Dkk-1-derived Synthetic Peptides and Lithium Chloride for the Control and Recovery of Adult Stem Cells from Bone Marrow*

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It is established that human mesenchymal stem cells (hMSCs) from bone marrow are a source of osteoblast progenitors in vivo and under appropriate conditions, differentiate into osteoblasts ex vivo. Because hMSCs are recovered by iliac crest aspirate and enriched by virtue of their adherence to tissue culture plastic, the cells provide a convenient ex vivo model for the study of osteogenic tissue repair in an experimentally accessible system. Recent advances in the field of skeletal development and osteogenesis have demonstrated that signaling through the canonical wingless (Wnt) pathway is critical for the differentiation of progenitor cell lines into osteoblasts. Inhibition of such signals can predispose MSCs to cell cycle entry and inhibit osteogenesis. Here, we report that synthetic peptides derived from the second cysteine-rich domain of the canonical Wnt inhibitor Dickkopf-1 (Dkk-1) have utility in controlling the growth and recovery of hMSCs from bone marrow stroma. Three peptides corresponding to residues 217–269 in Dkk-1 were each found to enhance the proliferation of hMSCs in culture over 2 days. The most active peptide exhibited agonistic characteristics in that it ablated the proliferation lag observed when cultures of hMSCs receive fresh medium. It also reduced the expression of endogenous Dkk-1 (Gregory, C. A., Singh, H., and Prockop, D. J. (2003) J. Biol. Chem. 278, 28067–28078). When the cytosolic level of β-catenin was elevated by addition of LiCl to cultures of hMSCs, the peptide also accelerated degradation of β-catenin on withdrawal of lithium. A second peptide, corresponding to residues 184–204 had preferential and high affinity for hMSCs in the log phase of proliferation. Peptide overlay assays on hMSC lysates confirmed that the peptide bound to a 184-kDa protein corresponding to the molecular mass of LRP6. Cells recovered by this peptide had enhanced osteogenic potential but less chondrogenic potential compared with controls. Because Wnt antagonists increase the number of non-committed hMSCs in culture, they may be of use in increasing the rate of osseous wound healing in vivo by increasing the level of systemically migrating hMSCs. Therefore, such molecules could contribute to the development of a novel family of pharmaceutical agents for the improvement of the healing process in humans.

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* The abbreviations used are: MSC, marrow stromal cells; hMSC, human marrow stromal cells; Dkk-1, Dickkopf-1; LRP6, low density lipoprotein-related protein 6; Cys2, carboxyl-terminal cysteine-rich (Cys2) domain; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; PBS, phosphate-buffered saline; BSA, bovine serum albumin; bis-Tria, 2-bis(2-hydroxyethylamino)-2-(hydroxymethyl)propane-1,3-diol; FACS, fluorescence-activated cell sorter; MES, 4-morpholineethanesulfonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; ALP, alkaline phosphatase.

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Peptide Agonists of Dkk-1 in Mesenchymal Stem Cells

MATERIALS AND METHODS

Tissue Culture of hMSCs—Bone marrow aspirates of ~2 ml were drawn from healthy donors ranging in age from 19 to 49 years under an Institutional Review Board approved protocol. The adherent nucleated cells were separated from the aspirate and cultured in α-minimal essential medium containing 20% (v/v) fetal calf serum as described previously (8, 25). After 14 days in culture, adherent cells were recovered by incubation with 0.25% (w/v) trypsin and 1 mM EDTA (Fisher) for 5 to 7 min at 37 °C (Fisher). For osteogenic differentiation of hMSCs at the log phase of growth, the cells were incubated with 1% (w/v) SDS, 1 mM EDTA (pH 8.0), and the equivalent of 1 half-tablet of Complete protease inhibitor (Roche). The lysate was sheared by passage through a 18-gauge needle and incubated at 4 °C for 20 min prior to electrophoresis and blotting. Fifteen μl (45,000 cells) were loaded onto a 4–12% NuPage bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA) and blotted onto polyvinylidene difluoride. The filter was washed in PBS (pH 7.4) containing 0.1% (v/v) Tween 20, blocked in 5% (w/v) nonfat dry milk, and then incubated in blocking buffer as the mobile phase. The membrane was washed thoroughly with water and allowed to dry. Colonies over 3000 cells per cm² were counted.

Peptides—Seven peptides (21 residues long) were synthesized by Tufts University Medical School Core Facility (Boston, MA) using an ABI 431 Peptide synthesizer employing FastMoc chemistry. To facilitate the synthesis of some of the peptides, some cysteine residues were substituted by serine residues. The peptides were biotinylated at the amino terminus and purified by reverse phase high performance liquid chromatography. To confirm purity and identity, the peptides were subjected to matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Ciphergen Chip Reader, Ciphergen Biosystems, CA) as described previously (10).

Immunocytochemistry—hMSCs were seeded at 1000 cells per cm² in 4-cm² chamber slides (Nunc, Fisher). When the hMSCs established a monolayer of the appropriate density, the slides were washed in PBS and the cells fixed in phosphate-buffered 4% (v/v) formaldehyde for 15 min.

Tissue Culture of hMSCs—hMSCs were re-plated in 54-cm² plates (Costar, Fisher) at a density of 100 cells/cm². For the analysis of hMSCs at the log phase of growth, the cells were re-plated in 54-cm² plates (Costar). The monolayers were fixed in phosphate-buffered 4% (v/v) formaldehyde for 2 min only. The monolayers were blocked in peptide block buffer consisting of PBS containing 5% (v/v) highly purified BSA (Sigma) for 1 h.

