Cytoskeletal Organization of Human Mesenchymal Stem Cells (MSC) Changes During Their Osteogenic Differentiation

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Abstract   Human MSCs have been studied to define the mechanisms involved in normal bone remodeling and the regulation of osteogenesis. During osteogenic differentiation, MSCs change from their characteristic fibroblast-like phenotype to near spherical shape. In this study, we analyzed the correlation between the organization of cytoskeleton of MSCs, changes in cell morphology, and the expression of specific markers (alkaline phosphatase activity and calcium deposition) of osteogenic differentiation. For osteoblastic differentiation, cells were cultured in a culture medium supplemented with 100 nM dexamethasone, 10 mM β- glycerophosphate, and 50 μg/ml ascorbic acid. The organization of microfilaments and microtubules was examined by inmunofluorescence using Alexa fluor 594 phalloidin and anti α-tubulin monoclonal antibody. Cytochalasin D and nocodazole were used to alter reversibly the cytoskeleton dynamic. A remarkable change in cytoskeleton organization was observed in human MSCs during osteogenic differentiation. Actin cytoskeleton changed from a large number of thin, parallel microfilament bundles extending across the entire cytoplasm in undifferentiated MSCs to a few thick actin filament bundles located at the outermost periphery in differentiated cells. Under osteogenic culture conditions, a reversible reorganization of microfilaments induced by an initial treatment with cytochalasin D but not with nocodazole reduced the expression of differentiation markers, without affecting the final morphology of the cells. The results indicate that changes in the assembly and disassembly kinetics of microfilaments dynamic of actin network formation may be critical in supporting the osteogenic differentiation of human MSCs; also indicated that the organization of microtubules appears to have a regulatory role on the kinetic of this process. J. Cell. Biochem. 93: 721–731, 2004.   © 2004 Wiley-Liss, Inc.

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The bone marrow stroma contains cells, the mesenchymal stem cells (MSCs) that have the potential to differentiate into bone, cartilage, adipocytes, muscle, or connective tissue [Caplan, 1991; Haynesworth et al., 1992a,b].

Recently, human MSCs have been studied in sufficient detail to define the mechanisms involved in normal bone remodeling and the regulation of osteogenesis under health and disease conditions, since altered dynamic responses of bone cells progenitors may be responsible for specific bone diseases [Gimble et al., 1996; Nuttall et al., 1998; Rodríguez et al., 1999]. Also, it is currently well accepted that the ability of MSCs to differentiate into various cell phenotypes may be critical in the progression of bone disease.

Osteoblasts develop in a linear sequence progressing from osteoprogenitors to preosteoblasts, osteoblasts, and osteocytes through the expression of a series of genes that are coordinately regulated [Yanaka et al., 2003]. During these processes, osteogenic cells secrete matrix proteins which are organized as an extracellular matrix (ECM) on which calcium phosphate is deposited as hydroxiapatite crystals [Long et al., 1995; Lecanda et al., 1997], which can be stimulated by promoting directly or indirectly the expression of alkaline phosphatase [Yanaka et al., 2003].

At the beginning of osteogenic differentiation, an important change in the morphology of MSCs is observed. The cells change from the characteristic fibroblast-like phenotype of MSCs to a nearly spherical form, that characterizes the differentiated stage. During this process, the clear correlation between cell shape and differentiation supports the hypothesis that cell shape and the cytoskeleton may affect cellular differentiation [Spiegelman and Farmer, 1982; Spiegelman and Ginty, 1983]. However, at present, it is not clearly established whether changes in cell morphology are prerequisite to the expression of osteogenic differentiation markers in human MSCs.

In this report we have used MSCs obtained from bone marrow of healthy postmenopausal women donors to address the correlation between the organization of cytoskeleton of these cells, changes in cell morphology, and the expression of specific markers (alkaline phosphatase activity and calcium deposition) of the osteogenic differentiation. This study should increase our knowledge regarding the features of MSCs and their relationship with the pathogenesis of some bone diseases providing potentially new tools to design novel therapeutic approaches.

MATERIALS AND METHODS

Subjects

Postmenopausal women (age range from 65 to 75 years), free of bone disease, who underwent therapeutic surgical procedures at the Traumatology Division, Hospital Sótero del Río in Santiago, Chile, to treat hip fractures produced as consequence of falls or traffic accidents, were selected as donors. None of them were under any therapies which may have affected the cells of the osteoblastic lineage. Bone marrow was obtained by iliac crest aspiration during the surgical procedure. For the purposes of this study, the donors were women $(n = 3)$ whose bone mineral density was higher than -2.5 standard deviations as compared with young adults [Raisz, 1997]. Bone mineral density was measured using dual-energy X-ray absorptiometry (DXA). DXA measures bone mass in the spine, the hip and the whole body by scanning and filtering X-rays from a stable source (LUNAR, Prodigy, General Electric Medical Systems, Madison, WI). Ethical approval was obtained from the Sótero del Río Hospital and INTA research ethics committees and written informed consent was obtained from all subjects.

