Alteration in Insulin Receptor Expression Accompanying Differentiation of HL-60 Leukemia Cells

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ABSTRACT

Differentiation of leukemic cells in vitro is characterized by the sequential appearance of morphological, functional, and biochemical markers of maturation. The interaction of insulin with its receptor may be a regulator of growth and differentiation of leukemic cells. Human promyelocytic leukemia cells (HL-60) demonstrate specific reversible insulin binding consistent with properties of human insulin receptor. HL-60 cells treated with 500 μM N6,O2-dibutyryl adenosine 3',5'-cyclic monophosphate, 1 μM 1α,25-dihydroxyvitamin D3, or 41 nm phorbol-12-myristate-13-acetate expressed monocyteic markers of differentiation and an increase in insulin receptor expression. The change in insulin receptor expression with 1 μM 1α,25-dihydroxyvitamin D3 and N6,O2-dibutyryl adenosine 3',5'-cyclic monophosphate induction was further characterized by Scatchard analysis. High affinity binding (Kd) constant was not altered, and the change in binding was attributed to receptor number. Commitment to increased insulin receptor expression was demonstrated after 1-h exposure to 1 μM 1α,25-dihydroxyvitamin D3. Agents which induced granulocytic differentiation, such as 160 mM dimethyl sulfoxide and 100 μM retinoic acid, significantly decreased insulin receptor expression compared to monocytic inducing agents. This difference in insulin receptor expression correlated with binding characteristics in normal human peripheral granulocyte and monocytes. The HL-60 cell line offers a model for the study of the molecular events which lead to the contrasting insulin receptor expression during myeloid and monocytic hematopoiesis.

INTRODUCTION

Specific receptors for insulin have been demonstrated on many human cells. Mature peripheral blood monocytes and granulocytes possess membrane receptors for insulin. Monocytes have a high insulin binding capacity (1), and granulocytes have a low insulin binding capacity (2). Changes in insulin binding on nucleated blood cells have been reported in diabetes and other pathophysiological conditions (3–5). Variability in insulin receptor expression has been related to insulin binding capacity consistent with properties of human insulin receptor. HL-60 cells treated with 500 μM N6,O2-dibutyryl adenosine 3',5'-cyclic monophosphate, 1 μM 1α,25-dihydroxyvitamin D3, or 41 nm phorbol-12-myristate-13-acetate expressed monocyteic markers of differentiation and an increase in insulin receptor expression. The change in insulin receptor expression with 1 μM 1α,25-dihydroxyvitamin D3 and N6,O2-dibutyryl adenosine 3',5'-cyclic monophosphate induction was further characterized by Scatchard analysis. High affinity binding (Kd) constant was not altered, and the change in binding was attributed to receptor number. Commitment to increased insulin receptor expression was demonstrated after 1-h exposure to 1 μM 1α,25-dihydroxyvitamin D3. Agents which induced granulocytic differentiation, such as 160 mM dimethyl sulfoxide and 100 μM retinoic acid, significantly decreased insulin receptor expression compared to monocytic inducing agents. This difference in insulin receptor expression correlated with binding characteristics in normal human peripheral granulocyte and monocytes.

The HL-60 cell line offers a model for the study of the molecular events which lead to the contrasting insulin receptor expression during myeloid and monocytic hematopoiesis.

MATERIALS AND METHODS

Materials. NBT, DMSO, PGE2, theophylline, dbcAMP, all-trans-retinoic acid, and PMA were obtained from Sigma Chemical Company (St. Louis, MO). Vit D was kindly donated by Dr. M. R. Uskokovic of Hoffman-LaRoche. Antibiotics, RPMI-1640 medium, and heat-inactivated fetal calf serum were obtained from Gibco (Grand Island Biological Company, Grand Island, NY). 125Iodine was from New England Nuclear (Boston, MA). Fraction V bovine serum albumin was obtained from Reheis Chemical Co., Kankakee, IL. Crystalline porcine insulin was provided by Dr. Ronald Chance of Lilly.

Cells and Culture Conditions. HL-60 cells, obtained from American Tissue Culture Collection, were grown in continuous suspension culture in RPMI-1640 medium, supplemented with 10 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM glutamine, 15% fetal calf serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml) (Gibco). Cells were divided every 2 days to maintain a density of 4 to 10 × 106 cells/ml. Doubling time for the HL-60 cell line was approximately 33 h.

