Benign Mesenchymal Stromal Cells in Human Sarcomas

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Abstract

Purpose: Recent evidence suggests that at least some sarcomas arise through aberrant differentiation of mesenchymal stromal cells (MSCs), but MSCs have never been isolated directly from human sarcoma specimens.

Experimental Design: We examined human sarcoma cell lines and primary adherent cultures derived from human sarcoma surgical samples for features of MSCs. We further characterized primary cultures as either benign or malignant by the presence of tumor-defining genetic lesions and tumor formation in immunocompromised mice.

Results: We show that a dedifferentiated liposarcoma cell line DDLS8817 possesses fat, bone, and cartilage trilineage differentiation potential characteristic of MSCs. Primary sarcoma cultures have the morphology, surface immunophenotype, and differentiation potential characteristic of MSCs. Surprisingly, many of these cultures are benign, as they do not form tumors in mice and lack sarcoma-defining genetic lesions. Consistent with the recently proposed pericyte origin of MSCs in normal human tissues, sarcoma-derived benign MSCs (SDBMSCs) express markers of pericytes and cooperate with endothelial cells in tube formation assays. In human sarcoma specimens, a subset of CD146-positive microvascular pericytes expresses CD105, an MSC marker, whereas malignant cells largely do not. In an in vitro coculture model, SDBMSCs as well as normal human pericytes markedly stimulate the growth of sarcoma cell lines.

Conclusions: SDBMSCs/pericytes represent a previously undescribed stromal cell type in sarcoma that may contribute to tumor formation.

Sarcomas are malignancies of mesenchymal tissues such as fat, bone, cartilage, and muscle. The incidence of bone and soft tissue sarcoma is estimated at approximately 13,000 cases a year in the United States, leading to more than 5,000 deaths (1). More than 50 types of sarcoma are known, broadly divided into translocation-associated (e.g., synovial sarcoma, Ewing sarcoma, myxoid/round cell liposarcoma) and genetically complex [e.g., osteosarcoma and malignant fibrous histiocytoma (MFH), also known as high-grade pleomorphic undifferentiated sarcoma; ref. 2)]. When diagnosed at a localized stage, 5-year survival is generally more than 80%. However, when metastases are present, 5-year survival is less than 30% (1), underscoring the need for new systemic therapies (3).

The candidate cell of origin of sarcoma is the mesenchymal stromal cell (MSC; ref. 4). Defined by their plastic-adherence, surface phenotype (CD45-CD34-CD73+CD105+CD90+CD44-CD34+CD73+CD105+CD90+CD44+), and differentiation into fat, bone, and cartilage in vitro (5), MSCs can be isolated from human bone marrow, adipose tissue, and other mesenchymal tissues (6). Recent indirect evidence supports MSC origin of sarcoma. Knockdown of EWSR1-FLI1 in Ewing sarcoma cell lines allows differentiation towards bone, cartilage, and adipocyte lineage, suggesting that the tumor-initiating translocation event originally occurs in an MSC (7). Murine (but not human) MSCs expressing human sarcoma translocations EWSR1-FLI1 and FUS-DDIT3 develop into Ewing and myxoid liposarcoma-like tumors, respectively, when injected into mice (8, 9). Various histologic types of liposarcoma can be associated with stages of MSC-to-adipocyte differentiation in vitro (10). Murine osteosarcoma seems to arise from MSC-like cells capable of differentiation into fat and bone (11).

Although MSCs are commonly isolated from the bone marrow, their identity in soft tissues has remained elusive until very recently. Indirect evidence over 2 decades showed that microvascular pericytes in a variety of tissues have MSC properties (12). Recently, several groups showed that CD146-positive microvascular pericytes isolated from a variety of normal tissues have all the features of MSCs, including multilineage differentiation in vitro and

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Translational Relevance

We show that, contrary to prior reports, mesenchymal stromal cells (MSCs) in sarcoma are often benign. This is the first characterization of benign stroma in sarcoma. We propose the endosialin-positive, CD105-positive pericyte as the location of MSCs in sarcoma. Agents targeting endosialin and CD105 are currently in development. We propose that they may be active in sarcoma in part by targeting the benign stromal elements.

