Signaling and biological effects of glucagon-like peptide 1 on the differentiation of mesenchymal stem cells from human bone marrow

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gon-like peptide 1 (GLP-1) functions as an incretin hormone with antidiabeticogenic properties. However, the role of GLP-1 in human bone marrow-derived mesenchymal stem cells (hMSCs), if any, remains unknown. The effects of GLP-1 on hMSCs were tested with regard to cell proliferation, cytoprotection, and cell differentiation into adipocytes. The signaling pathways involved in these processes were also analyzed. Cells were characterized with biochemical and morphological approaches before and after being induced to differentiate into adipocytes. PCNA protein levels were used as a proliferation index, whereas cell apoptosis was studied by deprivation of fetal bovine serum. Isolated hMSCs expressed stem cell markers as well as mRNA and GLP-1 receptor protein. GLP-1 increased the proliferation of hMSCs, which decreased when they were induced to differentiate into adipocytes. This process produced biochemical and morphological changes in cells expressing PPARγ, C/EBPβ, AP2, and LPL in a time-dependent pattern. Notably, GLP-1 significantly reduced the expression of PPARγ, C/EBPβ, and LPL. These effects were exerted at least through the MEK and PKC signaling pathways. In addition, GLP-1 significantly reduced cell apoptosis. Our data indicate that, in hMSCs, GLP-1 promotes cellular proliferation and cytoprotection and prevents cell differentiation into adipocytes. These latter findings underscore the potential therapeutic role of GLP-1 in preventing the adipocyte hyperplasia associated with obesity and, additionally, could bolster the maintenance of hMSC stores by promoting the proliferation and cytoprotection of undifferentiated hMSC.

human mesenchymal stem cell; proliferation; adipogenesis; cytoprotection

MESENCHYMAL STEM CELLS (MSCs) from bone marrow were initially described as clonal (30) cells capable of differentiating into adipocytes, chondrocytes, osteoblasts (30), and insulin-secreting cells (39). Although the ability of stem cells to proliferate and differentiate into several cell lines is well known, the mechanisms and the molecules involved in such processes are poorly understood. The candidates for such activity are likely to be a number of structurally related peptide hormones and neuropeptides that exert cytoprotective and proliferative effects through G protein-coupled receptor activation. Furthermore, several peptides related to the glucagon-secretin family, together with other peptides, exert either pro- or antiapoptotic actions on several cell types (10).

Glucagon-like peptide 1 (GLP-1) is encoded by the progluca-
gon gene, which is secreted by gut L cells and some brain neurons. It exerts multiple biological effects on peripheral tissues and the central nervous system (2, 3, 25, 18). Thus, GLP-1 stimulates glucose-dependent insulin secretion and has beneficial effects in the treatment of type-2 diabetes (18), although there are data demonstrating that at pharmacological concentrations the main effect of GLP-1 on postprandial glu-
cose homeostasis is mediated by a delay in gastric emptying rather than by a modulation of endocrine pancreatic secretion (27). It also acts as an anorexigenic peptide, reducing food intake and hence, body weight (28, 41). Strikingly, it causes an expansion of β-cell mass through the stimulation of pancreatic β-cell proliferation, the induction of islet neogenesis, and differentiation from exocrine cells or immature islet progenitors (5, 8) while at the same time providing protection from β-cell apoptosis. Furthermore, GLP-1 has a cell neuroprotector role (6, 11). Therefore, these findings concerning GLP-1, together with the properties of MSC (1), may prove useful for studying tissue regeneration, cytoprotection, and cell differentiation. Although GLP-1 receptor (GLP-1R) agonists activate signal pathways (42) related to both the stimulation of cell proliferation and the inhibition of apoptosis (10), and although Klinger et al. (24) have recently contributed significantly to clarifying the signaling pathways involved in cell proliferation, the effects of these glucagon-like peptides on such processes are still poorly understood.

