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Endothelin-1 Inhibits Human Osteoblastic Cell Differentiation: Influence of Connexin-43 Expression Level

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Abstract Gap junctional intercellular communication (GJIC) permits coordinated cellular activities during developmental and differentiation processes. In bone, the involvement of the gap junctional protein, connexin-43 (Cx43), and of GJIC in osteoblastic differentiation and mineralization of the extracellular matrix has been previously demonstrated. Former studies have shown that endothelin-1 (ET-1) was also implicated in the control of osteoblastic proliferation and differentiation. However, depending on the cellular models, ET-1 has been shown to decrease or increase osteoblastic differentiation markers. As no data were available on the ET-1 effect on GJIC and Cx43 expression in osteoblastic cells, we analyzed here the possible crosstalk between Cx43 and ET-1 in a human cell line (hFOB 1.19) which displays different Cx43 expression levels and phenotypes when cultured at 33.5 or 39°C. The presence of ET-1 (10^{-8} M) for 2–12 days of culture did not significantly alter the proliferation rate of hFOB cells whatever their phenotype. In contrast, ET-1 induced a differential inhibitory effect on the biochemical differentiation markers (alkaline phosphatase activity and osteocalcin expression) with a significant reduction in the differentiated phenotype at 39°C, whereas no effects were measured at 33.5°C. The inhibitory effect was linked to a decrease of GJIC and of Cx43 both at transcriptional and protein levels. Altogether, our results suggest that Cx43 expression level could influence the action of ET-1 on human osteoblastic cell differentiation. Our data also indicate that the gap junctional protein could play a pivotal role in the response of osteoblasts to mitogenic factors implicated in bone pathologies. J. Cell. Biochem. 103: 110–122, 2008.

Key words: gap junctional communication; connexin-43; osteoblast; differentiation process; endothelin

Gap junctions are clusters of transmembraneous channels composed of dodecamers of proteins named connexins (Cx) in closely apposed regions of cellular membranes in contact. These intercellular channels allow direct diffusion of small molecules including second messengers (cAMP, IP_3, cGMP, ...) and ions (K^+, Na^+, Ca^{2+}, ...). Cx are encoded by a multigenic family of at least 21 members in mammals and share a common topological structure [Willecke et al., 2002]. Nevertheless, Cx-formed junctional channels exhibit differences in biophysical properties (i.e., unitary conductance), functional abilities and regulation characteristics. Then, gap junctional intercellular communication (GJIC) can be finely regulated at several levels including Cx expression, Cx trafficking and gating of gap junctional channels by multiple factors such as ionic concentrations, second messengers, transjunctional potential, soluble factors, and protein phosphorylation [Saez et al., 2003; Segretain and Falk, 2004].

GJIC dysfunctions or mutations of Cx genes have been implicated in several human pathologies (i.e., X-linked Charcot-Marie-Tooth Disease, erythrokeratoderma variabilis, hereditary deafness) confirming an essential role for this communication in coordinating cellular activities during developmental and differentiation processes [Spray and Dermietzel, 1995;

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Lo, 1996; Di et al., 2002; Gerido and White, 2004]. GJIC has been also implicated in the control of cell proliferation and cellular differentiation in various normal tissues including bone [for review, see Stains and Civitelli, 2005a]. Among the multigenic Cx family, biochemical studies have documented the presence of two different gap junction proteins (Cx43 and Cx45) in the plasma membrane of bone-forming osteoblastic (OB) cells [Civitelli et al., 1993; Donahue, 2000], whereas a third connexin (Cx46) is retained in the late *trans*-Golgi network [Koval et al., 1997]. However, in this cell type, Cx43 is the predominant Cx and its function was directly related to the differentiation state of osteogenic cells [Schiller et al., 2001; Upham et al., 2003]. Moreover, alteration of Cx43 expression (by Cx45 transfection, antisense strategy, or mechanical/hormonal treatment) or of GJIC level affected the differentiation or mineralization processes [Civitelli et al., 1998; Lecanda et al., 1998; Ziambaras et al., 1998; Li et al., 2006]. The link between Cx43 expression and OB differentiation was clearly demonstrated in vivo by analyzing Cx43-deficient mice, in which bone build up by intramembraneous ossification suffered from severe hypoplasia [Lecanda et al., 2000].

Besides GJIC, soluble factors present in the bone microenvironment could largely influence the OB differentiation process in physiological or pathological conditions [Logothetis and Lin, 2005]. Endothelin-1 (ET-1), a 21 amino-acid peptide that displays a multitude of biological functions apart from vasoconstrictive effects [Yanagisawa et al., 1988] could play such a role. In bone, it was reported that ET-1 down-regulated the mineralization state of preosteoblastic MC3T3-E1 cells [Hiruma et al., 1998a]. Analyses of ET-1 knockout mice revealed that this peptide could modulate proliferation and migration of osteogenic cells [Kitano et al., 1998]. Although a well-described mitogenic effect, contradictory results have been reported showing either an inhibition [Takuwa et al., 1990; Kitten et al., 1997; Hiruma et al., 1998a,b; Someya et al., 2006] or a promotion [Kasperk et al., 1997; Von Schroeder et al., 2003] of osteoblastic differentiation by ET-1. Other studies documented physiopathological roles of ET-1 in several osseous human diseases [Tarquini et al., 1998], and prostate cancer-induced osteoblastic metastases [Jimeno and Carducci, 2004]. The essential role of GJIC in OB differentiation together with the well-established uncoupling action of ET-1 [Giaume et al., 1992; Spinella et al., 2003] led us to hypothesize that Cx43 expression in osteoblastic cells could influence the effect of this peptide on the differentiation process. In the present study, we have performed in vitro analyses on a temperature-sensitive human cell line (hFOB 1.19) and demonstrated that ET-1 induced a significant reduction of osteoblastic differentiation markers when Cx43 was expressed at high level at 39°C, whereas no effects were measured when Cx43 was weakly expressed at 33.5°C.

