Human Pluripotent and Progenitor Cells Display Cell Surface Cluster Differentiation Markers CD10, CD13, CD56, and MHC Class-I (44385)

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Abstract. **Each year millions of people suffer tissue loss or end-stage organ failure. While allogeneic therapies have saved and improved countless lives, they remain imperfect solutions. These therapies are limited by critical donor shortages, long-term morbidity, and mortality. A wide variety of transplants, congenital malformations, elective surgeries, and genetic disorders have the potential for treatment with autologous stem cells as a source of HLA-matched donor tissue. Our current research is aimed at characterizing cell surface cluster differentiation (CD) markers on human progenitor and pluripotent cells to aid in isolating comparatively purified populations of these cells. This study examined human pluripotent and progenitor cells isolated from fetal, mature, and geriatric individuals for the possible presence of 15 CD markers. The response to insulin and dexamethasone revealed that the cell isolates were composed of lineage-committed progenitor cells and lineage-uncommitted pluripotent cells. Flow cytometry showed cell populations positive for CD10, CD13, CD56, and MHC Class-I markers and negative for CD3, CD5, CD7, CD11b, CD14, CD15, CD16, CD19, CD25, CD45, and CD65 markers. Northern analysis revealed that CD13 and CD56 were actively transcribed at time of cell harvest. We report the first identification of CD10, CD13, CD56, and MHC Class-I cell surface antigens on these human cells.**

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Numerous studies have shown the existence of mesenchymal stem cells distributed widely throughout the connective tissue compartments of many animals. These cells provide for the continued maintenance and repair of tissues throughout the life span of the individual. Examples of these cells include the unipotent myosatellite myoblasts of muscle (1–3); the unipotent adipoblast cells of adipose tissue (4); the unipotent chondrogenic and osteogenic stem cells of the perichondrium and periosteum, respectively (5, 6); the bipotent adipofibroblast cells of adipose tissue (7); the bipotent chondrogenic/osteogenic stem cells of marrow (8–10); and the multipotent hematopoietic stem cells of bone marrow and peripheral blood (11–13).

Recent studies using serial dilution clonogenic analysis (14–17) have shown that mesenchymal stem cells consist of two uniquely different categories of cells: progenitor cells, committed to a variety of phenotypic lineages (see above), and pluripotent cells that are not committed to any particular lineage. Further analysis (6, 14) revealed that multiple lineage-specific progenitor cells as well as pluripotent cells were also present in the connective tissue compartments of various tissues. For example, the connective tissues of skeletal muscle contain not only myosatellite cells (the precur-

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sor cells for skeletal muscle) and fibroblasts (the precursor cells for connective tissues) but also adipoblasts (the precursor cells for fat), chondrogenic progenitor cells (the precursor cells for cartilage), osteogenic progenitor cells (the precursor cells for bone), as well as lineage-uncommitted pluripotent cells.

Lineage-committed progenitor cells conform to Hayflick's limit (18), having life spans limited to 50–70 cell doublings before programmed cell senescence and death occur. Progenitor cells differentiate into cell types limited to the lineage to which they are committed (see above). By contrast, pluripotent cells have the capacity for extended self-renewal beyond Hayflick's limit as long as they remain lineage-uncommitted. Pluripotent cells can commit to any tissue lineage within the embryonic mesodermal line. Once committed to a particular lineage, these cells assume all the attributes of progenitor cells.

We propose that progenitor and pluripotent cells could be of value in transplantation and/or gene therapies where donor tissue is in short supply. Indeed, Grande *et al.* (19) used rabbit pluripotent cells in the rabbit full thickness cartilage defect model. Dramatic results were reported in the resurfacing of articular cartilage as well as the reconstitution of adjacent subchondral and trabecular bone.

Previous studies (14–17) have shown that extended time periods are necessary to isolate and separate progenitor and pluripotent cells, either by limiting serial dilution clonogenic analysis (18–24 months) or propagation past Hayflick's limit (5–9 months). Improvements in the ease of isolation and induction of lineage commitment must be made for these cells to be useful in the clinical setting. Therefore, our current research is aimed at characterizing the cell surface antigens of human progenitor and pluripotent cells to shorten the time required for their isolation and separation.

Antibodies to cell surface cluster differentiation (CD) markers have been used in conjunction with flow cytometry to characterize cell surface antigens on hematopoietic cells. To date, more than 180 CD markers have been used to "fingerprint" hematopoietic cell lineages (20). The experiments reported in this paper involved characterizing 15 cell surface CD marker antigens on human male and female progenitor and pluripotent cells isolated from fetal, adult, and geriatric donors. We report the first identification of CD10, CD13, CD56, and MHC Class-I on human progenitor and pluripotent cells. Negative results were obtained for CD3, CD7, CD11b, CD14, CD15, CD16, CD19, CD25, CD45, and CD65 antigens. RNAs were extracted from the cells, electrophoresed, and probed with ^{32}P-labeled cDNAs to CD10, CD13, and CD56 using Northern analysis. CD13 and CD56 were being actively transcribed at time of cell harvest.

