Expression of α-Smooth Muscle Actin in Murine Bone Marrow Stromal Cells

By Amnon Peled, Dov Zipori, Oded Abramsky, Haim Ovadia, and Elias Shezen

Human fibrotic bone marrow (BM) stroma has been shown to contain α-smooth muscle actin (α-SMA)-positive cells. These closely resemble myofibroblasts that were described in other fibrotic tissues. We studied the expression of α-SMA in a series of murine BM-derived stromal cell lines to investigate the cellular origin and functional significance of myofibroblast-like cells in hematopoietic tissues. Although these cell lines differed in their biologic properties, most of them expressed α-SMA under certain conditions. Cells expressing α-SMA constituted a minor population in post-confluent, growth-arrested cultures. However, the incidence of cells expressing α-SMA increased significantly when cultures were transferred to nonconfluent conditions. A similar increase in α-SMA-positive cells occurred after a strip of cells was scraped away from the confluent cell layer; the cells of the affected area acquired α-SMA-positive contractile phenotype. The relationship between α-SMA expression and hematopoietic activity was studied using a cloned cell line of BM origin (14F1.1). The ability of these endothelial-adipocyte cells to support hematopoiesis in vitro was maximal under confluent conditions, whereas their expression of α-SMA under such conditions was residual. Moreover, in long-term BM cultures supported by confluent 14F1.1 cells, stromal areas associated with proliferating hematopoietic precursors, known as “cobblestone areas,” were devoid of α-SMA-positive cells. These observations suggest that the expression of α-SMA is reversible and inversely related to hematopoietic activity.

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DIFFERENT HEMATOPOIETIC disorders are commonly associated with fibrotic changes in bone marrow (BM) stroma. The contribution of fibrosis to the hematopoietic failure and the histogenesis and properties of cells that constitute the fibrotic BM stroma are unclear. Some of these cells display ultrastructural similarities to smooth muscle cells. The expression of α-smooth muscle actin (α-SMA), a differentiation marker of smooth muscle cells, was observed in BM stroma of different hematopoietic disorders such as BM involvement in metastatic carcinoma, Hodgkin’s disease, multiple myeloma, chronic myelomonocytic leukemia, and primary chronic myelofibrosis/osteomyelosclerosis. In all those cases, the incidence of cells expressing α-SMA correlated with the degree of fibrotic change. α-SMA-positive stromal cells were observed during fetal and early infant stages of BM development, but not in adult normal BM stroma. These observations show that the fibrotic BM stroma, like other fibrotic sites, contains cells displaying mixed fibroblastic and smooth muscle characteristics; these cells were referred to by Gabbiani et al as “myofibroblasts.”

Recently, it was shown by Charbord et al that α-SMA is constitutively expressed by stromal cells of hematopoietically active human Dexter cultures, but not by BM-derived fibroblasts. These findings were interpreted as substantiating the notion that the population of BM stromal cells that supports long-term hematopoiesis in vitro represents a cell lineage distinct from marrow fibroblasts, and has similarity to smooth muscle cells. However, the relevance of these findings to hematopoiesis in vivo remains unclear, because no expression of α-SMA was observed in hematopoietically active, intact BM stroma. Furthermore, expression of α-SMA in BM stroma is associated with a reduced hematopoietic activity, such as that which occurs in myelofibrosis or in tissue remodelling during fetal life.

We performed an in vitro study using cell lines representing functionally and morphologically distinct populations of murine BM stromal cells, in an attempt to show the origin of cells expressing α-SMA in BM stroma, the conditions regulating this expression, and the relevance to hematopoiesis. We found that different subpopulations of stromal cells are capable of reversible expression of a smooth muscle-like phenotype, and that the ability to support hematopoiesis is inversely related to α-SMA expression.

MATERIALS AND METHODS

Cell lines. Adherent stromal cell lines from mouse BM were developed and characterized in our laboratory. The following cell lines were used: 14F1.1 endothelial-adipocytes, which apparently represent the BM stromal cell population involved in maintenance of the hematopoietic stem cell pool; MBA-13 fibroendothelial cells; MBA-1.1.1 fibroblasts; MBA-2.1 endothelial-like cells; the non-characterized stromal cell clone MBA-22.2; and the osteogenic cell line MBA-15.

Cell cultures. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; BioLabs, Jerusalem, Israel). The cultures were incubated at 37°C in a humidified atmosphere of 10% CO₂ in air. Long-term BM cultures were supplemented with 10% horse serum (HS; BioLabs) and performed essentially as described previously.