Cell Preparation and Culture Methods of MSCs

MSCs were isolated from bone marrow as previously described [Jaiswal et al., 1997; Rodríguez et al., 1999]. Briefly, 10 ml of bone marrow aspirate were added to 20 ml of Dulbecco's Minimal Essential Medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (culture medium, basal condition), centrifuged to pellet cells, and the fat layer was discarded. Cells were suspended in culture medium and fractionated on a 70% Percoll (Sigma) density gradient. The MSCs-enriched low-density fraction was collected, rinsed with culture medium, and plated at $1-2 \times 10^7$ nucleated cells/100 mm dish (Nunc, Naperville, IL). Cells were cultured at $37°C$ in a humidified atmosphere of 5% CO_2. After 4 days in culture, non-adherent cells were removed and fresh culture medium was added. Culture medium was replaced weekly. When culture dishes became near confluent, cells were detached by mild treatment with trypsin (0.25%, 5 min, $37°C$) and plated at 1/3 the original density to allow for continued passaging. The experiments described here were performed after the fourth passage.

Differentiation of MSCs

For osteoblastic differentiation cells were maintained up to 10 days in culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 µg/ml ascorbic acid added daily (osteogenic medium). As biochemical markers of differentiation, we measured alkaline phosphatase activity and calcium phosphate deposition on the cell layer [Rodríguez et al., 1999].

Alkaline Phosphatase Assays

The alkaline phosphatase activity associated to the cell layer was measured in the cultures using p-nitrophenyl phosphate (Sigma 104 substrate) as substrate. At selected times (0, 1, 3, 5, 7, and 10 days), the cell layer was rinsed with TBS (20 mM Trizma Base, 150 mM NaCl, pH 7.5) and fixed in 3.7% formaldehide-90% ethanol for 30 s at room temperature. Fixed cells were incubated with 1 ml of alkaline phosphatase substrate P-nitrophenyl phosphate

(1 mg/ml) in 50 mM sodium bicarbonate buffer (pH 9.6) containing 1 mM $MgCl_2$ at 37°C. After 20 min, the reaction was stopped with 0.5 ml of 3 N NaOH and the amount of product (*p*-nitrophenol) was measured at 405 nm. Enzymatic activity was expressed as µg of *p*-nitrophenol produced/well [Martínez et al., 1996].

Calcium Phosphate Deposition

For calcium phosphate deposition, MSCs were maintained in basal or osteogenic culture medium for 7 days. The culture medium was then removed, the calcium phosphate crystals deposited on the cell layer were solubilized using a 0.5 N HCl solution, and the amount of calcium solubilized was measured by atomic absorption spectroscopy (423 nm) (Atomic Absorption Spectrophotometer Model 2280, Perkin-Elmer, Norwalk, CT) [Rodríguez et al., 2002].

Immunofluorescence Staining

The pattern of actin and tubulin distribution, in MSCs cultured in basal and osteogenic conditions, was analyzed by immunofluorescence staining. At selected times (0–7 days in culture), cells were washed three times with phosphate buffered saline (PBS), fixed for 10 min in 3.7% formaldehyde in PBS, and permeabilized for 5 min with 0.2% triton X-100 in 3.7% formaldehyde. The fixed cells were rehydrated with Tris buffered saline (TBS) and incubated for 1 h in blocking solution (3% BSA in TBS). To evidence the organization of microfilaments, cells were incubated with Alexa Fluor 594 phalloidin (Molecular Probes Inc., Eugene, OR) [González et al., 1998]. To evidence the microtubule organization cells were incubated sequentially with monoclonal anti α-tubulin antibody (Sigma) diluted 1:500 in 3% BSA–TBS, and with the secondary antibody, FITC-conjugated rabbit-antimouse IgG (Rockland, Gilbertsville, PA), at a 1:250 dilution in 3% BSA–TBS. Cells were incubated with alexa fluor 594 phalloidin, primary or secondary antibodies for 1 h at 37°C. Finally, cells were rinsed in TBS, mounted in DABCO/mowiol, and examined with a epifluorescence microscope (40× objective, Nikon, Labophot-2, Tokyo, Japan). As controls, experiments were performed in which the first or second antibodies were omitted. The morphology of MSCs was examined by using Giemsa-May Grünwald staining.

Cytochalasin D and Nocodazole Treatments of MSCs

MSCs were treated with different concentrations of cytochalasin D (from 0.1 up to 1 µg/ml) or nocodazole (from 1 up to 10 µg/ml) (Sigma) and its effect on the cytoskeleton was monitored from 0.5 to 48 h by immunofluorescence. This strategy allowed us to define the minimal concentration of each drug that perturbs microfilament or microtubule organization without affecting adhesion or viability. In our experimental conditions, actin organization was sensitive to a cytochalasin D concentration as low as 0.1 µg/ml and the arrangement of the microtubules was disorganized at a concentration of 1 µg/ml of nocodazole for 30 min. These drug concentrations were used for the experiment described in Figure 1. For the experiments described in Figures 2 and 3, the concentrations for cytochalasin D and nocodazole that permited to maintain the disorganization of the cytoskeletal structure for 48 h were 0.25 and 1 µg/ml, respectively, supplemented each 12 h. In the last experiment, treated cells were washed three times with PBS, then changed to osteogenic medium without the drug and cultured for five additional days. As control, cells were treated with 0.2% dimethyl sulfoxide (DMSO), which was used as vehicle of the drugs. No differences were observed on cell morphology and cytoskeletal integrity of untreated and DMSO treated cells (data not shown). At the indicated times, cells were processed for immunofluorescence as indicated above.

