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The Monoclonal Antibody SH-2, Raised against Human Mesenchymal Stem Cells, Recognizes an Epitope on Endoglin (CD105)

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Mesenchymal stem cells are multipotent cells resident in the bone marrow throughout adulthood which have the capacity to differentiate into cartilage, bone, fat, muscle, and tendon. A number of monoclonal antibodies raised against human MSCs have been shown to react with surface antigens on these cells *in vitro*. A protein of molecular mass 92 kDa was immunoprecipitated using the SH-2 monoclonal antibody. This was purified and identified by peptide sequencing analysis and mass spectrometry as endoglin (CD105), the TGF-β receptor III present on endothelial cells, syncytiotrophoblasts, macrophages, and connective tissue stromal cells. Endoglin on MSCs potentially plays a role in TGF-β signalling in the control of chondrogenic differentiation of MSCs and also in mediating interactions between MSCs and haematopoietic cells in the bone marrow microenvironment.

Mesenchymal stem cells (MSCs) are resident in the bone marrow stroma throughout adulthood. These cells have a multipotent capacity and can differentiate along a number of well characterized cell lineages including cartilage (1, 2) bone (3), adipose tissue (4), tendon (5) and muscle (6). MSCs have an adherent, fibroblastic phenotype and can be expanded in monolayer culture through many generations. The cells are easily isolated from bone marrow aspirates and, because of their multilineage potential, present exciting opportunities for cell-based therapeutic applications. In fact, therapeutic modalities have been described for the use of MSCs in cartilage (7), bone (8), tendon (5), and bone marrow stroma (9) regeneration and for the treatment of individuals with osteogenesis imperfecta (10).

Several monoclonal antibodies have been used as reagents in the isolation and characterization of MSCs. In general these have been raised against intact human MSCs (11, 12). Recently, one of these antibodies, SB-10, was shown to be reactive with an antigen present on undifferentiated MSCs which disappeared once the cells embarked upon an osteogenic pathway and began to express cell surface alkaline phosphatase. The specific SB-10 antigen was identified as Activated Leukocyte-Cell Adhesion Molecule (ALCAM, 13), which may play a role in the progression of osteogenic differentiation, although the precise mechanism remains to be elucidated (13). Other antibodies that have been described include SH-2, 3 and 4, also raised against human MSCs (12). These antibodies recognized epitopes present on the surface of MSCs and not on haematopoietic cells, and the antigens disappeared upon osteogenic or stromagenic differentiation. Initial studies suggested that each of the antibodies recognized a distinct antigen and that they might find use as reagents for the selection or screening of MSC populations isolated from bone marrow. Since the antigens disappeared upon induction of osteogenesis it also appeared that they would potentially cast light on the regulation of osteogenesis (12). To explore further the role which the SH-2 antigen might play in the biological control of MSC differentiation, and to determine its applicability as a cell surface marker for MSCs, we sought to determine its identity. A recent report described the immunoprecipitation of a glycoprotein of approximate mass 90 kDa from human MSCs (14). In the present study we describe the isolation and purification of the SH-2 antigen from human MSCs and its identification as the TGF-β receptor endoglin (CD105). The expression of endoglin by MSCs and by other cell types is described as well as its potential role in the control of stem cell differentiation.

Abbreviations used: MSC, mesenchymal stem cell; TGF, transforming growth factor; ALCAM, Activated Leukocyte-Cell Adhesion Molecule; MALDI-TOF, matrix-assisted laser desorption time-of-flight.

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METHODS

Isolation of mesenchymal stem cells. Samples of bone marrow were taken from the iliac crest of normal healthy adult volunteers and were processed as described (4, 15). Briefly, a sample of 20–40 ml marrow was collected in a syringe containing 6000 units of heparin. The sample was washed with phosphate-buffered saline and the cellular fraction was recovered by centrifugation. The cells were loaded onto a Percoll layer of density 1.073 g/ml and centrifuged at 1100*g* for 30 min. The nucleated cells were collected from the Percoll-cell layer interface and plated at 200,000 cells/cm^2. The cells were cultured in Dulbecco's modified Eagle's medium (low glucose) containing 10% fetal bovine serum. Medium was changed initially after 24 h and then every third day. MSCs were subcultured at 10 to 14 days by treatment with trypsin and EDTA and reseeded into T-185 flasks. The cells were grown to 80–100% confluence in monolayers at passage 2, 3, or 4. A total of 5×10^9 cells cultured from 8 donors (average age 27.5 y, range 19–35 y) was used for this study.