Antibodies. Monoclonal antibody (MoAb) to α-SMA (kindly provided by Bio-Makor, Rehovot, Israel) was used at optimal dilution of 1:40 for immunofluorescence, and at 1:200 for immunoperoxidase staining, immunoblots, and radioimmunoassay (RIA). MoAb V-13.2 to vimentin (Bio-Makor) was used at a dilution of 1:40 for immunofluorescence and at 1:100 for RIA and immunoblots. Keratin wide spectrum screening rabbit antiserum (Dako, Copenhagen, Denmark) and MoAb to desmin (Bio-Makor) were used for immunostaining at 1:100. All antibodies used in this study were specifically reactive with mouse tissues. In the immuno-
peroxidase assay, visualization of the antigen was performed using an extra avidin-biotin peroxidase system (Bio-Makor). The second antibody applied in immunofluorescence studies was affinity-purified goat antiamouse antibodies conjugated with Texas Red (Jackson Immunoresearch Labs, Inc, West Grove, PA) (1:100) and goat antirabbit antibodies conjugated with fluorescein isothiocyanate (FITC; Bio-Makor) (1:40).

**Immunocytochemistry.** Immunofluorescence studies were performed on cells grown in chamber slides (LabTek slides; Miles Scientific, Naperville, IN), and the immunoperoxidase assays were performed on cells grown in 60-mm culture dishes (Falcon; Becton Dickinson Labware, Oxnard, CA). The cells were fixed in absolute methanol at -20°C for 10 minutes and dried before being assayed. Methanol-fixed cells were incubated with 5% bovine serum albumin (BSA; Sigma, St Louis, MO) in phosphate-buffered saline (PBS) and the first antibodies were applied for 1 hour at room temperature (RT). In immunofluorescence studies, the fluorophore-conjugated antibody was applied for 45 minutes. Stained samples were dehydrated in absolute ethanol and mounted in Entellan (Eli Lilly, Indianapolis, IN). For immunoperoxidase stain, the standard procedure recommended by the manufacturer (Bio-Makor) was applied. The slides were examined using a Zeiss Photomicroscope III (Oberkochen, Germany), equipped for epifluorescence observations, with an oil immersion plane neofluar objective, at magnifications of ×25 and ×60.

**RIA.** Solid-phase RIA for cellular antigens was performed in 96-well plates (Dynatech Laboratories, VA); 2 × 10^5 cells in 200 μL of DMEM supplemented with 10% FCS were seeded in each well and allowed to adhere overnight. After washing with PBS, the cells were methanol-fixed at -20°C. Blocking was performed with 5% BSA-PBS solution. Cells were incubated with the tested antibodies for 1 hour at RT then washed three times for 5 minutes with 1% BSA in PBS. After washing, 125I-rabbit-antimouse antibody (Fab), 10^7 cpm (Jackson) was applied to each well. After 1 hour of incubation, the cells were washed with PBS and the plates dried. Counting was performed in a Kontron gamma counter (MR480; Zurich, Switzerland).

**Western blot.** Tissues and cells were solubilized in boiling buffer (2% sodium dodecyl sulfate, 100 mM/L tris [pH 6.9], and 10 mM/L dithithreitol [DTT]). The samples were run on 8% polyacrylamide gel (10 to 25 mg protein per well) and transferred for 4 hours to nitrocellulose paper. The blots were incubated with tris-buffered saline containing 0.1% Tween (TBST) and 5% BSA for 1 hour at RT. The blots were washed with TBST solution and 10^6 cpm/mL 125I-rabbit-antimouse antibody was added. After 45 minutes of incubation, the blots were washed three times with TBST and autoradiograms were prepared. Size markers (Bio-Rad Laboratories, Richmond, CA) and chicken gizzard actin (Sigma) were run in parallel to cell and tissue extracts.

In vitro "wounding." 14Fl1 cultures could be maintained at confluence for periods exceeding 6 months. The confluent cell monolayers were gently scraped with a rubber policeman. That procedure left a cell-free area ("wound") about 5 mm wide. Forty-eight and 96 hours after "wounding," the plates were fixed in methanol and stained for α-SMA.

