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Modulation of the Proliferation and Differentiation of Human Mesenchymal Stem Cells by Copper

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Abstract Copper plays important functional roles in bone metabolism and turnover. It is known that it is essential for normal growth and development of the skeleton in humans and in animals. Although at present the exact role that copper plays in bone metabolism is unknown, bone abnormalities are a feature of severe copper deficiency. Osteoblasts are derived from mesenchymal stem cells (MSCs) present in bone marrow stroma, which are able to differentiate into bone, adipocytes, and other cell phenotypes. Excess adipogenesis in postmenopausal women may occur at the expense of osteogenesis and, therefore, may be an important factor in the fragility of postmenopausal bone. The purpose of this study was to evaluate whether an increase of the extracellular concentration of copper affects the ability of MSCs to differentiate into osteoblasts or adipocytes. The results showed that copper modified both the differentiation and the proliferative activity of MSCs obtained from postmenopausal women. Copper (50 μM) diminished the proliferation rate of MSCs, increasing their ability to differentiate into the osteogenic and the adipogenic lineages. Copper induced a 2-fold increase in osteogenic differentiation of MSCs, measured as a increase in calcium deposition. Copper (5 and 50 μM) diminished the expression of alkaline phosphatase (50 and 80%, respectively), but induced a shift in the expression of this enzyme to earlier times during culture. Copper also induced a 1.3-fold increase in the adipogenic differentiation of MSCs. It is concluded that copper stimulates MSC differentiation, and that this is preferentially towards the osteogenic lineage. J. Cell. Biochem. 85: 92–100, 2002. © 2002 Wiley-Liss, Inc.

Key words: mesenchymal stem cells; copper; osteogenesis; adipogenesis; proliferation; differentiation; bone marrow

Post-natal bone formation is a complex process involving the proliferation of pluripotent progenitor cells, their differentiation into osteogenic progenitor cells, their migration to the bone surface, and their differentiation into osteoblasts. This results in the secretion of abundant extracellular bone matrix proteins and its eventual calcification [Long et al., 1995; Lecanda et al., 1997].

Although they are minor components of teeth and bone, trace elements play important functional roles in bone metabolism and turnover [Okano, 1996]. Copper, among other trace elements, is essential for the normal growth and development of the skeleton in humans and animals. The exact role of copper in bone metabolism is unknown, at present, but it is clear that bone abnormalities are relevant features of severe copper deficiency. Strain [1988] postulated that mild copper deficiency may contribute to the genesis of osteoporosis in humans. Bone defects characterized by osteoporotic-like lesions and bone fragility have been reported in ruminants in response to copper deprivation [Howell and Davidson, 1959]. Also, significant differences in zinc, copper, and manganese content have been reported in normal subjects compared with osteoporotic patients. Furthermore, Strause et al. [1994] demonstrated that supplements of calcium, copper, manganese, and zinc arrested spinal bone loss in postmenopausal women. More recently, the relevance of copper was further supported by a double blind study of 45–56-year-old women who received either a copper supplement or a placebo for two years [Eaton-Evans, 1994]; spinal bone density remained at the same levels in the supplemented group

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while it declined in the placebo group. Baker et al. [1999a] reported that markers of bone density and indicators of bone loss such as urinary pyridinium crosslinks were increased in men after 6 weeks on a low copper diet (0.67 mg/day). However, in healthy young men and women, biochemical markers of bone metabolism showed no effect after supplementation of 3 or 6 mg/day of copper for 6 weeks [Baker et al., 1999b].