Materials and Methods

Human Mesenchymal Stem Cells. Five populations of human cells, adult (female), fetal (male and female), and geriatric (male and female) were used for this study. All tissue harvesting protocols were approved by the Institutional Review Board at the Medical Center of Central Georgia, Macon, GA.

Adult female cells were purchased as a subconfluent culture of 25-year-old human dermal fibroblasts (NHDF, catalog # CC-0252, lot # 6F0600, Clonetics, San Diego, CA). Fetal male cells were purchased as a subconfluent culture of 22-week-old fetal skeletal muscle cells derived from the thigh muscle (CM-SkM, catalog # CC-0231, lot #6F0604, Clonetics). Fetal female cells were purchased as a subconfluent culture of 25-week-old fetal skeletal muscle cells derived from the triceps muscle (CF-SkM, catalog # CC-2561, lot # 14722, Clonetics). Upon arrival, the cells were transferred to plating medium-A (PM-A). PM-A consisted of 89% (v/v) Eagle's Minimal Essential Medium with Earle's salts (EMEM, GIBCO BRL, Grand Island, NY), 10% (v/v) preselected horse serum [lot nos. 17F-0218 (HS7) or 49F-0082 (HS4), Sigma Chemical Co., St. Louis, MO], and 1% (v/v) Penicillin/Streptomycin (10,000 units/ml penicillin and 10,000 mg/ml streptomycin, GIBCO), pH 7.4. Cells were incubated at 37°C in a 95% air/5% CO_2 humidified environment. After expansion, cells were released with 0.05% (w/v) trypsin (DIFCO, Detroit, MI) in Ca^{+2}-, Mg^{+2}-free Dulbecco's phosphate buffered saline (GIBCO) containing 0.0744% (w/v) ethylenediamine tetraacetic acid (EDTA, Sigma), centrifuged at 100*g* for 20 min, and the supernatant aspirated. The cell pellet was resuspended in PM-A and the cell suspension cryopreserved by slow freezing for storage at −70°C to −80°C in PM-A containing 7.5% (v/v) dimethyl sulfoxide (DMSO, Morton Thiokol, Danvers, MA) (21).

Geriatric cells were isolated from specimens of skeletal muscle obtained from a 67-year-old male patient and a 77-year-old female patient following standard protocols for the isolation of mesenchymal stem cells (6, 22). The male cells were designated "PAL#3", and the female cells "PAL#2." In brief, cells were liberated from the connective tissue compartment of skeletal muscle with collagenase (CLS-I, Worthington Biochemical Corp., Freehold, NJ) and dispase (catalog #40235, Collaborative Research Inc., Bedford, MA). Single cell suspensions were obtained by sequential filtration through 90-μm and 20-μm Nitex (Tetco Inc., Elmsford, NY). Cells were seeded at 10^5 cells/1% (w/v) gelatin-coated (EM Sciences, Gibbstown, NJ) T-75 flasks (Falcon, Becton-Dickinson Labware, Franklin Lakes, NJ) in PM-A and allowed to expand and differentiate prior to cryopreservation. Cells were incubated at 37°C in a 95% air/5% CO_2 humidified environment. After expansion, cells were released with trypsin, sieved as above to separate mononucleated cells from differentiated phenotypes (i.e., multinucleated myotubes, adipocyte colonies, cartilage nodules, bone nodules), and cryopreserved at −70° to −80°C in PM-A containing 7.5% (v/v) DMSO. Using the procedures outlined above, each subsequent cryopreservation step ef-

fectively removes more than 98% of contaminating fibroblasts and differentiated phenotypes from the stem cell preparation (21).

Further purification of progenitor and pluripotent cells was obtained by multiple expansion and cryopreservation steps using 1% gelatin-coated flasks with plating medium-B (PM-B). PM-B consisted of 89% (v/v) Opti-MEM based medium (catalog #22600–050, GIBCO) containing 0.01 m*M* β-mercaptoethanol (Sigma), 10% (v/v) horse serum (HS3, lot number 3M0338, BioWhittaker, Walkersville, MD), and 1% (v/v) antibiotic-antimycotic solution (GIBCO), pH 7.4. Cells were then propagated to 30 cell doublings, released with trypsin, and aliquoted for insulin/dexamethasone analysis, flow cytometry, and molecular analysis.

Insulin/Dexamethasone Analysis to Identify Progenitor and Pluripotent Cells. Isolated cells were examined using insulin and dexamethasone in a comparison/contrast analysis (15, 16, 23). This analysis compares and contrasts the separate effects of insulin and dexamethasone to identify the cells in question. Insulin will accelerate phenotypic expression in progenitor cells but will have no effect on pluripotent cells. By contrast, dexamethasone will induce lineage-commitment and expression in pluripotent cells, but will not alter phenotypic expression in progenitor cells. For example, if the same phenotype(s) at the same level are seen with both insulin and dexamethasone, then the culture contains only progenitor cells. In contrast, if different qualities and/or quantities of phenotypes are present in cultures treated with dexamethasone versus those treated with insulin, then the culture consists of a mixture of progenitor and pluripotent cells. Finally, if no phenotypes are seen following insulin treatment, but are present following dexamethasone treatment, then the culture contains only pluripotent cells. Thus, the response to insulin and dexamethasone can be used to determine whether progenitor and/or pluripotent cells are present within a population of cells.