Materials and Methods

Sample collection and processing

Sample collection was done according to a tissue acquisition protocol approved by the MSKCC Institutional Review Board. The samples were obtained through the MSKCC Pathology Department Tumor Procurement Service at the time of surgery, ensuring that sufficient sample was preserved for diagnostic purposes. Those samples in which margins were important were initially inked by the pathologist according to the standard protocol, but sterile ink was used. Under sterile conditions, the pathologist made an incision through the inked specimen and resected a sample, 1 cm³ or greater, from the inner portion of the tumor. In cases in which margins were deemed unimportant by the pathologist, such as metastasectomy or open biopsies, a representative portion of the resected specimen was collected for analysis, away from normal tissues or necrotic areas. Samples were minced and digested for 10 to 12 hours in MEMα supplemented with 20% FBS (Atlas Biologicals catalog number F-0500-A) and 2 mmol/L of L-glutamine, referred to as complete culture medium, or CCM (14), 250 ng/mL of amphotericin B, 100 units/mL of penicillin, 0.1 mg/mL of streptomycin, 2,500 units/mL of collagenase IV (Invitrogen catalog number 17104-019) in low-cell-binding culture dishes (Nunc catalog number 145383) to prevent premature attachment of cells. After digestion, tumor fragments softened and could easily be pipetted through a 5-mL tissue culture pipette, leading to disaggregation into a single-cell suspension with some residual undigested fragments. Viability by Trypan Blue exclusion ranged from 30% to greater than 90%. In many cases, the absence of significant debris and high viability indicated that most cells within the tumor were recovered. The entire sample was washed twice in CCM, filtered through a 40-μm mesh filter (Fisher Scientific catalog number 22363547; if residual tumor fragments remained) and plated on tissue-culture-treated polystyrene plates (BD Falcon catalog number 353003), analyzed by flow cytometry, injected into immunodeficient mice, or cryopreserved in 10% DMSO.

Cell culture, differentiation, and coculture assays

All cell cultures were maintained in CCM consisting of MEMα supplemented with 20% FBS (Atlas Biologicals catalog number F-0500-A) and 2 mmol/L of L-glutamine (14). Cells were examined 3 times weekly and passaged when they reached 80% confluence by brief treatment with 0.25% trypsin–2.21 mmol/L EDTA solution (Cellgro catalog number 25-053-CI). For the fat and bone differentiation assays, cells were plated in triplicate on tissue-culture-treated polystyrene plates (BD Falcon catalog number 353003) in CCM at approximately 20% confluence. After reaching 100% confluence, 1 well was switched into fat differentiation medium, 1 into bone differentiation medium, and 1 into fresh CCM. Cells were maintained for 3 weeks, with twice-weekly media changes.

Differentiation medium consisted of CCM with the following supplements according to published methods (14). For fat differentiation, 0.5 mmol/L of isobutylmethylxanthine, which increases cAMP levels, 50 μmol/L of indomethacin, which here acts as a PPARγ agonist, and 500 nmol/L of dexamethasone were used. Formation of lipid droplets positive for Oil Red O staining was used to confirm fat differentiation. Oil Red O staining was carried out according to standard methods (14). Alternatively, lipid droplets were visualized in live cells with BODIPY 493/503 (Invitrogen catalog number D-3922), a highly specific lipid dye (15) that was dissolved in DMSO to produce a 5-mmol/L stock solution, added directly to live cell culture for a final concentration of 0.5 μmol/L, and incubated for 10 minutes. Images were taken at low fluorescence intensity to avoid photobleaching.

