Aromatase activity of human mesenchymal stem cells is stimulated by early differentiation, vitamin D and leptin

Ana María Pino, Juan Manuel Rodríguez, Susana Ríos, Pablo Astudillo, Laura Leiva[1], Germán Seitz[2], Mireya Fernández and **J Pablo Rodríguez**

Laboratorio de Biología Celular, INTA, Universidad de Chile, El Líbano 5524, Macul. Casilla 138-11, Santiago, Chile

[1]Laboratorio de Envejecimiento y Enfermedades Crónicas Relacionadas con la Nutrición, INTA, Universidad de Chile, Santiago, Chile

[2]Servicio de Traumatología, Hospital Sótero del Río, Santiago, Chile

(Requests for offprints should be addressed to J P Rodríguez; Email: jprodrig@inta.cl)

Abstract

Human mesenchymal stem cells (hMSCs) are multipotent cells present in bone marrow, which differentiate into osteoblasts and adipocytes, among other lineages. Oestrogens play a critical role in bone metabolism; its action may affect the adipocyte to osteoblast ratio in the bone marrow. In hMSCs, oestrogens are synthesized from C19 steroids by the enzyme aromatase cytochrome P450. In this study, we assessed whether aromatase enzymatic activity varied through early osteogenic (OS) and adipogenic (AD) differentiation. Also, we studied the effect of leptin and 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) on aromatase cell activity. Finally, we analysed whether conditions that modify oestrogen generation by cells affected hMSCs differentiation. For these purposes, hMSCs derived from postmenopausal women (65–86 years old) were cultured under basal, OS or AD conditions, in the presence or the absence of leptin and $1,25(OH)_2D_3$. Aromatase activity was measured by the tritiated water release assay and by direct measurement of steroids synthesized from ^{3}H-labelled androstenedione or testosterone. Our results showed that different OS and AD patterns of aromatase activity developed during the first period of differentiation (up to 7 days). A massive and sharp surge of aromatase activity at 24 h characterized early OS differentiation, while increased but constant aromatase activity was increased through adipogenesis. Both leptin and vitamin D increased aromatase activity during osteogenesis, but not during adipogenesis; finally, we showed that favourable aromatase substrates concentration restrained MSCs adipogenesis but improved osteogenesis. Thus, it could be inferred that a high and early increase of local oestrogen concentration in hMSCs affects their commitment either restraining AD or facilitating OS differentiation, or both.

Introduction

Human bone marrow stroma contains mesenchymal stem cells (hMSCs) differentiating along osteogenic, chondrogenic, adipogenic and marrow stromal lineages (Caplan 1991, Bruder *et al.* 1997, Pittenger *et al.* 1999). Changes in the functional characteristics of hMSCs or in the regulation of the differentiation pathways may have consequences in some osteogenic disorders like human postmenopausal osteoporosis (Gimble *et al.* 1996, Nuttall *et al.* 1998, Bianco & Robey 1999, Rodríguez *et al.* 1999).

After the menopause, decreased endogenous oestradiol enhances bone turnover and this is accompanied by a shift in the adipocyte to osteoblast ratio, which favours fat tissue production in the bone marrow (Gambacciani *et al.* 1997, Justesen *et al.* 2001). The directive effect of oestrogen on the skeleton is supported by the developmental failure of bone in males with deficient oestrogen activity as a result of oestrogen receptor dysfunction or aromatase deficiency (Smith *et al.* 1994, Morishima *et al.* 1995), and the correlation between endogenous oestradiol concentrations and both mineral density and bone loss in men (Amin *et al.* 2000, Khosla *et al.* 2001). In addition, there is evidence that endogenous oestrogen production by CYP 19 aromatase as well as oestrogen receptor signalling play an important role in the development and the distribution of white adipose tissue in the body, as highlighted by analysis of the respective oestrogen receptor-α and ArKO mice (Heine *et al.* 2000, Jones *et al.* 2000).

The biosynthesis of oestrogen from C19 steroids is catalysed by aromatase cytochrome P450 encoded by the *CYP19* gene. In addition to gonads, this enzyme is found in different organs, including adipose tissue, brain, skin, endothelium and bone. Skeletal cells also express a number of other enzymes implicated in sex steroid metabolism (Schweikert *et al.* 1980, 1995, Janssen *et al.* 1999, Compston 2002, Ishida *et al.* 2002, Issa *et al.* 2002), supporting the concept that active androgens and oestrogens can be synthesized within the bone marrow cells from circulating

C19 precursors. Thus, besides contributing to the circulating oestrogen pool, the oestrogen synthesized within bone tissue compartments may be locally active in a paracrine or intracrine way (Labrie *et al.* 1997, Simpson 2000, Simpson & Davis 2001). Therefore, although the total amount of oestrogen synthesized at any given site could be small, local concentrations, could be substantial, giving it functional meaning. The extent, regulation and physiological significance of oestrogen synthesis within the bone remains almost unknown; however, this process could provide mechanisms for tissue-specific responses in the absence of changes in systemic hormone production, and for the preservation of homeostasis in the face of alterations in hormonal status, such as those originated during aging.

Aromatase has been reported to be expressed in hMSCs (Heim *et al.* 2004), in osteoblast or osteoblast-like cells from foetal and adult tissues (Purohit *et al.* 1992, Tanaka *et al.* 1993, Schweikert *et al.* 1995, Sasano *et al.* 1997, Janssen *et al.* 1999), in articular cartilage chondrocytes, in adipocytes adjacent to bone trabeculae, in osteocytes (Sasano *et al.* 1997) and in macrophage/osteoclast-like cells (Shozu *et al.* 1997). The expression of CYP 19 has been shown to be regulated by differential promoter usage, depending on the tissue context. In osteoblasts and adipocytes, aromatase is activated mainly through the I.4 promoter (Shozu & Simpson 1998, Simpson & Davis 2001, Enjuanes *et al.* 2003). In cultures of bone-derived osteoblast or osteoblast-like cells, the regulation of aromatase expression has been studied mainly at the transcriptional level, showing that dexamethasone, vitamin D, testosterone and phytoestrogen genistein, among others, may function as regulatory factors of CYP19 expression (Tanaka *et al.* 1996, Jakob *et al.* 1997, Shozu & Simpson 1998, Shozu *et al.* 2000, Enjuanes *et al.* 2003, Heim *et al.* 2004). Further, transcription of CYP19 has been reported to be induced by physiological or pathological conditions, such as bone differentiation and fractures (Lea *et al.* 1997, Janssen *et al.* 1999, Heim *et al.* 2004), pointing to the importance that local oestrogen generation may have for adequate triggering and ensuing of the differentiation pathway.