Aliquots of CM-SkM, CF-SkM, NHDF, PAL#3, and PAL#2 cells were thawed and plated individually at 10,000 cells/well in 1% gelatin-coated 24-well plates (Corning, Corning, NY) using PM-B. After 24 hr PM-B was removed and replaced with either control medium, insulin testing medium, or dexamethasone testing medium. Control medium consisted of 98% (v/v) Opti-MEM containing 0.01 m*M* β-mercapto-ethanol, 1% (v/v) HS3, and 1% antibiotic-antimycotic solution. Insulin testing medium consisted of control medium containing 2 μg/ml insulin (Sigma). Dexamethasone testing medium was composed of 98% Opti-MEM, 0.01 m*M* β-mercaptoethanol, 1% serum [HS3, HS9 (horse serum, lot number 90H-0701, Sigma) or FBS (fetal bovine serum, lot no. 3000L, Atlanta Biologicals, Norcross, GA)] and 1% antibiotic-antimycotic solution. This solution was made 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} *M* with respect to dexamethasone (Sigma) (6, 15, 16, 23). Media were changed three times per week for 6 weeks. Cultures were viewed twice per week for changes in phenotypic expression and photographed.

Discernible changes in phenotypic expression of the cells were assayed morphologically. These morphological tissue cellular types were identical to those previously noted in avian and mouse mesenchymal stem cells incubated with insulin or dexamethasone and extensively analyzed by histochemical and immunochemical procedures (6, 14–17, 23). Myogenic structures were identified at 1 week by their elongated multinucleated appearance. Adipogenic cells were identified at 2 weeks as polygonal cells containing multiple intracellular refractile vesicles. Chondrogenic cells were identified at 4 weeks as aggregations of round cells (either as sheets or discrete nodules) with refractile pericellular matrix halos. Osteogenic cells were identified at 6 weeks as three-dimensional extracellular matrices overlying cellular aggregations.

Flow Cytometry. Aliquots of CM-SkM, CF-SkM, NHDF, PAL#3, and PAL#2 cells were thawed and seeded at 10^5 cells/1% gelatin-coated T-75 flasks in PM-B, and allowed to expand at 37°C in a 95% air/5% CO_2 humidified environment. After expansion, cells were released with trypsin and resuspended in PM-B. The cells were then centrifuged and resuspended in wash buffer at a concentration of 1×10^6 cells/ml. Wash buffer consisted of phosphate buffer supplemented with 1% (v/v) FBS and 1% (w/v) sodium azide, NaN_3 (Sigma). Cell viability was >95% by the Trypan blue dye (GIBCO) exclusion technique (14, 21). One hundred microliters of cell preparation (1×10^5 cells) were stained with saturating concentrations of fluorescein isothiocyanate- (FITC), phycoerythrin- (PE), or perdinin chlorophyll protein- (PerCP) conjugated CD3, CD5, CD7, CD10, CD11b, CD13, CD14, CD15, CD16, CD19, CD25, CD45, CD56, CD65, MHC Class-I, or isotype matched controls (Becton-Dickinson, Inc., San Jose, CA). Briefly, cells were incubated in the dark for 30 min at 4°C. After incubation, cells were washed three times with wash buffer and resuspended in 0.5 ml of wash buffer for analysis on the flow cytometer. Flow cytometry was performed on a FACScan (Becton-Dickinson). Cells were identified by light scatter. Logarithmic fluorescence was evaluated (4 decade, 1024 channel scale) on 10 ,000 gated events. Analysis was performed using LYSYS II software (Becton-Dickinson), and the presence or absence of each antigen was determined by comparison to the appropriate isotype control. An antigenic event was gated if the fluorescence was greater than 25% above its isotype control. Absolute numbers of cells per 10,000 gated events are shown in Table II. A mean value above 1000 cells is considered positive for any CD marker.

Molecular Analysis. Aliquots of CF-SkM, NHDF, and PAL#3 cells were thawed and seeded at 10^5 cells/1% gelatin-coated T-75 flasks in PM-B, and allowed to expand at 37°C in a 95% air/5% CO_2 humidified environment. After expansion, cells were released with trypsin and centrifuged. The resulting supernatants were aspirated, and cell

Table I. Induction of the Expression of Different Mesodermal Morphologies by Dexamethasone and Insulin in Human Mesenchymal Stem Cells

	Insulin (2 μg/ml)				Dexamethasone (10^{-10}–10^{-6} *M*)			
	MT[a]	Adip	CN	BN	MT	Adip	CN	BN
Weeks[b]	1	2	4	6	1	2	4	6
CF-SkM	+[c]	+	+	+	++[d]	++	++	++
CM-SkM	+	+	+	+	++	++	++	++
NHDF	+	+	+	+	++	++	++	++
PAL#2	+	+	+	+	++	++	++	++
PAL#3	+	+	+	+	++	++	++	++

[a] MT, myotubes; Adip, adipocytes; CN, cartilage nodule; BN, bone nodule.
[b] Number of weeks of incubation for appearance of the cell type.
[c] Approximately 0%–5% of culture expressing each particular designated phenotype, with approximately 20% of culture exhibiting all four phenotypes after 6 weeks of incubation.
[d] Approximately 10% of culture expressing each particular designated phenotype, with ≥40% of culture expressing all four phenotypes after 6 weeks of incubation.