Peptide Overlay Assays—A monolayer of hMSCs was prepared from cultures at the log phase of growth. Approximately 3 million hMSCs on six 154-cm² tissue culture dishes were washed in PBS and scraped into 10 ml of PBS. The hMSCs were digested with 0.25% (v/v) trypsin and 1 mM EDTA at 37 °C for 10 min, and then incubated in 10 ml of fresh hMSC medium with hMSCs at the log phase of growth for 2 days. The most active peptide exhibited agonist activity.

Fluorescence-activated Cell Sorting—FACS sorting was achieved using an automated instrument (Cytomix FC 500, Beckman Coulter). The phycoerythrin-conjugated ABCG2 transporter antibody (clone 5D3) was purchased from eBioScience (San Diego, CA). All other fluorophore-conjugated antibodies used for FACS analysis, including isotype controls, were purchased from BD Pharmingen (San Diego, CA). Approximately 50,000 hMSCs were incubated in the presence of the manufacturer's recommended concentration of antibody diluted in PBS containing 1% (w/v) BSA and 1 mM EDTA for 1 h at room temperature. Prior to FACS analysis, the cells were washed in excess PBS containing 1% (v/v) BSA and 1 mM EDTA and 1% (v/v) paraformaldehyde.

Colonies Forming Unit Assay—Colonies forming unit assays were carried out as described previously (10). Briefly, hMSCs were counted by hemacytometer and 100 cells were transferred to a 154-cm² tissue culture dish (Costar, Fisher) containing 30 ml of culture medium. After 3 weeks, the dishes were washed in PBS and 1% (v/v) crystal violet in 50% methanol was used to stain actin filaments at a concentration of 1:1000 in PBS. Slides were dried and mounted with 4’,6-diamidino-2-phenylindole containing mounting reagent (Vector Laboratories, Burlingame, CA) and visualized on an upright epifluorescence microscope (Eclipse 800, Nikon). For peptide binding assays, the hMSCs were grown to a density of ~3000 cells per cm² (log phase) in 20,000 cells per cm² (fused) for 3 weeks. The colonies were dried and mounted with 4’,6-diamidino-2-phenylindole containing mounting reagent (Vector Laboratories, Burlingame, CA) and visualized on an upright epifluorescence microscope (Eclipse 800, Nikon). For peptide binding assays, the hMSCs were grown to a density of ~3000 cells per cm² (log phase) in 20,000 cells per cm² (fused) for 3 weeks.

Peptides corresponding to residues 217–269 in Wnt16 were identified as potential osteogenic differentiation agonists. These peptides were designed from a previously published study (10). To determine the synthesis of some of the peptides, some cysteine residues were substituted by serine residues. The peptides were biotinylated at the amino terminus and purified by reverse phase high performance liquid chromatography. To confirm purity and identity, the peptides were subjected to matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Ciphergen Chip Reader, Ciphergen Biosystems, CA) as described previously (10).

Immunocytochemistry—hMSCs were seeded at 1000 cells per cm² in 4-cm² chamber slides (Nunc, Fisher). When the hMSCs established a monolayer of the appropriate density, the slides were washed in PBS and the cells fixed in phosphate-buffered 4% (v/v) formaldehyde for 15 min. The cells were washed, then incubated in 1 h in a block buffer consisting of PBS containing 0.01% (v/v) Triton X-100 (Sigma) and 5% (v/v) normal goat serum (Chemicon International, Temecula, CA). After block, the slides were incubated in anti-β-catenin monoclonal antibody at a dilution of 1 in 5000 (clone 5H10, Chemicon) for 2 h. The slides were then washed 3 times in block buffer and incubated in Alexa Fluor 594- (red) or 488- (green)-conjugated anti-mouse secondary antibody (Molecular Probes). In negative controls, goat serum (Chemicon International, Temecula, CA) was used to stain actin filaments at a concentration of 1:1000 in PBS. Slides were dried and mounted with 4’,6-diamidino-2-phenylindole containing mounting reagent (Vector Laboratories, Burlingame, CA) and visualized on an upright epifluorescence microscope (Eclipse 800, Nikon). For peptide binding assays, the hMSCs were grown to a density of ~3000 cells per cm² (log phase) in 20,000 cells per cm² (fused) for 3 weeks. The colonies were dried and mounted with 4’,6-diamidino-2-phenylindole containing mounting reagent (Vector Laboratories, Burlingame, CA) and visualized on an upright epifluorescence microscope (Eclipse 800, Nikon). For peptide binding assays, the hMSCs were grown to a density of ~3000 cells per cm² (log phase) in 20,000 cells per cm² (fused) for 3 weeks.
For standard osteogenic differentiation, confluent monolayers of hMSCs were incubated in medium supplemented with 10^{-8} M dexamethasone, 50 \mu M ascorbic acid, and 5 mM \(-\)glycerol phosphate (Sigma) for 21 days with changes of medium every 5 days. For testing the effects of lithium, cultures were incubated under the same conditions but with the addition of 10 mM LiCl or KCl and incubation for up to 30 days. Quantification of staining was carried out using a modified version of a previously described procedure (27). Briefly, the cellular aggregates were washed in PBS, pressed flat with a Teflon-coated spatula, and fixed in formalin for 15 min. The cells were then stained with 40 mM Alizarin Red S for 30 min and washed 4 times with PBS. The stained cells were then transferred to a 2-ml screw-top microcentrifuge tube and incubated at 85 °C for 15 min in 1 ml of 10% (v/v) acetic acid overlaid with 0.5 ml of light mineral oil. The extraction was cooled on ice and then centrifuged at 21,000 g and 0.5 ml of the supernatant was transferred to a fresh tube containing 10% (v/v) 10% (v/v) ammonium hydroxide. The red solution was then transferred to a 96-well plate and read at 450 nm on a plate reader (Bio-Rad).