For bone differentiation, 5 mmol/L of β-glycerol phosphate, which promotes mineralization, 50 μg/mL of ascorbate-2-phosphate, which promotes ossification, and 1 mmol/L of dexamethasone were used. Bone differentiation was shown by the presence of Alizarin Red-positive deposits (14).
For the cartilage differentiation assay (14), 10^5 cells were pelleted in a 15-mL polypropylene tube resulting in a cell aggregate that was grown for 3 weeks in CCM supplemented with 10 ng/mL of TGFβ3, 100 nmol/L of dexamethasone, 50 µg/mL of ascorbate-2-phosphate, 40 µg/mL of proline, 100 µg/mL of pyruvate and 1 x ITS+1 supplement (1.0 mg/mL of insulin from bovine pancreas, 0.55 mg/mL of human transferrin, 0.5 µg/mL of sodium selenite, 50 mg/mL of bovine serum albumin, and 470 µg/mL of linoleic acid). All reagents were obtained from Sigma unless otherwise specified. The medium was changed and the tubes agitated twice a week to keep aggregates free-floating. After 3 weeks, cell aggregates were frozen in OCT (TissueTek catalog number 4583), cryosectioned, and stained with 1% Alcian Blue in 0.3% acetic acid to show cartilage differentiation. Negative controls without TGFβ3 were processed in parallel.

For coculture assays, an osteosarcoma cell line U2OS and an Ewing sarcoma cell line A673 were obtained from ATCC (HTB-96 and CRL-1598, respectively) and authenticated by STR typing. The cell lines were transduced with an RFP-encoding lentivirus according to standard methods at high titer, resulting in nearly 100% transduction. For in vitro mixing experiments, sarcoma cell lines were seeded at 300 cells per well of a 24-well plate in the same medium as described earlier (CCM) containing either the standard (20%) or reduced (2%) serum, either alone or mixed with a 10-fold excess of untransduced SDBMSC cultures, normal human brain vascular pericytes (HBVP; ScienCell catalog number 304022); CD140b-PE (PDGFRβ; BD Pharmingen catalog number 558821); CD146-Alexa 647 (Abd Serotec catalog number MCA2141A647); CD31-Pacific Blue (purified antibody, from BD Biosciences catalog number 355444, conjugated using the Pacific Blue conjugation kit, Invitrogen catalog number P30012). The following unconjugated antibodies were used: endosialin (clone RB5; courtesy of Dr. Lloyd Old, Memorial Sloan Kettering Cancer Center); NG2 (clone 7.1; Chemicon International catalog number MAB5520). Flow cytometry was carried out on the CyAn ADP flow cytometer (Dako) with Summit Software.

**Interphase FISH**

Cells were seeded in 2-chamber slides (Lab-Tek II; Nunc 177380). After attachment, medium was replaced with 2 mL of PBS, and 1 mL of 75 mmol/L KCl warmed to 37°C was added dropwise in 200-µL increments, each time waiting 2 minutes, followed by incubation in 75 mmol/L of KCl for 5 minutes. Ice-cold Carnoy’s fixative (methanol:acetic acid at a 3:1 ratio) was then added to the well gradually as described earlier for KCl. Chamber was removed and the slide was stored in ice-cold methanol at –20°C. FISH was conducted according to manufacturer’s instructions, using the following probes: for synovial sarcoma, SYT break-apart probe (Abbott Molecular catalog number 05S84-006); for liposarcoma, MDM2 probe (BAC clone RP11-61102, labeled with Orange-dUTP; Vysis/Abbott Molecular catalog number 0502N33).

**Xenograft studies**

Xenograft protocols were approved by the Institutional Animal Welfare Committee. To determine tumor-forming capacity of sarcoma-derived cultures, 1 million cells were injected per flank of NOD/SCID/IL2Rγ<sup>−/−</sup> (NOG) mice, which show the highest rates of engraftment of human tumors among known mouse strains (16). The mice were monitored for tumor formation for at least 4 months.

**Flow cytometry**

Single-cell–digested tumors were resuspended in ice-cold wash buffer (5% FBS in PBS) and incubated with fluorophore-conjugated primary antibodies and DAPI (Invitrogen catalog number D1306) as a viability stain. DAPI as well as CD45 and CD31 antibodies conjugated to Pacific Blue were mixed to create a “dump channel” analyzed on the UV laser to exclude dead, hematopoietic, and endothelial cells. In addition, pairwise combinations of an APC- and FITC- or PE-conjugated antibodies were used to avoid the need for compensation and FMO controls. The following directly conjugated monoclonal antibodies were used: CD73-PE (BD Biosciences catalog number 550257); CD105-APC (Southern Biotech catalog number 9811-11); CD44-PE (BD Biosciences catalog number 555479); CD90-APC (BD Biosciences catalog number 559869); CD45-Pacific Blue (Biolegend catalog number 304022); CD140b-PE (PDGFRβ; BD Pharmingen catalog number 558821); CD146-Alexa 647 (Abd Serotec catalog number MCA2141A647); CD31-Pacific Blue (purified antibody, from BD Biosciences catalog number 355444, conjugated using the Pacific Blue conjugation kit, Invitrogen catalog number P30012). The following unconjugated antibodies were used: endosialin (clone RB5; courtesy of Dr. Lloyd Old, Memorial Sloan Kettering Cancer Center); NG2 (clone 7.1; Chemicon International catalog number MAB5520). Flow cytometry was carried out on the CyAn ADP flow cytometer (Dako) with Summit Software.