pellets frozen and stored at −80°C. Cell pellets were thawed on ice, and total RNA was extracted from CF-SkM, NHDF, and PAL#3 cells using the Qiagen QIAshredder (catalog #79654, Qiagen, Chatsworth, CA) and RNeasy Total RNA Kits (catalog #74104, Qiagen) according to the manufacturer's instructions. I.M.A.G.E. Consortium (LLNL) cDNA clones (24) for CD10, CD13, CD56, and β-actin (I.M.A.G.E. Consortium Clone ID: 701606, 713961, 468885, and 586736, respectively, Research Genetics, Huntsville, Al) were obtained. The cDNA insert was excised from the plasmid by restriction digestion and separated by agarose gel electrophoresis according to standard procedures (25). The cDNA band was purified using the Qiaex II Gel Extraction Kit (catalog #20021, Qiagen) according to the manufacturer's instructions. The cDNA was labeled by incorporation of 3000 Ci/mM alpha-[32-P]-dCTP (catalog number AA0005, Amersham, Arlington Heights, IL) using the Prime-It Random Primer Labeling Kit (catalog #300385, Stratagene, La Jolla, CA).

Northern analysis. Total RNA(30 μg/lane/cell line) was electrophoresed through formaldehyde/agarose gels (formaldehyde, catalog #F79–500, and agarose, catalog #BP164–100, Fisher, Norcross, GA) and transferred to a nylon membrane (catalog #NJ0HYB0010 Magnagraph, Fisher) according to standard procedures (25). Hybridization was carried out in roller bottles at 68°C overnight in QuikHyb hybridization solution (catalog #201220, Stratagene). Washing was performed according to the manufacturer's instructions. Autoradiography (Fuji film, catalog #04–441–95, Fisher) was carried out at −70°C to −80°C, using an intensifying screen.

Results

Identification of Cells. The identity of the cells present within the human fetal, mature, and geriatric cell populations was examined using insulin and dexamethasone in a comparison/contrast analysis. Morphologies consistent with skeletal muscle myotubes, adipocytes, cartilage nodules, and bone nodules were produced by treatment with both insulin or dexamethasone in all five human cell populations. However, a greater percentage of morphologies was in-

Table II. CD Marker Expression[a]

	CM-SkM	CF-SkM	NHDF	PAL#3	PAL#2
CD3	150	140	13	19	0
CD5	23	38	26	26	0
CD7	29	66	2	2	0
CD10	4700	200	4676	4627	66
CD11b	4	126	31	31	0
CD13	9280	9638	9900	9976	8260
CD14	27	205	104	182	750
CD15	75	89	168	8	0
CD16	71	67	12	12	0
CD19	8	68	14	29	151
CD25	1	57	21	21	52
CD45	5	74	30	32	43
CD56	1120	2952	488	474	3980
CD65	210	87	8	10	0
Class-I	9542	9556	9542	8420	8416

[a] CD Marker expression detected by immuno-flow cytometry. Results are expressed as absolute numbers of cells exhibiting positive staining for cell surface CD markers from a gated population of 10,000 cells.

duced with dexamethasone than with insulin (Table I, Fig. 1). The data suggest that both progenitor cells (insulin accelerated morphologies) and pluripotent cells (dexamethasone induced morphologies) are present in human cells derived from 25-year-old female dermis, 22-week-old fetal male and 25-week-old fetal female (prenatal) skeletal muscle connective tissues, and 67-year-old male and 77-year-old female skeletal muscle connective tissues.

Flow Cytometric Analysis. Since the cell surface antigens expressed by human progenitor and pluripotent cells were unknown, we analyzed the five cell populations for the presence of CD3, CD5, CD7, CD10, CD11b, CD13, CD14, CD15, CD16, CD19, CD25, CD45, CD56, CD65, and MHC Class-I by immunochemistry coupled with flow cytometry. This powerful technique allowed us to examine large numbers of cells relatively quickly and easily. All human cell populations examined were positive for the cell surface expression of CD10, CD13, CD56, and MHC Class-I, and negative for CD3, CD5, CD7, CD11b, CD14, CD15, CD16, CD19, CD25, CD45, and CD65 (Table II, Figs. 2 and 3). The data demonstrate that CD10 (neutral endopeptidase), CD13 (aminopeptidase), CD56 (neural cell adhesion molecule, 140 kDa isoform), and major histocompatibility Class-I antigens are located on the cell surface of these human cells at fetal (male and female), adult (female), and geriatric (male and female) ages.

Molecular Analysis of CD10, CD13, and CD56. To determine whether CD10 (neutral endopeptidase), CD13 (aminopeptidase), and CD56 (neural cell adhesion molecule, 140 kDa isoform) were being transcribed by the cells at time of harvest, total RNA from CF-SkM, NHDF, and PAL#3 samples was analyzed by the Northern blot technique using fragments of human CD10, CD13, and CD56 ^{32}P-labeled cDNAs as probes. A variable pattern in the transcription of the CD markers at the time of cell harvest was observed (Table II, Fig. 4). Strong cDNA binding for CD56-mRNA was observed in all three cell lines, suggesting active transcription of neural cell adhesion molecule isoforms in all three cell lines. cDNA binding for CD13-mRNA was either weak (CF-SkM), strong (NHDF), or not present (PAL#3), suggesting that there are variations in the transcription of aminopeptidase within the different cell lines.