The properties of GLP-1, such as those cited above, may be considered to be antidiabetogenic (15) and contrary to an obese state (33), thus opening new avenues to study the role of GLP-1 in adipogenesis. Although the effects of glucagon-like peptides have been studied mainly in adult differentiated cells, our aim here was to investigate the role of GLP-1, if any, in the proliferation, differentiation into adipocytes, and cytoprotection of pluripotent human bone marrow-derived MSCs (hMSCs) as well as the mechanisms involved in these processes. The results indicate that the presence of GLP-1R in hMSCs permits an agonist-induced increase in cell proliferation, reduces differentiation into adipocytes, and prevents apoptosis. Further information is provided about the GLP-1R-signaling pathways involved in the proliferation and adipogenesis processes.

MATERIALS AND METHODS

hMSC isolation and cell culture. Adult hMSCs were obtained from the remaining part of bone marrow aspirated from the iliac crest donated by healthy subjects for cell transplantation (Hospital Univer-

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sitario La Princesa, Madrid, Spain). All procedures were carried out according to the Declaration of Helsinki II after permission had been obtained from the Hospital Universitario La Princesa Ethics Committee together with the volunteers’ consent. The isolation and expansion of hMSCs from 36 healthy donors (between 24 and 52 yr old) were carried out as described previously (31). Cells were grown in a standard culture medium consisting of Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG; Life Technologies), 15% fetal bovine serum (FBS) from selected batches (Hyclone, Madrid, Spain), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine and 12% bovine serum (FBS) from selected batches (Hyclone, Madrid, Spain), medium-low glucose (DMEM-LG; Life Technologies), 15% fetal serum-0.1% Triton X-100, 0.25 mM EDTA, pH 8.0, 0.2% NaF, and 0.1% sodium orthovanadate) and a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Western blots were performed essentially as described previously (33); 20 μg of total proteins was separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore). The membrane was blocked with TBS containing 0.1% Tween 20 and 5% nonfat dried milk before incubation with the specific primary antibodies: mouse anti-PCNA diluted 1:1,000, rabbit anti-phospho-ERK1/2 1:2,000 (MBL, Woburn, MA) and rabbit anti-ERK1/2 1:5,000 (Calbiochem), rabbit anti-PPARγ2 1:500 (Affinity BioReagents) and mouse anti-C/EBPβ 1:500 (Affinity BioReagents). Loading controls were performed with a mouse anti-β-actin antibody diluted 1:5,000 (Sigma-Aldrich). Secondary antibodies conjugated with peroxidase were purchased from Bio-Rad (Richmond, CA). Bound proteins were visualized using chemiluminescence horseradish peroxidase substrate (Millipore).

Proliferation and MAPK assays. For the proliferation and MAPK assays, the levels of PCNA and phosphorylated ERK1/2 were taken as an index of cell proliferation and MAPK pathway activation, respectively. hMSCs were cultured for 1 day in AIM (differentiating) or in a standard culture medium (undifferentiated) both in the absence of FBS as well as with or without 10 nM GLP-1 (Bachem) and kinase inhibitors. The kinase inhibitors were purchased from Calbiochem, and the concentrations used were 2 μM PD-98059, a selective inhibitor of MAPK kinase, 10 μM Ro 31-8220 (a PKC inhibitor), and 1 μM Ca2+/calmodulin kinase II inhibitor.

Detection of cell death using ELISA. A cell death detection ELISA kit (Roche Diagnostics) is employed in the relative quantification of apoptosis, differentiating apoptosis from necrosis by quantitative in vitro determination of cytoplasmic histone-associated DNA fragments from lysed cells. It discriminates apoptosis from necrosis since, for the detection of necrosis, histone-complexed DNA fragments are detected directly in the culture supernatant without cell lysis.