MATERIALS AND METHODS

Materials

ET-1, glutamine, geneticin, INDO-1-AM, ascorbic acid, β-glycerophosphate, methylene blue, sodium deoxycholate, sodium dodecyl sulfate (SDS), Triton X-100, protease inhibitors (P-8340), and 6-carboxyfluorescein diacetate were purchased from Sigma (St. Louis, MO), fetal calf serum (FCS) was from Cambrex BioSciences (Paris, France), monoclonal anti-Cx43 antibody was from Transduction Laboratory (Lexington, KY), anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC) from Chemicon International (Temecula, CA), nucleus counterstainer To-Pro®-3 from Molecular Probes Europe (Leiden, The Netherlands) and alkaline phosphatase-coupled anti-mouse antibody from Promega (Madison, WI). All other reagents were from standard suppliers.

Cell Model and Culture

The cellular model used in the present study was the human fetal osteoblastic cell line hFOB 1.19 (ATCC, CRL 11372, Manassas, VA,) immortalized with a gene encoding for a temperature-sensitive mutant of SV40 large T antigen [Harris et al., 1995]. The hFOB 1.19 cells provide a proliferation model system at permissive temperature (33.5°C) and have the ability to differentiate into mature osteoblasts expressing the normal osteoblast phenotype at restrictive temperature (39°C). Cells were seeded at a density of 6.5×10^4 cells/ml on 30 mm glass coverslips or on 35 mm plastic dishes (Nunclon, Nunc, Roskilde, Denmark). After a 4 h period, cells were washed in phosphate buffered saline (PBS) and maintained in DMEM/F12 (Invitrogen, Cergy Pontoise, France) supplemented with 2.5 mM

L-glutamine, 10% FCS, and 0.3 mg/ml geneticin treated with or without ET-1. The culture medium was renewed every 48 h until the end of the experimental period on days 2–12.

Recording of ($[Ca^{2+}]_i$) Transients

To demonstrate the presence of ET-1 receptors on hFOB cells, variations of free intracellular calcium concentration ($[Ca^{2+}]_i$) were measured by means of the ratiometric method with an inverted epifluorescence microscope (Olympus IX 70) after short ET-1 perifusion (10^{-7} M) as previously described [Niger et al., 2004]. Briefly, the Ca^{2+} indicator INDO-1 was used, for which fluorescence emissions of the Ca^{2+}-free (485 nm) and Ca^{2+}-bound (405 nm) forms of the indicator were collected using a dichroic filter and two photomultiplier tubes (excitation wavelength 355 nm). The calcium activity was estimated as the ratio of the 405/485 nm fluorescence emission intensities. For loading of the probe, hFOB cells were incubated for 45 min in the dark in Tyrode solution (144 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 5 mM HEPES and 5.6 mM glucose, pH 7.4) containing the lipophilic form of the dye (INDO-1/AM dissolved in DMSO 0.3%) at a concentration of 3 μM. After carefully washing off the unincorporated fluorogenic dye, cells were incubated in Tyrode solution for a further 15 min in the dark to obtain complete de-esterification of the dye. Variations of $[Ca^{2+}]_i$ with time were measured in a defined area located approximately in the center of the hFOB cells. By means of a homemade gravity-based microperfusion system, ET-1 was applied rapidly using a steamline flow directed onto the osteoblastic cells under investigation from the opening of a stainless steel capillary tube (internal diameter 50 μm) positioned in the bath. All experiments were conducted at room temperature ($20 \pm 1^\circ$C).

Proliferation Assay

Determination of cell proliferation was performed following a procedure adapted from Oliver et al. [1989]. After washing in PBS, hFOB cells were fixed in 0.5% glutaraldehyde and stained with a 1% methylene blue solution in borate buffer (pH = 8.5) for 1 h. Then, cells were rinsed in distilled water and dried at room temperature overnight. Fixed methylene blue was removed by exposure to 0.1 N HCl for 1 h and OD (optical density) of supernatants was determined at 620 nm. Comparison with cell number determined by counting gives a linear relationship and one OD unit corresponds to 50,000 cells.

Alkaline Phosphatase (ALP) Activity

ALP activity was determined colorimetrically using p-nitrophenylphosphate (pNPP; Biotron Diagnostic, Inc., Hemet, CA) as enzyme substrate. hFOB cells were scraped in a lysis buffer containing 10 mM tris-HCl, 2 mM $MgCl_2$, and 0.05% Triton X-100. Cell lysates were incubated in the presence of 16 mM pNPP at 37°C (pH = 10.2) and the enzymatic activity was measured at 405 nm and normalized against total cellular protein content determined by DC Protein Assay (BioRad, Marnes-la-Coquette, France).

Immunocytochemistry

To detect Cx43, cultured cells were rinsed with PBS, fixed in 2% paraformaldehyde at room temperature for 30 min and then washed three times in PBS. After incubation in a blocking solution consisting of 1% bovine serum albumin (BSA) and 1% Triton X-100 in PBS for 45 min, cells were washed three times in PBS and monoclonal anti-Cx43 antibody, diluted at 1:100, was applied for 1 h at room temperature. The second antibody conjugated to FITC and nucleus counterstainer To-Pro®-3 were then applied at 1:100 and 1:1,000, respectively and samples were examined on a BioRad MRC 1024 confocal microscope. All controls performed by omitting the primary antibody were negative.