The cells responsible for bone formation postnatally are the osteoblasts which derive from mesenchymal stem cells (MSCs) present in bone marrow stroma. MSCs are able to differentiate into bone, cartilage, adipose, and other connective tissues [Caplan, 1991]. At present, studies relating specific bone diseases to alterations in the dynamic response of bone progenitor cells, such as osteoporosis, are scarce [Gimble et al., 1996; Nuttall et al., 1998; Bianco and Robey, 1999; Rodríguez et al., 1999; Rodríguez et al., 2000]. 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. Excess adipogenesis in postmenopausal women may occur at the expense of osteogenesis and, therefore, may become a significant factor in determining the bone fragility in the elderly [Nuttall et al., 1998]. This phenomenon, together with the possibility that osteoblast and adipocyte differentiation pathways are regulated jointly, suggests that marrow adipogenesis has important implications in osteogenic disorders [Nuttall et al., 1998].

Understanding the factors and mechanisms involved in MSC commitment towards the osteogenic or adipocytic lineages and the regulation of their pathways is crucial for our understanding of the pathogenesis of osteoporosis. This study is aimed at evaluating the effect of copper on the functional characteristics of mature bone cells and their progenitors. We tested the hypothesis that an increase of extracellular copper concentration influences the capacity of MSCs to differentiate and that the differentiation favors the osteogenic more than the adipocytic lineages. Should the hypothesis prove correct, this study will provide relevant information on whether or not copper supplements should be provided to vulnerable groups (such as postmenopausal women) to prevent osteoporosis, or be used as part of therapeutics strategies to treat the disease.

For this purpose, MSCs were isolated from bone marrow obtained from apparently healthy postmenopausal women (ages 65–75 years), expanded in culture, and tested for functional effects of copper.

MATERIALS AND METHODS

Subjects

"Healthy" postmenopausal women (age range from 65 to 75 years), free of bone disease, were selected as donors from patients from the Traumatology Division, Hospital Sótero del Río in Santiago, Chile, who underwent therapeutic surgical procedures to treat hip fractures produced as consequence of falls or traffic accidents. Bone marrow was obtained by iliac crest aspiration during the surgical procedure ($n = 4$). The protocol was approved by the IRB and donors signed an informed consent prior to surgery.

Bone mineral density was assessed by bone densitometry using dual-energy X-ray absorptiometry (DXA). DXA measurements of bone mass were obtained in the spine, the hip, and total body by scanning and filtering X-rays from a stable source [Raisz, 1997]. Results ranged between a > -1.0 SD and < 2.5 SD in the four study subjects.

Cell Preparation and Culture Methods

MSCs were isolated from bone marrow as previously described [Jaiswal et al., 1997]. Briefly, 10 ml of bone marrow aspirate was added to 20 ml of culture medium consisting of Dulbecco's Minimal Essential Medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS). The suspension was centrifuged to pellet the cells and the fat layer was discarded. Cells were suspended in culture medium and fractionated on a 70% Percoll (Sigma) density gradient. The MSC-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 with 5% CO_2. After 4 days in culture, non-adherent cells were removed and fresh culture medium was added. The culture medium was replaced by fresh medium twice weekly. Cu, Fe, and Zn concentration in the culture medium was determined by total-reflection X-ray fluorescence spectrometry (TXRF) [González et al., 1999]. Concentrations were 0.44,

2.69, and 3.80 μM, respectively, for the three elements. When culture dishes became near confluent, cells were detached by mild treatment with trypsin (0.25%, 5 min, 37°C) and replated at 1/3 density for continued passage [Rodríguez et al., 1999; Rodríguez et al., 2000]. Cell proliferation assays and osteogenic and adipogenic differentiation experiments described below were performed after the fourth passage.

Cell Proliferation Assays

For cell proliferation studies, MSCs were plated on 35 mm dishes at a density of 2×10^4 cells/dish (Nunc) and cultured in 1 ml of culture medium. After 24 h in culture, non-adherent cells were removed and the medium was replaced by fresh medium supplemented with 0, 5, or 50 μM Cu-His. Copper was added as the copper-histidine complex (Cu-His, 1:10 molar ratio), from a stock solution containing 50 mM copper sulfate ($CuSO_4 \times 5H_2O$) (Sigma, St. Louis, MO) and 500 mM L-histidine monohydrochloride (Sigma) [Arredondo et al., 2000]. At selected times, cells were detached by a mild treatment with trypsin (0.25%, 5 min, 37°C), and released cells were counted in a hemocytometer. Cell proliferation was measured in triplicate cultures.