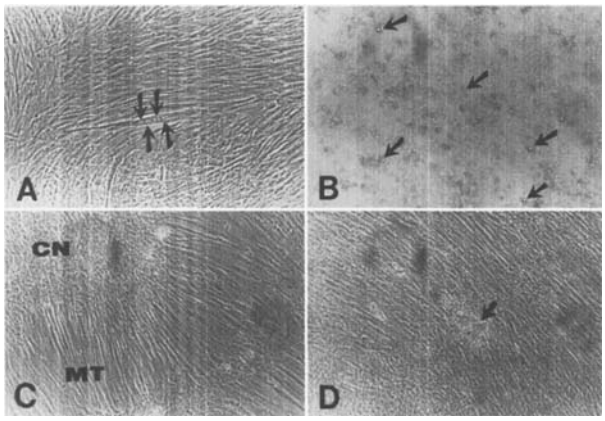

Figure 1. CF-SkM propagated to 30 cell doublings and incubated with insulin or dexamethasone for 0–6 weeks. Morphologies as noted. (A) Cells treated for 1 week with 2 μg/ml insulin. Note presence of four nuclei (arrows) within linear structure, indicative of a multinucleated myotube, MT. Orig. mag., 10X. (B) Cells treated for 2 weeks with 10^{-6} *M* dexamethasone. Note presence of clusters of cells (arrows) containing intracellular refractile vesicles indicative of adipogenic cells. Orig. mag., 10x. (C) Cells treated for 4 weeks with 10^{-6} *M* dexamethasone. Note presence of nodular mass of cells with pericellular matrix halos, indicative of cartilage nodule (CN) overlying multiple multinucleated linear structures indicative of myotubes (MTs). Orig. mag., 10x. (D) Cells treated for 6 weeks with 2 μg/ml insulin. Note presence of three-dimensional matrix (delineated by arrows) overlying cell cluster, indicative of bone nodule (BN). Orig. mag., 10x.

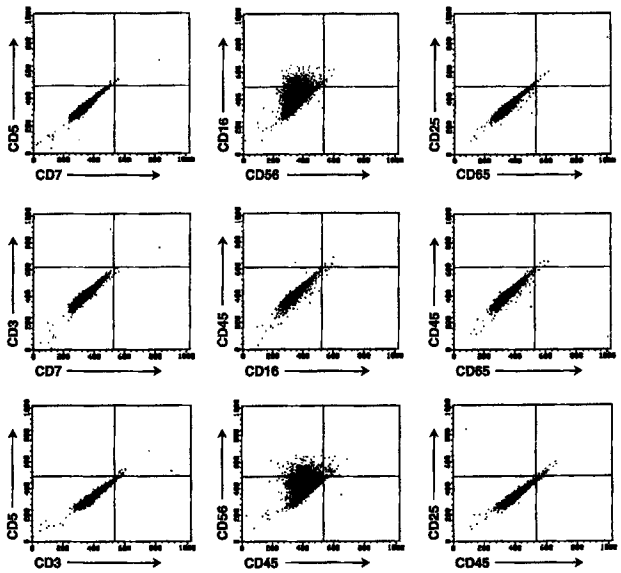

Figure 2. Flow cytometry of cluster differentiation markers. X-axis and Y-axis as noted on figure. NHDF propagated to 30 cell doublings and analyzed with antibodies to cell surface cluster differentiation markers.

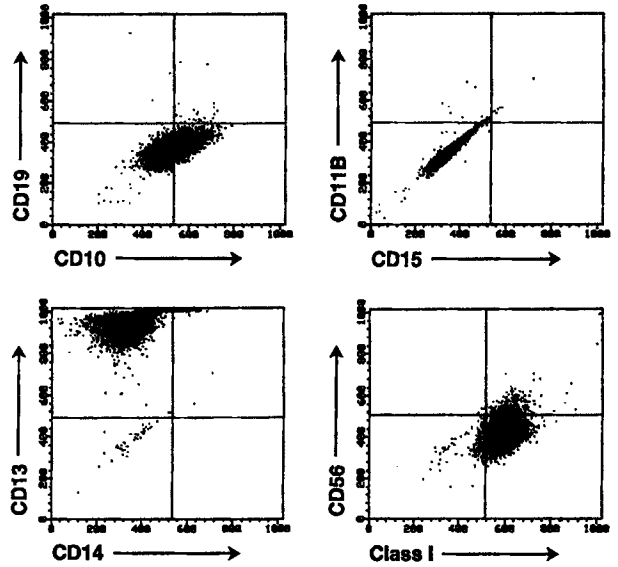

Figure 3. Flow cytometry of cluster differentiation markers. X-axis and Y-axis as noted on figure. NHDF propagated to 30 cell doublings and analyzed with antibodies to cell surface cluster differentiation markers.