**RESULTS**

Expression of smooth muscle-like phenotype in murine BM-derived stromal cell lines. An MoAb to α-SMA developed by Scalli et al was previously shown to react specifically with aortic SMA.9 Immunoblot experiments with this MoAb were performed using total protein extracts from different mouse tissues and purified actin from chicken gizzard. Extracts of heart, skeletal muscle, and chicken gizzard actin produce a faintly stained actin band, while an aortic actin protein band was strongly stained by the antibody (Fig 1B). A characteristic vascular stain in cryostat sections of normal murine tissues and a strong stromal stain of fibrotic and granulomatous murine tissues were observed (data not shown). These results are consistent with previously published data9,12 and testify to the specificity of the antibody used for murine α-SMA.

We further studied the cellular origin of α-SMA expressing BM stromal cells by using a series of cell lines that represent different subpopulations of murine BM stromal cells.9,12 These lines differ in their morphologic appearance, collagen synthesis profiles, and ability to support hematopoiesis (Table 1). In immunofluorescence and immunoperoxidase assays, all cell lines but one displayed a strong staining with α-SMA MoAb (Table 1). Western blot analysis (Fig 2A) confirmed the results obtained by immunocy-
Table 1. Properties of Murine BM-Derived Stromal Cell Lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Collagen Types</th>
<th>Support of Long-Term Hematopoiesis</th>
<th>α-SMA Expression</th>
</tr>
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<tbody>
<tr>
<td>14Fl.1*</td>
<td>I, IV</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MBA-15</td>
<td>I</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MBA-1.1,1*</td>
<td>I, III, IV, V</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MBA-13</td>
<td>I</td>
<td>-</td>
<td>+</td>
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<tr>
<td>MBA-22.2*</td>
<td>I</td>
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BM-derived stromal cell lines that differ in their morphologic appearance, collagen synthesis profiles, and ability to support hematopoiesis. Cloned cell lines.

BM-15 was the only cell line that did not express α-SMA, whereas extracted proteins from 14Fl.1, MBA-15 cells, and mouse aorta reacted identically with the antibody (Fig 2A and A1). All cell lines, including MBA-2,1, tested by immunostaining, RIA (data not shown), and Western blots (Fig 2B) expressed vimentin, an intermediate filament protein. No staining was observed with antibodies to keratin and desmin.

To investigate the pattern of α-SMA expression in relation to hematopoiesis we focused on the cloned endothelial-adipocyte cell line 14Fl.1. This line was shown previously to support long-term hematopoiesis in vitro. Sparse, freshly initiated 14Fl.1 cultures contain a large number of (＞30%) α-SMA-positive cells. The α-SMA positivity is mainly associated with giant flat-angled cells. α-SMA immunoreactivity was localized in stress fibers, which were extensive in these cells (Fig 3A). In confluent layers, 14Fl.1 cells displayed morphologic heterogeneity. These cultures contained adipocytes, small endothelial-like cells, and giant flat-angled cells. Using Western blot, immunoperoxidase, and RIA techniques, it was evident that the expression of α-SMA in cultures was reduced as a function of time and degree of confluence (Figs 3F, 4A, and 5A). The expression of vimentin in these cultures remained constant (Figs 4B and 5B). The same phenomenon was observed in other BM stromal cell lines expressing α-SMA (data not shown). The low number of α-SMA-positive cells (＜1%), observed in post-confluent stationary 14Fl.1 cultures, changed when the confluent cell layer was damaged. Scraping a strip of cells (in vitro “wounding”) caused the accumulation of cells bearing the α-SMA–positive contractile phenotype on the edge of the “wound.” This effect could be slightly observed 48 hours after scratching (Fig 3B), and at 96 hours these cells predominated in the populations colonizing the scraped area (Fig 3C). In these cultures, intermediate forms with adipocytic features and moderate levels of α-SMA were observed (Fig 3D). Mitotic figures were often seen among α-SMA–positive cells, colonizing the “wound” area (Fig 3E). The level of α-SMA in undamaged areas remained low.