Differentiation of MSCs

To study copper effects on osteoblastic and adipogenic differentiation processes, osteogenic, or adipogenic stimuli were applied as described below, at different copper concentrations.

For osteoblastic differentiation, cells were maintained during 14 days in culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid added daily (osteogenic medium), and 0, 5, and 50 μM of Cu-His. The degree of differentiation was evaluated by measuring alkaline phosphatase activity and the extent of calcium phosphate deposition on the cell layer [Rodríguez et al., 1999].

Adipogenic differentiation was assessed in cells cultured in a medium supplemented with 1 μM dexamethasone, 100 μg/ml 3-isobutyl-1-methylxanthine (IBMX) (adipogenic medium) and 0; 5, and 50 μM of Cu-His during 14 days. Cell differentiation was evaluated by conventional methods, staining the cells with oil Red O for 30 min, followed by a brief rinsing in 60% isopropanol, and counterstaining with hematoxylin [Nuttall et al., 1998; Rodríguez et al., 2000]. The number of adipocytes produced at different copper concentrations was evaluated by flow cytometric analysis as described below [Smyth and Wharthon, 1992].

Alkaline Phosphatase Assays

The alkaline phosphatase activity associated with the cell layer was measured in the cultures using *p*-nitrophenyl phophate (Sigma 104 substrate) as substrate. For these purposes, 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 seg at room temperature. Fixed cells were incubated with 1 ml of alkaline phosphatase substrate (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 O.D. at 405 nm/min × 10^6 cells) [Martínez et al., 1996].

Calcium Phosphate Deposition

The ability of MSCs to differentiate into the osteoblastic lineage in vitro was evaluated by the extent of calcium phosphate deposition on the cell layer. For this purpose, MSCs were maintained in culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid added daily (osteogenic medium), and different concentrations of Cu-His complex (0, 5, or 50 μM) for 16 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).

Flow Cytometry Analysis

The number of adipocytes produced from MSCs cultured in adipogenic medium and supplemented with different concentrations of Cu-His complex (0, 5, or 50 μM) was evaluated by flow cytometry analysis. After 14 days in culture, cells were detached by mild treatment with trypsin (0.25%, 5 min, 37°C), washed with PBS, and stained with Nile Red. After this treatment, cells cultured in the absence as well as in the presence of copper, showed 95–100% viability. Stock solutions of 1 mg/ml Nile red in DMSO were stored in the dark. Twenty minutes prior to analyzing the sample by flow cytometry,

the dye was diluted 1:1,000 in PBS and added to the cell preparation to provide a final concentration of 0.5 μg/ml. When Nile red is dissolved in neutral lipids, such as triglycerides, it emits a gold fluorescence; when dissolved in amphipathic lipids it emits red fluorescence. Cells were analyzed on a FACSCalibur flow cytometer, (Beckton Dickinson, CA), equipped with an argon-ion source operated at 488 nm. The green fluorescence produced by Nile Red was detected between 500 and 550 nm employing detector FL1. Gold fluorescence was detected between 560 and 590 nm employing a detector FL2. Between 20,000 and 50,000 cells were analyzed from each sample and duplicate samples were analyzed for each condition. Data analysis was performed using the software CellQuest [Smyth and Wharton, 1992].

Statistical Analysis

The statistical significance of the differences between control and the different concentrations of copper were determined by Student's *t*-test.