Immunoprecipitation. The SH-2 antibody used in this study was purified by chromatography on protein G–Sepharose. Immunoprecipitation of the antigen was carried out as follows: Cells in T-185 flasks were washed 3 times with 20 ml cold Tyrode's buffer and the washings were discarded. 15 ml antibody solution containing 1 μg/ml in Tyrode's buffer was added to each flask and incubated at 4°C for 5 h with shaking. The antibody solution was removed and discarded and the cells were washed again with 3 ml Tyrode's buffer which was again discarded. 10 ml fresh Tyrode's buffer was added and the cells were scraped and transferred to a conical tube. The flask was washed with a further 10 ml Tyrode's buffer which was added to the cell fraction. The cells were centrifuged at 2000*g* for 10 min and the supernatant was discarded. Lysis buffer (0.5% CHAPS in 20 mM Tris–Cl, pH 8.0 containing 0.14 M NaCl and 10 mM PMSF) was added at a ratio of 1 ml buffer per 3×10^7 cells. The lysate was incubated on ice for 45 min with regular agitation, then centrifuged at 12,000*g* for 30 min. The supernatant was removed and 550 μl of a 50% slurry of protein G Sepharose (Boehringer) equilibrated in lysis buffer without PMSF was added. The Protein G Sepharose-cell lysate was incubated with rocking at 4°C overnight, centrifuged briefly, washed with lysis buffer, and then centrifuged again. 200 μl SDS–PAGE sample buffer containing 2-mercaptoethanol was added and the material was boiled for 5 min, centrifuged and the supernatant was removed. This step was repeated once and the supernatants were combined. The supernatant containing the solubilized antigen-antibody complex was subjected to electrophoresis on a 12.5% SDS polyacrylamide gel. The gel was stained for 30–60 min with Coomassie blue G and destained in 10% methanol/10% acetic acid until bands were visible. At this point the gel was washed with water and stained bands were excised and stored at −20°C until further use.

In-gel tryptic digestion. Excised gel bands were cut into 1- to 2-mm pieces with a scalpel and incubated for 10 min at room temperature with 50% methanol. This washing procedure was repeated twice. All of the collected gel pieces were combined, dried on a Speedvac and then rehydrated by adding 400 μl 0.1 M Tris–Cl, pH 8.5/0.025% Tween 20 containing 0.5 μg/ml trypsin. After 2 h a further 0.5 ml of buffer was added and digestion was continued overnight at 37°C. As a control, an equal amount of unstained polyacrylamide from the same gel was treated with trypsin in an identical manner. The polyacrylamide pieces were centrifuged and the supernatant transferred to siliconized tubes. 0.5 ml 50% acetonitrile/0.1% trifluoroacetic acid was added to the gel pieces and incubated 1 h at 4°C. This procedure was repeated. The combined supernatants were pooled, dissolved in 0.1% trifluoroacetic acid, and used for peptide mapping. HPLC separation was carried out on a Hewlett–Packard 1090 LC with a 2.1 mm × 250 mm Vydac C18 column. Elution was with a gradient of 0–70% acetonitrile in 95 min and fractions were collected manually.

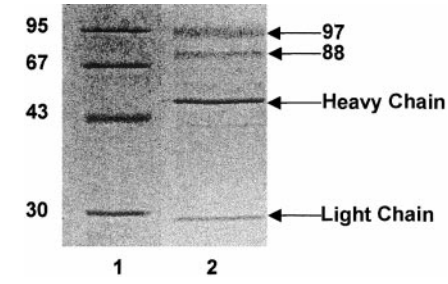

FIG. 1. SDS polyacrylamide gel electrophoresis of cell surface protein on human MSCs immunoprecipitated with SH-2 antibody. Lane 1 shows molecular weight standards and lane 2 shows immunoprecipitated proteins from a solubilized membrane fraction. The numbers on the left refer to the mass of molecular weight standards and on the right to the estimated mass of the immunoprecipitated proteins. The 92-kDa protein band was excised and analyzed further. The heavy and light chains of the antibody are indicated.