For adipogenic differentiation and quantification of Oil Red O staining—All reagents were purchased from Sigma. Confluent monolayers of hMSCs in 6-well plates (10 cm² per well) were incubated in medium supplemented with 10^{-8} M dexamethasone, 5 \times 10^{-8} M isobutylmethylxanthine, and 5 \times 10^{-7} M indomethacin. After 21 days, the adipogenic cultures were fixed in 10% formalin for 15 min and stained with fresh Oil Red-O solution in 60% (v/v) isopropyl alcohol in PBS for 20 min. The dishes were washed 3 times with excess PBS. Stained dishes were extracted with 2 ml of extraction buffer containing 50% (v/v) ethanol and 2% (w/v) SDS for 15 min at room temperature. Three aliquots (200 \mu l) of the extracted Oil Red O was transferred to a 96-well plate and quantified by absorbance measurement at 405 nm (Bio-Rad). The linear range of detection was determined by standard solutions of Oil Red O.

Chondrogenic Differentiation—Chondrogenic differentiation was carried out in accordance with Sekiya et al. (8) on 200,000 cells recovered by either peptide B recovery or standard means.

Evaluation of Cell Number—For assay of proliferation, cells were quantified by fluorescent labeling of nucleic acids (CyQuant dye; Molecular Probes) using a described previously method (10) and a microplate fluorescence reader (FLX800; Bio-Tek Instruments Inc., Winooski, VT) set to 480 nm excitation and 520 nm emission. Data from 3 separate assays were statistically analyzed using the two-tailed Student’s t test. Experiments were repeated using hMSCs from two donors.

Extraction of Cytoskeletal Fractions and Western Blotting—Triton-insoluble fractions were prepared using a protocol by Ko et al. (26) with modifications (10). Briefly, one million cells were suspended in 1 ml of ice-cold PBS with protease inhibitors (Roche Diagnostics) and 1% (v/v) Triton X-100 (Sigma). Lysis proceeded for 10 min on ice followed by a 60-s centrifugation at 800 g to remove particulate bodies. The cytoskeletal pellet was separated from the cytoplasmic fraction by centrifugation at 14,000 g for 15 min and resuspended in PBS containing 0.1% SDS. The concentration of protein was measured by Bradford assay (Sigma) prior to Western blotting. Electrophoresis was carried out using commercial reagents and systems (Novex; Invitrogen). Approximately 10 \mu g of protein were added to the appropriate volume of

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**FIG. 1.** Design of Dkk-1-derived peptides. Panel a, diagram of the Dkk-1 molecule illustrating the presence of two cysteine-rich domains separated by a “hinge” region. The LRP6 binding domain is in the second cysteine-rich (Cys2) domain. Panel b, the sequence of the second cysteine-rich domain of Dkk-1 (bold) with the sequences of seven overlapping peptides corresponding to the Dkk-1 Cys2 sequence. A list of the peptides synthesized and tested in this study is given below the sequence. Serines substituted from cysteines to facilitate synthesis are denoted in lowercase type. Panel c, MALDI-TOF mass spectrometry of the synthesized peptides demonstrate that the calculated molecular masses correspond to the observed masses to within 2 Da.

### Peptide Agonists of Dkk-1 in Mesenchymal Stem Cells

**Osteogenic Differentiation and Quantification of Alizarin Red S Staining**—For standard osteogenic differentiation, confluent monolayers of hMSCs were incubated in medium supplemented with 10^{-8} M dexamethasone, 50 \mu g ml^{-1} ascorbic acid, and 5 \mu M \(\beta\)-glycerol phosphate (Sigma) for 21 days with changes of medium every 5 days. For testing the effects of lithium, cultures were incubated under the same conditions but with the addition of 10 mM LiCl or KCl and incubation for up to 30 days. Quantification of staining was carried out using a modified version of a previously described procedure (27). Briefly, the cellular aggregates were washed in PBS, pressed flat with a Teflon-coated spatula, and fixed in formalin for 15 min. The cells were then stained with 40 mM Alizarin Red S for 30 min and washed 4 times with PBS. The stained cells were then transferred to a 2-ml screw-top microcentrifuge tube and incubated at 85 °C for 15 min in 1 ml of 10% (v/v) acetic acid overlaid with 0.5 ml of light mineral oil. The extraction was cooled on ice and then centrifuged at 21,000 g and 0.5 ml of the supernatant was transferred to a fresh tube containing 10% (v/v) 10% (v/v) ammonium hydroxide. The red solution was then transferred to a 96-well plate and read at 450 nm on a plate reader (Bio-Rad). The linear range of detection was determined by standard solutions of Oil Red O.

**Chondrogenic Differentiation**—Chondrogenic differentiation was carried out in accordance with Sekiya et al. (8) on 200,000 cells recovered by either peptide B recovery or standard means.
FIG. 2. Binding of hMSCs by the Dkk-1-derived peptides. Panel a, whole cell binding assay of peptide binding to intact hMSCs in monolayer culture at the log phase of growth. The biotinylated peptides were incubated with the cells, then binding was detected by fluorescently conjugated streptavidin. Fluorescent signal (left) is presented next to the phase image (right). The control (ctrl) was carried out in the absence of peptide. Panel b, peptide overlay assay demonstrating that peptide B and to a lesser extent, peptide E recognizes a 184-kDa protein on blotted whole cell lysates of hMSCs at the log phase of growth. The molecular mass of LRP6 is ~185 kDa. Equal loading of the blots was confirmed by Ponceau Red staining.
RESULTS