Culture of HL-60 cells was initiated at a concentration of 4 × 105 cells/ml. Cells were used during passages 14 to 60. Cell viability, as assessed by trypan blue exclusion, was greater than 95%. This was confirmed by functional markers such as adherence, chemotaxis, and superoxide generation. HL-60 cells were induced to differentiate using DMSO (160 μM), dbcAMP (500 μM), PGE2 (100 nM), theophylline (500 μM), retinoic acid (100 nM), PMA (41 nm), or Vit D (1 μM). The concentration of inducing agents was used to maintain 95% viability with maximum differentiating activity. All cultures were initiated at a concentration of 4 × 105 cells/ml.

Morphology, Cytochemistry, Volume, and Functional Tests. Cytocentrifuged samples were evaluated with Wright's stain, and morphological maturity was determined by multiple factors including decreased granulation, decreased nuclear:cytoplasmic ratio, loss of nucleoli, and impaired function.

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creased density, and indentation of nucleus. To distinguish maturation along the monocytic line, \( \alpha \)-naphthyl butyrate (nonspecific) esterase was activated cell sorter analyzed calibrated with microbeads (Becton Dickinson, Immunocytometry Systems, Mountainview, CA).

**Insulin Binding Studies.** Iodinated insulin was prepared using the chloramine-T method according to Cuatrecasas (20). \(^{125}\)I-insulin prepared in this manner retained full biological activity and was determined to be >95% pure by precipitation in 10% TCA. Specific activity of the iodinated insulin was 1 Ci/\( \mu \)mol.

Cells were collected by centrifugation (500 \( \times \) g) and washed twice in BAB [50 \( \mathrm{mm} \) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid:50 \( \mathrm{mm} \) Tris:10 \( \mathrm{mm} \) MgCl\(_2\):2 \( \mathrm{mm} \) EDTA:50 \( \mathrm{mm} \) NaCl:5 \( \mathrm{mm} \) KCl: 10 \( \mathrm{mm} \) CaCl\(_2\) bovine serum albumin, 0.1% pH 7.8]. Prior to incubation with 0.6 nm \(^{125}\)I-insulin, cells were suspended for 10 min in BAB with 0.5 \( \mu \)m phenylmethylsulfonyl fluoride to inhibit protein degradation. In siliconized microfuge tubes pretreated with BAB, 2.0 \( \times \) 10\(^6\) cells were incubated in a total volume of 0.2 ml of BAB for 3 h at 15\(^\circ\)C unless otherwise indicated. Binding was terminated by the addition of 1 ml of BAB at 4\(^\circ\)C, which then was centrifuged at 13,000 \( \times \) g for 1 min in a Fisher Model 235B microcentrifuge. The cell pellet was resuspended in BAB at 4\(^\circ\)C, centrifuged twice, and counted for \(^{125}\)I. Nonspecific binding was determined by an identical method except for the addition of 1 \( \mu \)m unlabeled insulin. All data are presented as specific binding. Specific binding is defined as the total insulin bound minus nonspecific binding. Data points were measured in triplicate with standard error less than 10%. \(^{125}\)I-insulin at 0.6 nm was used for comparison of insulin binding during differentiation. This concentration represented high affinity binding as shown by Scatchard plot. Statistical analysis of difference in binding was by the 2-tailed \( t \) test.

Insulin degradation in incubation buffer was determined by precipitation of intact insulin in 10% TCA (21). The percentage of degraded insulin was found by dividing the amount of non-TCA-precipitable radioactivity into total radioactivity in the supernatant.

**Internalization of Insulin.** Internalization of \(^{125}\)I-insulin was determined by washing the cells, following incubation under standard conditions at 4\(^\circ\)C, 15\(^\circ\)C, 25\(^\circ\)C, and 37\(^\circ\)C with \(^{125}\)I-insulin in 0.2 \( \mu \)m acetic acid and 0.5 \( \mu \)m NaCl, pH 3.0 (21). The percentage of degraded insulin internalized was calculated by dividing radioactive present in the cells washed in acetic acid by the amount of radioactivity bound by unwashed cells.