Sequence analysis and mass spectrometry. N-terminal sequence analysis of collected peptides was carried out using a Hewlett–Packard G1000A Protein Sequencer and mass spectrometric measurements were taken with a Hewlett-Packard G2026A matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometer. 95% of each fraction eluting from the HPLC was used for sequencing and 5% for MALDI-TOF. For MALDI analysis peptides were mixed with sinapinic acid solution (Hewlett Packard Co., Palo Alto, CA) to achieve a protein concentration of 1–10 pmol/μl. An aliquot (0.8 μl) of this solution was dried on the metal target, forming co-crystals of matrix and protein.

Immunocytochemistry. Embryonic human tissues were obtained from the Central Laboratory for Human Embryology, Department of Paediatrics, University of Washington, Seattle, Washington. Frozen sections of the femur and the hand were taken from tissue of estimated gestational age 84 days. The dissected tissue was placed in Tissue-Tek O.C.T., snap-frozen in a hexane/dry ice bath and cut into 7-μm sections using a cryostat at −29°C. The sections were mounted on Snowcoat glass slides (Surgipath, Richmond, IL) and air-dried at room temperature for 1 h and then stored at −80°C until stained. Staining was with fluorescein-conjugated SH-2 antibody at 10 μg/ml. Incubation was for 2 h at room temperature. Counterstaining was with propidium iodide (Molecular Probes), dissolved at 2 μg/ml. Stained sections were viewed on a Nikon Eclipse TE300 confocal microscope with excitation at 488 nm and detection at 515 and 565 nm.

RESULTS

Figure 1 shows a typical SDS PAGE gel with 2 stained bands of approximate molecular weight 92 and 80 kDa. Both bands were excised but only the 92-kDa material was used for subsequent analysis. Other bands corresponding to the IgG heavy and light chains were also evident on the gel. Figure 2A shows the tryptic map obtained after in-gel digestion of the 92 kDa band along with a control chromatogram (Fig. 2B). Collected fractions (marked with an asterisk) were used for N-terminal sequence analysis (Table I) and MALDI-TOF (Table II). A total of 12 collected HPLC fractions derived from the tryptic digest was used for sequence analysis. In eight of these sequence data were obtained and in the remaining four there was no de-