Peptide Design—Because Dkk-1 acts to prevent the coalescence of LR6P and Frizzled by binding to LR6P via its carboxy-terminal cysteine-rich (Cys2) domain (23, 24) (Fig. 1, panel a), 7 overlapping 21-mer peptides corresponding to the Cys2 domain of the ligand were synthesized (Fig. 1, panel b). The peptides were conjugated to biotin at the amino terminus and not directly related to hematopoietic tissue (34, 35) (Fig. 3, panel a). The binding fraction was seeded into 45-cm² tissue culture dishes and incubated for 2 days in the presence of 50% fresh hMSC tissue culture medium supplemented with 50% hMSC preconditioned tissue culture medium prepared as described under “Materials and Methods.” After 2 days, clusters of adherent cells could be visualized by phase-contrast microscopy (Fig. 3a, left frame) with a small number of non-adherent cells floating in the medium. At day 2, the medium was replaced by fresh 50% conditioned medium and incubated for a further 2 days prior to replacement with fresh unconditioned medium. Thereafter, the medium was replaced every 3 days for 2 weeks resulting in rapidly dividing adherent cells within colonies that rapidly coalesced to establish a partial monolayer (Fig. 3a, right frame). Negative control extractions that lacked peptide did not establish rich cultures, but there were a few adherent cells present that did not readily divide.

Data from FACS analysis of the adherent cells from 2-week cultures demonstrated a cell surface phenotype consistent with hMSCs. The cells expressed CD90 and HLA I but not CD34, CD45, HLA II, CD109, CD117 (Kit), or the Hoescht 33342 effluxing ABC transporter, suggesting that the cells are MSCs and not directly related to hematopoietic tissue (34, 35) (Fig. 3, panel b). To measure the frequency of hMSCs with affinity for peptide B, hMSCs bound to the peptide were extracted from 2 million nucleated bone marrow cells, allowed to adhere to tissue culture plastic for 15 h, and then counted by the fluorescence incorporation assay. Routinely, 200 cells were recovered in these experiments. To test the efficiency of the extraction, 2 million bone marrow cells were spiked with 10,000 rapidly dividing hMSCs that were recovered by scrap-
ing, then subjected to peptide B mediated recovery. Approximately 5,000 cells were recovered in each instance, suggesting that the procedure had an efficiency of about 50%. Correcting for an efficiency of 50%, the frequency of hMSCs in the nucleated fraction of bone marrow with affinity for peptide B was therefore \( \frac{1}{10,000} \) (Fig. 4, panel a). Recovery of hMSCs by the standard method of plastic adherence for 15 h in culture was also assayed by the same method. The percentage recovery ranged from 0.1 to 0.2%, roughly 5–10 times the recovery with peptide B. Although the plates were washed thoroughly with PBS to remove non-adherent cells, the MSCs frequently bind to hematopoietic cells when adhering to plastic and this phenomenon could contribute to the elevated fraction of cells detected by the original assay. Furthermore, larger, less clonogenic cells present in the aspirate may be recovered by plastic adherence but not by peptide B affinity, also resulting in higher initial recoveries by the original method.

The hMSCs Recovered by Peptide B Affinity Differentiate into Adipocytes and Osteoblasts—To determine whether the hMSCs recovered by peptide B affinity were multipotent, they were induced to differentiate into mesenchymal tissue lineages. Under standard conditions, the hMSCs differentiated into adipose tissue, as assayed by Oil Red O staining and osteoblasts, as assayed by Alizarin Red S staining and chondrocytes, as assayed by Toluidine Blue and Safranin O (Fig. 5, panels a–c). When compared with MSCs recovered from the same donor by standard methods, it was found that peptide B recovered cells more readily differentiated to osteoblasts with about 2-fold higher efficiency (Fig. 5, panel c) but this was at the expense of chondrogenic potential (Fig. 5, panel b). Indeed, peptide B recovered MSCs only partially differentiated into chondrocytes capable of depositing sulfated proteoglycan (purple with Toluidine Blue and Red with Safranin O) suggesting that the peptide B selection process enriched for cells with a propensity to form bone but not cartilage.

**Peptides Mapping to the Carboxyl-terminal End of the Dkk-1 Cys2 Domain Mimics the Activity of Dkk-1 in hMSCs**—Because Dkk-1 was previously shown to predispose hMSCs to entry into

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**FIG. 3.** Recovery of hMSCs from whole bone marrow aspirates by peptide B-mediated magnetic cell sorting. Panel a, strategy for recovery of hMSCs from whole bone marrow using magnetic activated cell sorting (above). The nucleated cells from bone marrow aspirates are incubated with biotinylated peptide B then recovered by paramagnetic beads coated with streptavidin. Phase-contrast micrographs of the recovered hMSCs after 48 h incubation (lower left) and 2 weeks (lower right) are presented. Panel b, fluorescence activated cell sorting of hMSCs after 2 weeks in culture. The cells express CD90 and HLA I but not CD34, CD45, HLA II, CD109, CD117 (Kit), or the Hoescht 33342 effluxing ABC transporter suggesting that the cells are MSCs and not directly related to hematopoietic tissue (34, 35). The appropriate isotype control profile is presented with each assay. The experiments were repeated on cells from two separate donors.
the cell cycle, the peptides were tested in hMSC proliferation assays (10). When hMSCs were plated at 500 cells per cm² in medium containing 2% (v/v) fetal bovine serum, and 10 μg ml⁻¹ of peptides corresponding to the carboxyl-terminal portion of the Cys2 domain (from Cys²¹⁷–Ala²⁶⁹, peptides D–F), proliferation was accelerated resulting in a 30% increase (p < 0.01) in cell number after 2 days (Fig. 6, panel a). The increase in hMSC recovery was probably attributable to an ablation of the 12-h
FIG. 5. Differentiation of peptide B recovered hMSCs into osteoblasts and adipocytes. Panel a, cultures of peptide B recovered and standard hMSCs were expanded to the stationary phase of growth and incubated in adipocytic medium for 21 days. The monolayers were then stained with Oil Red O to detect fat droplets. Micrographs (right) demonstrate lipid formation by peptide B-derived cells in the presence, but not
lag phase of growth observed on changes of medium when Dkk-1 levels are suboptimal (10). A similar lag phase ablation could be observed when hMSCs from two donors were exposed to medium containing 10 μg ml⁻¹ peptide E, whereas control cultures treated with peptide A exhibited the lag phase (Fig. 6, panel b). Furthermore, endogenous Dkk-1 transcription was down-regulated in response to peptide E treatment as assayed by RT-PCR (Fig. 6, panel c). This is consistent with the observation that Dkk-1 expression is regulated by negative feedback (10). Also peptide E induced an overall reduction of β-catenin in hMSCs when assayed by immunocytochemistry similar to the effect seen with recombinant Dkk-1 (10).