Although post-transcriptional modulation of CYP19 has been inferred to account for the differences in cell aromatase enzymatic levels (Tanaka *et al.* 1996, Janssen *et al.* 1999, Heim *et al.* 2004), post-translational modifications, protein stability or cofactor variations have scarcely been studied. These types of mechanisms may be especially relevant during commitment of the common precursor cell to the osteoblastic or adipocytic lineages. The osteogenic (OS) differentiation of cultured hMSCs has been shown to be dependent on the activation of runt-related transcription factor 2 (runx2) and extracellular signal-regulated kinase–mitogen-activated protein kinase (ERK–MAPK; Banerjee *et al.* 1997, Ducy *et al.* 1997); while the activation of p38-MAPK and peroxisome proliferators-activated receptor-γ2 accompanied by suppression of runx2 expression were shown to induce adipocytic (AD) differentiation (Lecka-Czernik *et al.* 1999). Recently, using selective inhibitors of MEK-1/2 (MAPK/ERK) in bone-derived osteoblast-like cells, it has been proposed that MAPK could play an important role in aromatase activation at the post-transcriptional level (Shozu *et al.* 2001).

Besides oestradiol, hormones like vitamin D and leptin are recognized as OS agents. Several *in vitro* studies indicate that stromal cells are responsive to leptin, which increases proliferation, differentiation to osteoblastic lineage and the number of mineralized nodules (Takahashi *et al.* 1997, Thomas *et al.* 1999, Reseland *et al.* 2001), but inhibits differentiation to adipocytes (Thomas *et al.* 1999, Hess *et al.* 2005). These observations suggest that leptin may participate in the regulation of bone mass, but the mechanism remains unclear. We have recently demonstrated the presence of high affinity leptin receptors associated with the cell membranes of hMSCs and a direct protective action of leptin on osteogenesis (Hess *et al.* 2005). On the other hand, vitamin D deficiency is an important risk factor for bone mass loss. The more severe deficiencies cause osteomalacia, decreased bone mineralization, bone pain and spontaneous fractures (Bouillo *et al.* 1995). Both 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and oestradiol may be involved in the regulation of hMSC differentiation (Komm *et al.* 1988, Bouillo *et al.* 1995). Further, $1,25(OH)_2D_3$ increases CYP19 transcripts level in bone cells (Tanaka *et al.* 1996, Enjuanes *et al.* 2003), but its effect on hMSCs aromatase activity has been least studied.

Two recent studies on the expression of CYP19 during MSCs differentiation point to the potential importance of distinctive local oestrogen production and action at OS and AD commitment (Janssen *et al.* 1999, Heim *et al.* 2004). Given that aromatase enzymatic activity has not been analysed during hMSCs commitment and early differentiation, and that each differentiation pathway may give rise to specific and exclusive regulation of aromatase activity, we studied in hMSCs: (1) whether early OS and AD differentiation give rise to definite cell aromatase activities; (2) the effect of two hormones involved in bone metabolism, leptin and $1,25(OH)_2D_3$, on aromatase cell activity and (3) whether AD differentiation is affected by defined oestrogenic conditions. Our results showed that during the first period of differentiation (up to 7 days), distinctive OS and AD patterns of aromatase activity developed and that a massive and sharp surge of aromatase activity characterized early OS differentiation, while increased but stable aromatase activity was associated with adipogenesis. Both leptin and $1,25(OH)_2D_3$ increased aromatase activity during osteogenesis, but not during adipogenesis; finally, we showed that steady oestrogenic conditions restrained MSCs adipogenesis.

Materials and Methods

Subjects

Postmenopausal women aged 65–86 years, patients from the Trauma Section, Hospital Sótero del Río, Santiago, Chile,

were selected as volunteer bone marrow donors. Written informed consent was obtained from all the subjects. Bone marrow was obtained by iliac crest aspiration during surgical procedures (Rodríguez *et al.* 1999); ethical approval was obtained from the Hospital Sótero del Río and INTA ethics committees. Donors considered themselves healthy, except for fractures and were not using glucocorticoids or oestrogen replacement therapy.

Reagents

Tissue culture reagents were obtained from Gibco/BRL; ICI 182780 (ICI) was purchased from Tocris Cookson Inc., Ellisville, MO, USA. Cell culture dishes were obtained from Nunc, Naperville, IL, USA. Androst-4-ene-3,17 dione, [1 β-^{3}H(N)]-25·3 Ci/mmol was purchased from Perkin-Elmer Sciences, Inc., Boston, MA, USA; androst-4-ene-3,17-dione, [1,2,6,7-^{3}H(N)] 85 Ci/mmol was from New England Nuclear, Du Pont Co., Wilmington, DE, USA, and [1,2,6,7-^{3}H(N)] testosterone 94 Ci/mmol was from Amersham Biosciences Limited, UK. 4-Androsten-4-ol-3,17-dione (Ar-Inh) and all other reagents were supplied by Sigma. Goat polyclonal antibody anti CYP19 (P450 arom) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA; fluorescein isothiocyanate (FITC)-conjugated rabbit-antigoat IgG and peroxidase-conjugated goat anti-rabbit secondary antibodies were from Rockland, Gilbertsville, PA, USA. ECL chemiluminescence reagents were from Amersham Pharmacia Biotech.

