and fresh uncultured BM (m.). Western blots were performed using Cald 8 antibody. The ratio was calculated using a procedure identical to that described for Fig 4. For fresh BM, six experiments gave a value of zero demonstrating the absence of h-caldesmon.

The expression of the α-SM actin isoform depends on time in culture. As previously shown for CGA-7-positive cells, the expression of α-SM-positive cells was time dependent (Table 2). At an early culture stage (for the first 2 weeks of incubation) few cells were α-SM positive (Fig 1A,B), although they expressed vimentin in a perinuclear network. As the number of adherent cells increased, so did their expression in α-SM actin (Fig 1C,D). In cultures where adherent layers were confluent (from week 3 until week 7), most cells presented linear microfilaments stained by the α-SM-1 MoAb. During this time span, the output of colony-forming units-granulocyte macrophage (CFU-GM) generated in the adherent layer was stable with values 7.5% to 17.5% of the value at culture inception (not shown). After week 7, some adherent cells frequently detached from the surface on which they were grown (culture flasks or chamber slides). The proportion of α-SM-positive cells at this culture time significantly decreased. The CFU-GM output in these "postconfluent" cultures usually declined (not shown).

The sharp increase in α-SM actin expression was also shown by Western blot studies (Fig 2). We compared the content in vimentin and α-SM actin from week 2 and week 5 adherent layers. Proteins were extracted from identical numbers of cells; ie, three flasks and one flask were sacrificed at weeks 2 and 5, respectively. Vimentin contents of week 2 and week 5 adherent layers were identical (panel B); on the contrary, at week 2, the α-SM actin band was barely visible, whereas, at week 5, a major band was revealed by the α-SM-1 antibody (panel A).

The content of α-SM actin in stromal cells from confluent cultures is similar to that of smooth muscle cells from uterus or from the A7r5 cell line. Proteins were extracted from (1) uterus, (2) cultured A7r5 smooth muscle cells; (3) the L2ori-stromal cell line; (4) stromal cells, ie, confluent adherent

**Fig 8.** Detection by Western blot of h-caldesmon in extracts from uterus (lane 1), cells from the L2ori-stromal cell line (lane 2), adherent layers devoid of hematopoietic cells (lane 3), and fresh uncultured BM (lane 4). Proteins loads were 30 μg for lanes 2, 3, and 4 and 15 μg for lane 1. Immunoblot was performed with the monoclonal anticaldesmon antibody h-CD.
layers devoid of hematopoietic cells; and (5) fresh uncul-tured BM cells. Samples were run on SDS-PAGE and actin content adjusted for each sample. Results of Western blots are shown on Fig 3. It can be observed that stromal cells (lane 4) contained amounts of α-SM actin similar to that of the uterus (lane 1) and A7r5 cells (lane 2), whereas the transformed L2ori-stromal cells (lane 3) expressed lower amounts of this actin isoform, and fresh marrow cells did not express α-SM actin (lane 5).

*Stromal cells contain the metavinculin isoform of the vinculin molecule.* Western blots were performed on stromal cell extracts using Vin 11.5. Two bands were apparent: a major 130-Kd band corresponding to vinculin and a minor 150-Kd band corresponding to metavinculin. The amounts of proteins expressed at both molecular weights were compared by measuring the thickness of the bands by densitometry. A similar procedure was used for extracts of the uterus, A7r5 cells, L2ori-cells, and fresh uncultured BM cells (Fig 4). Results of the densitometry studies are shown in Fig 5. For the uterus, the average metavinculin/vinculin ratio was 1:1. For A7r5 cells, the average ratio was 1:4, and for adherent layers (with or without hematopoietic cells) and L2ori-cells, it dropped to approximately 1:20. No metavinculin was found in fresh BM cells.

Immunofluorescence studies showed that only focal adhesions were observed with antivinculin MoAb 1B2 (Fig 6). We may infer from this observation that metavinculin was located at places similar to that of vinculin, as previously reported for vascular smooth muscle cells.α

*Stromal cells contain the 150 Kd caldesmon isoform.* Western blots carried out on adherent layer extracts using Cald 8 MoAb showed two bands: a major band of 75 Kd and a minor band of 150 Kd. Proteins contents for both molecular weights bands were measured by densitometry as described for metavinculin and vinculin. As shown in Fig 7, the 150 Kd isoform was predominant in the uterus and A7r5 cells, and there were approximately two heavy caldesmon molecules per each light one. In adherent layer extracts with or without hemopoietic cells, an inverse ratio was obtained: there was approximately two light caldesmon molecules per each heavy one.

The presence of a 150-Kd heavy caldesmon isoform in...
stromal cells was confirmed by using the MoAb h-CD, which recognizes solely this isofrom.20 A band at 150 Kd was clearly visible in extracts of stromal cells (lane 3) and L2ori- cells (lane 2), although it was less intense than in the uterus (lane 1) because for this lane the protein load was half that of the other lanes. For fresh marrow cells, no band at 150 Kd was detected (lane 4).