Immunoblot Analysis

hFOB cells were washed with ice-cold PBS, scraped and digested in PBS containing 0.5% sodium deoxycholate, 20% SDS, 0.1% Triton X-100 and protease inhibitors at 4°C. The lysates were boiled for 3 min. Samples were then aliquoted and stored at −20°C until use. Protein concentration was determined according to Bradford's method (BioRad) using BSA as the standard. Proteins (60 μg) were subjected to electrophoretic separation using 12% polyacrylamide–SDS gel and transferred to nitrocellulose membrane. Membranes were blocked by incubation in a saline solution (pH 8.0), containing 0.05% Tween-20 and 5% non-fat dry milk, for 1 h at room temperature. Blots were then incubated overnight at 4°C with a monoclonal

antibody against mouse Cx43 diluted at 1:1,000. The corresponding antigen was detected after incubation with an alkaline phosphatase-coupled secondary antibody at 1:10,000. Densitometric analyses were performed using VisioL@b2000 software (Biocom, Les Ulis, France).

Semi-Quantitative RT-PCR And Real-Time PCR

After a 6–8 days period of culture, total RNA was extracted using RNeasy method (Qiagen, Hilden, Germany). cDNA was synthesized using oligo$(dT)_{12-18}$ as primer and superScriptII reverse transcriptase (Roche, Meylan, France) for Cx43 analysis and Ambion RETROscript kit (Ambion Ltd, Huntingdon, UK) for osteocalcin analysis.

The mRNA levels of Cx43 expression were then analyzed by semi-quantitative RT-PCR. One-fifth of the reaction mixture was amplified with *Taq* polymerase (Invitrogen) in a final volume of 50 μl. For the semi-quantitative PCR, each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and primer extension at 72°C for 1 min. To examine the quality and quantity of synthesized cDNA, primers of GAPDH were added at cycle 8 and PCR products (10 μl) were appropriated at cycles 23, 24, 25, 26, and 27. Primers were designed from previously published sequence data [Defamie et al., 2003]: the human GAPDH 5′ sense CTG CAC CAC CAA CTG CTT AG-3′ and 5′ antisense AGG TCC ACC ACT GAC ACG TT-3′; human Cx43 5′ sense AGT CTA TCT TTG AGG TGG CC-3′, and 5′ antisense GGC TGT AAT TCA TGT CCA GC-3′. PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide. Sizes of the expected amplification products were 1148 base pairs (bp) for Cx43 and 275 bp for GAPDH.

Analysis of osteocalcin transcripts by qPCR was used after 10 days of culture as late criterion for OB differentiation. Specific primers for OCN and PO were synthesized by Eurogentec and checked by a BLAST search of GenBank (osteocalcin precursor bone gla protein (U11542): OCN-forward 5′-ACC TCA CAG ATG CCA AGC CCC-3′ and OCN-reverse 5′-TCA CTT GTC TGA GGC CGC GT-3′ and housekeeping gene encoding ribosomal protein P0 (NM_007475): P0-forward 5′-ATG CCC AGG GAA GAC AGG GC-3′ and P0-reverse 5′-CCA TCA GCA CCA CAG CCT TC-3′).

The reactions were setup in duplicates in 20 μl total volume with 5 pmol of each primer, 10 μl of 2X SYBRgreen Master Mix and 1 μl of template. The PCR cycle was as follows: 95°C for 10 min, 35 cycles of 95°C for 15 s, 60°C for 1 min, and a melt curve analysis was performed at the end of each experiment to verify that a single product per primer pair was amplified. Furthermore, the sizes of the amplified DNA fragments were verified by gel electrophoresis on a 1.5% agarose gel. The amplification and analysis were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems). Samples were compared using the relative C_T method. The fold increase or decrease was determined relative to a vehicle-treated control after normalizing to a housekeeping gene (P0) using $2^{-\Delta\Delta CT}$, in which ΔC_T is (gene of interest C_T)—(PO C_T) and $\Delta\Delta C_T$ is (ΔC_T treated)—(ΔC_T control). Two normalizing controls (PO and 18S) were always included in the real-time reverse transcription (RT)-PCR experiments and yielded similar results. Data shown were normalized to the PO housekeeping gene.

Gap-FRAP

The degree of cell-to-cell communication between hFOB cells was measured by means of the gap-FRAP method [Wade et al., 1986] on a confocal microscope (FV 1000 Olympus IX-81, Tokyo, Japan). After washing, cultured hFOB cells were loaded for 15 min at room temperature in Tyrode solution containing the membrane-permeant molecule 6-carboxyfluorescein diacetate (7 μg/ml in 0.25% DMSO). The highly fluorescent and membrane impermeable 6-carboxyfluorescein moiety is released and accumulates within cells. After washing off the excess of extracellular fluoregenic ester to avoid further loading, the fluorescence of some selected cells adjacent to others cells was photobleached by applying strong light pulses from an argon laser set at 405 nm. Digital images of the fluorescent emission excited at 488 nm by weak laser pulses were recorded at regular intervals for 9 min (each time period = 30 s) and stored for subsequent analysis. In each experiment, one labeled, isolated cell was left unbleached as a reference for the loss of fluorescence due to repeated scanning and dye leakage. When the bleached cell was interconnected by open gap junctional channels to unbleached contiguous cells, a fluorescence recovery following a slow exponential time

course was measured. In case of the presence of cytoplasmic bridges, the fluorescence recovery is characterized by a fast step-like course which is not prevented by an exposure to a known junctional uncoupler like heptanol [Cronier et al., 1994, 1997]. In our experimental conditions, GJIC was investigated (coupled cells or not) in a population of cells in contact, leading to a percentage of coupled cells. An estimation of the relative permeability of the gap junctions is given by the diffusion rate constant k determined from recovery curves using the following equation: $(Fi - Ft)/(Fi - F0) = e^{-kt}$ where Fi, Ft, and F0 are fluorescence intensities before bleaching, at time t and at $t = 0$, respectively.