RESULTS

Cell Proliferation

Up to day 4 in culture, MSCs proliferation rate remained similar with and without copper and at the different copper concentrations (Fig. 1). After day 4, proliferation rate decreased when MSCs were cultured in media containing either 5 or 50 μM copper. This decrease was concentration dependent and was more evident at the highest concentration of copper tested (50 μM). Thus, after 10 days in cultures, the cell number in the presence of 50 μM copper was 50–55% compared to the cell number in the absence of copper. Several explanations may account for these differences. Copper may increase the size of the cells, which would then reach confluency with a lesser number of cells, and cell proliferation would be inhibited by cell to cell contact. However, this possibility is unlikely, since flow cytometry analysis showed no differences in cell size in the absence/presence of different copper concentrations (data not shown). One may also postulate a cytotoxic effect of copper on the MSCs cells. However, this is unlikely because our analysis of cell viability in MSCs exposed up to 250 μM copper during 3 days showed that it remained between 95–100%. Also, 95–100% viability was observed up to 10 days at the

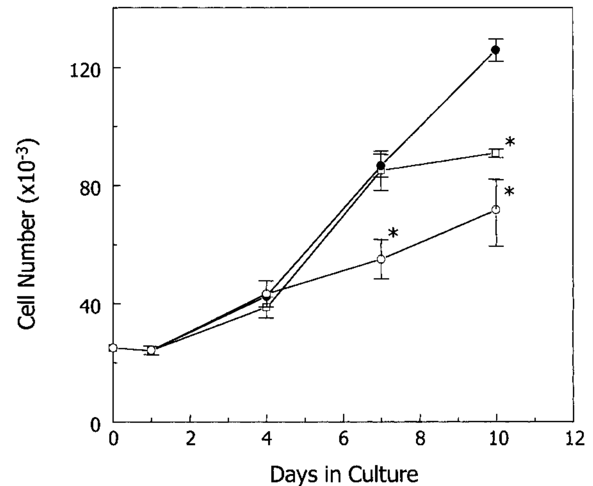

Fig. 1. Cell proliferation. MSCs were cultured in the absence of copper (●-●) and in the presence of 5 μM (□-□) or 50 μM (o-o) copper, as described in Materials and Methods. At selected times, culture medium was removed, cells were released by a mild treatment with trypsin and counted in an hemocytometer. Each experiment was performed in triplicate. The results were expressed as mean ± SD. *$P < 0.05$.

different copper concentration treatments. On the basis of these results, it is probable that copper produces an effect on cell proliferation rate by acting at one or more stages of the cell cycle.

Effects of Copper on MSCs Differentiation

Because MSCs are able to differentiate into several cell phenotypes, it is relevant to determine whether copper induces changes in the two related differentiation pathways assessed in this study: osteoblastogenesis and adipogenesis.

The degree of differentiation towards the osteogenic lineage was evaluated by measuring alkaline phosphatase activity and by the extent of calcium phosphate deposition on the cell layer. In addition, the extent of adipogenic differentiation was also measured (by flow cytometry), by counting the adipocytes produced at the different copper concentrations tested.

Osteogenic Differentiation

(a) Alkaline phosphatase activity. As in previous reports [Rodríguez et al., 1999], alkaline phosphatase activity produced by MSCs increased 2-3 times, when cells were cultured in osteogenic medium, reaching the maximum value at day 12 in culture (data not shown).

Alkaline phosphatase activity produced by MSCs cultured in osteogenic medium after