**RESULTS**

**Characterization of HL-60 Differentiation.** As demonstrated by other investigators, specific chemical agents induced the promyelocytic HL-60 cell line to granulocytes or monocytes (9, 10, 22, 23). Differentiation of HL-60 cells was assessed by multiple parameters such as a reduction of NBT, appearance of nonspecific esterase marker, and morphological changes (Table 1). These cells, when induced with PMA, Vit D\(_3\), or dbcAMP, differentiated along the monocytic line. When induced with retinoic acid, PGE\(_2\):theophylline, or DMSO, the HL-60 cells demonstrated typical granulocytic maturation. Cellular volume was determined to correlate change in ligand binding during the course of differentiation. Fluorescent-activated cell sorter analysis demonstrated an average 40% decrease in cellular volume as HL-60 cells were induced to differentiate (Table 1).

**Expression of Insulin Receptors in HL-60 Cells.** Undifferentiated HL-60 cells in culture specifically and reversibly bound \(^{125}\)I-insulin. At 15\(^\circ\)C, HL-60 cells rapidly increased specific binding of 0.6 nm \(^{125}\)I-insulin with a plateau in binding at approximately 2 h. Nonspecific binding in all experiments ranged from 5 to 15% of total binding. When binding data were analyzed by Scatchard plot, a curvilinear line was obtained (Fig. 1a). The dissociation constant (\( K_d \)) for the high affinity binding site for HL-60 cells was 1.4 nm with 16,544 receptors/cell as shown in Table 2. The addition of 1 \( \mu \)m unlabeled insulin after 3 h of incubation at 15\(^\circ\)C in the presence of \(^{125}\)I-insulin resulted in the displacement of 70%
of bound $^{125}$I-insulin from induced and uninduced cells. Internalization of $^{125}$I-insulin at 15°C accounted for less than 5% of specific binding. Insulin degradation determined by TCA precipitation of intact insulin was found to be less than 5% in the uninduced and most differentiated HL-60 cells.

Insulin is one of the growth factors required for maintaining HL-60 cells in suspension culture. The cell line has been maintained in serum-free medium supplemented with transferrin (5 μg/ml) and insulin (5 μg/ml) (11). Our study demonstrated that proliferation and differentiation of the HL-60 cells were not affected by medium supplemented with 1 μM to 1 nM insulin. Insulin receptor expression was similar in cells maintained in standard medium (1.54 fmol of insulin per 10$^6$ cells), serum-free medium for 48 h (1.60 fmol of insulin per 10$^6$ cells), and with fetal calf serum added to the standard binding assay (1.6 fmol of insulin per 10$^6$ cells).

### Expression of Insulin Receptors in Differentiated HL-60 Cells

HL-60 cells in continuous culture expressed under standard binding conditions insulin receptor binding of 1.54 fmol of insulin per 10$^6$ cells. In our laboratory, insulin receptor expression was 0.08 fmol of insulin per 10$^6$ cells on monocytes. Induction of HL-60 cells with 1 μM Vit D or 41 nM PMA for 3 days resulted in a significant increase (P < 0.05) in insulin receptor expression compared to control HL-60 cell expression (Fig. 2a).

As early as 8 h after treatment with 1 μM Vit D, an increase in insulin receptor expression was demonstrated. From Days 3 to 5 of Vit D treatment, $^{125}$I-insulin binding was maintained at 3.9 fmol of insulin per 10$^6$ cells. This expression on modified HL-60 cell surface membrane was effectively increased, since cellular volume progressively decreased during differentiation (Table 1).

A progressive decrease in insulin receptor expression was found in cells treated with 1.25% DMSO (P < 0.05), 100 nM retinoic acid, and 100 nM PGE$_2$:500 μM theophylline (P < 0.05) (Fig. 2b) compared to controls. These agents induced typical granulocytic morphology. With 500 μM dbcAMP, the initial increase in insulin receptor expression was followed by a decrease through Day 5. This agent is known to induce both granulocytic and monocytic phenotypes (Table 1) (23). Statistical analysis demonstrated that differences in $^{125}$I-insulin binding on HL-60 induced with granulocytic agents as compared to monocytic agents were significant at the P < 0.01 level.