Statistical Analysis

All reported data are expressed as mean ± SEM and significant differences were identified by unpaired Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Cultured hFOB 1.19 Cells

As shown in Figure 1, after 6 days of culture at 39°C (Fig. 1B), a characteristic punctuate Cx43 immunostaining was observed both at the membrane level and inside the osteoblastic cells. After the same period at 33.5°C (Fig. 1A), the Cx43 staining was decreased. Western blot analyses confirmed that Cx43 was less expressed when hFOB cells were cultured at 33.5°C and showed a large increase in Cx43 levels at 39°C between days 2 and 6 of culture (Fig. 1C,D). Then, gap junction functionality was measured by means of the gap-FRAP method (Fig. 1E–G). Coupled cells were characterized by an exponential time course of fluorescence recovery from neighboring cells into a photobleached test cell (Fig. 1F) as previously described [Cronier et al., 2002]. A functional communication was measured between hFOB cells in both conditions and the percentages of coupled cells were 44.5% ($n = 77$) and 58.1% ($n = 93$) at 33.5°C and 39°C, respectively (Fig. 1G). In addition, the mean values of diffusion rate constants were 0.038 ± 0.0027 min^{-1} at 33.5°C and 0.044 ± 0.0037 min^{-1} at 39°C, demonstrating the absence of a significant difference in the degree of coupling when intercellular communication was established. To validate the link between Cx43 expression and the differentiation characteristics of our cellular model, proliferation and ALP activity were measured from days 2 to 13 of culture (Fig. 2). At 33.5°C, hFOB cells proliferated until 13 days following a sigmoid curve whereas at 39°C, no evolution of cell number was observed. At 33.5°C, ALP activity was very weak and decreased with the time of culture (0.75 ± 0.05 UI/L/μg proteins at day 2 to 0.26 ± 0.03 UI/L/μg proteins at day 13) whereas at 39°C the enzymatic activity increased during the first 8 days of culture (0.87 ± 0.03 UI/L/μg proteins at day 2 to reach 2.35 ± 0.01 UI/L/μg proteins at day 8) and then decreased afterwards (1.58 ± 0.04 UI/L/μg proteins at day 13).

When hFOB cells were cultured at 33.5°C, a proliferative phenotype was observed whereas at 39°C a differentiated but not proliferative phenotype was obtained. Thus, depending on the temperature culture conditions, hFOB cells exhibit two different levels of Cx43 expression and function corresponding to two distinct phenotypes, proliferative at 33.5°C and differentiated at 39°C.

ET-1 Action on hFOB Proliferation and Differentiation

First, the presence of ET-1 receptors was physiologically demonstrated. In our experimental conditions, the perifusion of ET-1 (10^{-7} M) in the vicinity of cells was effective in inducing an intracellular calcium ($[Ca^{2+}]_i$) rise in 66.7% and 63.6% of investigated cells at 33.5 and 39°C, respectively (Fig. 3). However, the ET-1-induced $[Ca^{2+}]_i$ peak was significantly lower ($P < 0.05$) in the differentiated phenotype ($+37.5 \pm 3.87\%$ compared to Ca^{2+} baseline; $n = 57$) than in the proliferative phenotype ($+69.4 \pm 26.9\%$ compared to Ca^{2+} baseline; $n = 49$). A plateau phase was exclusively observed in the differentiated phenotype reinforcing the hypothesis that the mechanisms implicated in the control of Ca^{2+} homeostasis were different between the two phenotypes. Results obtained with BQ123 (10^{-6} M), an ETA receptor antagonist, and BQ788 (10^{-6} M), an ETB receptor antagonist, supported the involvement of both receptor subtypes in the Ca^{2+} rise in hFOB cells (data not shown). Further studies are needed to validate the dual involvement of ETA and ETB receptors in this short-term effect of ET-1. The presence of ET-1 (10^{-8} M) in the culture medium for 2–12 days did not significantly alter the proliferation rate of