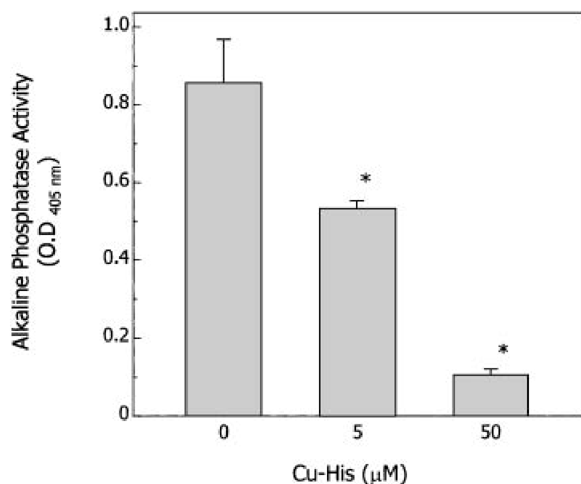

Fig. 2. Differentiation in vitro. Alkaline phosphatase activity. Cells were cultured in a culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid; and in the absence and in the presence of 5 or 50 μM copper. At day 12, the medium was removed and alkaline phosphatase activity was measured as described in Materials and Methods. Each experiment was performed in triplicate. The results were expressed as mean ± SD. $^*P < 0.05$.

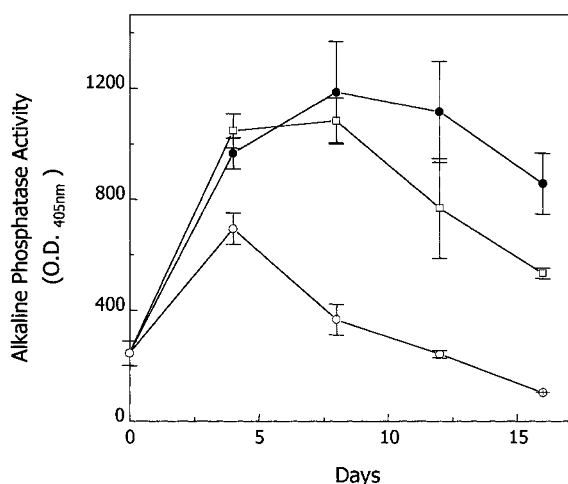

Fig. 3. Differentiation in vitro. Alkaline phosphatase activity. Cells were cultured in a culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid; and in the absence (●-●) and in the presence of 5 (□-□) and 50 μM (o-o) copper. At days 0, 4, 8, and 12, the medium was removed, and alkaline phosphatase activity was measured as described in Materials and Methods. Each experiment was performed in triplicate. The results were expressed as mean ± SD.

14 days was dependent of the presence of Cu-His in the culture medium. Thus, 5 and 50 μM Cu-His in the osteogenic medium produced 45–50% and 82–88% decrease, respectively, in comparison to the activity measured in the absence of copper (Fig. 2). Addition of 50 μM copper induced a similar effect in cells cultured in control medium. It is important to point out that copper addition to the reaction mixture did not inhibit enzymatic activity (data not shown).

To determine the temporal pattern of changes observed in copper exposed cells, we performed a more detailed analysis measuring the enzyme activity periodically, at selected times during culture (4, 8, 12, and 16 days). As shown in Figure 3, extracellular copper modified the expression of alkaline phosphatase activity with time. Our results indicate that 50 μM copper decreased by 50% the maximum value of alkaline phosphatase activity and also induced a shift in the expression of the enzyme towards earlier days of culture. The presence of 5 μM copper did not significantly change the enzymatic activity as compare with the values observed in cells cultured without copper supplement. However, the presence of 50 μM copper was associated with an earlier expression and the maximum value was reached after shorter cultures (the maximum value with 5 μM or without copper was reached after 8–10 days in culture, while with 50 μM copper this was attained after 4 days).

Expression of alkaline phosphatase activity precedes calcium deposition in bone cell cultures. Therefore, these results suggest that copper effects on mineralization are not only to increase the extent but also modify the kinetics of this process, decreasing the time required to initiate calcium phosphate deposition.

(b) Calcium phosphate deposition. MSCs cultured in the absence or presence of copper respond differently to osteogenic stimuli. As we reported previously [Rodríguez et al., 1999], MSC culture in osteogenic medium increases calcium phosphate deposition 2.5 times compared with MSC cultured in control medium. Calcium phosphate deposition by cells cultured in control medium was slightly inhibited (10–30%) by both copper concentrations tested (data not shown). When cells were cultured in osteogenic medium the addition of 5 μM copper did not produced significant differences in calcium phosphate deposition. However, when MSCs were maintained in osteogenic medium supplemented with 50 μM copper, calcium phosphate deposition increased 1.8–2.0 times compared to cells cultured without copper (Fig. 4).