Cells were kept for 1 day in DMEM-LG at different FBS concentrations (0–20%) or 3 days in the absence of serum plus 10 nM GLP-1. They were then homogenized in an incubation buffer, and assays were performed, following the manufacturer’s instructions (Roche Diagnostics) and as described by Sanz and colleagues (34, 35).

cAMP measurements. hMSCs were washed with HEPES-buffered saline (HBS) medium (130 mM NaCl, 0.9 mM NaH2PO4, 0.8 mM MgSO4, 5.4 mM KCl, 1.8 mM CaCl2, 20 mM HEPES, pH 7.4, and 25 mM glucose) and incubated for 8 min in HBS containing 1 mM IBMX and 0.1% BSA with GLP-1 in a concentration range of 0 to 100 nM or with forskolin (10 μM). For cAMP measurements, a commercial enzyme immunoassay kit (Amersham) was used, and the values were normalized with the total protein concentration in each sample.

Statistical analyses. All values are presented as means ± SE. Comparisons among groups were made using analysis of variance followed by the Tukey post hoc test. P < 0.05 was considered statistically significant.

RESULTS

Characterization and in vitro growth of hMSCs. hMSCs kept in a standard culture medium containing 15% FBS pro-

anti-rabbit antibody (Calbiochem) (both diluted 1:200 in blocking solution) for PCNA and GLP-1R detection, respectively. The specificity of GLP-1R immunodetection was confirmed by adding preimmune serum or by antibody preabsorption with the keyhole limpet hemocyanin-conjugated synthetic peptide. Nuclei were stained with 1 μg/ml DAPI-PBS, and cells were mounted with Fluoromount G mounting medium (EMS, Hatfield, PA). Images were taken with a TCS SP2 confocal laser microscopy system (Leica) equipped with an inverted DMIRE2 Leica microscope.

Western blot and immunodetection. hMSCs were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 1% sodium deoxycholate, 1% Triton X-100, 0.25 mM EDTA, pH 8.0, 0.2% NaF, and 0.1% sodium orthovanadate) and a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Western blots were performed essentially as described previously (33); 20 μg of total proteins was separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Millipore). The membrane was blocked with TBS containing 0.1% Tween 20 and 5% nonfat dried milk before incubation with the specific primary antibodies: mouse anti-PCNA diluted 1:1,000, rabbit anti-phospho-ERK1/2 1:2,000 (MBL, Woburn, MA) and rabbit anti-ERK1/2 1:5,000 (Calbiochem), rabbit anti-PPARγ2 1:500 (Affinity BioReagents) and mouse anti-C/EBPβ 1:500 (Affinity BioReagents). Loading controls were performed with a mouse anti-β-actin antibody diluted 1:5,000 (Sigma-Aldrich). Secondary antibodies conjugated with peroxidase were purchased from Bio-Rad (Richmond, CA). Bound proteins were visualized using chemiluminescence horseradish peroxidase substrate (Millipore).

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RESULTS

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liferated, as indicated by the higher number of positives for PCNA, identified by immunocytochemistry (Fig. 1A) and by Western blot immunodetection (Fig. 1B), were compared with cells cultured in the absence of serum. RT-PCR analysis revealed that hMSCs kept in a standard culture medium expressed mRNA from stem cell markers such as stem cell factor (SCF) and its receptor (c-kit), Nestin, and Thy-1 (Fig. 1C). GLP-1R mRNA was also detected with RT-PCR (Fig. 1C) and