Cell preparation and culture methods

hMSCs were isolated from bone marrow as previously described (Jaiswal *et al.* 1997). Briefly, 10 ml bone marrow aspirate were added to 20 ml Dulbecco's modified Eagle's medium high glucose containing 10% foetal bovine serum (basal medium), and it was then centrifuged to pellet the cells, discarding the fat layer. Cells were suspended in basal medium and fractionated on a 70% Percoll density gradient. The hMSCs-enriched low-density fraction was collected, rinsed with culture medium and plated at a density of $1–2\times10^7$ nucleated cells/100 mm dishes. 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 by fresh medium twice weekly. When cultures reached near confluence, cells were detached by a mild treatment with trypsin (0·25%, 5 min, 37 °C) and plated at one-third the original density to allow for continued passage. The experiments were performed after the fourth cell passage.

MCF-7 epithelial cell line (Human tumor cell Bank 22, American Type Culture Collection, Rockville, MD, USA), used as control in western blot studies, were cultured as described (Catalano *et al.* 2003).

Osteogenic differentiation

hMSCs ($1–1·5\times10^5$) were maintained in OS culture medium: basal medium supplemented with 0·1 μM dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid (added daily). The medium was changed twice weekly (Rodríguez *et al.* 1999). The ability of hMSCs to differentiate into the osteoblastic lineage *in vitro* was evaluated by measuring alkaline phosphatase activity, as an early osteogenic differentiation marker (Hu *et al.* 2003). At the indicated time (7 days of culture), the culture medium was removed and alkaline phosphatase activity was measured as previously described (Rodríguez *et al.* 2002).

Adipogenic differentiation

hMSCs ($1–1·5\times10^5$ cells/dish, 35 mm) were maintained in AD medium: basal medium supplemented with 1 μM dexamethasone, 10 μg/ml insulin, 0·45 mM isobutyl-methyl-xanthine and 0·1 mM indomethacin, and this was replaced by fresh medium every 4 days. hMSCs were tested for their lipid content after 14 days of AD treatment by flow cytometry. Cells were placed in freshly diluted Nile Red (1 mg/ml) and analysed by flow cytometry (FACSCalibur, Becton Dickinson, Franklin, NJ, USA; Dennis *et al.* 1999).

Aromatase activity assay

The aromatase activity in hMSCs under specified culture and time conditions was measured by the tritiated water release assay using 8–100 nM [1-β-^{3}H] androst-4-ene-3, 17-dione as substrate, for 2 h at 37 °C (Lephart & Simpson 1991). Cell numbers and protein concentrations were measured in cell monolayers.

Determination of steroid formation from aromatase substrates: $1–1·2\times10^5$ cells/dish were incubated in OS or AD medium in the presence of 30 or 100 nM androstenedione plus 0·3 μCi ^{3}H-androstenedione or 100 nM testosterone plus 0·3 μCi ^{3}H-testosterone, as aromatase substrates. Cells were incubated for 24 h with treatments; the reaction was stopped by placing the plates on ice. The medium was removed, placed in corresponding glass stoppered test tubes. All hormones were extracted four times with a three-fold volume of ethyl ether and the ether phases were pooled. All samples were evaporated to dryness under nitrogen and re-dissolved in 150 μl ethanol, immediately prior to spotting on TLC plate. Hormones were separated by thin layer chromatography (TLC) using aluminium-backed silica gel-coated plates (60F254, EM Science, Darmstadt, Germany). Each sample included 0·1 μM standards of oestradiol, oestrone and androstenedione for identification of sample bands. The solvent system (mobile phase) consisted of chloroform/ethyl acetate/(4:1, v/v). Extraction consistencies were controlled using blank incubations without cells that contained known amounts of the radioactive hormones.

Immunofluorescence staining

Cells were seeded on sterile glass coverslips ($1{\cdot}25\times10^4$ cells/cm^2) and placed into 15 mm wells containing basal medium. After 3–5 days, cells were incubated with basal, OS or AD media for 24 h. Cells were washed thrice with PBS, and fixed with ice cold methanol for 20 min at −20 °C. The fixed cells were re-hydrated with Tris buffer saline (TBS) and incubated for 1 h in blocking solution (3% BSA in TBS) at room temperature. Cells were incubated with goat polyclonal antibody anti CYP19 (P450 arom), at 1:1000 dilution in 3% BSA–TBS, during 45 min at 37 °C and subsequently with the secondary antibody, FITC-conjugated rabbit-antigoat IgG, at a 1:250 dilution in 3% BSA–TBS. Finally, the cells were rinsed in TBS, mounted in DABCO/mowiol and examined with an epifluorescence microscope (100× objectives, Nikon, Labophot-2, Tokyo, Japan). In the controls, the first or the second antibodies were omitted.

Western-blot analysis

hMCSs cells were grown in 100 mm dishes to 70–80% confluence. At selected times, cells were lysed in 500 µl of 50 mM HEPES, pH 7·5, 150 mM NaCl, 1·5 mM MgCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100 and a mixture of proteases inhibitors (aprotinin, *p*-methylsulphonylfluoride and sodium orthovanadate). Further 12 µg protein were separated on 10% SDS-PAGE under reducing conditions. Afterwards, gels were blotted onto polyvinylidene fluoride (PDVF) membrane (Bio-Rad) and aromatase was detected using goat polyclonal antibody anti-CYP19 (P450 arom). Peroxidase-conjugated rabbit anti-goat secondary antibodies were used. Immunoreactivity was determined using the ECL chemiluminescence reaction. MCF-7 cells were used as a positive control.

Statistical analysis

Statistically significant differences between groups were detected using ANOVA and an *a posteriori* Tukey test. All analyses were performed using STATISTICA 6·1 (StatSoft, Inc.,Tulsa, OK, USA; 2004, www.statsoft.com). In all cases, $P<0{\cdot}05$ was considered significant.