**Immunohistochemistry**

The immunofluorescence detection was conducted at the Molecular Cytology Core Facility, Memorial Sloan Kettering Cancer Center, using Discovery XT processor from Ventana Medical Systems. Five-micron sections of formalin-fixed, paraffin-embedded archived patient materials were obtained. CD146 antibody (mouse monoclonal, clone N1238; Abcam catalog number ab49492) was used at 1:10 dilution. The detection was carried out with secondary antibody blocker, Blocker D, streptavidin-HRP D (Ventana Medical Systems), followed by incubation with Tyramide-Alexa Fluor 488, 568, or 647 (Invitrogen catalog number T20922, T20914, T20916). Images were acquired on a Zeiss Axioplan 2 imaging microscope equipped with the AxiosCamMR camera, Plan Neofluar 40 x/0.75 (DIC II) objective, and AxioVision v 4.6 software.
Matrigel tube formation assay
Wells of a precooled 24-well plate were coated with Matrigel (BD catalog number 356234) mixed in a 1:1 ratio with ice-cold endothelial growth medium 2 (Lonza catalog number CC-3162) and allowed to solidify for 45 minutes at 37°C (17). GFP-transduced human bone-marrow-derived endothelial cells immortalized with hTERT (18) and SDBMSC cultures labeled with Dil (Invitrogen catalog number V-22885) were plated onto the surface of the Matrigel, either individually or in combination, ensuring that the total number of cells remained the same. Serial immunofluorescent images were taken over several hours. For live nuclear staining, 50 μg/mL of Hoechst 33342 dye (Invitrogen catalog number H1399) was added.

Results

MSC properties of sarcoma cell lines
Because MSC is the proposed cell of origin of sarcoma, we reasoned that sarcoma cell lines might have properties of MSCs. Most cell lines we examined showed bone differentiation but lacked fat differentiation potential (data not shown). However, a liposarcoma cell line DDLS8817 (19) showed fat, bone, and cartilage trilineage differentiation potential characteristic of MSCs and expressed MSC surface markers, CD73, CD105, CD44, and CD90 [Supplementary data, Fig. S1]. When injected into mice, DDLS8817 cells formed tumors indistinguishable from the dedifferentiated component of dedifferentiated liposarcoma (data not shown). Thus, we describe for the first time a malignant sarcoma cell line with MSC properties. However, other sarcoma cell lines we tested did not show multilineage differentiation potential characteristic of MSCs.

Isolation of MSCs from sarcoma surgical specimens
We next turned to the analysis of surgical samples from sarcoma patients. Because MSC markers are defined by flow cytometry and not by immunohistochemistry (IHC), we digested sarcoma surgical samples into a single-cell suspension by using collagenase treatment, which preserves most surface epitopes (data not shown). We then carried out flow cytometry analysis on these samples by using MSC markers. We found [Supplementary data, Table S1] that MSC markers CD73, CD105, CD90, and CD44 were expressed in a fraction of tumor cells averaging about 50% across a variety of sarcoma types.