Fig. 1. Characterization of human bone marrow-derived mesenchymal stem cells (hMSCs) and effects of glucagon-like peptide 1 (GLP-1) on the mRNA expression of specific stem cell markers. hMSCs were cultured for 1 day in Dulbecco’s modified Eagle’s medium-low glucose (DMEM-LG) in the absence of FBS (NS) or in the presence of 15% FBS, and the growth was determined with PCNA detection by immunocytochemical (A) and Western blot (B) analyses. A: the micrographs show (in green) the PCNA immunofluorescence using a mouse anti-PCNA antibody and an anti-mouse conjugated to Alexa 488. Nuclear staining was performed in blue with 4,6-diamino-2-phenylindole (DAPI). Arrows indicate proliferating cells positive for PCNA. C: mRNA expression of GLP-1 receptor (GLP-1R) and different stem cell markers in hMSC detected by RT-PCR. Reverse transcriptase minus controls [RT(−)] was included. D: PCNA-positive cells and GLP-1R location in hMSCs grown with 15% FBS. These were detected by immunocytochemistry using PCNA (green fluorescence) and GLP-1R antibody (red fluorescence) with Alexa 488 or Texas Red coupled to secondary antibodies, respectively. Arrow indicates a double-positive cell. Nuclei were stained with DAPI (blue fluorescence). D, a and b: the immunostaining controls obtained by cross-preabsorption of the antibody with the blocking peptide or with cells incubated with preimmune serum, respectively. E: hMSCs were incubated for 1 day in DMEM-LG in the absence (−GLP-1) or presence of 10 nM GLP-1 (+GLP-1). The RT-PCR amplification of specific fragments for stem cell markers is shown. RT(−) was included in the figure. SCF, stem cell factor.
protein by immunocytochemistry in either proliferating or nonproliferating hMSCs (Fig. 1D).

RT-PCR analysis of hMSCs cultured for 1 day in the absence (−GLP-1) or presence of 10 nM GLP-1 demonstrated that GLP-1 treatment did not alter the expression of stem cell markers (SCF, Thy-1, and Nestin; Fig. 1E). In all of the cases, the PCR amplifications of RT(−) controls were negatives.

Adipogenic potential of hMSCs. Previous studies have demonstrated the osteogenic capacity of the same batches of cells used here (29). To study the capacity of hMSCs to differentiate into adipocytes, between three and nine different batches of adult hMSCs were induced by the addition of AIM. After 3 days of induction (Dif 3d), RT-PCR was used to view the expression of several adipocyte markers: PPARγ, C/EBPβ, adipocyte fatty acid-binding protein (AP-2), and LPL (Fig. 2A). During this period, GLP-1R was consistently present in hMSCs (Fig. 2A). Morphological modifications, such as the loss of a flat fibroblast phenotype, were also visible after 3 days of adipocyte differentiation (Fig. 2B, b) as well as the appearance of lipid droplets at 14 days of adipocyte differentiation (Fig. 2B, d). To quantitate the mRNA levels of the early adipocyte markers C/EBPβ, PPARγ, and LPL over the time course, we used quantitative real-time PCR performed in duplicate (Fig. 2, C–E) and observed that the expression of these genes was time dependent. After 8 h of incubation with AIM, C/EBPβ gene expression appeared, and maximum mRNA levels (2.5 ± 0.6-fold) were reached between the 1st and 3rd day after cell induction (Fig. 2C). However, the

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**Fig. 2. Adipogenic induction of hMSCs.** A: mRNA expression of adipocyte markers and GLP-1R in differentiating hMSCs analyzed by RT-PCR after 3 days of adipogenic induction (Dif 3d) compared with nondifferentiated (ND) cells. RT(−) was included in the figure. B: contrast-phase microphotographs from undifferentiated hMSCs (ND; a and c) or 3 or 14 days after the addition of the adipogenesis induction medium (Dif 3d and Dif 14d; b and d, respectively). B, C and D: cells stained with red oil. C–E: time-dependent mRNA expression of CCAAT/enhancer-binding protein-β (C/EBPβ), PPARγ, or lipoprotein lipase (LPL) analyzed by RT-quantitative real-time PCR in hMSCs cultured with (Dif) or without (ND) the adipogenesis induction medium. Values represent means ± SE of 3–9 different batches performed in duplicate and are given as the mRNA expression relative to the values found at 0 h. AP-2, adipocyte fatty acid-binding protein.
expression of PPARγ occurred in a time frame of between 1 and 9 days after the induction of adipocyte differentiation, the maximum expression (30 ± 7.5-fold) being reached between the 3rd and 7th day of differentiation (Fig. 2D). Finally, LPL mRNA appeared after 1 day of induction, but maximum expression was attained on the 7th day, and high levels persisted along the time course studied (Fig. 2E). These results indicate that LPL and PPARγ expression appear later, and with higher mRNA levels, than does C/EBPβ.