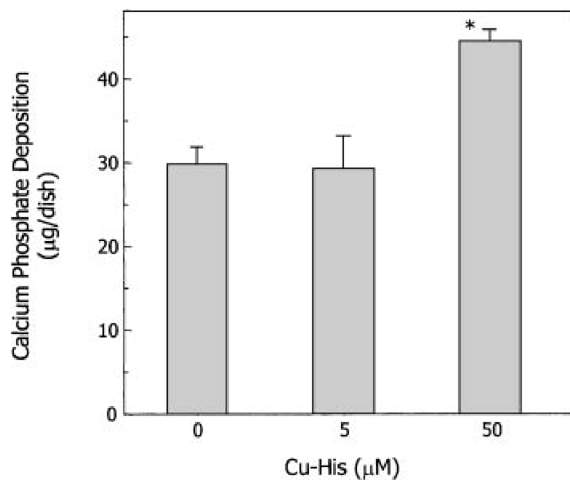

Fig. 4. Mineralization in vitro. Calcium deposition. Cells were cultured in a culture medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid; and in the absence and in the presence of 5 and 50 μM copper. At day 12, the medium was removed, the crystals of calcium phosphate deposited on the cell layer solubilized, and the amount of calcium measured by atomic absorption spectrometry. Each experiment was performed in triplicate. The results were expressed as mean ± SD. $*P < 0.05$.

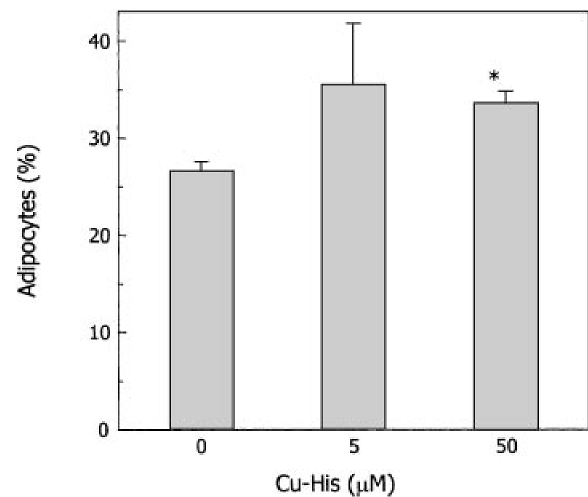

Fig. 5. Adipogenic differentiation. Cells were cultured in a culture medium supplemented with 1 μM dexamethasone, 100 μg/ml 3-isobutyl-1-methylxanthine (IBMX) in the absence and in the presence of 5 and 50 μM copper. After 14 days in culture, cells were detached by a mild treatment with trypsin (0.25%, 5 min, 37°C), washed with PBS, and stained with Nile Red at a final concentration of 0.5 μg/ml. Samples were analyzed by flow cytometry as described in Materials and Methods. Each experiment was performed in duplicate. The results were expressed as mean ± SD. $*P < 0.05$.

Since only differentiated bone cells are able to induce in vitro mineralization, the amount of calcium deposited reflects the extent of osteogenic differentiation of MSCs. Results strongly suggest that the highest copper concentration tested (50 μM) was able to increase MSC differentiation towards the osteoblastic lineage.

Adipogenic Differentiation

MSCs cultured in the absence or in the presence of copper were able to respond to adipogenic stimuli and to differentiate into adipocytes. As it was observed in osteogenic differentiation, the addition of 50 μM copper to the adipogenic differentiation medium produced a significant increase in the extent of adipogenic differentiation (1.2–1.4 times) (Fig. 5). In the absence of the adipogenic stimulus, the percentage of adipocytes was less than 1%.