We then attempted to isolate MSCs from human sarcoma specimens by using the protocol used for the isolation of bone marrow MSCs, relying on selective plastic adherence of MSCs in the presence of serum (14). Sarcoma surgical samples were digested with collagenase in MSC-qualified, serum-containing medium in ultralow-adhesion plates to prevent premature attachment of cells. After digestion, the cells were grown in MSC medium in tissue culture–treated plastic dishes. For most tumors, after several days, adherent cells were visible, either as individual colonies or as a subconfluent layer of cells. The cells had spindle-shaped morphology, forming sheets in a storiform arrangement recognizable by the naked eye as repeated patterns approximately 2 to 4 mm in size. Some cells remained in suspension and were passaged into fresh dishes; these cells did not proliferate and eventually died. Adherent cultures were expanded and characterized. We noted that cultures derived from various types of sarcoma often had identical morphology at high density (Fig. 1A), remarkably similar to that of MSCs (20). Table 1 summarizes the cultures derived from a consecutive cohort of sarcoma samples. Most sarcoma-derived cultures could be passaged for more than 10 passages, maintaining a doubling time of several days. Most cultures showed differentiation into fat and bone when grown in the appropriate medium; cartilage differentiation was also shown for the 3 cultures tested (Table 1, Fig. 1B). Most cultures that were evaluated by flow cytometry expressed MSC markers in the majority of cells and lacked hematopoietic (CD45) and endothelial (CD31, CD34) markers (Table 1).

Sarcoma-derived MSCs are frequently benign
We then assessed whether the cultures were malignant as evidenced both by the presence of characteristic genetic lesions and by the tumor-forming ability in immunodeficient mice. Translocations involving SYT are present in more than 95% of synovial sarcomas (21). Similarly, MDM2 amplification has recently been shown to be a diagnostic marker of liposarcoma (22). Unexpectedly, 3 of 4 synovial sarcoma cultures were negative for the translocation (Table 1), even though the original tumor
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<td>CS, high grade</td>
<td>No</td>
<td>&gt;10</td>
<td>5–7 d</td>
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Note: Preop Tx, preoperative therapy (chemotherapy, XRT, none); Pass in vitro, number of passages in culture; Fat, Bone, Cart diff, ability to differentiate into fat, bone, and cartilage in appropriate differentiation medium; Tumors in NOG, tumor formation in NOG mice ("No" indicates no tumor formation by at least a million cells in at least 3 months). FISH+, percentage of positive cells for SYT-SSX translocation (for synovial sarcoma) or for MDM2 amplification (for liposarcoma). Conclusion is based on FISH and/or tumor formation ability. Flow cytometry data indicates % of positive cells. Osteo, osteosarcoma; MPNST, malignant peripheral nerve sheath tumor; LMS, leiomyosarcoma; lipo, liposarcoma; WD, well-differentiated; DD, dedifferentiated; CS, chondrosarcoma. Blank cell, not done.
been proposed as the component of the tumor microenvironment, not the malignant tumors. We termed these cultures SDBMSCs derived malignant MSC cultures, as evidenced both by the lack of tumor formation in immunodeficient mice. One FISH-positive culture (904) grew very slowly and could not be expanded and thus could not be tested for fat and bone differentiation.

In liposarcoma, we isolated 1 culture (348) that was benign by FISH (lacked MDM2 amplification), 1 (351) that was malignant (100% positive for MDM2 amplification), and 1 that was mixed (10% of cells positive). Tumor formation assays confirmed these findings. Sarcomas other than liposarcoma and synovial sarcoma in Table 1 are less amenable to interphase FISH because of complex karyotypes. On the basis of tumor formation in mice, we tentatively classified 1 osteosarcoma culture (298) and 1 MFH culture (458) as benign and 1 MFH culture as malignant (344).

We thus describe several benign and several sarcoma-derived malignant MSC cultures, as evidenced both by the presence or absence of genetic changes found in the original tumor and by tumor-forming ability in mice.

"Benign" here is not meant as "premalignant" but rather as synonymous with "stromal," that is, a cellular component of the tumor microenvironment, not the malignant cells within the tumor. We termed these cultures SDBMSCs and focused on understanding their origin and their role within the tumor.