The Effect of LiCl and Dkk-1-derived Peptides on hMSCs in Culture—In previous studies, BMP2 has been shown to induce osteogenic differentiation in a canonical Wnt-dependent manner in a number of mesenchymal progenitor cell lines (12, 13). Furthermore, inhibition of the canonical Wnt signaling pathway by Dkk-1 was shown to inhibit such differentiation (12–14) and predispose hMSCs to cell cycle entry (10). To investigate the effect of canonical Wnt signaling on hMSC differentiation and to evaluate the effects of Dkk-1-derived peptides further, Wnt signaling was mimicked by the addition of LiCl to rapidly dividing cultures of hMSCs (31). In initial assays of proliferation, 10–15 mM lithium reduced the rate of hMSC division in a dose-dependent manner over 7 days, whereas control cultures treated with KCl were unaffected (Fig. 7, panel a). Concentrations above 20 mM caused complete cell death over 7 days (data not shown). To test whether LiCl treatment had an effect on the levels of cytosolic β-catenin, a fractionation technique was employed based on detergent extraction and Western blot assays of the cytoskeletal and cytosolic proteins (Fig. 7, panel b) (26). The fractionation was evaluated by simultaneous Western blot assays of actin and GAPDH. GAPDH was not detected in the cytoskeletal fraction where the majority of actin was detected. The cytosolic fraction contained all of the detectable GAPDH and some actin, presumably not associated with microfilaments. In the KCl-treated control cultures, the majority of the β-catenin was present in the insoluble fraction, possibly as actin-associated adherens junctions, and trace levels were detectable in the soluble fraction of the cells (Fig. 7, panel b). LiCl-treated cells also had high levels of β-catenin associated with the insoluble fraction but there were also high soluble levels. The observation that cytosolic β-catenin was increased in response to lithium is consistent with the conclusion that activation of canonical Wnt signaling inhibits hMSC proliferation. Because positive canonical Wnt signaling seems to enhance osteogenic differentiation of a number of mesenchymal cell lines (12–13), the effect of lithium on hMSCs in inhibiting proliferation probably represents an initial stage of osteogenic differentiation.

The effect of peptide E on β-catenin distribution was evaluated in hMSCs pretreated with LiCl. After pretreatment of hMSCs with lithium to increase cytosolic β-catenin, the hMSCs were transferred to fresh medium containing 10 μg ml⁻¹ of peptide E or peptide A as a control. After 6 h, the hMSCs were recovered and assayed for β-catenin distribution. The level of soluble β-catenin was reduced in peptide E-treated hMSCs, suggesting that its degradation had been accelerated (Fig. 7, panel c). This observation is consistent with the prediction that peptide E inhibits canonical Wnt signaling by preventing the Wnt-mediated formation of the Frizzled/LRP co-receptor complex and thus reducing the activity of Dishevelled in inhibiting glycogen-synthetase kinase 3β.

The Effect of LiCl on Osteogenesis in an Osteogenic Differentiation Assay—To investigate the effect of lithium directly on osteogenesis, the hMSCs were grown to confluence and treated for up to 30 days with an osteogenic medium containing a 100-fold lower than standard concentration of dexamethasone and 10 mM LiCl or 10 mM KCl. Lower levels of dexamethasone were used to improve the detection of differences in osteogenesis induced by LiCl. Under these conditions, the hMSC monolayer detached from the plastic and spontaneously curled into a roughly spherical cellular aggregate. In the presence of LiCl, the aggregate was formed after about 7 days of treatment, whereas in the presence of KCl, the effect was seen after about 12 days. The aggregates were fixed, paraffin embedded, sectioned, and stained for calcified deposits (Alizarin Red S), sulfated proteoglycan (Toluidine Blue), and collagenous deposits (Trichrome). On sectioning and staining, the overall morphology of the LiCl-treated aggregates were surprisingly different from the controls in that they were much more compact (Fig. 8, panel a), suggesting that the intercellular contacts were much more adherent. Control micromasses had a more open, lamellar, morphology (Fig. 8, panel a). Staining of the sections with Alizarin Red S revealed that mineralization was much more evident in the LiCl-treated aggregates (Fig. 8, panel a) than in the control with dense patches of mineral detected throughout the sections of the LiCl-treated cells. In both cases, there was little evidence of sulfated proteoglycan deposition as demonstrated by Toluidine Blue (data not shown). The matrix adjacent to the constituent cells in the LiCl-treated aggregates appeared to be collagenous in nature as demonstrated by Trichrome staining (data not shown).