Stromal cells contain smooth muscle myosin heavy chains (SMMHCs) and calponin. Western blots performed on stromal cell extracts using the MoAb SMMS-1 described by Frid et al20 showed a 200/204-Kd doublet (Fig 9, lane 4). This doublet was observed for a protein load 10 times superior to that of the uterus (lane 1), which indicated that although detectable, the amounts of SMMHCs were low. Similar amounts of SMMHCs were detected in the A7r5 cell lane (lane 2), whereas in L2ori- transformed stromal cell bands were barely visible (lane 3). In fresh BM, no band was detected (lane 5). Immunofluorescence studies showed that the anti-SMMHC MoAb stained stress fibers (Fig 10) confirming the presence of actomyosin within these cell structures.

Using the anticalponin MoAb clone CALP,20 a 34-Kd band (Fig 11) was observed on immunoblots of stromal (lane 4) and L2ori- cell (lane 3) extracts, uterus extracts (lane 1), and A7r5 cell extracts (lane 2). In fresh BM extracts, a band also was observed (lane 5). It was due to a platelet contamination (lane 6).

Cells positive for the α-SM actin isoform are found in marrow biopsies from patients without hematologic abnormalities. We studied five biopsies collected from patients with stage IA or IIA Hodgkin’s disease. In all specimens, α-SM–positive cells were found at diverse locations.

As expected, there were α-SM–positive vascular smooth muscle cells in the media of arteries and arterioles (Fig 12A). However, most α-SM–positive cells were isolated and elongated cells lining capillaries (Fig 12B) and sinuses (Fig 12C) or located within hematopoietic logettes without obvious spatial relationship to the vasculature (Fig 12D). Sinus-lining cells were located on the abluminal side of endothelial cells. More or less mature cells of the granulocytic series were in intimate contact with sinus-lining cells (Fig 12C) and cells within hematopoietic logettes (Fig 12D). Both cell types have already been described as myoid cells.24 Eventually, endosteal cells were also α-SM positive (Fig 12E,F). These very flattened cells extended along bone trabeculae.

**DISCUSSION**

This report shows that in stromal cells generated in human long-term marrow cultures (1) the expression of α-SM actin isoform is time dependent and (2) other cytoskeletal proteins considered as specific for smooth muscle cells are detected when the adherent layer is confluent.

In the days following culture inception, α-SM actin is not detected in the early adherent spindle-shaped cells that display an intermediate filament perinuclear network of vimentin. Adherent cells containing α-SM actin in stress fibers increase with time. The number of positive cells reaches a maximum when a confluent adherent layer is observed (from week 3 until week 7). During this span of time, 60% to 90% of the large and interdigitated cells making up most of the adherent layer are α-SM positive while still expressing vimentin in intermediate filaments. Beyond week 7, as cultures decay (shown by partial detachment of the adherent layer), a significantly lower number of cells contain α-SM–positive stress fibers.

These data strongly suggest that few α-SM–positive cells, if any, are inoculated at culture inception. A previous estimation of the maximal percentage of α-SM–positive cells at culture inception was <1%.6 It is probable that under culture conditions, the gene coding for α-SM actin is activated, which results in the progressive expression of the protein. Our results also suggest that there is a positive correlation between the generation of α-SM–positive cells and that of granulopoiesis within the adherent layer. During the first 2 weeks of culture, most of the CFU-GM are found in the culture supernatant. From week 3 to week 7, most of the CFU-GM are generated in the adherent layer and the CFU-GM output is stable with a value 7.5% to 17.5% of that at culture inception. Cobblestone areas consisting of granulocytic cells are regularly found in confluent adherent layers.

This positive correlation is also supported by results of cocultures of CD34+ cells and Stro-1+ cells recently performed in our laboratory. We have observed that Stro-1+ stromal cells27 from confluent cultures are able to support CFU-GM and long-term culture-initiating cell formation from cocultured CD34+ cells. Stro-1+ stromal cells are a subset of stromal cells: (1) that comprises 5% to 50% of the cells from the adherent layer, depending on the time in culture; and (2) that consists of a majority of α-SM–positive cells.