Pericyte features of SDBMSCs

CD146-positive microvascular pericytes have recently been proposed as the in vivo identity of MSCs in normal tissues (13). We therefore hypothesized that SDBMSCs may be of pericyte origin. At low density, the cells had cytoplasmic projections characteristic of pericytes (23). Indeed, we found that each of the SDBMSC cultures we examined expressed several markers of normal pericytes (CD146, NG2, PDGFRβ) and tumor pericytes (endosialin; ref. 24; Fig. 2A; Supplementary Table S2). Another feature of pericytes is the ability to cooperate with endothelial cells in Matrigel tube formation assays. We used immortalized bone marrow endothelial cells (BMECs; ref. 18), which alone do not form tubes in Matrigel (Fig. 2C). However, when mixed with an SDBMSC culture (197), dramatic tube formation was seen, composed of both endothelial cells and SDBMSCs (Fig. 2C). Thus, SDBMSCs show the morphology, surface marker expression, and functional properties of pericytes in vitro.

Immunohistochemical detection of pericytes in formalin-fixed sarcoma tissue

Because we isolated benign pericyte-like cells from sarcoma, we sought to examine by IHC whether pericytes can be identified in formalin fixed, paraffin-embedded, archived surgical specimens. In carcinoma, pericyte coverage of microvessels is thought to be abnormal (25). The existence or the nature of pericytes in human sarcoma has never been investigated except for a recent study in chondrosarcoma, using smooth muscle actin (SMA) as a marker (26). However, SMA is not ideal for pericyte detection, as capillary pericytes are SMA negative whereas stromal myofibroblasts are SMA positive. We instead turned to CD146, which was previously thought to be a purely endothelial marker and found to be expressed in the vasculature in synovial sarcoma, MFH, and liposarcoma (27). However, in recent studies conducted on normal tissues, CD146 was shown to be expressed by microvascular pericytes in addition to endothelial cells (13, 28). The clone of the CD146 antibody used in these studies (P1H12) does not recognize CD146 in formalin fixed tissue, which hampers the analysis of archived tumor material. We evaluated a different clone of the CD146 antibody (N1238) that works on formalin fixed tissue (29). To validate this antibody, we first showed expression in microvascular pericytes in normal tissues (Fig. 3A). We used CD31 as an endothelial marker and not CD34, which was used by Crisan et al., as some sarcomas are CD34 positive, which would complicate the analysis, and since CD31 is a more sensitive marker of tumor endothelium than CD34 (30). We then used this CD146–CD31 combination to carry out staining in sarcoma formalin fixed archived material. We found high numbers of CD146+ pericytes, which surround CD31-positive endothelial cells both in myxofibrosarcoma and in synovial sarcoma (Fig. 3B). Thus, our findings show that CD146 is a pericyte as well as endothelial marker in sarcoma, in contrast to a prior report describing it as an endothelial restricted marker (27). These benign CD146-positive pericytes may be the source of SDBMSCs that we have isolated from surgical sarcoma specimens.
Expression of CD105, an MSC marker, is limited to sarcoma vasculature

MSC marker expression has never been investigated in sarcoma by IHC. We examined the expression of one MSC marker, CD105, to confirm our hypothesis that benign MSCs are associated with the vasculature in sarcoma. Crisan et al. showed the expression of MSC markers, including CD105, in tissue microvascular pericytes. We carried out CD105 IHC on a set of sarcoma archived tissue that corresponds to samples we immunophenotyped after single-cell digestion (Supplementary data, Table S1). We found that CD105 expression is limited to tumor vasculature in most tumors we examined (Fig. 4A; Table 2). Triple immunofluorescence using CD31, CD105, and CD146 identified CD105+CD146+CD31− pericytes in osteosarcoma sample (Fig. 4B). Of note, CD105 expression was not detected in surrounding normal tissues, showing that CD105 is a tumor-specific vascular marker in sarcoma as was previously observed in carcinoma (31).

Thus, we show for the first time that CD105 is expressed in sarcoma vasculature. However, it is not expressed in malignant cells in most sarcomas we examined, consistent with the perivascular location of MSCs in sarcoma.

SDBMSCs stimulate the growth of sarcoma cell lines in an in vitro coculture model

To evaluate the effect of SDBMSCs on the growth of malignant sarcoma cells, we performed a coculture experiment. We transduced 2 sarcoma cell lines, U2OS and A673, with red fluorescent protein–encoding lentivirus to generate RFP+ SDBMSCs. We then cocultured RFP+ SDBMSCs with sarcoma cell lines in 24-well plates. After 5 days of coculture, we evaluated the proliferation of SAR/GFP+ sarcoma cell lines using fluorescent microscopy. We found that RFP+ SDBMSCs stimulated the growth of sarcoma cell lines in an in vitro coculture model (Fig. 4C). This suggests that SDBMSCs may play a role in the progression of sarcoma by stimulating the growth of sarcoma cell lines.