Results

Aromatase activity in hMSCs under OS and AD differentiation conditions

Aromatase activity of hMSCs showed significant variations after OS and AD differentiation treatments. Very low aromatase activity was observed in cells under basal conditions, its value being slightly higher than blank values (14±5 fmol/mg). However, after OS or AD stimulation, aromatase activity increased significantly. As shown in Fig. 1A, increased aromatase activity was already observed at 12 h of OS differentiation, reaching the maximum level at 24 h of treatment; afterwards the activity decreased sharply and remained low for the rest of the 7 days of treatment. During AD differentiation, a different temporal pattern of aromatase activity was detected, in that increased aromatase activity was maintained up to 3 days, then declining until 7 days of treatment (Fig. 1B).

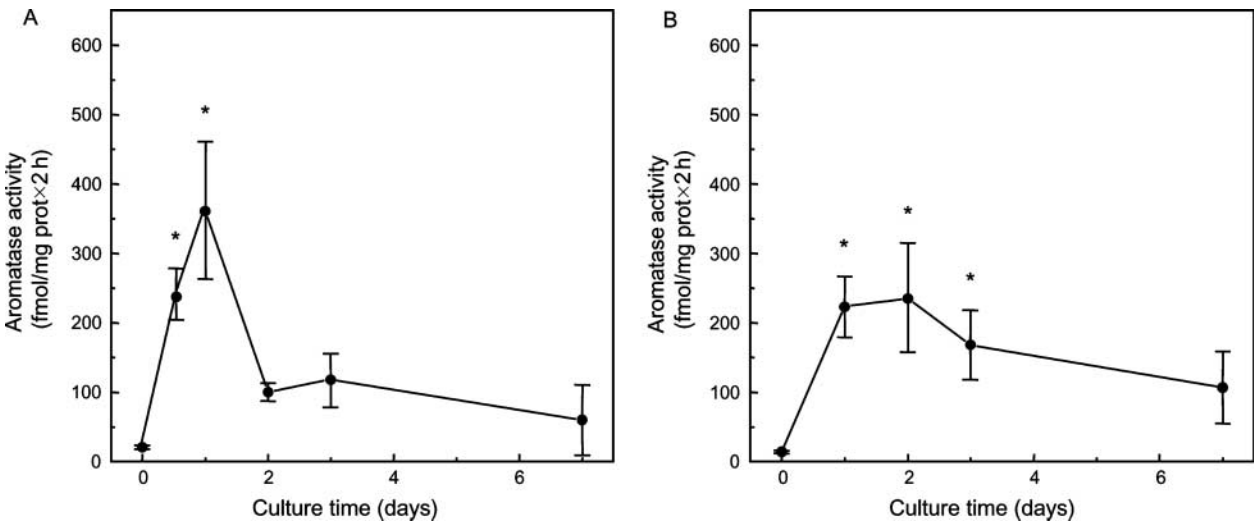

Figure 1 Aromatase activity during osteogenic (OS) and adipogenic (AD) differentiation of hMSCs. Cells were cultured in OS (A) and AD (B) medium as described in Materials and Methods. At indicated times, the aromatase activity was evaluated by measuring tritiated water released by cells after incubation with 30 nM [1-β-^{3}H] androst-4-ene-3, 17-dione as substrate, for 2 h at 37 °C. Results were expressed as femtamoles of [^{3}H]H_2O produced/milligram of protein×2 h. Experiments were performed in triplicate from four different samples. Results are the mean±S.D. *$P<0{\cdot}05$ compared with basal value.

Further characterization of the enzyme was performed in cells after 24 h of OS or AD differentiation treatments. Aromatase activity increased in relation to androstenedione concentration, so that the observed Michaelis–Menten constant (K_m) was 9·22±0·3 nM, although variations in maximal activity were noticed when comparing samples from different donors.

In addition, hMSCs were immunostained for specific aromatase cytochrome P450. Immunostaining occurred in the cytoplasm with a similar distribution in cells cultured under basal OS or AD conditions. No difference in staining intensity of cells was appreciated between basal or differentiation conditions (data not shown).

Results in Table 1 demonstrate that oestradiol and oestrone are found in the incubation medium of hMSCs cultured under OS or AD conditions. Under OS conditions, the amount of oestrogens formed from androstenedione was clearly substrate concentration dependent. As expected, testosterone as substrate (100 nM) allowed the formation of oestradiol as much as that originated from androstenedione (30 nM), but oestrone production was low. No significant difference was appreciated between oestrogens produced under OS and AD conditions at this time point of cell differentiation.

Effect of leptin and 1,25(OH)$_2$D$_3$ on aromatase activity

Neither leptin nor 1,25(OH)$_2$D$_3$ affected aromatase activity of hMSCs under basal conditions; however, the addition of these compounds to the culture media during OS cell differentiation was associated with increased aromatase activity. At 24 h of OS differentiation the effect was dose dependent (Fig. 2A and B).

After 24 h of OS differentiation 200 nM leptin increased aromatase activity 1·6-fold, compared with the activity in the absence of leptin. The addition of leptin to hMSCs during AD differentiation did not modify aromatase activity compared with the activity in the absence of leptin (Fig. 3A). On the other hand, 1,25(OH)$_2$D$_3$ (10 nM) increased 1·8-fold hMSCs aromatase activity after 24 h of OS differentiation, compared with cells cultured in the absence of the seco-steroid hormone; this effect was not observed in hMSCs under AD conditions (Fig. 3B).

Protein aromatase levels were analysed by western blot; no difference in the level of protein immunostained for aromatase cytochrome P450 under basal, OS, AD, OS plus leptin or OS plus 1,25(OH)$_2$D$_3$ conditions were detected (Fig. 4A and B). Western blots of proteins from hMSCs demonstrated similar protein migration as the pattern obtained with MCF-7 cells proteins (Fig. 4A).

Figure 5 shows that at 24 h of OS differentiation, increased aromatase activity is dependent, in part, on MAPK-dependent activities, since the inhibitor 2′-amino-3′ methoxyflavone (PD 98059) (25 μM) abolished 60–80% of the aromatase activity. The inhibitor also decreased the stimulatory effect of leptin.