RESULTS

As a first step to understand the role of the cytoskeleton on the mechanisms involved in normal bone remodeling we examined the importance of microfilaments and microtubules in osteogenic differentiation. We used indirect immunofluorescence to study cytoskeletal rearrangements in MSCs from postmenopausal women. During the in vitro osteogenic differentiation of these cells, alkaline phosphatase activity and calcium deposition were measured as biochemical markers of this process.

Changes in Cytoskeletal Organization of MSCs During Their Osteogenic Differentiation

MSCs cultured in osteogenic conditions exhibited a progressive change in cell shape (Fig. 1, lower panels) that involved principally
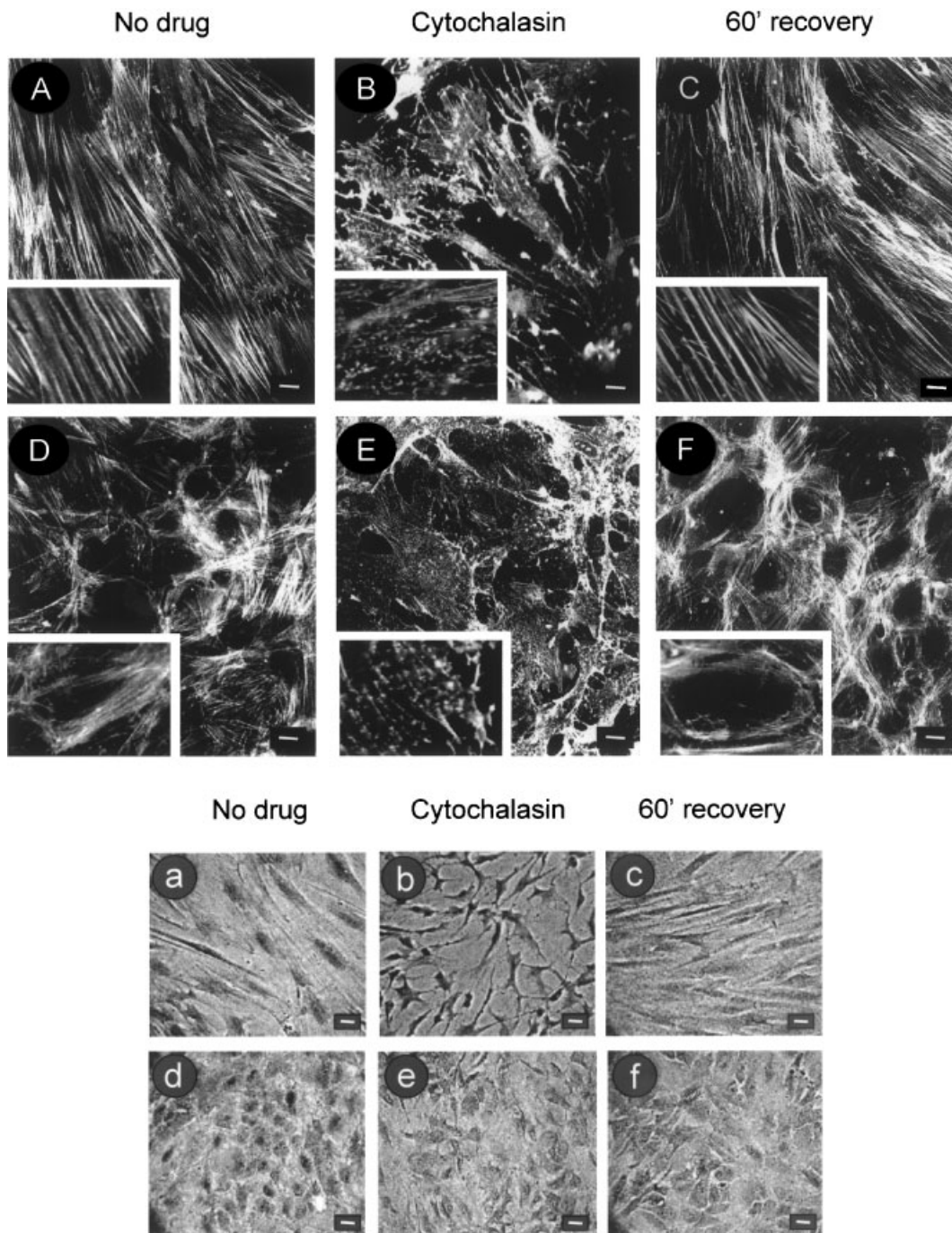
No drug   Cytochalasin   60' recovery