Table 2. Summary of CD105 staining in sarcoma

<table>
<thead>
<tr>
<th>Histology</th>
<th>CD105 (tumor vessels)</th>
<th>CD105 (malignant tumor cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondrosarcoma</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Liposarcoma</td>
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<td>−</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MFH</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>MFH</td>
<td>+</td>
<td>(rare)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Myxofibrosarcoma</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Synovial sarcoma</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>Synovial sarcoma</td>
<td>+++</td>
<td>−</td>
</tr>
</tbody>
</table>

*Positive perivascular cells in addition to endothelial cells are clearly seen.
*Surrounding normal muscle negative.
*Surrounding normal liver negative.
*Cytoplasmic background staining.
*Surrounding normal lung negative.
and mixed them with a 10-fold excess of unlabelled SDBMSC cultures in low-serum medium. After 7 days of coculture, we observed a marked increase in sarcoma cell number when grown in the presence of SDBMSCs as compared with sarcoma cells grown alone (Fig. 5A). Each SDBMSC culture, regardless of the sarcoma of origin, showed a stimulatory effect on both U2OS (an osteosarcoma cell line) and A673 (an Ewing sarcoma cell line) (Fig. 5B). The increased sarcoma cell number was also observed in coculture with normal HBVPs but not with immortalized human bronchial epithelium (Beas2B), showing that the stimulatory effect of the stroma was not simply due to increased total cell number (Fig. 5A and B). In the presence of SDBMSCs, sarcoma cells were seen embedded within the stromal cells and following along the whorl-like growth pattern of the stromal cells (Fig. 5A). In contrast, in the presence of Beas2B human epithelial cells, sarcoma cells remained in colony-like foci similar to sarcoma cells grown alone. Compared with the starting cell numbers, a significant increase in cell number was seen in the presence of SDBMSCs, and apoptotic cells were not observed when sarcoma cells were cultured alone (Fig. 5A), suggesting that the stimulatory effect of the stroma is due to an increase in proliferation rather than protection from apoptosis. Of note, the stimulatory effect of the stromal cells was not seen when coculture was done in 20% serum (data not shown), suggesting that the stromal cells may secrete a factor that is present in the serum and which is required for proliferation of sarcoma cells.

**Discussion**

Spindle cell overgrowth in tumor cultures has long been appreciated in carcinoma, in which these cells are thought
endothelial cells and pericytes, are quickly replaced by a microenvironment where sarcoma cells clearly outgrow the cells we isolate. Our culture conditions do not adequately mimic the tissue microenvironment where sarcoma cells clearly outgrow the cells we isolate. This has been proposed as an explanation for the paucity of sarcoma cell lines.

We now show that sarcoma-derived cultures have properties of MSCs, namely, surface marker expression, morphology, and in vitro differentiation potential. The finding of benign MSCs in sarcoma was unexpected. Despite the accumulating evidence for MSC origin of sarcoma, there are no published reports describing the expression of MSC markers in sarcoma or the isolation of MSCs from sarcoma cell lines or primary sarcoma samples. Adherent cells from single-cell–digested liposarcoma surgical specimens have been shown to differentiate along the adipocyte lineage with the addition of a PPARγ agonist (36, 37), but bone and cartilage differentiation potential or MSC marker expression was not investigated in these cultures. In retrospect, some of these cultures may have been benign SDBMSCs we describe in the current study.

In fact, even in carcinoma, in which tumor-associated fibroblasts are well studied (38, 39), their relationship with MSCs remains unclear. Heterologous bone marrow–derived MSCs have been shown to stimulate tumor metastasis (40) and home to tumors in mice when injected intravenously (41). However, fibroblasts isolated from human carcinoma specimens have never been shown to have MSC properties such as differentiation into mesenchymal lineages and surface marker expression.