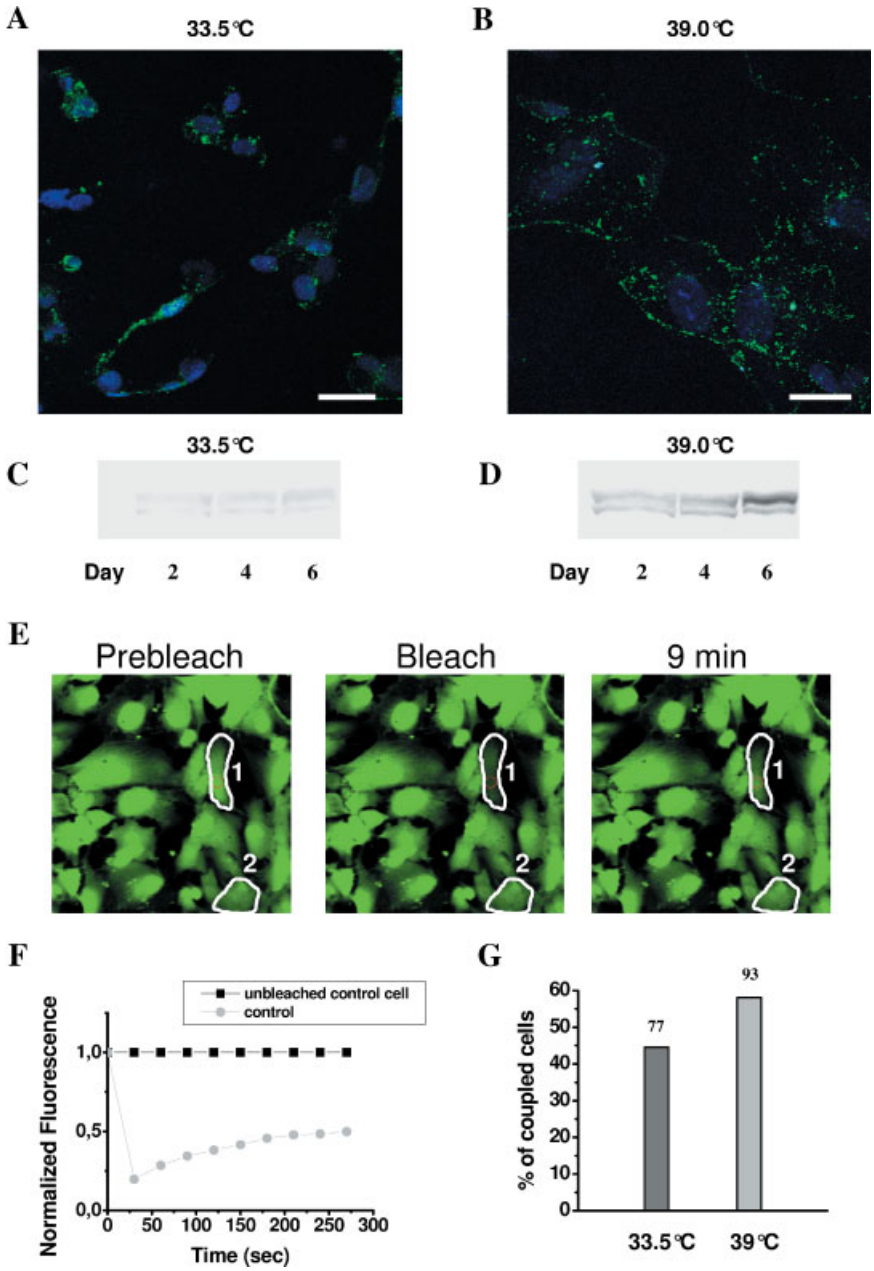

Fig. 1. Gap junctional properties of hFOB 1.19 cells in culture. **A**,**B**: Immunolocalization of Cx43 protein (green fluorescence) and counterstain of nuclei (blue fluorescence) after 6 days of culture. Strong Cx43 spots were observed in osteoblastic plasma membranes and in perinuclear regions mainly at 39°C (B). Scale bars = 50 μm. **C**,**D**: Cx43 expression level versus time and temperature of culture. Note the increase of Cx43 immunoblotting with time only at 39°C (D). **E**: Images of fluorescence distribution in hFOB cells cultured at restrictive temperature measured during gap-FRAP experiment. After a prebleach scan (PREBLEACH), the fluorescent dye was photobleached in some selected cells (polygons 1 and 2) by a strong laser illumination (see BLEACH). Polygon 2 was used as an unbleached control cell. The evolution of fluorescence intensities was measured for 9 min with a scanning period of 30 s. **F**: Example of corrected recovery curves expressed as the percentage of prebleach value versus time in selected cells. As previously described [Cronier et al., 1994], fluorescence recoveries in bleached cells follow a closely exponential time course, reflecting the presence of open gap junctional channels. **G**: Functional coupling in hFOB 1.19 cells after 2 days of culture at 33.5 and 39°C measured by means of gap-FRAP method expressed as percentage of coupled cells. Numbers of investigated cells are indicated on top of the bars.

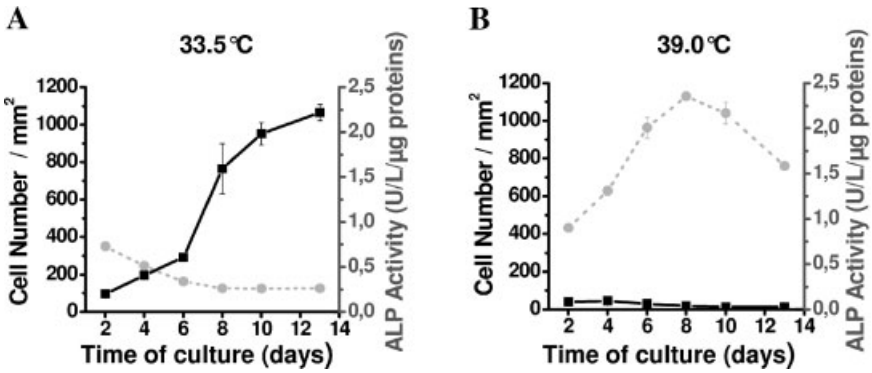

Fig. 2. Proliferative and differentiating properties of hFOB 1.19 cells in culture. **A**,**B**: Influence of culture temperature on ALP activity (dotted lines) and proliferation rate (solid lines) in hFOB 1.19 cells. A dramatic increase in cell number was measured at 33.5°C between days 2 and 13 of culture (A) with a decrease in ALP activity per µg of proteins. In contrast, at 39°C (B), a biphasic evolution of the differentiation marker was obtained without any rise in proliferation rate. Values represent the mean ± SEM of five distinct determinations.

