CGA-7 and HHF, Two Monoclonal Antibodies That Recognize Muscle Actin and React With Adherent Cells in Human Long-Term Bone Marrow Cultures

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The CGA-7, a monoclonal antibody that reacts with smooth muscle cell actin but not with endothelial cell or fibroblast actin, and HHF, a monoclonal antibody that reacts with smooth muscle, skeletal muscle, and cardiac muscle actin, both recognize microfilaments present within adherent cells from actively hematopoietic human long-term marrow cultures. Macrophages, monocytes, and cultured marrow fibroblasts do not react with either antibody. These data suggest that the anti-actin antibodies may serve as useful markers for in vitro microenvironmental cells and lend support to the hypothesis that stromal cells from long-term marrow cultures are different from marrow fibroblasts and may constitute a unique cell lineage.

HUMAN LONG-TERM marrow cultures are dependent on the establishment of a layer of adherent cells that have been described as endothelial-like, fibloblastoid, and epithelioid on the basis of their microscopic appearance.[1] Several features of these adherent cells suggest their potential uniqueness. Recent data indicate that at least some stromal cells are donor in origin after allogeneic marrow transplantation and thus, like other hematopoietic cells, may constitute a transplantable population.[2] In addition, studies on the origin of adherent cells in long-term marrow cultures from four glucose-6-phosphate dehydrogenase heterozygous patients with leukemias originating in multipotent stem cells indicate the potential derivation of these adherent cells from hematopoietic stem cells.[3] These studies suggest that the predominant cell population in the adherent cell layers from long-term marrow cultures is dissimilar from cultured, passaged marrow fibroblasts, since fibroblasts are not transplanted after allogeneic marrow transplantation[4,5] and are derived from nonclonal progenitors in neoplastic diseases originating in multipotent stem cells.[6-8] Furthermore, unlike the adherent cells from long-term marrow cultures, fibroblasts are unable to support hematopoietic stem cell proliferation.[9] Recent data also indicate that long-term marrow culture adherent cells differ in biosynthetic properties from fibroblasts. Specifically, long-term marrow culture adherent cells synthesize type IV collagen as well as types I and III, which are characteristic of marrow fibroblasts, and exhibit a qualitatively more complex and a quantitatively greater proteoglycan biosynthesis profile than do marrow fibroblasts (reviewed in reference 10).

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Because the preceding data suggested that the adherent cells in long-term marrow cultures may constitute a different lineage than marrow fibroblasts, we examined adherent cells from long-term marrow cultures and cultured marrow fibroblasts with a series of monoclonal antibodies directed against cytoskeletal proteins. These antibodies have been used to characterize other mesenchymal cell populations, such as smooth muscle cells, endothelial cells, and non-marrow fibroblasts.[11] The present data indicate that two of the anti-cytoskeletal protein monoclonal antibodies, the CGA-7 and the HHF, react strongly with microfilaments in most of the spindle-shaped and flat angulated cells from long-term marrow cultures but do not react with cultured marrow fibroblasts. The CGA-7, an IgG antibody developed after immunization with chicken gizzard actin, reacts only with human smooth muscle and with myoepithelial cell actin but not with cultured human endothelial cells or fibroblasts.[12] HHF is a monoclonal antibody raised against cardiac muscle actin that reacts with actin filaments in skeletal, cardiac, and smooth muscle cells but, like the CGA-7, does not react with endothelial cells or fibroblasts. Neither antibody reacts with hematopoietic cells.[11,12] These antibodies may therefore be useful in the identification of the major cellular component of the adherent cell populations of long-term marrow cultures.

MATERIALS AND METHODS

Marrow samples were obtained from normal individuals at the time of marrow donation for transplantation under protocols approved by the human investigations committee of the Fred Hutchinson Cancer Research Center.

Non-Passaged Adherent Cells From Long-term Marrow Cultures

Long-term bone marrow cultures were established by a modification of the method of Gartner and Kaplan[13] as reported previously.[2] Marrow buffy coat cells were placed in chamber slides with 1-cm^2 wells (Miles Laboratories, Naperville, Ill) at a cell concentration of 2×10^6/mL and incubated at 33 °C in a 5% CO_2 incubator. One half of the supernatant cells were removed weekly when the cultures were fed and plated for committed granulocytic progenitor (CFU-GM) growth. Approximately 90% of normal marrows cultured by this method produce CFU-GM for more than ten weeks.