Oestrogens produced by hMSCs affect their differentiation capacity

Table 2 summarizes the extent of AD differentiation of hMSCs under different oestrogenic conditions after 14 days of treatment. Under plain AD condition, the mean number of adipocytes detected by flow cytometry was 1114±384. This number was not affected by the presence of 50 nM oestradiol in the AD medium. The addition of 0·1 μM androstenedione or 0·5 μM testosterone, substrates for aromatase, during AD differentiation produced a significant inhibition (60%) in the number of adipocytes differentiated from hMSCs. Moreover, this inhibitory effect was blocked by either 0·5 μM 4-androsten-4-ol-3,17-dione or 0·1 μM ICI-182,780, specific inhibitors of the enzyme aromatase (Ar-Inh) and of the oestrogen receptors respectively. On the other hand, the presence of 0·1 μM ICI-182,780 did not have a significant effect on AD differentiation.

A similar experiment was performed to evaluate the effect of the oestrogenic substrates androstenedione and testosterone on OS differentiation of hMSCs. Table 3 shows that under these substrate conditions, OS differentiation is enhanced as evidenced by the alkaline phosphatase activity measurements. This positive effect was decreased by the presence of the inhibitors Ar-Inh and ICI-182,780. Similarly with the AD differentiation, no effect on OS differentiation was observed by the addition of 50 nM oestradiol to the culture medium.

Control studies showed that the presence of inhibitors Ar-Inh and ICI-182,780 did not have a significant effect on OS and AD differentiation (data not shown).

Table 1 Oestrogens produced by hMSCs cultured under OS and AD conditions. Results are the mean±S.D.

	Substrate (concentration μM)	**Oestradiol** (pmol/mg protein)	**Oestrone** (pmol/mg protein)
Culture condition			
OS medium	Androstenedione (0·03)	4·90±0·27	7·96±1·33
OS medium	Androstenedione (0·1)	11·9±1·8*	17·9±2·4*
OS medium	Testosterone (0·1)	5·95±0·78†	0·93±0·29†
AD medium	Androstenedione (0·1)	8·8±1·3	15·1±4·48

1–1·2×10^5 hMSCs were cultured under osteogenic or adipogenic conditions during 24 h. Oestradiol and oestrone produced were measured using ^{3}H-labelled substrates as described in Materials and Methods. Experiments were performed in duplicate from two different samples. *P<0·05 compared with corresponding value of androstenedione (30 nM). †P<0·05 compared with corresponding value of androstenedione (100 nM).

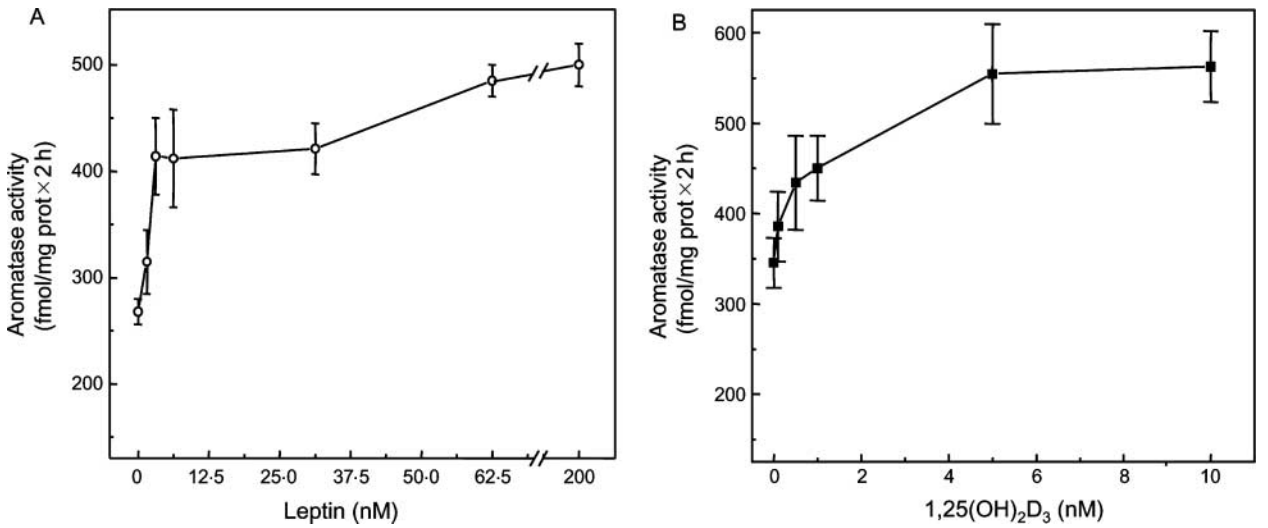

Figure 2 Aromatase activity of hMSCs in response to leptin and $1,25(OH)_2D_3$ in different concentrations. hMSCs were cultured in osteogenic conditions for 24 h, supplemented with specified leptin (A) or $1,25(OH)_2D_3$ (B) concentrations. The aromatase activity was measured by the tritiated water release assay, as described in legend of Fig. 1. Experiments were performed in triplicate from four different samples. Results are the mean ± S.D.

Discussion

Differentiation of hMSCs towards osteoblasts or adipocytes requires the sequential expression of genes associated with each of the resulting cell phenotypes (Ren *et al.* 2002, Kobayashi & Kronenberg 2005). The regulation of gene expression or activation during these processes is modulated by several endocrine, paracrine, autocrine and intracrine factors, which determine the phenotype to which the progenitor cells differentiate. Among these factors, oestrogens play an important role. Moreover, it appears that local aromatization of C19 precursors in bone may contribute significantly to skeletal homeostasis (Labrie *et al.* 1998, Simpson 2000, Simpson & Davis 2001), suggesting that the regulation of aromatase activity by factors present in the local environment play a decisive role in adjusting the levels of bioavailable oestrogenic hormone.