No drug   Cytochalasin   60' recovery

Fig. 1. Actin and tubulin distribution pattern in MSCs cultured in basal and osteogenic medium: effect of cytochalasin D or nocodazole. **Upper panels**: MSCs were cultured in basal (**A–C** and **G–I**) or osteogenic conditions for 7 days (**D–F** and **J–L**). After that, cells were treated with cytochalasin D (0.1 μg/ml) (row 2; B, E) or nocodazole (1 μg/ml) (row 2; H, K) during 30 min and cells were allowed to recover after drug treatment for 1 h (C, F, I, and L). Microfilaments and microtubules were stained as described in Materials and Methods. Images correspond to a representative experiment showing the characteristic pattern observed in samples derived from three different donors. Bar = 10 μm. Magnification, ×40. Insets show higher magnifications, ×100. **Lower panels**: Microphotographs show the morphology of hMSCs in the culture conditions described for microphotographs shown in upper panels. Cells were analyzed using phase-contrast microscopy. Bar = 10 μm. Magnification, ×10.

No drug   Nocodazole   60' recovery

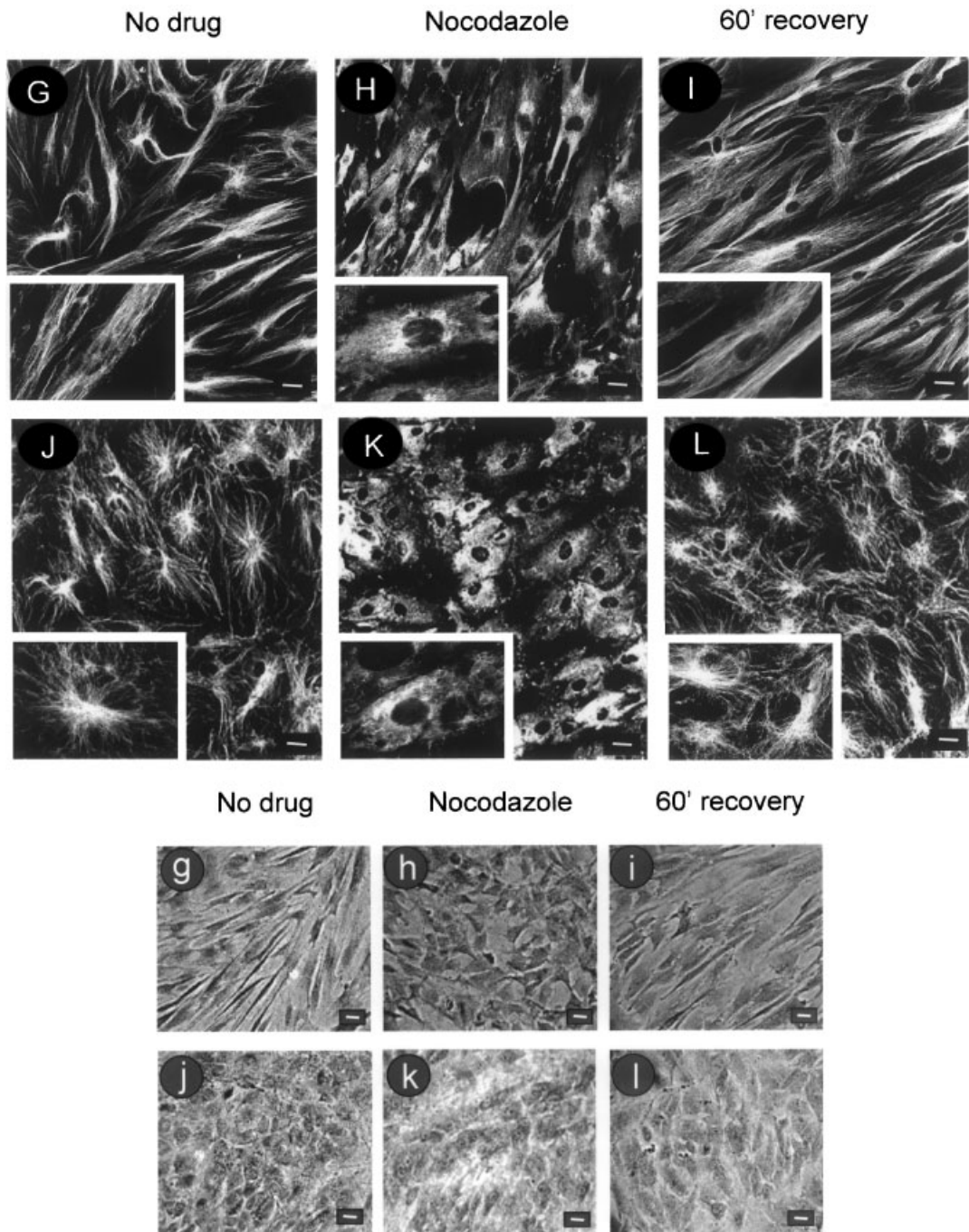No drug   Nocodazole   60' recovery

Fig. 1. (*Continued*)