Brune et al. recently reported in abstract form the isolation of MSCs from osteosarcoma by using a similar approach to ours. They also find that osteosarcoma-derived fibroblasts are cytogenetically normal and therefore likely benign (42). The authors conclude that the benign MSCs likely represent “tumor stroma.” Another abstract reports isolation of MSCs from glioma (43). To our knowledge, these are the only studies reporting the isolation of MSCs from human tumors of any histology.

Suva et al. used a cancer stem cell marker, CD133, to isolate a tumor-initiating fraction in Ewing sarcoma. CD133+ cells showed MSC-like differentiation and over-expressed FLI1, indicative of the presence of the EWS–FLI1 translocation (44). We have not analyzed any Ewing sarcoma samples in the current study. However, in a flow cytometric analysis of more than 30 single-cell–digested surgical sarcoma samples of various histologies, we saw no CD133-expressing cells when CD45+ and CD31+ cells are excluded (data not shown).

Selective survival of benign MSCs in vitro suggests that our culture conditions do not adequately mimic the tissue microenvironment where sarcoma cells clearly outgrow benign cells. In fact, human stromal cells, including endothelial cells and pericytes, are quickly replaced by mouse vasculature in tumor xenografts, suggesting that in the mouse environment, human malignant cells survive whereas human benign cells do not (45).

The presence of benign MSCs within sarcomas underscores the need to analyze primary tumor cultures for the presence of benign cells. In studies attempting to identify the cancer stem cell fractions in sarcoma (46), it is important to ensure that tumor-initiating ability of individual tumor fractions is normalized to the numbers of malignant cells by using a technique such as interphase FISH. Similarly, expression profiling on whole tumor preparations may detect the MSC signature of benign cells.

The tumor microenvironment is increasingly recognized as a novel therapeutic target (47). Tumor stroma is broadly defined to include tumor microvasculature (endothelial cells and pericytes), tumor-associated fibroblasts, and tumor-infiltrating hematopoietic cells (38, 48). In sarcoma, benign cells include hematopoietic cells, endothelial cells, and fibroblasts (recognized by their lack of nuclear atypia), and pericytes. We find that SDBMSCs have properties of pericytes, such as the expression of pericyte markers and cooperation with endothelial cells in forming capillaries. Given the recent finding that normal tissue pericytes have MSC properties, we hypothesize that in sarcoma, benign MSCs also reside in a perivascular location. Indeed, we show that one MSC marker, CD105, is expressed by endothelial cells and pericytes in a variety of sarcomas but not in malignant cells.

Some surgical sarcoma samples we analyzed, such as 334, 904, 355, 394, did not yield any MSC cultures (Table 1). One of them (355) has been irradiated. Because of a small number of samples, we are unable to make correlations between the yield of MSC cultures or their benign versus malignant nature and clinical characteristics of the tumor.

Two roles of pericytes in sarcoma can be envisioned. First, as the cells in direct contact with malignant sarcoma cells, pericytes may constitute a previously unrecognized stromal supporting element. In accordance with this model, we show that under low-serum conditions, SDBMSCs as well as pericytes from healthy donors markedly stimulate the growth of sarcoma cell lines. In other malignancies such as leukemia, stromal cocultures are well established and have been used to study leukemia–stroma interactions. To our knowledge, this is the first demonstration of such a stromal coculture model in sarcoma. Such a coculture system may represent a more physiologically relevant model for evaluating therapeutic agents in vitro than sarcoma cell lines grown alone in high serum concentration. Some agents may exhibit activity in sarcoma not by targeting the malignant cells but by abrogating the supporting influence of the pericytes. Such agents may be identified through a high-throughput screen by using our coculture model; this effort is currently underway in our laboratory. Potential therapeutic strategies to target pericytes in sarcoma may involve antibodies against CD105 (31, 49), which we show to be largely restricted
to sarcoma vasculature, and endosialin, which is expressed on tumor pericytes and malignant cells (24).

Second, malignant sarcoma cells share features of pericytes, such as differentiation along mesenchymal lineages and expression of pericyte markers such as NG2 (50) and endosialin (24), pointing to the pericyte as a candidate cell of origin of sarcoma. Experiments to address this model are under way in our laboratory.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing financial interests.

References


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