In this study, we demonstrate in hMSCs that OS differentiation promoted an early peak of aromatase CYP 19 activity, followed by a marked decrease of enzyme activity

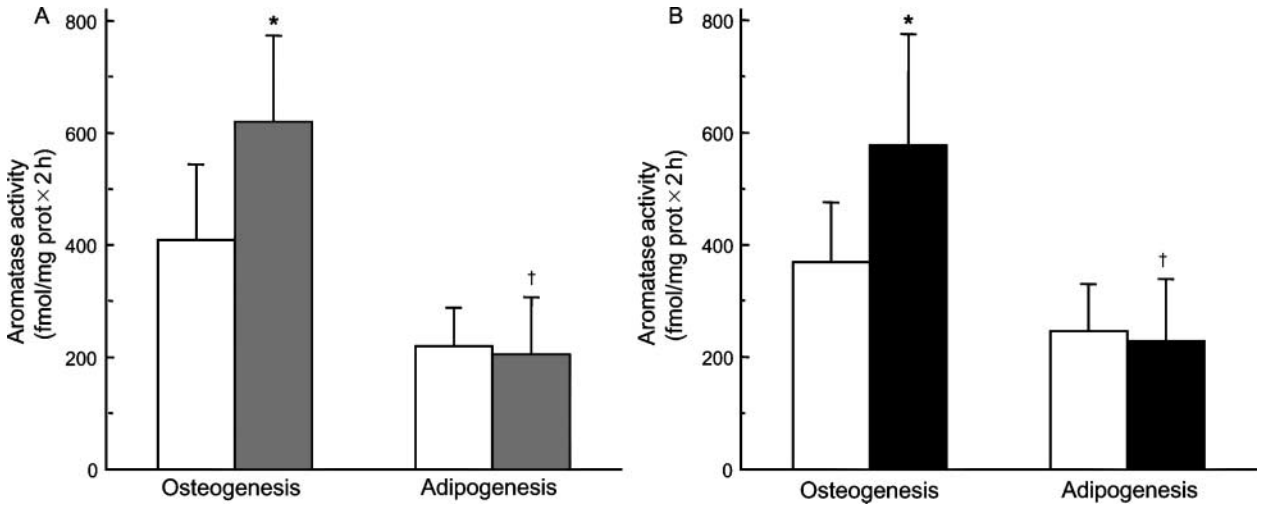

Figure 3 Effect of leptin and $1,25(OH)_2D_3$ on aromatase activity of hMSCs. Cells were cultured for 24 h in osteogenic or adipogenic conditions, supplemented with 200 nM leptin (A) or 10 nM $1,25(OH)_2D_3$ (B) and the aromatase activity was measured by the tritiated water release assay as specified in legend of Fig. 1. White bars, no addition; grey bars, leptin; black bars, $1,25(OH)_2D_3$. Experiments were performed in triplicate from four different samples. Results are the mean ± S.D. *$P<0{\cdot}05$ compared with values in the absence of hormone. †$P<0{\cdot}05$ compared with osteogenesis values in the presence of hormones.

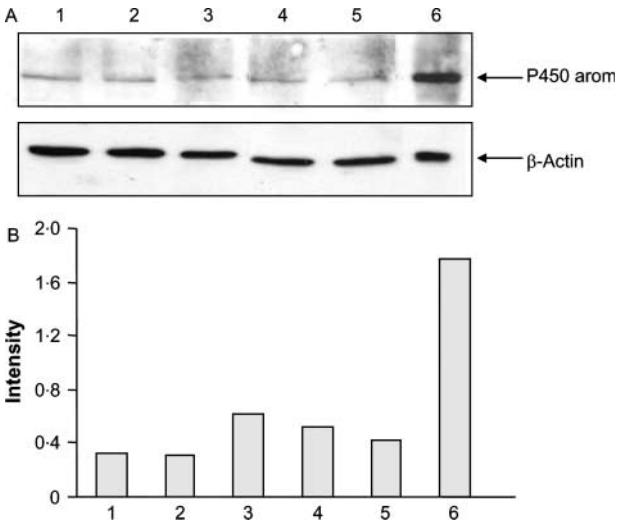

Figure 4 Western-blot analysis of aromatase in hMSCs. (A) hMSCs were cultured under basal (lane 1), OS (lane 2), AD (lane 3), OS + leptin (lane 4), and OS +1,25$(OH)_2D_3$, (lane 5) conditions, positive control (lane 6, MCF-7 cells). Western-blot analysis was performed using an anti CYP19 (P450 arom). (B) Densitometric analysis performed as described in Materials and Methods and normalized with β-actin. Representative data from three independent experiments are shown.

after 48 h; thereafter aromatase activity remained above basal values for up to 7 days. As far as we know, this is the first observation of aromatase activity during commitment and early stages of OS differentiation. A former study, carried out in a human foetal osteoblast cell line (SV-HFO) showed by

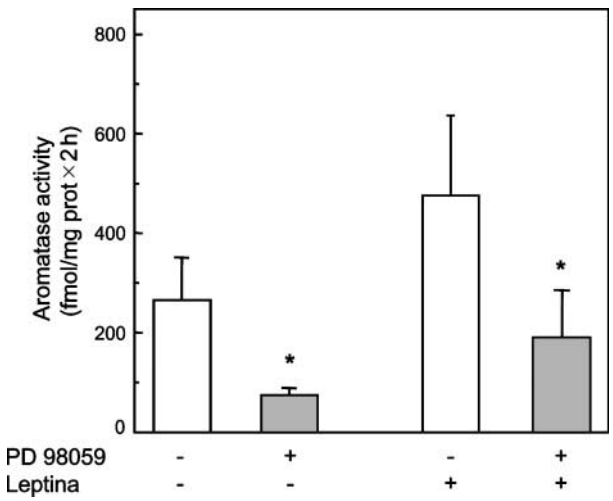

Figure 5 Effect of PD98059 on aromatase activity of hMSCs. Cells were cultured for 24 h in osteogenic conditions, supplemented or not with 200 nM leptin, and in the presence or the absence of 25 μM PD98059. The aromatase activity was measured by the tritiated water release assay as specified in legend of Fig. 1. Experiments were performed in triplicate from four different samples. Results are the mean ± S.D. *$P<0{\cdot}05$ compared with values without PD98059.

semi-quantitative analysis that aromatase mRNA expression did not change, while aromatase activity decreased during late stages of OS differentiation (measurements were done from 6 to 21 days of differentiation; Janssen *et al.* 1999). In addition, Heim *et al.* (2004), observed no variation in aromatase transcript levels in the first 7 days of differentiation of hMSCs, however, they did not study aromatase activity. Increased levels of transcripts were observed later during OS differentiation, supporting previous reports of aromatase expression and activity in mature osteoblasts (Sasano *et al.* 1997, Shozu & Simpson 1998). Considering both the results of Heim *et al.* (2004) and ours, it may be concluded that post-transcriptional mechanisms could play an important role in regulating aromatase activity during early OS differentiation.