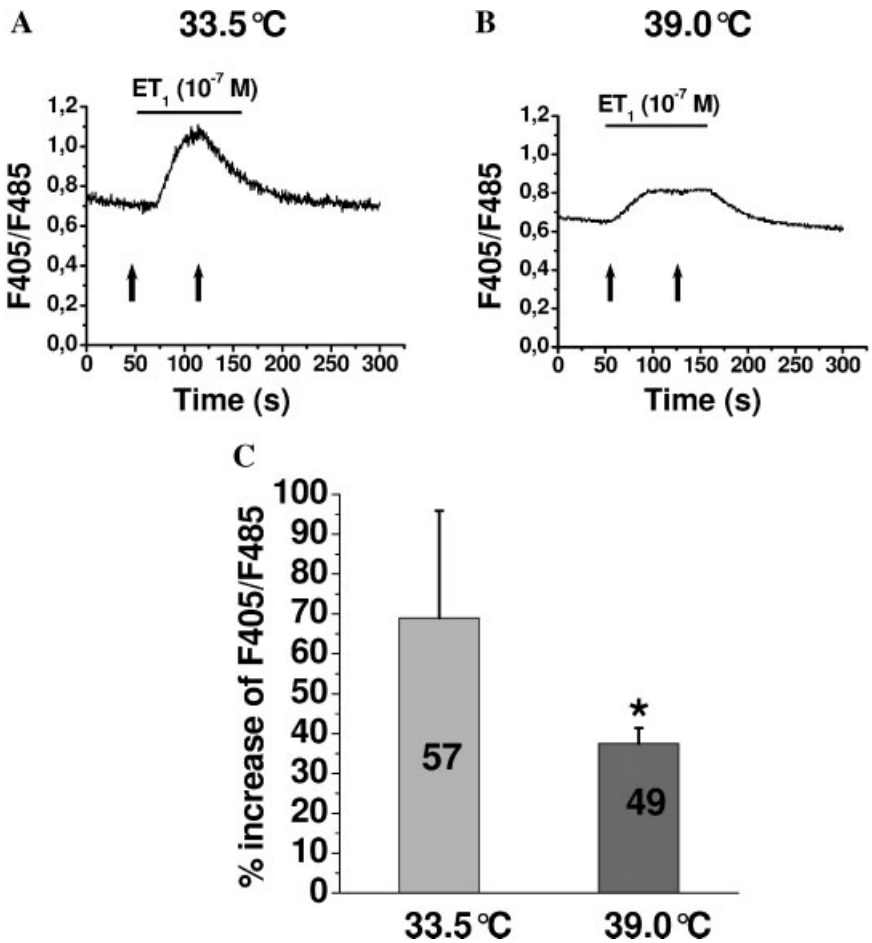

Fig. 3. Presence of ET-1 receptors in hFOB 1.19 cells. **A**,**B**: Recordings of $[Ca^{2+}]_i$ changes in response to 10^{-7} M ET-1 perifusion (100 s) in hFOB cells cultured for 6 days at 33.5°C (A) or at 39°C (B). Arrows indicate the time of measurements for calculation of the percentage change in ratio expressed in (**C**). C: Averaged data for the percentage increase in fluorescence ratio (F405/F485). Data are mean ± SEM and numbers of experiments are indicated inside of the bars; $*P < 0.05$.

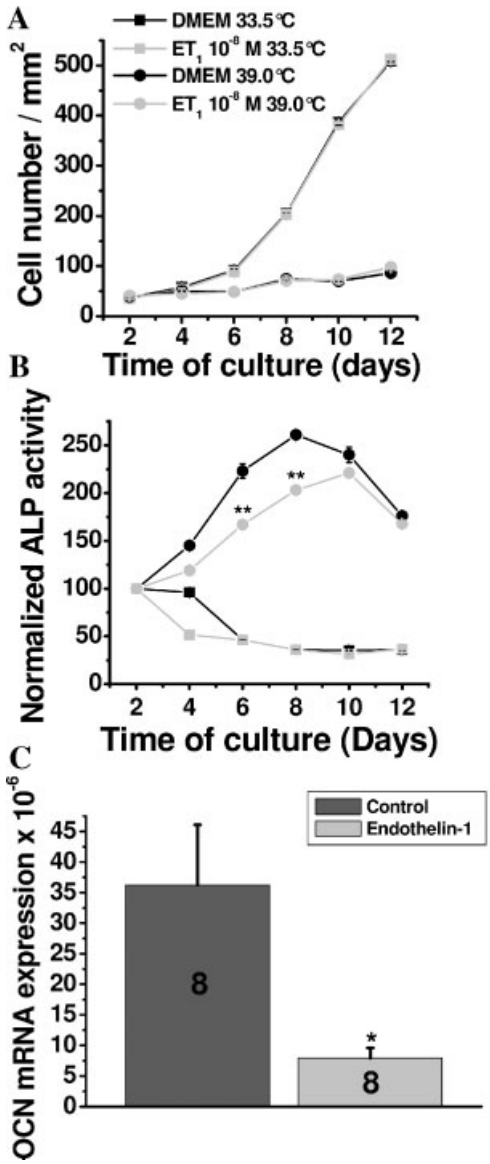

Fig. 4. Effects of ET-1 exposure on hFOB 1.19 cell proliferation and differentiation in culture. **A**: Cell number/mm^2 was not significantly affected by the presence of 10^{-8} M ET-1 (gray lines) in the culture medium in both temperature conditions. **B**: At 33.5°C, ET-1 (10^{-8} M) did not alter the ALP activity with progression of culture (squares). However, a significant reduction of this early biochemical marker was determined after 6 and 8 days of culture at 39°C (circles) in the presence of ET-1 (10^{-8} M). Data correspond to the mean ± SEM of five independent triplicate experiments; $^{**}P < 0.01$. **C**: In the differentiated phenotype, real-time PCR revealed a significant reduction of OCN mRNA levels when OB cells were cultured 10 days in the presence of 10^{-8} M ET-1 compared to control. Data correspond to the mean ± SEM of eight independent triplicate experiments; $^{*}P < 0.05$.

hFOB in proliferative as well as in differentiated phenotype (Fig. 4A). In contrast, ET-1 (10^{-8} M) induced a differential inhibitory effect on the ALP activity (Fig. 4B). At 39°C, a significant reduction of this biochemical marker was demonstrated after 6 and 8 days of culture in the presence of ET-1 ($P < 0.05$) whereas no effect was observed in the proliferative phenotype. Moreover, real-time PCR analyses on the differentiated phenotype revealed a significantly reduced ($P < 0.05$) expression of osteocalcin mRNA, a widely used marker of the OB late differentiation (Fig. 4C).