Inverse relationship between α-SMA expression and hematopoietic activity of 14Fl.1 cells. Although most of the BM stromal cell lines were capable of expressing α-SMA under certain conditions, only 14Fl.1 cells were able to induce and support long-term proliferation of BM hematopoietic precursors (Table 1). Early hematopoietic precursors proliferated in close contact with supporting 14Fl.1 cells and form characteristic, well ordered “cobblestone” areas. This pattern of hematopoietic proliferation was similar to that of long-term BM cultures supported by primary stromal cell populations, ie, Dexter-type cultures. Immunostaining of post-confluent 14Fl.1 cells cocultured with BM cells showed a low frequency of α-SMA–positive cells (＜1%). The “cobblestone” areas

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Fig 2. Reactivity of MoAbs to α-SMA (A, A1) and vimentin (B) with protein extracts (equal amount) from different BM stromal cell lines (MBA-2.1, MBA-15, 14Fl.1 [A]) and from aorta muscle (A1), analyzed by Western blotting. Size markers are as indicated in Fig 1.
Fig 3. Immunoperoxidase staining of methanol-fixed 14F1.1 cell cultures reacted with MoAbs to α-SMA. (A) Nonconfluent 14F1.1 cells demonstrating α-SMA organized in stress fibers (arrow). (B) The appearance of α-SMA-positive cells in the border of the scraped area 48 hours after wounding the culture (arrow). (C) Panoramic view of post-confluent culture 96 hours after "wounding," showing α-SMA-positive cells accumulating in the wounded site (enclosed area). (D) 14F1.1 cells showing a transient combined phenotype of adipocyte and α-SMA-positive cells (arrow). (E) α-SMA-positive mitotic 14F1.1 cell observed in the "wounded" area (arrow). (F) Confluent 14F1.1 cells cocultured with BM cells. Enclosed area "h" indicates position of a cobblestone area, shown at higher magnification in G. (B, C, and F, original magnification ×160; A, D, E, and G, original magnification ×403.)
antibody in murine tissues suggested that the distribution of this antigen was identical to that observed in the above-mentioned species.

In our cultured cell lines, the expression of α-SMA was inducible. Experiments with cloned adipocyte-endothelial line 14F1.1 and other lines demonstrated that only a minor pool of α-SMA-positive cells could be detected in post-confluent growth-arrested cultures. However, the incidence of this phenotype rose under sparse culture conditions and during repopulation of “wounds” inflicted on confluent cell layers. These results were compatible with the fact that appearance of myofibroblasts in BM stroma in vivo is associated with conditions of extensive proliferation of stromal elements. Such conditions prevail during the establishment of hematopoiesis in the BM during fetal development as well as under pathologic conditions associated with the fibrotic reaction of BM stroma, i.e., spread of carcinoma within the BM, hematopoietic malignancies, and primary idiopathic myelofibrosis. α-SMA expression patterns observed in our stromal cell cultures contrasted with those described in cultured rat smooth muscle cell populations, where maximal expression of this contractile protein was associated with growth arrest. These differences may serve as criteria to distinguish between myofibroblastic and genuine smooth muscle cell populations.

The coexpression of α-SMA and vimentin was associated with a characteristic contractile morphology of positive cells (large cells with angulated shape and extensive stress fibers). This phenotype closely resembled myofibroblasts described in granulation and fibrotic tissues in vivo. Of the six cell lines tested, the MBA-2.1 cell line was the only one found to be α-SMA negative. In contrast to the other lines, this cell line is tumorigenic (Yankelevich and Zipori, unpublished). The observation that expression of α-SMA was inhibited in virally transformed rodent fibroblasts may explain this exception.

Immunocytochemical studies in long-term BM cultures supported by confluent layers of 14F1.1 cells showed no association between α-SMA-positive cells and areas of proliferating hematopoietic precursors, i.e., “cobblestone areas”. Our previous data indicated that the hematopoietic supporting activity of 14F1.1 line was most pronounced in post-confluent nonproliferating cultures (Zipori, unpublished), where the α-SMA-positive phenotype was minimal. Our data are in line with studies showing that stroma of...
hematopoietically intact adult BM displays no α-SMA positivity, whereas such a phenotype arises under conditions associated with deterioration of hematopoiesis.

Both phenomena may result from changes in growth factors operating in the BM microenvironment. Recently, it was shown that transcription of actin genes and c-fos proto-oncogene is induced by serum growth factors.

The appearance of stromal cells bearing adipocytic phenotype is enhanced by hydrocortisone and is considered essential for the establishment of long-term hematopoiesis in vitro. Our preliminary results indicated that hydrocortisone inhibits the expression of α-SMA in 14Fl.1 cells.

The observations discussed here point to a similarity between α-SMA-positive cells in vitro and myoid cells of in vivo fibrotic BM stroma. We suggest that both in vivo and in vitro reactive conditions activate similar mechanisms of expression of a contractile α-SMA-positive phenotype.

ACKNOWLEDGMENT

We thank Dr R. Moor for his help and valuable advice.

REFERENCES

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