To semiquantify the apparent enhancement of Alizarin staining in LiCl-treated cultures of hMSCs, Alizarin Red S was extracted and measured over a time course of osteogenic differentiation. Over 27 days, the LiCl-treated cultures exhibited an enhanced rate of mineralization when compared with KCl-treated cells as determined by Alizarin Red S staining (Fig. 8, panel b). Untreated hMSCs in the absence of osteogenic supplements also exhibited a degree of mineralization but to a lesser extent than the cultures incubated in osteogenic medium (Fig. 8, panel b), demonstrating that osteogenesis is the default fate of hMSCs grown for long periods in stationary cultures. To investigate the effect of LiCl treatment more closely, total RNA was extracted from the mineralizing micromasses at 10-day intervals. The transcription of genes known to play a role in osteogenic development was assayed by RT-PCR. Because of the surprising enhancement of cellular adhesion, the transcription of gap junctional proteins, connexin 43 and connexin 45 were assayed, because both of these proteins are known to play a role in osteogenesis (32). The levels of connexin 43 remained constant throughout the time course of osteogenesis. Interestingly, connexin 45 was present in untreated cultures but was

absence, of adipocytic medium. Back extraction of the dye at weekly intervals and colorimetric quantification (left) demonstrate extensive Oil Red O staining throughout both the monolayers (n = 6, error bars = 1 S.D.) Duplicate experiments were carried out on two separate donors. Panel b, pellets (200,000 cells) of peptide B recovered and standard hMSCs were incubated for 2 days in chondrocyte medium. The pellets were then stained with Toluidine Blue and Safranin O to evaluate proteoglycan deposition. Panel c, cultures of peptide B recovered and standard hMSCs were expanded to the stationary phase of growth and incubated in osteogenic medium for 21 days. The monolayers were then stained with Alizarin Red S to detect mineral. Micrographs (right) demonstrate mineral deposition by peptide B-derived cells in the presence, but not absence, of osteogenic medium. Back extraction of the dye at weekly intervals and colorimetric quantification (left) demonstrate extensive Alizarin Red S staining throughout the monolayer (n = 6, error bars = 1 S.D.) that was more intense in the peptide B recovered cells than the control.

p values: p < 0.05 (*) , p < 0.01 (**) for n = 6. Duplicate experiments were carried out on cells from two separate donors.
FIG. 6. Peptide E mimics the action of Dkk-1 on hMSCs. **Panel a**, fluorescence incorporation assay demonstrating that overlapping peptides corresponding to amino acids 217–269 (peptides D, E, and F) increase the rate of hMSC proliferation. Log phase cultures of hMSCs were incubated for 2 days in medium containing 2% (v/v) fetal calf serum in the presence of vehicle or 10 µg ml⁻¹ of each peptide. The cells were then recovered and counted. *p* values versus control: *p* < 0.05 (*), *p* < 0.01 (**) for *n* = 3. Experiments were duplicated with cells from two donors. **Panel b**, fluorescence incorporation assay demonstrating that peptide E ablates the lag phase of hMSC growth observed on replacement of medium. Log phase cultures of hMSCs were incubated for 24 h in fresh medium containing 2% (v/v) fetal calf serum in the presence of vehicle or 10 µg ml⁻¹ of peptide E or peptide A (control). After 12 and 24 h, hMSCs were recovered and counted. Data on cells from two donors are expressed as the mean of 3 counts with error bars representing 1 S.D. Statistically significant differences in data are *p* < 0.05 (*). **Panel c**, RT-PCR assay demonstrating that peptide E reduced the rate of endogenous Dkk-1 expression. Log phase cultures of hMSCs were incubated for 8 h in fresh medium containing 2% (v/v) fetal calf serum in the presence of vehicle or 10 µg ml⁻¹ of peptide E or peptide A (control). Data on cells from 2 donors are presented in duplicate with GAPDH levels to control for mRNA quality. **Panel d**, immunohistochemistry of late log phase cultures of hMSCs demonstrates that peptide E reduces the level of cytoplasmic β-catenin. β-Catenin is stained green, actin is stained red, and the nuclei are stained blue.
completely down-regulated on exposure to osteogenic medium (Fig. 8, panel c). Surprisingly, LiCl failed to effect transcription of either connexin 43 or connexin 45 and therefore did not appear to account for the enhanced cellular adherence or mineralization on treatment with LiCl. RT-PCR assays for osteonectin demonstrated that lithium treatment reduced the rate of its transcription at all of the time intervals tested (Fig. 8, panel c). Because lithium has roughly the same effect as the
FIG. 8. Long-term treatment of hMSCs with 10 mM LiCl improves the differentiation of hMSCs into osteoblasts. Panel a, ARS sections of mineralizing micromass cultures treated with 10 mM LiCl or KCl (control). Note that lithium treatment results in a more tightly packed and intensely stained culture. Panel b, extraction and colorimetric quantification of ARS confirms that lithium increases the rate of osteogenic differentiation by hMSCs. *p < 0.05, **p < 0.01 for n = 6. Experiments were duplicated with 2 donors. Supplemented medium refers to complete medium containing osteogenic supplements as defined under “Materials and Methods.” Panel c, RT-PCR assays for ALP demonstrates that addition of 10 mM lithium causes earlier and higher levels of ALP expression in mineralizing cultures of hMSCs. Data are presented with cells from 2 separate donors. Conn43 and Conn45, connexins 43 and 45.
EF-hand domain of osteonectin on the development of *Xenopus laevis* embryos (33), it is possible that lithium treatment results in negative feedback of the expression of osteonectin. The transcription of type X collagen was up-regulated in osteogenic medium but unaffected by the addition of lithium, whereas alkaline phosphatase (ALP) activity was profoundly up-regulated. In KCl-treated cultures, ALP transcription was maximally up-regulated after 30 days of mineralization, whereas in the presence of lithium, the level of ALP transcription reached maximal transcription by day 10 (Fig. 8, panel c). These observations are in agreement with previous work demonstrating the requirement for sustained levels of cytosolic and nuclear β-catenin for ALP transcription (12).