ET-1 Action on Cx43 Expression and GJIC

Western Blot analyses revealed a significant inhibitory effect of ET-1 (10^{-7} and 10^{-8} M) on Cx43 expression in hFOB cells cultured for 6 days at 39°C (Fig. 5A,B). No significant reduction of Cx43 level has been observed in the proliferative phenotype in the presence of ET-1 (data not shown). Interestingly, the reduced protein expression in the presence of the peptide was associated with a decrease in Cx43 transcript level in the differentiated phenotype measured by semi-quantitative RT-PCR (Fig. 5C) whereas no significant variation was obtained at 33.5°C. The inhibitory effect of ET-1 on Cx43 expression in the differentiated OB cells induced a decrease in gap junction functionality as assessed by gap-FRAP method (Fig. 5D). Thus, a 50% reduction of the percentage of coupled cells was measured (58.1% in control medium; 29% in the presence of ET-1). Moreover, a significant reduction ($P < 0.05$) of the mean values of diffusion rate constant could be observed (0.0307 ± 0.0003 min^{-1} in control medium; 0.0204 ± 0.0002 min^{-1} in the presence of 10^{-8} M ET-1). Altogether, our data suggest that ET-1 induced a reduction of Cx43 expression level and GJIC leading to a delayed differentiation process of hFOB 1.19 cells.

DISCUSSION

We demonstrated for the first time that the inhibitory effect of ET-1 on the OB differentiation process depends on the level of Cx43 expression with a significant effect only in hFOB cells that exhibit a differentiated phenotype. In addition, our study clearly showed a reduction of GJIC and of Cx43 both at protein and transcriptional levels in hFOB cells in the presence of ET-1. In the present study, we also confirmed that Cx43 protein expression, GJIC and ALP activity were significantly reduced in proliferative hFOB 1.19 cells cultured at 33.5°C compared to the differentiated phenotype at

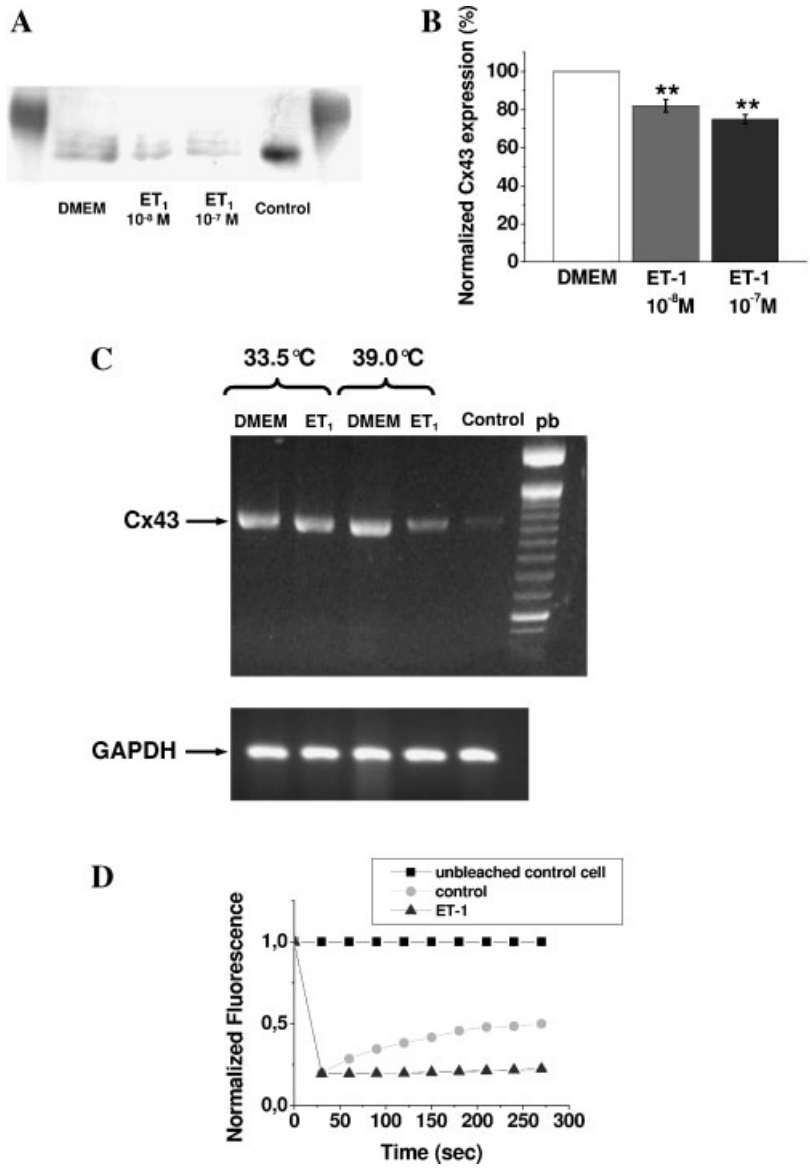

Fig. 5. Long-term effects of ET-1 on Cx43 expression and gap junctional intercellular communication in hFOB 1.19 cells. **A**: Western blot analysis of Cx43 after exposure to ET-1 (10^{-8} and 10^{-7} M) for 6 days at 39°C. Control lane represents total protein extract from rat brain. Results are representative examples of four separate experiments. **B**: Normalized Cx43 expression revealed a significant effect of ET-1; $^{**}P < 0.01$. **C**: ET-1 action on Cx43 mRNA expression as detected by semi-quantitative RT-PCR in hFOB cells cultured at 33.5 and 39°C for 6 days without (DMEM) or with ET-1 at 10^{-8} M (ET-1). Sizes of the amplification products are 1,148 bp for Cx43 and 275 bp for GAPDH. Control corresponds to amplified sequence of rat Cx43 cDNA. A 100 bp DNA ladder was used for size analysis (pb). The results shown are representative of three independent experiments. **D**: Example of corrected recovery curves expressed as the percentage of prebleach value versus time in selected cells. OB cells treated with ET-1 (dark gray triangle) for 6 days exhibit a significantly decreased fluorescence recovery compared to control (light gray circle) as soon as a 270 s period.