Preliminary studies showed that the extent of adipogenic differentiation attained by MSCs after 4 days of 50 μM cooper exposure corresponded to 65–70% of the maximum differentiation observed after 14 days of culture in the presence of the same concentration of copper. This observation indicates that a short copper pulse (4 days) was able to produce an important fraction of the maximum effect, and that longer exposure (14 days) did not significantly increase the effect. These results suggest that copper may influence the initial steps of the differentiation process and it will not be involved in further steps leading to mature adipocytes. Currently, further experiments are in progress in our laboratory to better understand the effect of copper on the adipogenic differentiation.

Although copper stimulated both osteogenic and adipogenic differentiation, the degree of these effects differed, since we observed a greater effect on the stimulation towards osteogenic differentiation. Moreover, high concentration (50 μM) of copper not only affected the extent of osteogenic differentiation but also modified the kinetics of this process, inducing earlier differentiation, as evidenced by the shifting of alkaline phosphatase activity expression towards earlier times in culture.

DISCUSSION

Our results show that copper was able to modify both the proliferation and differentiation behavior of MSCs in vitro. Cell proliferation decreased when cells were cultured in a copper-supplemented medium. In addition, the differentiation extent to both the osteogenic and

adipogenic lineages were modified. The fact that copper decreased the proliferation rate of MSCs and increased their ability to differentiate is in agreement with observations reported previously indicating that both processes would be regulated in an opposite manner [Fromigué et al., 1998].

Measurements of calcium phosphate deposition and alkaline phosphatase activity showed that the presence of 50 μM copper modulated MSCs osteogenic differentiation, increasing the amount of calcium phosphate crystals deposited at the end of the culture period and also affected the timing of the expression of alkaline phosphatase activity, a biochemical marker of osteogenic differentiation, which reached its maximum value earlier. During osteogenic differentiation, the expression of alkaline phosphatase associated to the cell membrane precedes calcium phosphate deposition [Stein et al., 1990; Stein and Lian, 1993], therefore, the shifting of enzymatic activity expression observed implies an earlier beginning of calcium phosphate deposition in the presence of copper (50 μM). This may explain the increased amount of calcium deposit observed at the end of the culture period (16 days). The effect of high concentrations of copper on shifting alkaline phosphatase expression seems to be more important than the effect observed on the decrease of enzyme activity. These results suggest that copper would affect the mineralization process, which begins earlier.

Copper exposure also induced an increase in MSCs differentiation towards the adipogenic lineage, as evidenced by the increased number of adipocytes found after stimulation. According to these results, copper would affect both processes, osteogenic, and adipogenic differentiation, but the extent of the effect would be different. The increase observed in adipogenesis (1.2–1.4 times) in the presence of 50 μM copper may be statistically significant, but it may not be physiologically significant. However, the positive effect on osteogenic differentiation not only is greater than the adipogenic differentiation, but also it begins earlier.

The evidence indicates that MSCs may differentiate into several cell phenotypes, such as osteoblasts, adipocytes, and chondrocytes [Caplan, 1991]. A number of studies strongly suggest that the increased differentiation of MSCs towards the adipocytic lineage observed in the bone marrow of osteoporotic patients occurs in detriment of the differentiation towards the osteogenic lineage [Bennett et al., 1991; Beresford et al., 1992; Nuttall et al., 1998; Nuttall and Gimble, 2000; Rodríguez et al., 2000]. If this is correct, both differentiation pathways should be reciprocally regulated. In this context, our results seems to be paradoxical., since copper appears to positively affect both the osteogenic and adipogenic differentiation process, differing only on the magnitude of the effects.