Effects of GLP-1 after hMSCs have been induced to differentiate into adipocytes. To examine the role of GLP-1 on adipocyte hMSC differentiation, we induced between two and six different batches of hMSCs to differentiate in the presence or absence of 10 nM GLP-1. The mRNA levels of PPARγ, C/EBPβ, and LPL were quantified with quantitative real-time PCRs performed in duplicate, using cDNAs transcribed from total RNAs isolated from differentiating hMSCs. When the hMSCs were stimulated to differentiate with AIM in the presence of GLP-1, this peptide inhibited C/EBPβ and PPARγ expression (Fig. 3, A and B), which became significant at the time at which the expression of both was maximum (the 1st and 3rd days, respectively; Fig. 2, C and D). It was noteworthy that LPL expression was strongly reduced by GLP-1 from the 3rd to the 7th day of differentiation (Fig. 3C). Interestingly, the inhibitory effect of GLP-1 was sequential and additive, as occurred with the time expression pattern of adipocyte markers (Fig. 2, C–E).

The pathways involved in GLP-1R signaling that might mediate the inhibitory effect of GLP-1 on the early adipocyte differentiation of hMSCs were studied. hMSCs were induced with AIM for 1 day in the presence or absence of GLP-1 with or without different kinase inhibitors, and PPARγ mRNA levels were quantified by real-time PCR. The reduction in PPARγ mRNA levels (or LPL mRNA; data not shown) by GLP-1 in hMSCs (Table 1 and Fig. 3B) was reversed by the inhibition of MAPK signaling and strengthened by PKC inhibition (Table 1). These observations suggest that the activation of MAPK signaling induced by GLP-1 (Fig. 4B) reduces the

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**Fig. 3. Effects of GLP-1 on the expression of mRNA adipocyte markers.** The hMSCs were induced to differentiate with adipogenic induction medium in the absence (−GLP-1) or presence of 10 nM GLP-1, which was renewed once a day. The graphics represent the mRNA levels determined by RT-quantitative real-time PCR of C/EBPβ (A), PPARγ (B), and LPL (C) in differentiating hMSCs normalized by 18S. The data plotted for each time point represent relative mRNA levels in cells treated with GLP-1 at the indicated time divided by the relative RNA levels in untreated cells at the same time. Bars represent the means ± SE of 2–6 experiments performed in duplicate. D: representative Western blotting detection of C/EBPβ and PPARγ proteins either in undifferentiated (ND) or in 1- or 3-day differentiating hMSCs (Dif 1d or Dif 3d) cultured in the presence (+) or absence (−) of 10 nM GLP-1. In all cases, β-actin was used to standardize lane-to-lane loading variations. Statistical significance: *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 (10 nM, GLP-1 vs. −GLP-1).
GLP-1 reduces cell death in hMSCs induced by serum starvation. To quantify serum starvation-induced cell death in hMSCs, we used an ELISA assay that specifically detects apoptotic cell death (Fig. 6). Cell death increased significantly in hMSCs cultured in the absence of serum for 1 day (0% FBS condition, 100% cell death, arbitrary value) compared with hMSCs cultured in the presence of complete medium containing 20% fetal bovine serum (Fig. 6A). The addition of GLP-1 (10 nM) partly prevented the cell death induced by 3 days of serum deprivation (Fig. 6B), although it did not exert the cytoprotective effect of adding 20% FBS. Furthermore, the cytoprotective effect of GLP-1 was additive to FBS.