Passaged Adherent Cells From Long-term Cultures

Adherent cells from long-term marrow cultures were passaged weekly after achieving confluence after mechanical disruption with a rubber policeman. The culture medium used was the same as that for long-term marrow cultures. Adherent cells were tested after the fifth

and tenth passages for expression of microfilaments reactive with the anti-actin antibodies.

Passaged Marrow Fibroblasts

Fibroblast cultures were initiated with normal marrow buffy coat cells cultured at 2×10^6/mL in Waymouth's medium and 10% fetal calf serum. This medium is less complete than the McCoy's medium used for long-term cultures and was not supplemented with horse serum or hydrocortisone. The cultures were split after trypsinization when they reached confluence, generally in ten to 20 days. Only fibroblasts beyond fourth passage were used in the present study. Previous data indicates that these cells are incapable of supporting long-term granulopoiesis[9] and do not synthesize type IV collagen characteristic of stromal cells from long-term marrow cultures.[10]

Immunofluorescence Studies

CGA-7 was prepared by fusing spleen cells from BALB/c mice immunized against partially purified chicken gizzard actin with NS-1 myeloma cells.[12] Hybrid cells producing supernatants reacting with rat intestine muscularis were subjected to a two-step cloning procedure as previously described.[12] HHF was made using purified human cardiac muscle actin as the immunogen. The ascites fluids finally obtained were used at a 1:50 dilution for this study. The CGA-7 is an IgG antibody that recognizes the actin of smooth muscle cells.[12] The HHF is an IgG antibody that reacts with actin present in cardiac, smooth, and skeletal muscle cells. It does not react with endothelial cells or fibroblasts (A.M.G., unpublished observations, October 1984)

Each week, two long-term marrow cultures were sacrificed. The supernatant cells were tested for the presence of CFU-GM and the adherent cells were washed twice with phosphate-buffered saline (PBS) and fixed in absolute methanol (five minutes at −20 °C). The 1:50 dilution of the antibodies (0.1 mL) or PBS alone were added to the wells and incubated for 30 minutes at room temperature. After washing twice, a fluorescein-conjugated goat antimouse IgG $F(ab')_2$ (Cappel Laboratories, Malvern, Pa) diluted 1:150 in PBS was added for 30 minutes at room temperature. After washing twice, the upper plastic parts of the wells were removed. The glass slides were moistened with PBS and covered with a glass coverslip.

In addition to use of the second antibody alone, negative controls included irrelevant IgG monoclonal antibodies that react with either epidermal cell membranes (donated by Dr T. Huang, University of Washington, Seattle) or cytokeratin, 34BE12.[11] In addition, non-fixed cells and cells fixed in methanol after treatment with the CGA-7 were examined. A positive control was included in each experiment and consisted of the 43BE8 antibody,[11] which is an IgM monoclonal antibody that reacts with vimentin present in both fibroblasts and adherent cells from long-term marrow cultures. The slides were examined using a Leitz Labor 11 microscope equipped for epifluorescence with appropriate excitation and emission filters. The magnification of the objective was 50 and its numerical aperture 1.00.

In some experiments, chondroitinase ABC (Sigma Chemical Co, St Louis) (2.5 U/mL in 0.2 mol/L Tris buffer, pH 7.4) was added to the wells before the use of CGA-7 to remove the dense layer of proteoglycans covering the adherent cells.[9] The wells were incubated with the enzyme for 30 minutes at 37 °C. Wells incubated with buffer alone served as controls.