an extensive reorganization of actin filaments (Fig. 1, upper panels). Under basal culture conditions, cells exhibited a characteristic fibroblast-like phenotype (Fig. 1a) with parallel actin stress fibers extending across the entire cytoplasm, as revealed by Alexa Fluor 594 phalloidin staining (Fig. 1A). After 7 days of culture in osteogenic conditions, more than 95% of the MSCs (N = 320) acquired a cuboidal shape (Fig. 1d). This cell shape change was accompanied by a rearrangement of the actin cytoskeleton as thick actin bundles were observed at the outermost periphery of the cells (Fig. 1D). To characterize the organization of the microtubule network, MSCs cultured in basal (Fig. 1G,g) or osteogenic conditions (Fig. 1J,j)
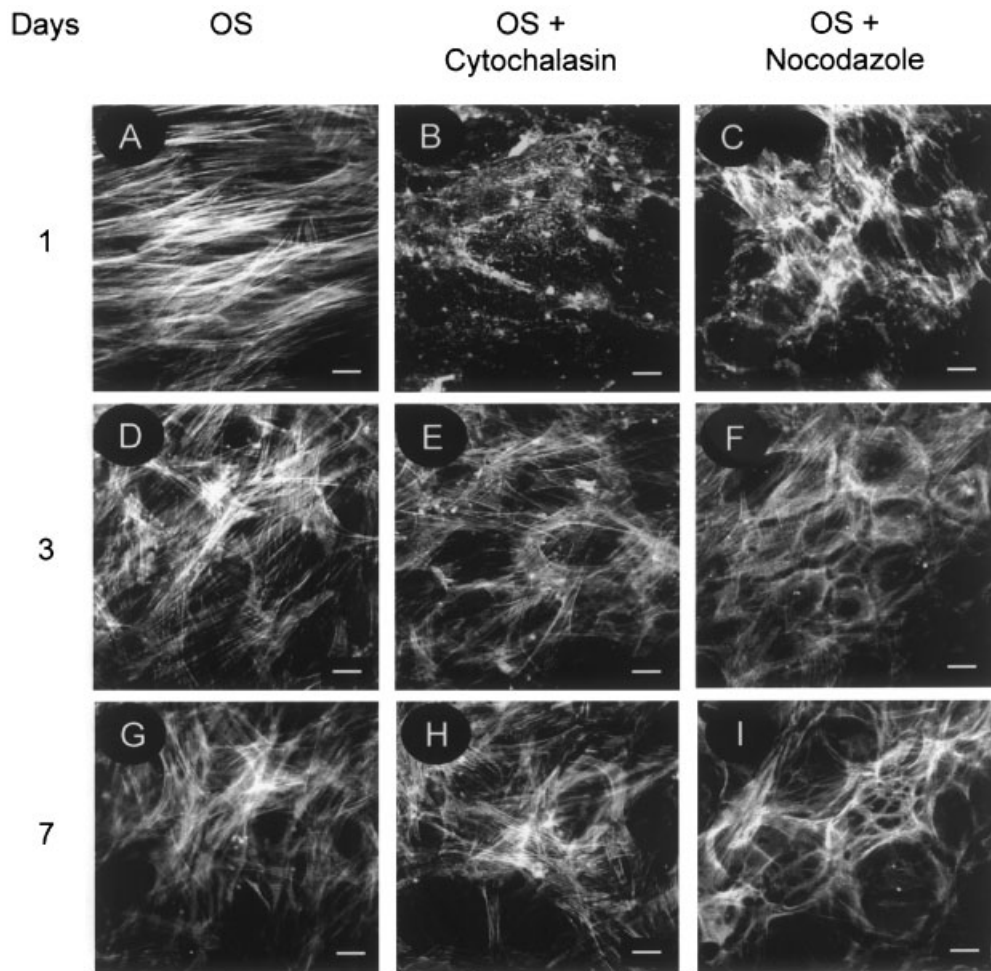
Fig. 2. Reorganization of microfilaments during osteogenic differentiation of MSCs transiently treated with cytochalasin D or nocodazole. MSCs cultured in osteogenic conditions (OS) were treated with cytochalasin D (0.25 µg/ml) (**B**, **E**, **H**) and nocodazole (1 µg/ml) (**C**, **F**, **I**) for the firsts 48 h. Actin was stained at the selected times (1, 3, and 7 days). Images labeled Day 1 were obtained while the cells were being treated with differentiation medium without (**A**) or with the drugs (B, C). Day 3 and 7 of differentiation correspond to day 1 and 5 after removal of the drugs, respectively. Representative images showing the characteristic pattern observed in samples derived from three different donors. Bar = 10 µm. Magnification, ×40.