Table 1. Effects of PD-98059 and RO 31-8220 (inhibitors for MEK and PKC in parenthesis) in the expression of PPARγ in 1-day adipocyte-differentiating hMSCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Differentiating hMSCs</th>
</tr>
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<tbody>
<tr>
<td>CT</td>
<td>1.00</td>
</tr>
<tr>
<td>GLP-1</td>
<td>0.78 ± 0.12 (n = 5)</td>
</tr>
<tr>
<td>PD-98059 (MEK)</td>
<td>0.89 ± 0.31 (n = 3)</td>
</tr>
<tr>
<td>GLP-1 + PD-98059</td>
<td>1.35 ± 0.40 (n = 6)*</td>
</tr>
<tr>
<td>Ro 31-8220 (PKC)</td>
<td>1.35 ± 0.39 (n = 6)</td>
</tr>
<tr>
<td>GLP-1 + Ro 31-8220</td>
<td>0.04 ± 0.01 (n = 2)*</td>
</tr>
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</table>

Values are means ± SE. hMSCs, human bone marrow-derived mesenchymal stem cells; CT, no additives; GLP-1, glucagon-like peptide 1. *P < 0.05 (+GLP-1 inhibitor vs. GLP-1 treatment).

expression of PPARγ and hence, adipocyte differentiation. Additionally, PKC signaling also mediated the GLP-1 effect on adipocyte differentiation in a manner contrary to that of the MAPK pathway.

Effects of GLP-1 on the proliferation of MSCs and signaling pathways involved in this process. The proliferation of hMSCs was tested by immunodetection of PCNA levels, which decreased significantly when these cells were induced to differentiate (Fig. 4A). GLP-1 increased the proliferation of undifferentiated cells, although it was unable to elevate the proliferation in differentiating cells. In parallel to this, we observed the induction of the MAPK pathway by GLP-1 in both undifferentiated and differentiating cells, as indicated by the detection of phosphorylated ERK1/2 (Fig. 4B). It is also worth noting that ERK1/2 activation was significantly reduced in differentiating hMSCs compared with the undifferentiated type.

In 10 nM GLP-1 hMSCs, a slight but significant (P < 0.05) rise in cAMP levels occurred (1.48 ± 0.12, n = 3, vs. 1.0 ± 0.08, n = 3, in untreated cells), measured as fold rise of cAMP levels in treated cells vs. untreated cells, with the latter being considered 1 (Supplemental Fig. S1). No significant increase in cAMP levels was caused by GLP-1 in 14-day differentiating cells (data not shown). The next step was to search for the signaling pathways involved in the GLP-1-induced proliferation of undifferentiated hMSCs. Undifferentiated and 1-day differentiating cells were incubated in the presence of 10 nM GLP-1 plus inhibitors or inhibitors alone for 24 h (Fig. 5, A and B). No effects on proliferation were found after adding the inhibitors alone (Fig. 5B). The inhibition of the MAPK pathway by PD-98059 blocked the proliferation induced by GLP-1 in undifferentiated hMSCs (Fig. 5A), indicating that the proliferative effect of GLP-1 on hMSCs was mediated by activation of the MAPK pathway. The inhibition of Ca2+/calmodulin kinase did not significantly alter PCNA levels in either undifferentiated or 1-day differentiating hMSCs. However, the inhibition of PKC restored proliferation in differentiating GLP-1-treated cells (Fig. 5A). These results indicated that, when hMSCs were undergoing differentiation, the proliferation rate was lower than under undifferentiated conditions and that GLP-1 could not activate this process. This phenomenon in 1-day differentiating cells must be mediated by PKC pathway activation, since the blocking effect on this pathway together with GLP-1 addition restored the proliferation rate of undifferentiated cells.