Cell block studies. Marrow stromal cells from long-term cultures, passaged marrow stromal cells, and passaged marrow fibroblasts were cultured to subconfluence. Cells were removed from the flasks using 230 U/mL of collagenase I (Sigma) in Hanks' saline solution and resuspended in Carnoy's fixative (60% methanol, 30% chloroform, and 10% glacial acetic acid). After four hours' fixation, the tissues were placed sequentially in 100% methanol, xylene, and molten paraffin, the latter at 56 °C. Standard 5- to 6-μm paraffin sections were cut, and the immunoperoxidase procedure was performed as previously described with a primary antibody dilution (as ascites fluid) of 1:1,000.[12]

Expression of the results. In most experiments, the cells expressing material reacting with the antibodies appeared to be syncytial, and it was not possible to determine the precise number of positive and negative cells. Therefore, we used a grid that delineated areas of 0.0936 mm^2. The percentage of grids with positive cells was determined by counting at least 40 such fields. A field was considered to be positive only if cells contained brightly fluorescent cytoplasmic fibrillar material.

One-dimensional "Western Blot" experiments. Confluent cultures of stromal cells (approximately 1×10^6 cells) were washed in PBS, sonicated and solubilized in 2% sodium dodecyl sulfate (SDS), 100 mmol/L Tris (pH 6.8) and together with control material (rat colon muscularis or human uterus) were run on an 8% polyacrylamide gel and transferred overnight to nitrocellulose paper.[11] The paper was incubated with a 1:50 diluted CGA-7 or HHF ascites fluid, and binding was revealed with a 1:200 diluted glucose oxidase-conjugated goat antimouse IgG (Accurate Chemical and Scientific Corp).

RESULTS

Flat angulated or spindle-shaped adherent cells appeared by the fourth day in the long-term marrow culture. Their number steadily increased with time. At one week, most of these cells were isolated. By two weeks, they began to show a more confluent pattern. By four weeks, most cultures had confluent adherent layers with fat-containing cells, macrophage-like cells with ingested debris, and flat angulated and spindle-shaped cells. Hematopoietic islands were apparent after three weeks overlying confluent areas of adherent cells and had a "cobblestone" appearance. When adherent cells from the long-term marrow cultures were serially passaged, they retained the morphological appearance of the parent cultures. However, macrophages and hemopoietic cells were not observed after the third passage.

Only the flat angulated and the spindle-shaped cells showed specific positive fluorescence with either the CGA-7 or the HHF monoclonal antibodies (Fig 1). Both antibodies reacted with thin filamentous structures within the cytoplasm of these cells that had the appearance of stress fibers (Fig 1). Passaged marrow stromal cells also contained anti-actin antibody-positive fibers in most cells. The smaller, round hematopoietic cells present within the adherent layer did not react with either anti-actin antibody.

The generation of CGA-7–positive cells in long-term cultures over time is shown in Table 1. The frequency of microscope fields containing CGA-7–positive cells increased with time, and by four weeks, 69% ± 5% of the fields examined reacted with this antibody. The generation of HHF-positive fibers over time in long-term culture was similar to that obtained with the CGA-7 (data not shown).

Because it was possible that the dense layer of proteoglycans coating the adherent cell layers in long-term cultures[10] may have inhibited antibody access to the cells, we examined the effect of treating cultures with chondroitinase ABC before immunofluorescent staining. This enzyme is capable of degrading both chondroitin sulfate and dermatan sul-

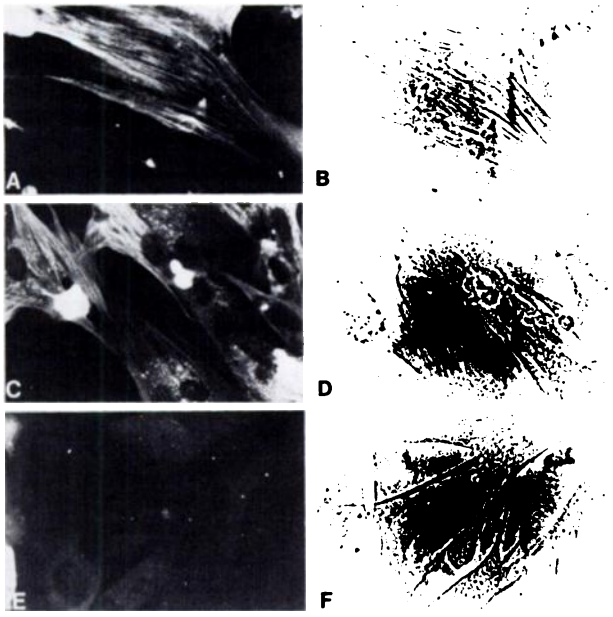

Fig 1. Microfilaments as revealed by the CGA-7 and HHF monoclonal antibodies. Adherent cells from long-term cultures contain fibrillar material that react with the CGA-7 (A) or HHF (C) antibodies. Fourth-passaged marrow fibroblasts are negative with either antibody. The CGA-7 is shown (E). B, D, and F are the corresponding phase-contrast photomicrographs.