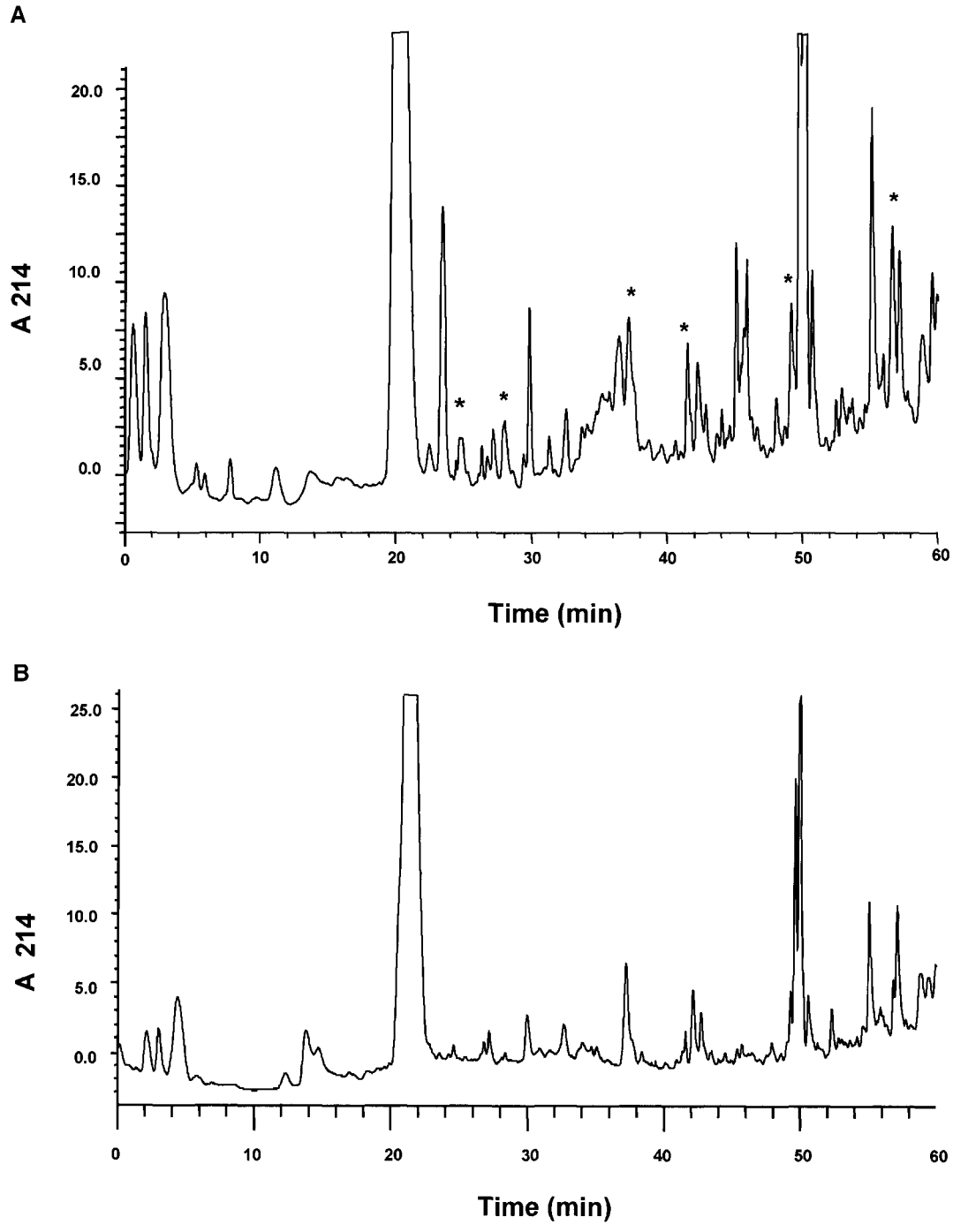

FIG. 2. Separation by reverse-phase HPLC of tryptic peptides derived from digestion in situ of the 92 kDa protein immunoprecipitated with the SH-2 antibody. Peptides were applied to a 2.1 mm × 250 mm Vydac C18 column and eluted with a gradient of 0–70% acetonitrile in 95 min. Separation of the tryptic digest is shown (A) and a control extract taken from an unstained portion of the gel (B).

tectable sequence (Table I). In some fractions there were several detectable sequences. The peptide sequence information obtained was used to search the Genbank database for alignments using the Blitz search routine (16) and all of these showed identity with the human endoglin sequence (17). A total of 12 peptide sequences was determined and aligned with the endoglin sequence (Table I). Seven of the collected HPLC fractions were analyzed by MALDI-TOF (Table II) and in two fractions, those eluting at 35.12 and 41.46 min, several peptide masses were detected. For two of these peptide masses, 2932.8 and 2164.6 Da, there were several possible assignments, all of which are shown in Table II. In a number of cases the mea-

TABLE I

Sequence Information Obtained from Peptides Derived from a Tryptic Digest of SH-2 Antigen

Fraction number	Retention time (min)	Sequence[a]
7	24.71	(210)VLPGHSAGPR
10	28.00	(187)TPALVR
		(204)EAHILR
		(268)IFPEXX
11	29.82	No sequence
13	32.51	(13)ETVHCDXQPVXP
15	35.00	(531)TGTLSX
		(387)GDKFV
		(611)EPVVAVAAP
17	37.12	(13)ETVHXDLQPVGXERGXVTY
21	43.99	(131)TQILEW
22	45.04	No sequence
23	45.81	No sequence
25	49.07	(276)GFKLPDTPQGGLLGEA
32	56.58	(131)TQILEWAAERGPITSAAELN
35	59.55	No sequence

[a] The number in parentheses refers to the residue number that begins the aligned sequence of human endoglin (see Fig. 2).

sured mass suggested an acrylamide or phosphate adduct, as indicated. The mass data obtained were used to search the Genbank database utilizing the mass fitting software MS-FIT (18) and the results of this search indicated that all of the peptides for which definitive mass data were obtained were derived from human endoglin. A total of 12 peptides was identified by MALDI-TOF and these could be aligned with the human endoglin sequence when the measured masses were corrected for the presence of acrylamide or phosphate adducts (Table II). When these were taken into account the measured and calculated peptide masses were comparable within a low level of error (0.71 ± 1.25).

These results all indicated that the SH-2 antigen present on the surface of human MSCs was in fact human endoglin or CD105. Endoglin is a dimeric glycoprotein present on human vascular endothelium, with monomeric mass of 95 kDa based on SDS-PAGE analysis (17). It is a component of the TGF-β signalling system (19), acting as a type III receptor. It has also been detected in bone marrow stroma (20). There is an alternatively spliced variant of human endoglin, referred to as endoglin-S (21), which lacks a 33-residue cytoplasmic tail. This is likely to be the 80 kDa species that we observed on SDS PAGE (Fig. 1).