Although the specific factor responsible for sharply increasing aromatase activity during early OS differentiation is not known, it could result from the dexamethasone present in the OS medium, since a similar increase of aromatase activity has been observed in primary cultured human osteoblasts after 12 h of dexamethasone treatment (Tanaka *et al.* 1996). Moreover, it has been concluded that glucocorticoids regulate transcription of the aromatase gene in bone, adipose and ovarian cells. (Simpson *et al.* 1981, Purohit *et al.* 1992, Shimodaira *et al.* 1996, Tanaka *et al.* 1996, Shozu & Simpson 1998, Enjuanes *et al.* 2003). However, post-transcriptional regulation of the enzyme after dexamethasone has also been suggested by the effects of inhibitors of protein synthesis or MAPK pathway phosphorylation (Tanaka *et al.* 1996, Shozu *et al.* 2001). Thus, the diminished aromatase activity observed after 48 h might result from modulatory changes during osteogenesis.

During early AD differentiation, aromatase activity levels were lower than during OS differentiation, but the enzyme activity remained significantly increased upto 72 h of treatment, suggesting that in addition to the initial effect of dexamethasone, interplay of signals developed during differentiation of MSCs may contribute to define the ensuing level of aromatase activity. Thus, a different pattern of enzyme activity developed after AD treatment of MSCs, despite the fact that the AD medium also contains dexamethasone. There are no previous observations on aromatase activity during early AD differentiation of MSCs, although increased CYP19 transcript levels were observed at this time, compared with control, that decreased considerably by the end of AD maturation (Heim *et al.* 2004). Thus, taking into consideration Heim's report (Heim *et al.* 2004), our observations could indicate that during early adipogenesis, aromatase cell activity could result mainly from transcriptional regulation.

Kinetic parameters of hMSCs aromatase enzyme agree with the values found in human osteoblasts (Tanaka *et al.* 1996), both in the range of apparent maximal velocity and in apparent K_m for androstenedione. Thus, in bone cells, the kinetic properties of both hMSCs and mature osteoblasts (Tanaka *et al.* 1996) indicate a high capacity for conversion of circulating androgens. Direct measurements of oestrogens produced by hMSCs support this conclusion and demonstrate

Table 2 Adipogenic differentiation of hMSCs under different culture conditions. Results are expressed as the relative adipocyte number compared with value obtained in plain adipogenic medium (AD). Experiments were performed in triplicate from four different samples

	Adipocytes (relative number)
Culture condition	
AD medium	1·00
+50 nM Oestradiol	1·03±0·19
+0·1 μM Androstenedione	0·62±0·36*
+0·5 μM Testosterone	0·53±0·10*
+0·1 μM Androstenedione +0·5 μM Ar-Inh	1·20±0·24
+0·1 μM Androstenedione +0·1 μM ICI-182,780	1·08±0·19
+0·5 μM Testosterone + +0·5 μM Ar-Inh	0·76±0·04*
+0·5 μM Testosterone +0·1 μM ICI-182,780	0·99±0·10

hMSCs were cultured under adipogenic conditions during 14 days. The adipocyte number was determined by flow cytometric analysis. Aromatase inhibitor (Ar-Inh): 4-androsten-4-ol-3,17-dione. Results are the mean±S.D. *$P<0·05$ compared with the plain adipogenic condition.

that significant concentration of oestradiol (1–3±0·56 nM) and oestrone (2–5±0·8 nM) is attained, depending on the substrate availability. Our results indicate that during the early stages of differentiation, hMSCs actively biosynthesize oestrogens as described for differentiated osteoblasts. Moreover, the different oestrogen-generating capabilities found among differentiating MSCs suggest that accurate oestrogens signalling may be important for appropriate early bone marrow cell differentiation.

Leptin and $1,25(OH)_2D_3$, significantly increased aromatase activity only through early OS differentiation. Neither leptin nor $1,25(OH)_2D_3$ affected aromatase activity of hMSCs under basal conditions, nor during AD differentiation. The dose–response curves support the *in vivo* action of these agents as modulators of aromatase activity, since effective concentrations used in this study are in the physiological range of circulating leptin and $1,25(OH)_2D_3$.

There are no previous reports on the effect of leptin on bone aromatase cell activity, although there are studies on leptin effects on aromatase gene expression and/or cell activity in luteinized granulose cells (Kitawaki *et al.* 1999), adipose stromal cells (Magoffin *et al.* 1999) and MCF-7 cell line (Catalano *et al.* 2003). Leptin activity on immortalized stroma cells from human bone marrow increased their differentiation to osteoblasts, while it inhibited their differentiation to adipocytes, suggesting a role for leptin in bone metabolism (Thomas *et al.* 1999). Previously, we have demonstrated the presence of membrane leptin receptors through early hMSCs differentiation, as well as its direct protective effect on their OS differentiation process (Hess *et al.* 2005). Therefore, from our results it may be suggested that part of the protective influence of leptin on bone tissue may result from its effect on aromatase activity during early differentiation of hMSCs.