were analyzed with anti α-tubulin monoclonal antibody. In both conditions, microtubules were observed to radiate from a perinuclear location to fill the entire cell body. Thus, the results indicate that despite the changes of cell shape that take place during osteogenic differentiation, there was not a substantial reorganization of the microtubule network, suggesting that actin filament network was the principal component of the cytoskeleton that changed. Cytochalasin D disrupts the actin cytoskeleton, and thus it is thought to affect the cell shape. To test such an effect, MSCs maintained in basal or osteogenic conditions for seven days were treated with a low concentration of cytochalasin D (0.1 µg/ml) for 30 min, and the organization of

their actin filaments was examined by immunofluoresence microscopy (Fig. 1B,E). In treated cells, the staining pattern of Alexa Fluor 594 phalloidin was diffuse compared to the control cells (Fig. 1A) with a few bright patches of intracellular fluorescence indicating the complete disorganization of the actin filaments. Both in basal and osteogenic culture conditions, the actin cytoskeleton was similarly disturbed (Fig. 1B,E). Even though the main changes in cell shape were observed in cells cultured under basal conditions, as revealed by cell body collapse and decrease in size (Fig. 1b), differentiated MSCs displayed the same alterations in shape and size, but to a lesser extent (Fig. 1e). If cytochalasin D was removed from the
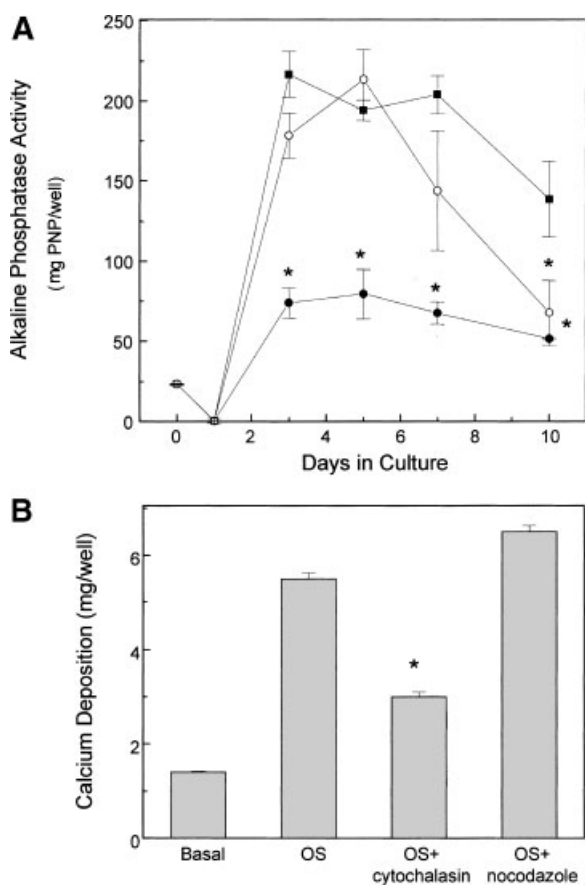
Fig. 3. Alkaline phosphatase activity and calcium deposition during osteogenic differentiation of MSCs. MSCs were cultured in osteogenic conditions (OS) (-■-) in the presence of cytochalasin D (0.25 µg/ml) (-●-) or nocodazole (1 µg/ml) (-○-) for the firsts 48 h. **A**: Alkaline phosphatase activity was measured at 0, 1, 3, 5, and 10 days. Days 3, 5, 7, and 10 correspond to day 1, 3, 5, and 8 after removal of the drug, respectively. Activity was expressed as µg of *p*-nitrophenol (PNP) produced by well. *$P < 0.05$. **B**: Calcium deposition (µg/well) was measured after 7 days of culture, which correspond to day 5 after removal of the drugs. Results were obtained from three different donors. Each experiments was performed in triplicate and results were expressed as mean ± SD. All results are significant as compared with basal conditions. *$P < 0.05$ as compared with untreated cells cultured in osteogenic conditions.

medium, the cells recovered their initial cell shape (Fig. 1c,f) and their actin filament organization within 60 min (Fig. 1C,F) whether they were cultured in basal or in osteogenic conditions.

Treatment of MSCs, maintained in basal or osteogenic medium, with nocodazole (1 µg/ml) during 30 min completely disrupts microtubules arrangement with little effect on cell morphology (Fig. 1H,h,K,k). At this time, microfilaments organization appeared not to be

affected (data not shown). Removal of the drug favored a gradual recovery of the microtubule network in the cells in both culture conditions. Thus after 60 min in fresh medium, MSCs display the initial pattern of tubulin without exhibiting any obvious changes in their previous cell morphology (Fig. 1I,i,L,l). The results after treatment with drugs that affect the cytoskeletal dynamic suggest that the sensitivity of the microfilament and microtubule networks of MSCs to cytochalasin D and nocodazole and their capacity to recover the original pattern of distribution were independent of their degree of differentiation.