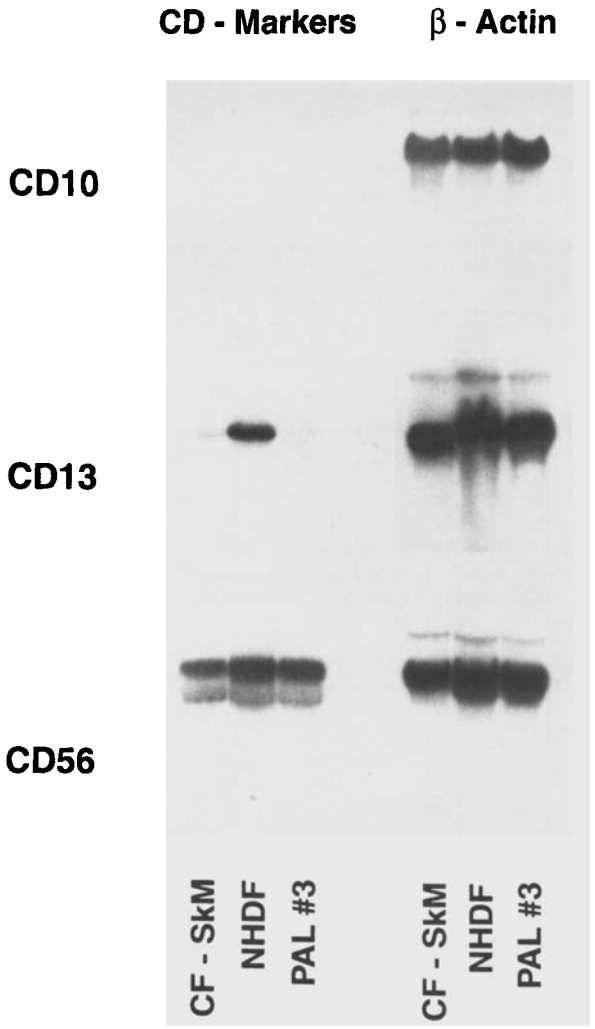

Figure 4. Northern analysis of cluster differentiation markers CD10, CD13, and CD56 for cell lines CF-SkM, NHDF, and PAL#3. Cells were propagated to 30 cell doublings, harvested, total RNAs extracted, electrophoresed, and probed with ^{32}P-labeled cDNAs to CD10, CD13, CD56, and β-actin (control). As shown, mRNAs for CD13, CD56, and β-actin were being actively transcribed at time of cell harvest.

No cDNA binding for CD10 mRNA was present in any of the three cell lines examined. This finding suggests two possibilities: either the mRNA for CD10 was not transcribed at the time of harvest, or the amount of mRNA for CD10 was below the limits of detection of the assay.

Discussion

Every year millions of people suffer tissue loss or end-stage organ failure (26). The total national US health care costs for these patients exceeds $400 billion per year. Currently over 8 million surgical procedures requiring 40–90 million hospital days are performed annually in the United States to treat these disorders. Although these surgical procedures have saved and improved countless lives, they remain imperfect solutions. Options such as tissue transplantation and surgical intervention are severely limited by critical donor shortages, long-term morbidity, and mortality. Donor shortages worsen every year, and increasing numbers of patients die while on waiting lists for needed organs. A wide variety of transplants, congenital malformations, elective surgeries, diseases, and genetic disorders have the potential for treatment with autologous stem cells as the source of donor tissue, either alone or in combination with other agents. A preferred treatment is the treatment of tissue loss where the object is to increase the number of cells available for transplantation, thereby replacing the missing tissues or providing sufficient numbers of cells for *ex vivo* gene therapy. The use of autologous cells should result in an identical HLA match, obviating the morbidity and mortality associated with allogeneic transplants and immunosuppressive therapy.

Previous studies have demonstrated the existence of mesodermal stem cells located within the connective tissue matrices of many animal species, including humans (6, 17, 22, 27–30). The existence of two categories of these cells has been demonstrated by serial limiting dilution clonogenic analysis (14, 23). Lineage-committed progenitor cells are either unipotent (forming tissues of a single lineage such as the myogenic, fibrogenic, adipogenic, chondrogenic, or osteogenic lineages), bipotent (forming tissues of two lineages such as the chondro-osteogenic or adipofibrogenic lineage), or multipotent (forming multiple tissues or cells within the same lineage, such as the hematopoietic lineage). Lineage-committed progenitor cells are capable of self-replication but have a life span limited to approximately 50–70 cell doublings before programmed cell senescence occurs. Individual clones of progenitor cells demonstrate lineage restriction by giving rise to progeny of separate lineages (e.g., myogenic, fibrogenic, adipogenic, chondrogenic, and osteogenic). One unique characteristic of progenitor cells is that their phenotypic expression can be accelerated by treatment with progression factors such as insulin, insulin-like growth factor-1 (IGF-1), or insulin-like growth factor-2 (IGF-2) (15, 23). By contrast, pluripotent cells are capable of extended self-renewal and the ability to generate various lineage-committed progenitor cells from a single clone. For example, a prenatal pluripotent mouse clone was induced by long-term treatment with dexamethasone to form lineage-committed progenitor cells that exhibited morphological and phenotypic expression markers characteristic of skeletal muscle, fat, cartilage, and bone after more than 690 cell doublings (15). Similar results were obtained using a postnatal pluripotent rat clone after more than 300 cell doublings (23). Differentiation-inducing factors, such as dexamethasone, bone morphogenetic protein (BMP), and muscle morphogenetic protein (MMP), are necessary to induce lineage-commitment (15, 23). Progression factors such as insulin, IGF-1, or IGF-2 have no effect on pluripotent cells (23). Once pluripotent cells commit to a particular lineage (i.e., become lineage-committed progenitor cells), their ability to replicate is limited to approximately 50–70 cell doublings before programmed cell senescence occurs. These newly generated progenitor stem cells can proliferate (under