39°C [Donahue et al., 2000]. It was demonstrated in the same cellular model that stable Cx43 antisense cDNA altered expression of differentiation markers like ALP activity, osteocalcin and cbfa-1 mRNA levels suggesting that Cx43 expression and GJIC represent targets for altering OB differentiation [Li et al., 2006]. Finally, our study suggests that calcium mobilization elicited by ET-1 seems to be different depending on the phenotype. The difference in the calcium homeostasis could be explained by the differential expression and/or function of different calcium or cationic channels between both phenotypes especially voltage sensitive

and/or capacitative calcium channels [Santillán et al., 2004; Bergh et al., 2006].

Cellular coordination through gap junctions during cell differentiation can be observed in most developing tissues in early embryo as well as in adult. Previous studies showed that Cx43 participate in differentiation of several cell types such as glial cells [Naus et al., 1997], myoblasts [Constantin and Cronier, 2000], or trophoblasts [Cronier et al., 2003]. Among the connexin family, Cx43 exhibited developmental functions as demonstrated by antisense or knockout strategies [Reaume et al., 1995; Becker et al., 1999; Lecanda et al., 2000; Frendo et al., 2003]. Analyses of Cx43-deficient mice clearly demonstrated a delayed mineralization and craniofacial abnormalities, further suggesting that Cx43 was critical for normal osteogenesis [Lecanda et al., 2000]. In the same way, GJIC dysfunctions or Cx43 gene mutations have been associated with human oculodentodigital dysplasia (ODDD) [Paznekas et al., 2003; Lai et al., 2006]. Altogether, these studies and the current data suggest that Cx43 expression and function largely contribute to normal osteoblastic differentiation.

There is evidence that Cx expression and GJIC vary in response to treatments with biologically active substances such as growth factors and hormones [Bruzzone et al., 1996; Cronier et al., 1997]. ET-1, a known physiological disruptor of GJIC [Giaume et al., 1992; Spinella et al., 2003] was able to inhibit communication in other differentiation processes implicating Cx43 [Cronier et al., 1999]. In bone, it was reported that ET-1 decreased osteocalcin mRNA levels and matrix calcium deposition by MC3T3-E1 cells via a specific interaction with the ETA receptor [Suzuki et al., 1997; Hiruma et al., 1998a; Someya et al., 2006]. Moreover, other in vitro studies clearly showed an inhibitory effect of ET-1 in primary rat calvaria osteoblasts [Takuwa et al., 1990]. In vivo, a severe hypoplasia was described in facial bones of ET-1 knockout mice, suggesting a disruption in the matrix mineralization process [Kitano et al., 1998]. However, it should be noted that in two primary OB models, ET-1 was able to promote osteoprogenitor differentiation [Kasperk et al., 1997; Von Schroeder et al., 2003]. In the present study, the fact that the inhibitory action of ET-1 was correlated to a reduced expression of Cx43 mRNA and protein led us to hypothesize that Cx43 expression level could influence the action of ET-1 on human osteoblastic cell differentiation. Similar chronic effects of ET-1 on Cx43 expression have been previously reported in human trophoblastic cells [Cronier et al., 1999], carcinoma cells [Spinella et al., 2003], and cortical astrocytes [Rozyczka et al., 2005]. However, in our model, no significant effect of ET-1 was demonstrated in the two distinct phenotypes (proliferative and differentiated) on the proliferation rate despite the well-known mitogenic ability of this peptide. The present study demonstrates that the induced decrease of Cx43 expression did not seem to be secondary to increased cell proliferation as described for certain other mitogens [Reuss et al., 2000]. Further studies must be undertaken to analyze the full action of ET-1 on the proliferation and the differentiation processes of OB cells especially the molecular aspects of the interaction between Cx43 and ET-1-induced inhibitory effect. Among the multiple messenger signals affected by ET-1 [Stern et al., 1995] which are known to interfere with Cx43 expression and/or function [Stains and Civitelli, 2005b], it has been previously described in the OB model, the involvement of PKC and phosphatidyl inositol turnover, MAP kinase and PI_3 kinase pathways. Other soluble factors present during bone remodeling have been shown to modulate Cx43 expression and GJIC in osteoblastic cells [Rudkin et al., 1996; Civitelli et al., 1998]. Parathyroid hormone (PTH) increased both the steady-state levels of Cx43 mRNA and GJIC with a process involving cAMP in several osteoblastic cell lines [Schiller et al., 1992; Civitelli et al., 1998]. In this context, endothelin represents one of the rare inhibitory modulators of inter-osteoblastic communication.

The source of ET-1 in the environment of osteoblastic cells in vivo is probably multiple with a possible production in physiological conditions by osteoblasts [Kitten and Andrews, 2001] or by endothelial cells leading to an autocrine/paracrine action in addition to the systemic ET-1. In pathological conditions, significant higher ET-1 circulating levels were measured in patients with Paget's bone disease compared to healthy subjects [Tarquini et al., 1998]. Moreover, ET-1 has been implicated in the progression of prostate cancer as well as in the development of the osteogenic bone lesions during metastasis [Nelson et al., 1996; Jimeno and Carducci, 2004].

In conclusion, our results suggest that Cx43 expression level could influence the action of ET-1 on differentiation of human OB cells. This observation could explain contradictory results previously obtained in vitro on different cellular models. This study also indicates that the gap junctional protein could play a pivotal role in the response of osteoblasts to mitogenic factors implicated in bone pathologies. Further studies are needed to investigate the ET-1 signal transduction pathways implicated in the Cx43 modulation in proliferative and differentiating phenotypes, especially the calcium homeostasis investigated in the present study which displayed significant differences between both phenotypes.

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