The Effect of Peptide E on hMSC Osteogenesis—Initial experiments on stationary cultures of hMSCs in mineralizing conditions failed to detect an effect of peptide E on osteogenic differentiation (data not shown). Because peptide E was previously shown to act on rapidly dividing hMSCs (Fig. 6, panel b), log phase cultures were assayed. LRP6 expression is highest in rapidly dividing hMSCs and therefore the effect of peptide E in agonizing the action of Dkk-1 on LRP6 should be more readily detected. Cells were plated at 1000 cells per cm² in 6-well plates with complete medium and allowed to adhere for 15 h. The next day, medium was replaced by osteogenic medium containing 50 μg ml⁻¹ peptide E or peptide A as a control. At 7-day intervals, the hMSCs were recovered from 3 wells of a 6-well plate and assayed for cell number. The cells in the remaining 3 wells were fixed and stained with Alizarin Red S for dye extraction and quantification of mineralization. The level of Alizarin Red S staining per cell was calculated and plotted for 21 days. In this modified assay, the hMSCs remained as a monolayer and mineralized with far less efficiency (Fig. 2, panel c). Furthermore, peptide B bound to a few isolated cells in stationary phase cultures, had affinity for a few isolated cells in stationary phase cultures, which subsequently failed to detect an effect of peptide E on osteoblast differentiation after 7 days of treatment (Fig. 9, inset). On quantifying the Alizarin Red staining, peptide E reduced the rate of osteogenic differentiation by hMSCs when compared with peptide A (Fig. 9), providing further evidence that peptide E inhibits the effect of canonical Wnt signaling during osteogenesis.

**DISCUSSION**

Because hMSCs can differentiate into numerous mesenchymal tissue lineages including osteoblasts, chondrocytes, adipocytes, and neural precursors (1–8), there has been much interest in the cells and their potential application in cytotherapy, bioengineering, and gene therapy (9). The rapid expansion of hMSCs under simple culture conditions is an attractive characteristic of the cells and it has been the subject of much investigation (1, 8, 10). In investigating secreted factors that regulate the growth of hMSCs, it was found that synthesis of the canonical Wnt inhibitor Dkk-1 was required before the cells entered the cell cycle (10). Furthermore, Wnt signaling apparently plays an essential role in the differentiation of pluripotent cell lines into osteoblasts (12–14). Indeed, it has been shown that a high systemic level of Dkk-1 contributes to the persistence of osteolytic lesions in cases of multiple myeloma (14), and mutations in the LRP6 receptor that prevents the binding of Dkk-1 cause abnormally high bone density (15).

In this study, we sought to design overlapping peptides derived from the Cys2 domain of Dkk-1 and characterize their effects on hMSC proliferation and differentiation. In brief, four peptides of interest were identified. One peptide (Leu184–Ser204), peptide B) had a high specific affinity for the receptor LRP6 and rapidly dividing hMSCs. Furthermore, it was effective as an affinity molecule for the recovery of highly clonogenic hMSCs from whole bone marrow aspirates. A further three peptides, corresponding to the extreme carboxyl terminus (Cys217–Arg237, peptide D; Cys233–Cys253, peptide E; and Glu249–Ala269, peptide F), increased the rate of proliferation of hMSCs. In *ex vivo* bioassays, peptide E was found to robustly exhibit bioactivity consistent with it acting as a Dkk-1 agonist.

Peptide B was initially identified by virtue of its high affinity for fixed intact hMSCs in monolayer culture (Fig. 2, panel a). In the same assay, the peptide was found to be specific for semi-confluent, rapidly dividing hMSCs and although the peptide had affinity for a few isolated cells in stationary phase cultures, the peptide did not bind the majority of cells in the confluent monolayer (Fig. 2, panel c). Furthermore, peptide B bound to a 184-kDa species when used to probe blotted lysates of log phase hMSCs (Fig. 2, panel b), suggesting that the peptide had affinity for its intended target, the 184-kDa LRP6 receptor. These data are in agreement with the observation that LRP6 expression is high in rapidly dividing hMSC cultures and low in stationary phase cultures (10). The detection of a few isolated hMSCs with affinity for peptide B on stationary phase mono-