Our data are at variance with those of Peled et al.28 These investigators have found that only a minority of cells from the 14 F 1.1 “adipose endothelial” murine stromal line expressed α-SM, and, more specifically, that α-SM–positive cells were not present where cobblestone areas were observed. The difference between the culture systems (murine vs human) and stromal cells (selected stromal cell line vs primary cultures) may explain the difference in results. Nevertheless, to clarify the point as to whether the expression of α-SM actin in stromal cells is crucial for the maintenance of
Fig 12. Cells positive for \( \alpha \)-SM actin isoform in trephine bone marrow biopsies. (A) Smooth muscle cells in the media of an artery (original magnification \( \times \) 400). These oval or rounded cells are aligned in several rows (in this case two rows are visible) delineating the lumen bordered by \( \alpha \)-SM–negative endothelial cells. (B) Pericapillary pericytes (\( \times \) 400). These elongated cells line (more or less continuously) the capillary forming two opposite rows. They are located on the abluminal side of \( \alpha \)-SM–negative endothelial cells. (C) Sinus-lining myoid cells \( \times \) 400). These elongated cells with a protrusive oval nucleus are found along the sinus border on the abluminal side of endothelial cells. One such cell is seen on the left-hand side of a small sinus (whose lumen contains two band cells and a polymorph). One may notice that the cytoplasm extends away from the sinus within the hematopoietic cord. An endothelial cell (on the right-hand side of the sinus) is \( \alpha \)-SM negative. (D) Myoid cells within the hematopoietic logette (\( \times \) 320). These very elongated cells with a protrusive nucleus are found within the cord, without obvious spatial relationship with the vasculature. One such cell, whose nucleus is located in the left-hand side of the figure, runs parallel to (but not in contact with) a small sinus (on the upper left side of the figure). (E) Pericapillary pericytes and endosteal cells \( \times \) 100). Two capillaries (left-hand side of the figure) are seen at distance from a bone trabecula. Very thin endosteal cells are observed along the inferior border of the trabecula (lower right-hand side of the figure). (F) Endosteal cells \( \times \) 400). This figure is a magnification of figure E. The endosteal cell appears as a very flattened cell with a barely visible nucleus and a thin \( \alpha \)-SM–positive cytoplasm lining the bone surface.
granulopoiesis, other approaches should be used: addition of antisense RNA to long-term marrow cultures and production of stromal cell lines lacking functional α-SM actin genes by homologous recombination (double knock-outs).

The expression of α-SM actin in cultured cells is not restricted to smooth muscle cells: Many human and rodent fibroblast cell lines contain cells that can express α-SM actin after certain time in culture. This is why we looked for other smooth muscle–specific cytoskeletal proteins. We have indeed found that metavinculin, 150-Kd caldesmon, SMMHCs, and calponin were expressed by stromal cells, which confirm the smooth muscle differentiation of these cells. Comparison of the ratios of metavinculin to vinculin and of h-caldesmon to l-caldesmon to those found in the different layers of human adult aorta and in fetal aorta lead to the conclusion that stromal cells are very similar to immature fetal smooth muscle cells and subendothelial smooth muscle intimal cells. These latter cells may be considered as immature smooth muscle cells with a limited development following birth and an active recruitment when an atherosclerotic process occurs. We did not find other cytoskeletal markers of smooth muscle cell differentiation. The absence of desmin intermediate filaments was expected because this molecule is rarely found in vascular smooth muscle cells. We did not either find cytokeratin 8, although this molecule has been observed in immature smooth muscle cells. The results of the study of the extra-cellular matrix glycoproteins fit with the immature smooth muscle phenotype: collagens I, III, IV, laminin, thrombospondin, and tenasin are regularly found in adherent layers; and stromal cells synthesize a fibronectin variant distinct from soluble fibronectin and including the alternatively spliced EDα region (H. Lerat et al, manuscript in preparation).

Our description of stromal cells as vascular smooth muscle–like cells underscores a distinctive differentiation of marrow mesenchymal cells borrowing features of the vascular smooth muscle cell repertoire.

What may be the relevance of stromal cells with such a smooth muscle phenotype to in vivo studies? We have found that within human adult BM, α-SM–positive cells are smooth muscle cells within the media of arterioles, pericytes delineating capillaries, and myoid cells frequently found in the vicinity of sinuses. Sinus-lining myoid cells are located at the abluminal side of endothelial cells, whereas other myoid cells are found within hemopoietic logettes, without obvious spatial relationship to the vasculature. Sinus-lining myoid cells or myoid cells within the hemopoietic cord appear as elongated cells with a prominent nucleus and one or more very thin cytoplasmic extensions in intimate contact with cells of the granulocytic series. Myoid cells may therefore be the in vivo counterpart of α-SM–positive stromal cells generated in long-term marrow cultures. It is noteworthy that these already-described cells are more frequently in fetal BM and in adults in cases of inflammatory disease and hemopathies. Myoid cells appear to be predominant when the production of hemopoietic cells is increased either during development or because of an inflammatory process or the generation of cells from a neo-plastic stem cell clone. A murine equivalent of myoid cells may be barrier cells that also contain myofibrils and whose number and location depend on the state of stimulation of hemopoiesis.

Taken together our observations suggest that marrow mesenchymal cells, whose differentiation in cultures results in immature vascular smooth muscle–like stromal cells, give rise in vivo to myoid cells or barrier cells whose number and location may vary with the hemopoietic cell need.

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Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway

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