Because endoglin is an abundant protein associated with endothelial tissues we looked for reactivity of the SH-2 antibody towards different cell types throughout several embryonic tissues. The purpose of this was to compare the reactivity of cells within the bone marrow compartment with cells in endothelial and other tissues. The SH-2 antibody was previously reported to react with fibroblastic cells within the marrow stromal matrix, and to show no reactivity with osteoblasts, chondrocytes or skeletal muscle (12). A more recent assessment of reactivity towards different cells types indicated the presence of the antigen on vascular cells of developing dermis (14).

Fixed specimens of human fetal tissue of estimated gestational age 84 days were probed for reactivity with the antibody and the results are shown in Fig. 3. Positive antibody staining was detected within the bone marrow compartment of the femur and also in the periosteal layer (Figs. 3A and 3B). In the knee joint there was no apparent staining associated with cartilage chondrocytes or with meniscal cells, but positive staining was seen in the cells surrounding the developing vasculature in the meniscus (Fig. 3C). In the soft tissue of the hand (Figs. 3D and 3E) staining was

TABLE II

MALDI-TOF Analysis of Peptides Collected from a Tryptic Digest of the Antigen Immunoprecipitated with SH-2 Monoclonal Antibody

Fraction	Retention time (min)	Measured *m/z*	Calculated *m/z*	% Error	Sequence	Position	Modification
7	24.71	990.7	990.1	0.06	VLPGHSAGPR	210–219	
13	32.51	1651.9	1651.9	0.0	ETVHCDLQPVGPER	1–14	Acryl
15	35.00	1418.5	1418.7	0.01	TGTLSCTVALRPK	531–543	Acryl
17	35.12	1700.3	1699.9	0.02	GFKLPDTPQGLLGEAR	276–291	
		2932.8	2931.4	0.05	MLNA. . . CGGR	292–320	
			2928.3	0.15	ETVH. . .ERGE. . .QVSK	1–26	Acryl
19	41.46	1899.4	1899.1	0.02	AAKG. . .GDPR	498–516	
		2164.6	2163.5	0.05	NIRG. . .GEAR	273–291	Phosphate
			2163.4	0.06	TVFM. . .CTSK	554–572	Phosphate
22	45.04	2319.0	2319.6		EAHI. . .VTVK	204–224	Phosphate
32	56.58	3094.1	3094.5		TQIL. . .ERGP. . .ILLR	131–158	

Note. The mass search program MS-Fit (18) was used to search the Genbank database for identity. Two collected fractions, 17 and 19, gave spectra with two mass signals and the measured masses matched the calculated masses of several tryptic peptides derived from endoglin. All of the possible matches are included.