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**FIG. 9.** Treatment of log phase hMSCs with peptide E inhibits the differentiation of hMSCs into osteoblasts. Extraction and colorimetric quantification of Alizarin Red S demonstrates that peptide E decreases the rate of osteogenic differentiation by dividing hMSCs. Data are expressed as the mean of 3 Alizarin Red S readings divided by the mean of three CyQuant cell counts. Error bars represent the maximum error possible based on the 2 S.D. Statistically significant differences in data are *p* < 0.01 (**). Phase micrographs are presented (inset) demonstrating the presence of clusters of mineralizing cells at day 14 in the control that are not present on treatment with peptide E.
layers demonstrates variable but generally low levels of expression of LRP6 under these conditions. The peptide was used to recover hMSCs with high clonogenic potential from the nucleated fraction of bone marrow. MSCs recovered by this method constituted about 0.02% of the total number of cells in the bone marrow extract, ~5–10-fold lower than the traditional recovery technique involving only adherence to plastic. The higher recovery when solely adherence is used is probably because of the presence of hMSCs with a lower clonogenic potential that would not be selected by peptide B. Furthermore, it is likely that hematopoietic cells are carried through with the hMSCs in the initial stages of plating, also accounting for the increased initial cell recovery. Peptide B-recovered cells had a higher clonogenic potential than standard preparations of hMSCs (Fig. 4, panel b), confirming that the peptide had excluded hMSCs with lower clonogenic potential. The highly clonogenic hMSCs were also pluripotent in that they readily differentiated into bone, adipose, and chondrocytes (Fig. 5). When compared with control MSCs recovered by standard means, the peptide B-recovered cells more efficiently deposited a calcium-rich matrix during osteogenic assays in vitro, but they less readily differentiated into chondrocytes capable of depositing sulfated proteoglycan. Because the selection process enriches for cells expressing the LRP6 receptor, a known regulator of osteogenesis in humans (15, 16), it is likely that the peptide enriches for an osteogenic progenitor subpopulation with a propensity to form bone but not cartilage. Because peptide B recovers hMSCs with a significantly higher clonogenic potential, it is likely to have future utility in hMSC isolation and also measurement of hMSC levels in bone marrow and blood.

In assays of hMSC proliferation a further three peptides exhibited bioactivity consistent with a Dkk-1 agonist. The peptides corresponding to the extreme carboxyl terminus (Cys217–Arg237, peptide D; Cys232–Cys253, peptide E; and Glu249–Ala269, peptide F), increase the initial rate of proliferation of hMSCs in ex vivo bioassays (Fig. 6, panel a). The action of the peptides was short lived and could be detected only for up to 2 days in time courses, presumably because of the short half-life in the medium. Peptide E also behaved in a similar manner to Dkk-1 by abating the 12-h lag phase in proliferation detected on a change of culture medium (Fig. 6, panel b) (10) and by reducing the endogenous level of Dkk-1 transcription. Based on these observations it is probable that peptide E mimics the activity of Dkk-1 by preventing the binding of Wnt ligands or the co-receptor Frizzled to the target molecule, LRP6. To explore the action of peptide E further, its effect on β-catenin levels and distribution was examined. Because inhibition of canonical Wnt signaling has a profound effect on β-catenin levels, a Dkk-1-like inhibitor of Wnt signaling would therefore act to release the inhibition of glycosyl synthetase kinase 3 and accelerate β-catenin phosphorylation and degradation. Addition of lithium to hMSCs mimicked positive Wnt signaling has a profound effect on β-catenin levels. Lithium addition of peptide E, the lithium-induced accumulation of cytosolic β-catenin was degraded more rapidly than in cultures treated with a control peptide. These data strongly suggest that peptide E acts as a Wnt inhibitor in a manner similar to Dkk-1 itself.

In previous studies, BMP2 has been shown to induce osteogenic differentiation in a canonical Wnt-dependent manner in a number of mesenchymal progenitor cell lines (12, 13). Furthermore, inhibition of the canonical Wnt signaling pathway by Dkk-1 was shown to inhibit such differentiation (12–14) and predispose hMSCs to cell cycle entry (10). Consistent with these observations, we observed that addition of lithium to cells at the late phase of growth reduced the rate of proliferation in a dose-dependent manner (Fig. 7, panel a). Incubation of confluent cultures in the presence of standard osteogenic medium with 100-fold lower than standard concentration of dexamethasone (1 $\times$ 10$^{-10}$ M) resulted in detachment of the monolayer from the plastic and formation of a roughly spherical aggregate of hMSCs that formed a calcified matrix as determined by Alizarin Red S staining. Incubation of hMSCs under the same conditions but with 10–15 mM LiCl formed a similar, but much more compact ball of mineralizing cells. Quantiﬁcation of mineral by colorimetric measurement of Alizarin Red S staining demonstrated that LiCl-treated cultures produced mineralized matrix more rapidly than the controls. Analysis of gene expression by the differentiating hMSCs revealed that transcripts commonly associated with osteogenesis increased over time in osteogenic medium both in the presence and absence of LiCl but there was a striking up-regulation of alkaline phosphatase transcription. Alkaline phosphatase transcription was maximal after 10 days in lithium-treated culture compared with 30 days in the controls, an observation consistent with cell line studies (12, 13).

Because inhibition of canonical Wnt signaling has been shown to inhibit the differentiation of osteogenic precursors to osteoblasts in a number of systems (12–14), we therefore decided to test the effects of peptide E on osteogenic differentiation of hMSCs. In initial experiments on stationary cultures of hMSCs, peptide E had no effect on osteogenic differentiation (data not shown). The reason for this is unclear, but it is likely that in the process of becoming a stationary phase culture, hMSCs irreversibly commit to an osteogenic lineage. Indeed, long-term cultures of hMSCs spontaneously form mineral even in the absence of osteo-inductive supplements (27). It is likely that inhibitors of Wnt signaling work to sustain the undifferentiated state of hMSCs before an irreversible cascade of osteogenic commitment, therefore log phase cultures were assayed. Although hMSCs did not efficiently form mineralized deposits in proliferative conditions, peptide E treatment resulted in even lower levels of mineralization suggesting that the action of peptide E was confined to non-committed and dividing hMSCs.

In this study, we describe the production of synthetic inhibitors of the canonical Wnt signaling pathway based on peptide analogues of Dkk-1. The agents described here that affect the canonical Wnt pathway may be useful for controlling hMSCs in the treatment of bone fractures and skeletal lesions such as those observed in multiple myeloma. They also may have utility in producing subpopulations of MSCs with a greater propensity for osteogenesis.

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