the influence of proliferation factors, such as platelet-derived growth factors) for a maximum of 50–70 cell doublings. They can also differentiate further (under the influence of progression factors) along separate mesodermal lines (14–17, 23).

Because of both the proliferative and differentiative potential of these cells, we propose that they could be of value in various transplantation and/or gene therapies where donor tissue is in short supply. Indeed, using our protocols (22, 29) for the isolation of mammalian pluripotent cells, Grande *et al.* (19) have demonstrated dramatic results in the reconstitution of articular cartilage as well as subchondral and trabecular bone in the treatment of full thickness articular cartilage defects in rabbits.

The time required for pluripotent cell isolation, propagation, and induction of lineage commitment must be relatively short for these cells to be used in many clinical situations in which the cells are removed, treated, and reintroduced into the patient's body. Indeed, previous studies from our laboratories (14–17, 23) have shown that extended periods are necessary to isolate and purify pluripotent cells. Isolation of these cells may be accomplished by two different methods. In the first procedure, a purified population of pluripotent cells is obtained by propagating isolated cells from a primary harvest past Hayflick's limit (50–70 cell doublings) (18). This procedure requires 5–9 months. In the second procedure, individual clones of pluripotent and progenitor cells are obtained by serial dilution clonogenic analysis. This procedure requires 18–24 months. We would like to minimize the time required for isolating these cells. Our current research is aimed at characterizing cell surface antigens on human progenitor and pluripotent cells. This knowledge is intended to reduce the time required to isolate more highly purified populations of these cells.

This is the first study to demonstrate the cell surface localization of neutral endopeptidase (CD10), aminopeptidase (CD13), neural cell adhesion molecule, 140-kDa isoform (CD56), and MHC Class-I for human progenitor and pluripotent cells. We suggest that these cell surface CD antibodies could be used in conjunction with flow cytometry and fluorescence-activated cell sorting or magnetic bead technology as an initial step to isolate more purified populations of human cells from an initial cell harvest. Starting with a population enriched with these autologous cells would significantly decrease the culture time and cost required to obtain an adequate number of progenitor and pluripotent cells for various transplantation and/or gene therapies.

Positive Staining for CD Markers in Human Mesodermal Cells. The functional significance of the particular cell surface moieties CD10, CD13, CD56, and MHC Class-I expressed by the human fetal, adult, and geriatric cells used in this study remains unknown at this time. However, CD10, CD13, and CD56 are known to be expressed on both differentiated cells and early stem cells within the hematopoietic system (20). Cell surface neutral endopeptidase (CD10) has been used with antibodies to cluster differentiation (CD) markers and flow cytometry as a method for the identification of common acute lymphoblastic leukemia antigen (CALLA) cells, early lymphoid progenitor cells, mature granulocytes, and neutrophils (20). This membrane-associated zinc-metallopeptidase has been shown to inactivate a wide variety of regulatory peptide hormones, including enkephalin, chemotactic peptide, substance P, neurotensin, oxytocin, bradykinin, bombesin, and angiotensins I and II (31–34).

Cell surface aminopeptidase (CD13) has been utilized with flow cytometry to identify early committed progenitors of granulocytes and monocytes (CFU-GM). It is expressed by all cells of these lineages as they mature (20). CD13 is also expressed on a small proportion of large granular lymphocytes, but not other lymphocytes (20). CD13 is identical in structure to aminopeptidase N (EC 3.4.11.2), a membrane-bound zinc-binding metalloprotease (35, 36). This enzyme is known to catalyze the removal of NH_2-terminal amino acids from regulatory peptides produced by diverse cell types (36, 37).

One possible function of the cell surface enzymes, neutral endopeptidase (CD10) and aminopeptidase (CD13), on these stem cells is that they may serve to regulate the differentiation process by preferentially degrading autocrine, paracrine, and/or endocrine regulatory peptides (e.g., lineage-commitment agents, progression factors, and proliferation agents) that may affect these cells. Young *et al.* (15) demonstrated the ability of various paracrine and endocrine regulatory peptides to alter proliferation, lineage-commitment, and lineage progression in progenitor and pluripotent stem cells. These compounds included those that affected proliferation (platelet-derived growth factors-AA and -BB), lineage-induction (dexamethasone and MMP), and progression (insulin, IGF-1 and IGF-2). Their study suggested that the ability of stem cells to respond to specific regulatory peptides is more tightly controlled as differentiation proceeds from a lineage-uncommitted pluripotent stem cell to a lineage-committed progenitor stem cell.