It is known that the first of differentiation of pluripotent progenitor cells implies, the commitment of these cells towards a specific differentiation pathway followed by maturational steps until the mature phenotype is reached. Results of this study raise the question whether copper acts in the commitment, in the maturation stages or in both. Preliminary results obtained in our laboratory suggest that at least for adipogenic differentiation, the presence of copper at the early stages of the process is important, and thus it may influence the commitment of the cells. It is not known how copper affects cell differentiation, however, several lines of evidences indicate that copper may exert its effect by acting on the extracellular matrix (ECM). It is well known that both the composition and the structure of the ECM play important roles in cell proliferation and differentiation [Bortell et al., 1994; Panagakos and Kumar, 1995; Rodríguez et al., 2000]. Enzymes involved in post translational processing of collagen fibers, like lysyl oxidase, are copper-dependent [Gacheru et al., 1990; Smith-Mungo and Kagan, 1998; Rucker et al., 1998]. Dietary copper deficiency rapidly depresses lysyl oxidase activity in chick aorta [Harris et al., 1974] and lung [Harris, 1986] causing increases in non-cross-linked collagen and elastin. In this context, resistance to proteolysis occurs within a short period of copper repletion in several organisms [Tinker et al., 1990]. With these facts in mind, we speculate that the presence of copper in the microenvironment may modify ECM maturation and consequently the proliferation and differentiation processes. This is in agreement with the results presented in this work. Studies in progress in our laboratory are evaluating the effect of copper on both the ECM and the MSCs as well as their interrelationships.

Several studies confirm that copper is required for infant growth, host defense

mechanisms, red and white cell maturation, bone resistance, iron transport, and brain development. [Danks, 1988a, 1988b].

The clinical features observed in Menkes' patients result from a deficiency of copper and its effects on the function of copper-dependent enzymes [Kodama, 1993; Bankier, 1995]. The primary defect is a marked reduction in the first phase of copper transport. As a result, in most tissues, enzymes that depend upon copper for their function are inactive or have reduced activity. The symptoms of the syndrome include connective tissue abnormalities with deformities of the skull, long bones, and ribs. Decreased activity of lysyl oxidase reduces the strength of connective tissue in numerous tissues, accounting for many of the abnormalities of the Menke's syndrome. Occipital horn syndrome, which was formerly known as X-linked cutis laxa is also a mutation in the *Menkes* gene [Kodama and Murata, 1999]. The bones are osteoporotic with flared metaphyses of the long bones, rib fracture, and possible wormian bones (small irregular bones in the sutures between the bones of the skull). In severe occipital horn syndrome, the main feature is the growth of bone spurs, perhaps because of disordered connective tissue function and neuronal problems [Kaler et al., 1994].

In humans [Strain, 1988] and animals [Howell and Davidson, 1959], it has been suggested that mild copper deficiency may contribute to bone defects characterized as osteoporotic-like lesions and bone fragility, and that bone loss may be arrested with supplements of calcium, copper, manganese, and zinc [Strause et al., 1994]. These facts support the importance of understanding the mechanisms of action of copper on bone progenitor cells. However, the fact that combined supplements was more effective than either calcium alone or the trace element mixture alone [Strause et al., 1994] suggests that, in the future, it is necessary to study the effects of copper in the presence of calcium and zinc.

Recently, we demonstrated that MSCs derived from osteoporotic postmenopausal patients have important functional differences as compared with MSCs from control donors, such as their proliferation rate, their ability to differentiate towards osteogenic and adipogenic lineages, their capacity to synthesize collagen, and others, [Rodríguez et al., 1999; Rodríguez et al., 2000]. Thus, it is important to study the effect of copper on the proliferation rate and differentiation pattern of MSCs derived from osteoporotic patients, because these cells may respond to copper in a different manner than the cells derived from normal donors. The comparison between the results obtained from both types of cells may contribute to the understanding of the mechanisms through which copper exerts its action and, may give evidence as to whether or not a diet supplemented with copper may be useful in the prevention and treatment of osteoporosis.

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