Table 1. Generation of CGA-7–Positive Adherent Cells in Long-term Marrow Cultures: The Effect of Culture Age

Age of Culture (wk)	No. of Experiments	Degree of Confluence	CGA-7-Positive Fields (%)	
			Range	Mean ± SEM
2	7	1 to 2	10-90	42 ± 12
3	11	1 to 3	0-98	59 ± 10
5	11	2 to 3	43-90	69 ± 5

The degree of confluence of adherent cells was graded on a scale of 1 to 3: (1) isolated adherent cells only; (2) some contact between adherent cells is present; (3) adherent cells constitute a syncitium. At least 40 0.0936 mm^2 microscope fields were evaluated for each specimen. A field was considered positive when one or more bright stress fiber was observed within an adherent cell.

phate, the major proteoglycans produced by long-term human marrow cultures.[10] Two experiments were performed on separate cultures analyzing the frequency of CGA-7–positive fields before and after treatment with chondroitinase ABC. Treatment of three-week-old long-term marrow cultures with this enzyme released many of the round cells and significantly increased the frequency of CGA-7–positive fields from mean values of 39% and 45% before enzyme treatment to values of 74% and 81% after enzyme treatment ($P < .001$).

When marrow stromal cells were serially passaged and examined after the fifth and tenth passages for expression of CGA-7–positive microfilaments, they were found to be present in approximately the same frequency as in non-passaged stromal cells using both peroxidase cell block and immuno-

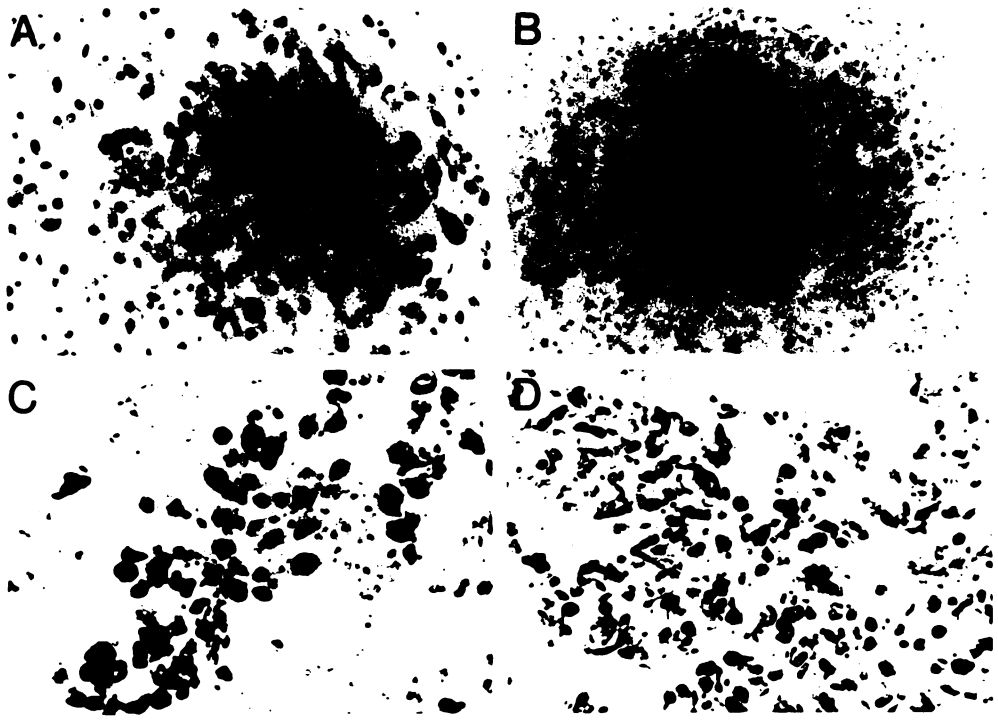

Fig 2. Immunoperoxidase staining of paraffin-embedded sections of cell blocks of marrow stromal cells. (A) Hematoxylin-eosin stain. (B) Negative control with antibody 34BE12 (anti-cytokeratin). (C) CGA-7 (anti-smooth muscle actin). (D) Positive control with antibody 43BE8 (antivimentin).