**Fig. 4. Effects of GLP-1 on proliferation and MAPK pathway activation in undifferentiated or in 1-day adipose-differentiating hMSCs.** Undifferentiated or 1-day differentiating hMSCs were cultured for 1 day in DMEM-LG in the absence of serum, with or without 10 nM GLP-1. Cells were then harvested and total protein isolated for Western blotting detection of PCNA protein (A) or ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2) proteins (B). In all cases, β-actin was used to standardize lane-to-lane loading variations. p-ERK1/2 protein levels were corrected with ERK1/2 and β-actin protein levels. Bars represent means ± SE (n = 5 different batches performed in duplicate) of protein levels relative to undifferentiated hMSCs without additives. *P < 0.05, **P < 0.01, and ***P < 0.001.
DISCUSSION

GLP-1 is an incretin hormone with multiple functions, including the promotion of proliferation, cytoprotection, and cell neogenesis in several tissues (10). Although the signaling pathways and biological actions of GLP-1 have been studied extensively in established cell lines, isolated tissues, and in vivo studies of models of adult organisms, less is known about the effects of GLP-1, if any, on the events occurring during stem cell differentiation. Accordingly, we used mesenchymal stem cells from human bone marrow to study the action of GLP-1 on the differentiation, proliferation, and cytoprotection of these cells.

The characteristics of the same batches of hMSCs used here have been reported in previous studies (31) and confirmed by internal control experiments showing the appearance of osteocytic and chondrocytic phenotypes in response to standard protocols (31). The mRNA and protein of the GLP-1R were identified in hMSCs, which is consistent with the actions of its agonists on the processes we have described. In the present study, we show that isolated hMSCs expressed the Thy-1 stem cell marker, the SCF stem cell factor, and its receptor c-kit and nestin throughout the culture period. However, no adipocyte markers were found, showing that the undifferentiated phenotype of hMSCs persisted. It is known that although polypeptides stimulate cell growth they also block cell differentiation, as occurs with PDGF, EGF, and FGF, all of which inhibit fat cell differentiation in culture and in vivo (20, 29, 38). Furthermore, several cytokines, including TNFα, interleukin-1, interleukin-6, transforming growth factor-β, and interferon-γ, inhibit cell differentiation into adipocytes (32, 40). Here, we show that GLP-1, through GLP-1R signal transduction in hMSCs, increased cell proliferation and promoted the inhibition of the early adipogenesis steps of these cells. The proliferation of hMSCs, determined by PCNA detection, was sig-
components stronger than PKA-dependent pathways might activate ERK1/2 and cell proliferation. In this sense, the involvement of the cAMP-Epac cascade or c-Src (9, 23, 24) should not be dismissed.

Additionally, GLP-1 did not significantly increase proliferation in differentiating hMSCs, suggesting that PKC could mediate this effect, because the PKC inhibitor (Ro 31-8220) restored the proliferation rate in differentiating cells up to the values found in undifferentiated hMSCs.

We detected the differentiation of MSCs into adipocyte-like cells, which were characterized by phenotypic changes, such as the appearance of lipid vesicles, and by biochemical markers. At least two families of transcription factors, C/EBPβ and PPARγ, are induced early on during adipocyte differentiation. It is known that a rise in C/EBPβ above a threshold level induces the expression of PPARγ (16) and cooperatively activates adipocyte genes such as LPL, thereby triggering adipocyte differentiation. Thus, after the addition of an adipocyte differentiation cocktail, we observed a significantly higher expression of C/EBPβ and PPARγ as well as of LPL and AP-2, leading to the appearance of lipid droplets. These markers appeared sequentially, following a time-dependent expression pattern, as occurs in the initial steps in adipocyte differentiation in vivo (16). We also found that GLP-1 significantly reduced the expression of C/EBPβ, PPARγ and LPL, and it was notable that the inhibitory effect was dependent on the scheduled expression of C/EBPβ, PPARγ, and LPL. It is worth mentioning that correct adipocyte differentiation depends on the coordinated regulation of early adipocyte-specific gene expression, which may explain why the relatively minor inhibition of C/EBPβ and PPARγ expression by GLP-1 (33 and 30%, respectively) later led to a considerable reduction in LPL expression (90%).

