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

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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 D3 (1,25(OH)2D3) on aromatase cell activity. Finally, we analysed whether conditions that modify oestrogen generation by cells affected hMSCs differentiation. For these purposes, hMSCs derived from post-menopausal 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)2D3. Aromatase activity was measured by the tritiated water release assay and by direct measurement of steroids synthesized from 3H-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 androstenedione or testosterone.
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 CYP19 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 phytooestrogen 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 D3 (1,25(OH)2D3) and oestradiol may be involved in the regulation of hMSC differentiation (Komm et al. 1988, Bouillo et al. 1995). Further, 1,25(OH)2D3 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)2D3 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)2D3 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 (Rodriguez 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, [1β,2,6,7-3H(N)] 25.3 Ci/mmol was from Perkin-Elmer Sciences, Inc., Boston, MA, USA; androst-4-ene-3,17-dione, [1,2,6,7-3H(N)] 85 Ci/mmol was from New England Nuclear, Du Pont Co., Wilmington, DE, USA, and [1,2,6,7-3H(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-antiggo 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 supplemented with 0.1 mM 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).

Adipogenic differentiation

hMSCs (1–1.5 × 10^5 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,3H] androst-4-ene-3, 17-dione as substrate, for 2 h at 37 °C (Lephardt & Simpson 1991). Cell numbers and protein concentrations were measured in cell monolayers.

Determination of steroid formation from aromatase substrates: 1–1.2 × 10^5 cells/dish were incubated in OS or AD medium in the presence of 30 or 100 nM androstenedione plus 0.3 μCi 3H-androstenedione or 100 nM testosterone plus 0.3 μCi 3H-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 (1.25 × 10^4 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 (PVDF) 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 < 0.05 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 [1,2-3H] androst-4-ene-3,17-dione as substrate, for 2 h at 37 °C. Results were expressed as femtomoles of [3H]H2O produced/milligram of protein × 2 h. Experiments were performed in triplicate from four different samples. Results are the mean ± s.d. *P < 0.05 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).

<table>
<thead>
<tr>
<th>Substrate (concentration µM)</th>
<th>Oestradiol (pmol/mg protein)</th>
<th>Oestrone (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione (0-03)</td>
<td>4.90 ± 0.27</td>
<td>7.96 ± 1.33</td>
</tr>
<tr>
<td>Androstenedione (0-1)</td>
<td>11.9 ± 1.8*</td>
<td>17.9 ± 2.4*</td>
</tr>
<tr>
<td>Testosterone (0-1)</td>
<td>5.95 ± 0.78†</td>
<td>0.93 ± 0.29†</td>
</tr>
<tr>
<td>Androstenedione (0-1)</td>
<td>8.8 ± 1.3</td>
<td>15.1 ± 4.48</td>
</tr>
</tbody>
</table>

Table 1: Oestrogens produced by hMSCs cultured under OS and AD conditions. Results are the mean ± s.d.

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).

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′-aminoo-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).
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 CYP19 activity, followed by a marked decrease of enzyme activity.

Figure 2 Aromatase activity of hMSCs in response to leptin and 1,25(OH)2D3 in different concentrations. hMSCs were cultured in osteogenic conditions for 24 h, supplemented with specified leptin (A) or 1,25(OH)2D3 (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.

Figure 3 Effect of leptin and 1,25(OH)2D3 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)2D3 (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)2D3. Experiments were performed in triplicate from four different samples. Results are the mean ± s.d. *P<0.05 compared with values in the absence of hormone. †P<0.05 compared with osteogenesis values in the presence of hormones.


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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 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 up to 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

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Adipocytes (relative number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD medium</td>
<td>1.00</td>
</tr>
<tr>
<td>+50 nM Oestradiol</td>
<td>1.03 ± 0.19</td>
</tr>
<tr>
<td>+0.1 μM Androstenedione</td>
<td>0.62 ± 0.36*</td>
</tr>
<tr>
<td>+0.5 μM Testosterone</td>
<td>0.53 ± 0.10*</td>
</tr>
<tr>
<td>+0.1 μM Androstenedione + 0.5 μM Ar-Inh</td>
<td>1.20 ± 0.24</td>
</tr>
<tr>
<td>+0.1 μM Androstenedione + 0.1 μM ICI-182,780</td>
<td>1.08 ± 0.19</td>
</tr>
<tr>
<td>+0.5 μM Testosterone + 0.5 μM Ar-Inh</td>
<td>0.76 ± 0.04*</td>
</tr>
<tr>
<td>+0.5 μM Testosterone + 0.1 μM ICI-182,780</td>
<td>0.99 ± 0.10</td>
</tr>
</tbody>
</table>

hMSCs were cultured under adipogenic conditions during 14 days. The adipocyte number was determined by flow cytometric analysis. Aromatase inhibitor (Ar-Inh): 4-androst-4-en-3,17-dione. Results are the mean ± s.d. *P<0.05 compared with the plain adipogenic condition.

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

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Alkaline phosphatase activity (μg p-nitrophenol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.53 ± 0.18</td>
</tr>
<tr>
<td>OS medium</td>
<td>4.84 ± 0.55*</td>
</tr>
<tr>
<td>+50 nM Oestradiol</td>
<td>5.24 ± 0.61</td>
</tr>
<tr>
<td>+0.1 μM Androstenedione</td>
<td>8.03 ± 0.46*</td>
</tr>
<tr>
<td>+0.5 μM Androstenedione + 0.1 μM ICI-182,780</td>
<td>6.54 ± 1.85*</td>
</tr>
<tr>
<td>+0.1 μM Androstenedione + 0.5 μM Ar-Inh</td>
<td>6.48 ± 0.15*</td>
</tr>
<tr>
<td>+0.1 μM Testosterone</td>
<td>6.21 ± 1.03*</td>
</tr>
<tr>
<td>+0.1 μM Testosterone + 0.5 μM Ar-Inh</td>
<td>5.43 ± 1.64</td>
</tr>
<tr>
<td>+0.1 μM Testosterone + 0.1 μM ICI-182,780</td>
<td>5.32 ± 0.23</td>
</tr>
</tbody>
</table>

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-androst-4-en-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)\textsubscript{2}D\textsubscript{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)\textsubscript{2}D\textsubscript{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)\textsubscript{2}D\textsubscript{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. 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.

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