fluorescent analyses. In contrast, when marrow fibroblasts passaged four or more times were examined with CGA-7 or with HHF, less than 1% of microscope fields contained positive material (Fig 1). Treating marrow fibroblast cultures with chondroitinase ABC did not increase the frequency of CGA-7–positive cells. With the antivimentin antibody (43BE8), which was used as a positive control, the mean percentage of positive microscope fields (± SEM) was 82 ± 3 (N = 9) for marrow fibroblasts and 76 ± 4 (N = 20) for long-term marrow culture stromal cells.

In the immunoperoxidase studies on fixed, embedded sections of cell block material (Fig 2), while both marrow stromal cells and fibroblasts were uniformly positive with the antivimentin antibody 43BE8, only the marrow stromal cells had a strongly positive subset of cells with antibodies CGA-7 or HHF. The frequency of positive cells ranged from 25% to 70%, depending on the area of the block examined.

In "Western blot" experiments, CGA-7 detects a 43-kd protein in preparations of purified chicken gizzard actin, extracts of human uterus, and extracts of monkey ileum but does not blot to any band on extracts of marrow stromal cells (data not shown). HHF recognizes the 43-kd actin band in SDS-polyacrylamide gel electrophoresis separations of proteins from marrow stromal cells as well as from human uterus (Fig 3). HHF does not react with marrow fibroblast actin (data not shown).

DISCUSSION

The light and electron microscopic appearance of the adherent cell layer from human long-term bone marrow cultures has been reviewed elsewhere.[1,10] The layers are composed of layers of flat angulated cells, fat-containing cells and occasional spindle cells surrounded by an extracellular matrix consisting of collagen types I, III, IV, and V, fibronectin, laminin, and large amounts of proteoglycans. Although the adherent cell layer is essential for maintenance of hematopoiesis, the hematopoietic role of the cellular and extracellular components has not yet been adequately defined. Even the derivation of the cellular elements is currently unresolved. Due to the complexity of the system and the lack of suitable markers useful in identifying cells within the adherent layer, it has been difficult to dissect out the individual elements of the system.

The present data suggest that the CGA-7, a monoclonal antibody previously thought to react only with actin in smooth muscle and myoepithelial cells, and the HHF, an antibody reactive with actin present in all types of muscle cells, may prove useful in analyzing "microenvironmental" cell populations. Expression of the actin filament recognized by these antibodies can be noted in isolated spindle-shaped or flat angulated adherent cells within one week of culture inception. By three to four weeks, when the adherent cell layer is nearly confluent, most angulated and spindle-shaped adherent cells manifest thin stress filaments that react with the anti-actin antibodies. The number of positive filaments per cell, as well as the frequency of positive cells, appears to increase with time in culture.

Extracellular proteoglycans appear to impede antibody access to the adherent cells. Therefore, when we treated the adherent cell layers with chondroitinase ABC to remove the dense layer of proteoglycans covering these cells and allow better antibody access, we significantly increased the proportion of CGA-7–positive cells.

The presence of muscle-type actin in adherent cells from long-term cultures provides further data for the uniqueness