The induction of aromatase activity we observed in hMSCs in response to $1,25(OH)_2D_3$ is consistent with the previous studies in other bone cell types showing that the effect of the hormone on aromatase is dependent on previous or concomitant glucocorticoid treatment (Tanaka *et al.* 1996, Enjuanes *et al.* 2003, Yanase *et al.* 2003). Our results show that the stimulatory effect of $1,25(OH)_2D_3$ is restricted to early osteogenic hMSCs differentiation and is characterized by a massive increase of aromatase activity that decreases after 48 h

Table 3 Osteogenic differentiation of hMSCs under different culture conditions. Results are the mean±S.D.

	Alkaline phosphatase activity (μg *p*-nitrophenol/well)
Culture conditions	
Basal	0·53±0·18
OS medium	4·84±0·55*
+50 nM Oestradiol	5·24±0·61
+0·1 μM Androstenedione	8·03±0·46†
+0·1 μM Androstenedione +0·5 μM Ar-Inh	6·54±1·85‡
+0·1 μM Androstenedione +0·1 μM ICI-182,780	6·48±0·15†,‡
+0·1 μM Testosterone	6·21±1·03†
+0·1 μM Testosterone +0·5 μM Ar-Inh	5·43±1·64
+0·1 μM Testosterone +0·1 μM ICI-182,780	5·32±0·23

hMSCs were cultured under osteogenic conditions during 14 days. Alkaline phosphatase activity was measured at 7 days of culture. Experiments were performed in duplicate from three different samples. Aromatase inhibitor (Ar-Inh): 4-androsten-4-ol-3,17-dione. *$P<0·05$ compared with basal medium. †$P<0·05$ compared with the plain osteogenic condition. ‡$P<0·05$ compared with osteogenic medium+androstenedione.

of differentiation. This effect of $1,25(OH)_2D_3$ on aromatase cell activity contrasts with the rather modest increase of aromatase mRNA (Enjuanes *et al.* 2003), supporting a post-transcriptional modulation of aromatase by $1,25(OH)_2D_3$ (Tanaka *et al.* 1996, Yanase *et al.* 2003).

Interestingly, western-blot analysis of hMSCs under basal, OS, OS plus leptin, OS plus $1,25(OH)_2D_3$ and AD differentiation showed no difference in the expression of protein level associated with immunostained aromatase. Therefore, the amount of protein appeared unrelated to the increase observed in enzyme activity suggesting that aromatase activity increased without changes in the protein concentration.

We observed that much of the increased aromatase activity after 24 h of OS stimulation was abolished by PD 98059, a selective inhibitor of MEK-1/2, supporting the proposition that aromatase activity might be acutely regulated by phosphorylation–dephosphorylation reactions during hMSCs differentiation. This type of post-transcriptional modulation of aromatase activity has been deduced from the inhibitory effects of the selective MEK-1/2 inhibitor on osteoblast-like cells, THP-1 (human peripheral blood) and JEG-3 (human choriocarcinoma)cell lines (Shozu *et al.* 2001). Therefore, specific OS and AD signals could trigger rapid and characteristic changes in aromatase cell activity, avoiding significant variation in aromatase protein and mRNA levels. This may explain the discordance between aromatase immunostained protein and enzyme activity.

We also evaluated whether compounds that modify either oestrogen synthesis or response affected OS and AD capacity of hMSCs. When substrate conditions that favoured aromatase activity existed (addition of androstenedione or testosterone), adipogenesis was significantly inhibited suggesting that high local oestrogen production restrains the process. Further, the effect was abolished by specific inhibitors for either aromatase or the oestrogen receptors, corroborating that increased oestrogen action is required to hold down AD.

During OS differentiation, we noticed that androstenedione added to medium had a positive effect on alkaline phosphatase activity, an early marker of OS differentiation. This favourable effect was diminished by the presence of specific inhibitors of aromatase or oestrogen action.

Osteoblasts (Arts *et al.* 1997, Heim *et al.* 2004) and adipocytes (Mizutani *et al.* 1994, Crandall *et al.* 1998) express oestrogen receptors showing different receptor expression patterns along hMSCs OS and AD differentiation (Heim *et al.* 2004). There is evidence supporting the role of oestrogen as a negative regulator for adipogenesis. *In vivo*, oestrogen receptors knockout mice (Heine *et al.* 2000) and aromatase-deficient mice (Jones *et al.* 2000) have been reported to manifest increased adiposity, although bone marrow adipocytes were not investigated in these reports. Two *in vitro* studies in mouse bone marrow stromal ST2 cell lines (Okazaki *et al.* 2002) and hMSCs (Heim *et al.* 2004) reported reciprocal regulation by oestrogen of osteoblastic and adipocytic differentiation from a common progenitor cell population. Our results confirm these observations and further underline the effect of oestradiol synthesized from C19 substrates by aromatase activity. In our experiments, these substrates repressed adipogenesis and favoured osteogenesis, while no effect was detected when pharmacological concentration of oestradiol was added to the medium. The lack of a direct effect of oestradiol could result from the presence of both 10% FCS and phenol red in the culture medium. These experimental conditions could provide suboptimal oestrogenic conditions even for cells in basal conditions. We could neither diminish FCS concentration nor use carbon-dextrane-treated serum to reduce oestrogen content in medium, since both treatments diminished viability of hMSCs. Thus, these observations suggest that locally produced oestradiol exerts great impact on hMSCs differentiation. Furthermore, these results support the hypothesis of a threshold oestradiol level for normal skeletal remodelling (Riggs *et al.* 2002, Gennari *et al.* 2004), which could be attained by the activity of endogenous aromatase on appropriate C19 precursors.

Overall, these observations point to critical requirements for the regulation of aromatase activity during the commitment and differentiation of bone hMSCs, suggesting that local production of oestrogen may hold appropriate cell differentiation, its production subjected to subtle adjustments depending on specific local signals. It could be inferred that high and early increases of oestrogen concentration in hMSCs affect their commitment by either restraining AD or facilitating OS differentiation, or both. During aging and some bone disorders, both substrate availability and aromatase regulation might affect the differentiation processes.

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