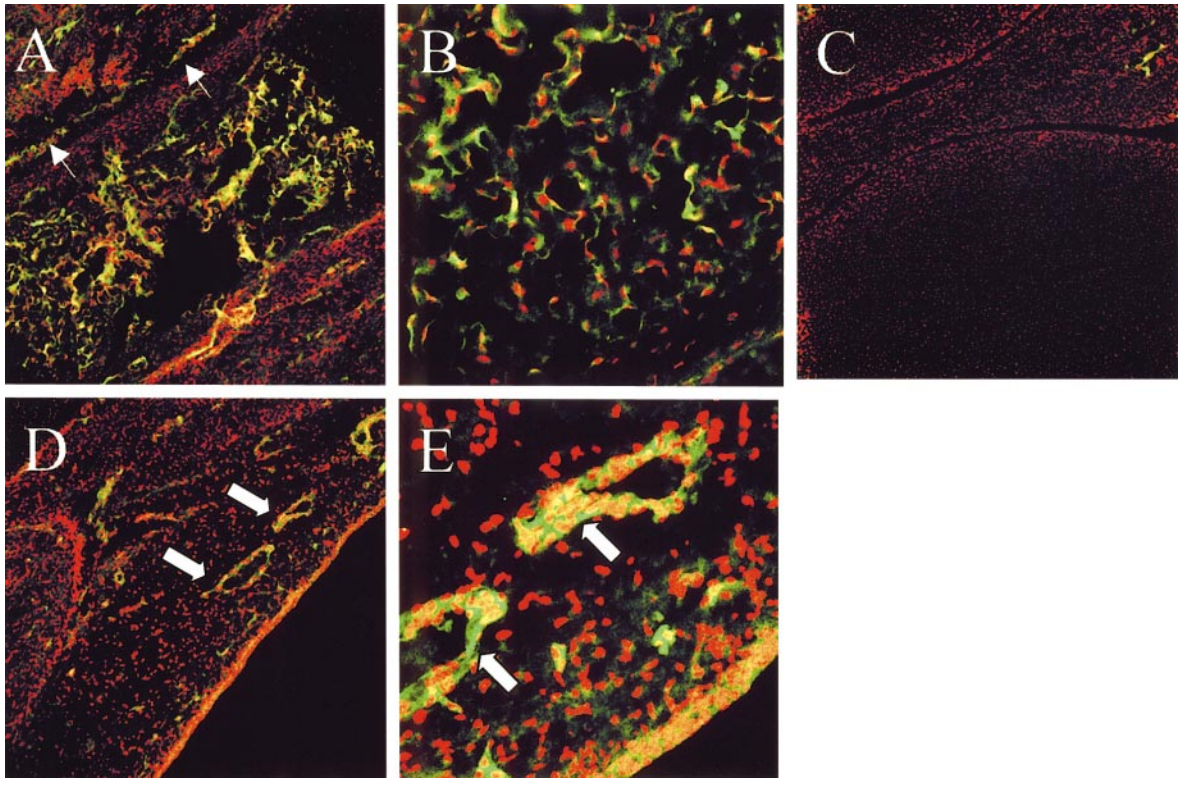

FIG. 3. SH-2 staining in human fetal tissues. Positive antibody staining was detected within the bone marrow compartment of the femur shown at 10× original magnification (A) and 40× original magnification (B). In (A) antibody staining was also evident in the periosteal layer. In the knee joint (C) there was no apparent staining associated with chondrocytes or with meniscal cells, but positive staining was seen in the cells surrounding the developing vasculature in the meniscus (arrow). In the soft tissue of the hand (D) staining was evident in the endothelial cells that surround developing blood vessels (arrows). These are shown in higher magnification (E) to show the specific staining of endothelial cells.

evident in the endothelial cells on developing blood vessels.

DISCUSSION

In this study we have identified the SH-2 antigen as endoglin (CD105), a TGF-β type III receptor that is present on many cell types. The identification was made by determination of the sequence and mass of a number of peptides derived from the protein that was specifically immunoprecipitated from human MSCs with the antibody.

The monomeric mass of the core protein of endoglin is 68,000 Da based on amino acid sequence data. The additional mass of the mature protein (97 Da) can be accounted for by N- and O-glycosylation. In fact endoglin contains 4 N-linkage sites and a putative O-glycosylation domain, all of these sites occurring on the extracellular portion of the molecule (17). Gouges and Letarte (22) showed that endoglin from the pre-B leukemic HOON cell line was reduced by 20,000 Da after treatment with *N*-glycosidase or endoglycosidase F and by 15,000 Da following *O*-glycanase and neuraminidase treatment. Our results are consistent with those measurements. Fleming *et al.* (14) showed a similar reduction in size after treatment with N-glycanase but not with *O*-glycanase.

The role of endoglin in signal transduction has been well characterized. Cheifetz *et al.* (19) showed that endoglin binds TGF-β1 and TGF-β3 with high affinity but not TGF-β2. In this sense endoglin is distinguished from betaglycan which binds to all three isoforms of TGF-β (19). It appears to form a complex with TGF-β receptors I and II (23) and presumably can play a role similar to that played by betaglycan (24) in regulating access to TGF-β. The key role played by endoglin in endothelial cell interactions or vascular development is underlined by the finding that hereditary haemorrhagic telangiectasia, an autosomal dominant disorder characterized by widespread vascular failure and haemorrhage, is caused by a mutation in the endoglin gene (25).

Endoglin may play a role in mediating interactions between haematopoietic and mesenchymal stem cells in the marrow. Since endoglin is expressed on early B-lineage precursor cells (CD19+ and CD34+), proerythroblasts (CD71+ and glycophorin A+) in fetal bone marrow (26), it is possible that interactions are

mediated via the cell-binding domains. Another possible role for endoglin on MSCs may be in mediating TGF-β signalling during chondrogenic differentiation. All TGFβ isoforms (2, 27) are capable of inducing MSCs from human and other species along the chondrogenic pathway. TGFβ-2 is also capable of initiating chondrogenesis but does not bind to endoglin, suggesting that other mechanisms are also possible.

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