Differential Effect of Cytochalasin D and Nocodazole on Cytoskeleton Structure During Osteogenic Differentiation of MSCs

To examine whether an initial disturbance of cytoskeletal integrity might affect the progressive changes in the organization of actin filaments that take place during osteogenic differentiation, MSCs were treated with drugs that affect microtubule and microfilaments assembly (Fig. 2). We defined previously that during the first 48 h, supplementation of the osteogenic culture medium with 1 µg/ml of nocodazole or 0.25 µg/ml cytochalasin D produced a sustained disorganization of actin filaments and microtubules without affecting the viability of MSCs. After drug treatment, cells were maintained for 5 days to allow the recovery of the cytoskeleton organization. Figure 2 shows that after 24 h in osteogenic conditions (without drugs) the actin filament staining of MSCs (Fig. 2A) was similar to that observed in cells cultured in basal conditions (Fig. 1A). This pattern was completely disrupted in cells treated with cytochalasin D (Fig. 2B). Cells that were cultured in osteogenic conditions and treated with the microtubule-disrupting drug nocodazole showed change in their shape from a fibroblast-like phenotype to the characteristic osteogenic differentiated shape (Fig. 1d,j) along with an increment in the actin filament staining at the periphery of the cells, indicating a reorganization of the actin cytoskeleton after microtubule disassembly (Fig. 2C). This cell shape change was not observed when undifferentiated cells were treated with nocodazole in the absence of an osteogenic medium (data not shown), suggesting an additive effect of microtubule disruption and differentiation signals on the morphological changes

observed during MSCs osteogenic differentiation. Removal of cytochalasin D and nocodazole favored the gradual recovery of a structured microfilament and microtubule network. One day after removal of the drug (day 3), treated cells showed a cortical distribution of actin, which was more evident in cells treated with nocodazole (Fig. 2D,E,F). After 5 days removal of the drug, day seven of differentiation no obvious differences of actin distribution pattern were detected in control and drug treated cells (Fig. 2, panels G, H, and I). These results indicate that cells treated with nocodazole seem to acquire the cortical organization of actin earlier than untreated or cytochalasin D treated cells, while the later were able to reach the same microfilaments organization of untreated cells at the end of the differentiation period.

Differential Effect of Cytochalasin D and Nocodazole on Alkaline Phosphatase Activity and Calcium Deposition During Osteogenic Differentation of MSCs

To investigate whether cytoskeleton reorganization during osteogenic differentiation affects the expression of biochemical markers of osteogenesis, we disrupted cytoskeletal integrity of MSCs cultured in osteogenic medium as described in Figure 2 and alkaline phosphatase activity and calcium deposition were measured.

As shown in Figure 3A, cells that were treated for 48 h with cytochalasin D and then allowed to recover for 24 h (3 days in culture) showed a significantly lower level of alkaline phosphatase activity when compared to untreated cells. These cells displayed low levels of activity until day 10 (8 days after drugs treatment). Under the same conditions, cells were able to recover the actin organization that characterizes the differentiated phenotype (Fig. 2E). In addition, we observed that calcium deposition was decreased in cells treated with cytochalasin D compared to untreated cells (3.5 vs. 5.5 µg/well, respectively; $P < 0.05$) (Fig. 3B). These results indicate that despite the recovery in cell shape and actin cytoskeleton organization observed after cytochalasin D, MSCs treated with this drug were unable to attain the level of metabolic markers expression of control cells during the differentiation process. In contrast, during the firsts 5 days of culture cells treated with nocodazole displayed the same level of alkaline phosphatase activity as measured in untreated cells.

However, after day 5 the activity of the enzyme in the cells treated with nocodazole decreased before that of the activity of untreated cells, and by day 10 it reached similar values to those observed in cytochalasin D treated cells. In addition, after nocodazole treatment these cells exhibited no significant differences in calcium deposition when compared to untreated cells (Fig. 3A,B). Taken together, our results indicate that changes in the organization of two components of the cytoskeleton affect differentially the biochemical markers of osteogenic differentiation, in particular the integrity of microfilaments during the initial phase of this process appears to be crucial to express these differentiation markers.

DISCUSSION

Changes in cell morphology have been extensively studied, mainly because they are commonly, if not universally, correlated with mammalian cell differentiation. Many of these changes can be understood in terms of a dynamic reorganization of the cytoskeletal network. The cytoskeleton, composed by a three-dimensional network assembled from microtubules, actin filaments, and intermediate-size filaments, is involved in a wide variety of cell functions associated with the differentiation process, including the determination of cell shape, the spatial organization of cell organelles, intracellular membrane traffic, modulation of surface receptors, and mitosis [Maccioni and Cambiazo, 1995]. In particular, the evident correlation between cell shape and differentiation supports the hypothesis that the cytoskeleton may affect cellular differentiation [Spiegelman and Farmer, 1982; Spiegelman and Ginty, 1983]. At present, it is not clearly established whether the modifications in cytoskeleton organization that take place during osteogenic differentiation are a prerequisite to the cell shape changes and/or for the expression of characteristic biochemical markers of osteoblast differentiation. In this study, we have examined the morphological changes of human MSCs during osteogenic differentiation in vitro and assessed its association with changes in cytoskeleton organization.