In an attempt to investigate the transduction signals involved in the effect of GLP-1 in the differentiation of hMSCs into adipocytes, we treated cells with the MEK inhibitor PD-98059. The blockade of the MAPK signal pathways abolished the inhibitory effects of GLP-1 on differentiation, indicating that the effects of GLP-1 are mediated, at least in part, by ERK1/2 activation (22). However, inhibition of PKC promoted the inhibitory effects of GLP-1 on hMSC differentiation while keeping the proliferative potential of differentiating cells at the same level as the undifferentiated. It is important to remark that the studies were carried out during early adipocytic differentiation (1 day of differentiation), when not all cells were undergoing differentiation. In this stage, the culture is a mixture of differentiated and undifferentiated cells. In this sense, the presence of GLP-1, together with the PKC inhibitor, could be contributing to the inhibition of the adipocyte differentiation and consequently yielding a higher quantity of proliferative undifferentiated cells. The ability of GLP-1 to inhibit differentiation and promote proliferation through PKC inhibition suggests that PKC could be involved in GLP-1R signaling as a pivotal molecule balancing the processes of proliferation and differentiation.

These results seem to be in accordance with those of Fleming et al. (13), who demonstrated the involvement of different protein kinase C isoforms in the early clonal expansion of differentiating cells and the later persistence of the adipocyte phenotype.
In contrast, neither PKA nor Ca\(^{2+}/\)calmodulin kinase inhibitors were found to mediate the effects of GLP-1 on adipocytic differentiation.

Our results indicate that GLP-1 receptors transduce at least two signaling pathways involved in hMSC differentiation into adipocytes; one of them leads to the activation of MAPK, which is essential for mitotic clonal expansion, and the second pathway involves the PKC effect mediating GLP-1 signaling in both the differentiation and proliferation processes.

Another physiological effect of GLP-1 in hMSCs was the prevention of apoptosis, as occurs in \(\beta\)-cell lines and in isolated pancreatic islets (12, 26). GLP-1 has beneficial effects on the carbohydrate and lipid alterations found in obesity and diabetes. Obesity is an important risk factor for type 2 diabetes mellitus, and hence, a reduction in body weight should be the first step in preventing this disease, whereas in the late stages, antidiabetic drugs become more important (19). In this regard, the first step in preventing this disease, whereas in the late stages, antidiabetic drugs become more important (19).

In contrast, neither PKA nor Ca\(^{2+}/\)calmodulin kinase inhibitors were found to mediate the effects of GLP-1 on adipocytic differentiation. Another physiological effect of GLP-1 in hMSCs was the inhibition of cell apoptosis and improvement of glucose responsiveness of freshly isolated human islets. Our findings reported here, reinforce the pathophysiological interest of GLP-1. In this sense, mice lacking dipeptidyl peptidase IV (which inactivates GLP-1 and many other substrates by cleavage) are protected against obesity and insulin resistance (7). Thus, the fat mass and percentage of fat content in DPP-IV\(^{-/-}\) mice fed a high-fat diet are lower than in wild-type animals. Morbid and childhood obesity are characterized by an excessive accumulation of adipose tissue mass that results from the enlargement of existing differentiated adipocytes or fat cells (hypertrophic obesity) as well as from the acquisition of new adipocytes from the proliferation and subsequent differentiation of preadipocytes (hyperplastic obesity) (14). Moreover, other authors have reported the lower lipogenic effect and higher lipolytic action of GLP-1 in obese subjects (33). Our results concerning the actions of GLP-1 in preventing adipogenesis, together with the properties mentioned above, may strengthen the potential therapeutic role of GLP-1, preventing the adipocyte hyperplasia associated with obesity (21), and could additionally reinforce the maintenance of hMSC stores by promoting the proliferation and cytoprotection of undifferentiated hMSCs.

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**DISCLOSURES**

We declare that no conflict of interest that might compromise the impartiality of this scientific study exists.

**REFERENCES**