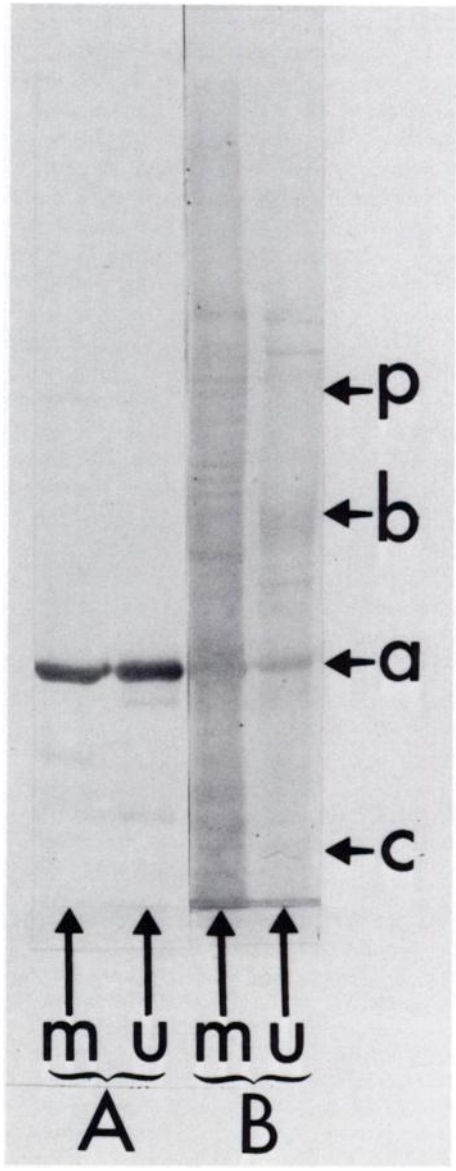

Fig 3. Western blots with HHF. (A) Glucose oxidase-conjugated goat antimouse antibody reaction with marrow stromal cells (m) or human uterus (u) after exposure to HHF. (B) Amido-black stain with marrow stromal cells (m) or human uterus (u). An equivalent amount of actin solubilized in 2% SDS in the presence of 2-mercaptoethanol was applied to 8% polyacrylamide gels for both tissues although the amount of marrow stromal cell protein was approximately 20 times that of uterus. The locations of the marker proteins (c: carbonic anhydrase, 29 kd; a: skeletal actin, 43 kd; b: bovine serum albumin, 68 kd; and p: phosphorylase, 97.5 kd) are shown on the right.

of this cell population. Previous studies demonstrated that proteoglycan biosynthesis by adherent cell layers from long-term marrow cultures have greater similarities to the proteoglycans synthesized by cultured smooth muscle cells than to those synthesized by either marrow fibroblasts or human umbilical vein endothelial cells.[10] In this regard, since cultured marrow fibroblasts do not express the antigens recognized by either the CGA-7 or the HHF and the majority of the cells with a "fibroblastic" appearance in the long-term marrow cultures were actin positive, the present study provides additional data regarding the low frequency of true fibroblasts within these adherent cell layers. Nevertheless, it is certainly probable that cells of several lineages that do not express the actin-like antigen, such as fibroblasts and endothelial cells, are present within the adherent layers of long-term marrow cultures and that their frequency can be increased by altering culture conditions. For example, if the horse serum and hydrocortisone are removed from the long-term marrow culture medium, the adherent cell layers become predominantly fibroblastic and within two weeks no longer contain cells that express CGA-7–positive fibers (data not shown). Under conditions in which the cultures are actively hematopoietic, the CGA-7–negative cells, which are presumably of fibroblast origin, constitute a minority population within the adherent layers.

We have not determined why CGA-7 fails to detect in marrow stromal cells the 43-kd band found in smooth muscle cells. In other studies,[12] antibody CGA-7 always reacted weakly on immunoblots compared with a rabbit polyclonal antibody to actin, which did not distinguish between various isoforms. Reactivity may depend on tertiary or quarternary structures, which are only partially recovered in proteins transferred out of SDS onto nitrocellulose paper. However, a second monoclonal antibody that recognizes the actin expressed in cardiac and skeletal muscle as well as smooth muscle, HHF, recognized a 43-kd band in marrow stromal cell digests. Because with immunofluorescence the morphological appearance of the microfilaments in stromal cells recognized by the two antibodies appears identical, it is highly probable that both are reacting with different epitopes of the same actin molecule.

A potential use for monoclonal antibodies such as the CGA-7 and the HHF, which react with the cells that provide the "microenvironment" in long-term cultures, is to examine multipotential hematopoietic colonies for the presence of positive cells. If it can be demonstrated that multipotent clonal colonies contain cells that express muscle actin, then such data would provide additional evidence for the hematopoietic derivation of microenvironmental cells.

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