The 140-kDa isoform of neural cell adhesion molecule (NCAM, CD56) has been utilized with flow cytometry as the prototypic marker to identify natural killer (NK) cells and (CD4 +/CD8 +) T cells (20). Although its function has not been demonstrated convincingly with hematopoietic cells, it may be involved in homophilic adhesion for NK and T cells due to the C2-set Ig regions and fibronectin regions within its extracellular domain (38, 39). With respect to nonhematopoietic tissues, homophilic and heterophilic adhesion by NCAM has been proposed to regulate both cell-cell and cell-matrix interactions. This may be due in part to its ability to interact with type I collagen in its associated extracellular matrix, a key element in adhesion and migration of cells (40). NCAM appears on early embryonic cells and is important in the formation of cell collectives and their boundaries at sites of morphogenesis (41). Later in development it is found on various differentiated tissues.

Previous studies (6, 14, 22, 23) demonstrated the potential for mesenchymal stem cells to form tissues of mesodermal origin such as skeletal muscle, cardiac muscle, smooth muscle, and bone (osteoblasts). These particular differentiated cell types have been shown to use NCAM for cell-cell and cell-matrix interactions leading to their differentiation (42–47). Of particular interest is the percentage of mesenchymal stem cells within the five cell lines displaying CD56 (Table II). The differences in numbers of cells exhibiting CD56 may reflect the chronological age or the functional capability of the cells at time of harvest. It is also possible that the percentage of cells exhibiting CD56 in each cell line may reflect the absolute numbers of progenitor versus pluripotent stem cells within their respective populations. Cell surface NCAM functions during normal embryological development to regulate the required cell-cell and cell-matrix interactions in preparation for further differentiation of mesenchymal stem cells along their respective tissue lineage pathways. It may also have a similar function in the adult.

Cell surface major histocompatibility complex (MHC) Class-I molecules have been shown to be present on all vertebrate species and to be expressed on almost every nucleated cell in the body (48). While MHC Class-I molecules play a central role in the phenomena of antigen processing and presentation (48, 49), they have also been studied extensively to understand the mechanisms of immune responses that discriminate between self and nonself. Mesenchymal stem cells have been proposed as a source of cells for tissue engineering, either as donor tissue for transplantation or as a delivery vehicle for gene therapy (1, 3). As shown in Table II, greater than 80% of the cells within the populations of stem cells isolated from fetal, adult, and geriatric aged individuals express MHC Class-I antigens. This indicates that those particular Class-I antigen-expressing cells would be recognized as foreign in an MHC-mismatched immunocompetent individual, and thus should only be used for autologous or syngeneic transplants. In contrast, there were approximately 5% of fetal and adult stem cells and approximately 15% of geriatric stem cells that did not express MHC Class-I antigens. This apparent decrease in MHC Class-I antigen expression may have been due to quantities of cell surface Class-I antigens below the limits detectable by the immunochemical/flow cytometric procedure used, or complete absence of these molecules from the surface of a particular subset of stem cells. The significance of this finding is unknown at this time. The presence or absence of cell surface MHC Class-I molecules on these stem cells may signify the "differentiated" state of that particular cell (i.e., the more differentiated (progenitor) stem cell exhibiting MHC Class-I antigens and the more primitive (pluripotent) stem cell not expressing these particular cell surface antigens). Alternatively, the "differentiated" state of a particular stem cell may have nothing to do with the expression of MHC Class-I antigens on its cell surface. In this instance there may be a subset of stem cells without MHC Class-I antigens that are essentially invisible to the immune system and thus may be candidates for a universal tissue transplant. Such a particular subset of cells might be useful in allograft transplant procedures.

Negative Staining for CD Markers in Human Mesenchymal Stem Cells. In contrast to the above four positive staining cell surface antigens, the following 11 antigens were found absent on the cell surface of fetal, adult, and geriatric human mesenchymal stem cells. These markers were CD3, CD5, CD7, CD11b, CD14, CD15, CD16, CD19, CD25, CD45, and CD65. The significance of these findings is unknown at this time. However, these particular cell surface antigens have been ascribed only to differentiated cells within the hematopoietic system (20) (i.e., T-cells: CD3, CD5, CD7, CD11b, CD25, CD45; B-cells: CD5, CD11b, CD19, CD25, CD45; thymocytes: CD7; granulocytes: CD11b, CD14, CD15, CD16, CD45, CD65; monocytes: CD11b, CD14, CD16, CD25, CD45; natural killer cells: CD11b, CD16, CD45; follicular dendritic cells: CD19; and mature erythrocytes: CD45).

In conclusion, this is the first study to demonstrate the cell surface localization of neutral endopeptidase (CD10), aminopeptidase (CD13), neural cell adhesion molecule isoform (CD56), and MHC Class-I in human progenitor and pluripotent cells. We suggest that these cell surface CD markers could be used in conjunction with flow cytometry and fluorescent-activated cell sorting as an initial step to isolate more purified populations of human progenitor and pluripotent cells from an initial cell harvest. Starting with a population enriched for mesodermal cells would significantly decrease both culture time and supply costs, plus improve the yield on the requisite progenitor and pluripotent cells needed for various transplantation and/or gene therapies.

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