Our results show that osteogenic differentiation of human MSCs was accompanied by considerable alterations of morphological and cytoskeletal organization. In MSCs maintained under osteogenic differentiation medium, the

microfilaments change from parallel-oriented actin stress fibers extending across the entire cytoplasm to a cortical organization. This change was similar those described for intermediate mouse osteoblastic cells (MC3T3-E1) [Luegmayr et al., 1996]. The molecular mechanism explaining the changes in cytoskeletal organization observed under these conditions may be explained by the rapid changes in the monomer/polymer equilibrium of the microfilaments that have been described as part of the mechanism by which hormones induce early cellular responses [Koukouritaki et al., 1997]. In this regard, it has been described that hormones like dexamethasone [Hughes-Fulford et al., 1992], growth hormone [Egan et al., 1991], parathyroid hormone [Takahashi et al., 1999], mechanical stimulation [Pavalko et al., 1998], or microgravity [Hughes-Fulford and Lewis, 1996] among others, induce changes of cytoskeletal organization in osteoblasts regulating the dynamics of actin polymerization and microfilament organization, which are important for the physiological role of osteoblasts. Based on these reports we can assume that some molecules present in the osteogenic medium (e.g., dexamethasone), may participate in promoting these changes. In support of this possibility, it has been shown that dexamethasone induces alterations of the dynamics of the actin cytoskeleton and these are closely associated with rapid changes of cellular cAMP levels [Koukouritaki et al., 1996]. Recently, a novel membrane protein containing glycerophosphodiester phosphodiesterase activity has been described [Yanaka et al., 2003], which is transiently expressed during osteogenic differentiation, co-localizes with actin, and whose over-expression induces morphological changes resulting in dramatic increases in alkaline phosphatase activity and calcium deposits. Additionally, activation by several kinases of proteins that interact with the actin-based cytoskeleton has also been implicated in such processes [Koukouritaki et al., 1999]. Accordingly, these findings may indicate that activation of specific proteins, controlled by cAMP-dependent or other signal transduction cascades may be part of the mechanisms regulating the alterations of actin microfilament dynamics following dexamethasone treatment [Koukouritaki et al., 1997].

The disruption of the tubulin or actin-cytoskeleton organization of MSCs was shown to be reversible after the drugs were removed and the cells recovered their initial cytoskeleton organization whether they were cultured in basal or in osteogenic conditions for seven days. These results indicate that the cytoskeletal organization that MSCs recover after treatment with the cytoskeleton disrupting drugs depends on the differentiation status of the cells. Although the mechanisms involved are not clearly established, we can speculate that this may be due to the intrinsic characteristics of the cells in the undifferentiated and differentiated states or as a response to stimuli from the microenvironment. It is possible to speculate about the role that the ECM or the neighbor cells may play as inducers of the cytoskeletal organization of MSCs. In this regard, the important role that ECM plays in defining the differentiation pathways of MSCs is known [Bellows et al., 1986; Rodríguez et al., 2000].

During osteogenic differentiation, we observed that the alterations in the cytoskeletal organization affect the expression of osteogenic differentiation markers. Our results show that disruption of the actin cytoskeleton by cytochalasin D decreased significantly the alkaline phosphatase activity and calcium deposition as compared with untreated cells. These results strongly suggest that in spite of MSCs acquired the actin arrangement characteristic of differentiated cells, they induce a lower level of alkaline phosphatase activity and calcium deposition than untreated cells. Thus, our results show that it is possible to attain the differentiated morphology of the cells without a complete expression of these differentiation markers. The alterations in osteoblast shape, induced by the differentiation stimulus, must involve relatively rapid changes in the cytoskeletal organization of actin. Our results indicate that a perturbation in actin organization during the first 48 h of differentiation was sufficient to prevent the expression of the biochemical markers of differentiation. This suggests that microfilament integrity is important in the initial steps of the osteogenic differentiation to stimulate the expression of the differentiated phenotype. This seems to be specific for actin, because disruption of tubulin assembly and its subsequent reorganization does not affect the increase of the expression of alkaline phosphatase activity and calcium deposition. However, disruption of tubulin organization affected the kinetics of actin organization (Fig. 2). The

cortical organization of actin occurred earlier in cells treated with nocodazole than in cells untreated or treated with cytochalasin D. These results indicated an intimate interplay between both cytoskeletal components and suggests that the integrity of microtubules (or proteins associated to microtubules) appears to have a regulatory role on this process.

Finally, it is important to mention that the alterations in actin cytoskeletal organization in cells derived from osteopetrotic [Watanabe et al., 1997, 1998] and osteoporotic bones [Perinpanayagam et al., 2001] have been previously reported. In addition, we reported that MSCs derived from healthy control and postmenopausal osteoporotic women share some functional characteristics but differ importantly in others [Rodríguez et al., 1999, 2000]. Although we continue studying MSCs derived from control and osteoporotic donors to understand the origin of the differential behavior of the cells at present we have not evidence about cellular or molecular mechanisms explaining the differences observed. Considering the existence of a correlation of actin cytoskeletal organization abnormality with some bone human diseases, it appears attractive to analyze the cytoskeletal organization of MSCs derived from osteoporotic donors to obtain new evidences that may contribute to better understand the pathogenesis of this disease.

ACKNOWLEDGMENTS

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