HL-60 cells incubated simultaneously with Vit D and dbcAMP demonstrated the largest increase in insulin receptor expression (Fig. 2a). By Day 3 of Vit D and dbcAMP treatment, insulin receptor expression was increased to 5.85 fmol of insulin per 10$^6$ cells with 95% cellular viability. This 400% increase in receptor expression over control cells represented an additive effect with these two agents. These cells also showed an increase in markers, such as nonspecific esterase and NBT reduction.

Scatchard analysis was performed on untreated cells (Fig 1a) and on cells exposed to 500 μM dbcAMP for 2 or 5 days or 1 μM Vit D for 5 days (Fig. 1b). Treated and untreated cells demonstrated both high and low affinity binding sites typical of the two site insulin receptor model. Cells treated with Vit D maintained high (Kd 1.2 nM) and low affinity binding characteristics as compared to uninduced cells (Table 2). The increase in $^{125}$I-insulin binding, achieved by treatment with Vit D, was attributed to an increase in receptor number (P < 0.05). Cells treated for 2 days with dbcAMP resulted in an increase in receptor number (Table 2). By Day 5 of culture with dbcAMP, receptor number decreased, but the Kd was similar to uninduced HL-60

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**Table 2**

Analysis of Scatchard plot of $^{125}$I-insulin binding on HL-60 cells during differentiation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>dbcAMP (Day 2)</th>
<th>dbcAMP (Day 5)</th>
<th>Vit D (Day 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_1$</td>
<td>1.4 nm</td>
<td>1.7 nm</td>
<td>1.7 nm</td>
<td>1.2 nm</td>
</tr>
<tr>
<td>$K_2$</td>
<td>340 pm</td>
<td>190 pm</td>
<td>370 pm</td>
<td>350 pm</td>
</tr>
<tr>
<td>Receptor no./cell</td>
<td>16,544 ± 1,534$^a$</td>
<td>19,296 ± 2,058</td>
<td>12,663 ± 3,114</td>
<td>23,517 ± 2,735$^b$</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SE.

$^b$ Statistical significance as compared to control (P < 0.05).
cells. dbcAMP induced consistent change in binding, but statistical significance compared to control was not demonstrated.

**Commitment to Vit D Induced Insulin Receptor Expression.**
Insulin receptor expression was determined for HL-60 cells treated with Vit D for variable intervals. In these experiments, cells were exposed to inducer for specific time intervals, then washed, and resuspended in new medium without inducer. These cells were compared to uninduced cells and cells continuously exposed to Vit D.

HL-60 cells treated with 1 μM Vit D for 1 h showed commitment to increase in insulin receptor expression (Fig. 3). A progressive increase in insulin binding was demonstrated when cells were exposed for 1-, 4-, or 12-h time intervals. Continuous exposure to Vit D was required for a sustained increase in 125I-insulin binding. Commitment to differentiation was also determined by the appearance of nonspecific esterase marker and NBT reduction after 5 days of Vit D exposure. A proportion of cells (33%) was committed to differentiation as shown by NBT reduction after 1 h of exposure to Vit D. The proportion of cells expressing this marker did not significantly change when exposed for longer intervals. After 48 h of Vit D treatment, insulin receptor expression, NBT reduction, and nonspecific esterase positivity approached the levels of cells continuously treated with inducer.

**DISCUSSION**

Since the establishment of the HL-60 cell line by Collins et al. (8), details of myelocytic and monocytic maturation have been delineated. Although a unifying mechanism of induction of differentiation has not been established, alteration of receptor expression and functional properties of the phagocyte have served as useful markers of maturation. However, the study of nonhematopoietic cellular surface markers in leukemic cells is limited.

The insulin receptor is one of the best characterized membrane proteins in cellular biology. HL-60 cells, as shown in this study and by other investigators, have specific insulin receptors (13-17). Insulin binding to HL-60 cells is time, temperature, and pH dependent. Insulin bound to HL-60 cells at 15°C is rapidly dissociated by the addition of cold insulin, and degradation of extracellular insulin is minimal. At physiological temperatures, insulin is internalized, while at 4°C and 15°C, internalization is minimal. Scatchard analysis of HL-60 cells demonstrates a curvilinear plot, typical of insulin receptor binding in human tissues.

Monocyte and granulocyte membranes have distinctly different insulin binding characteristics (1, 2). Our study establishes the simultaneous alteration of insulin receptor expression in a bipotential cell line with agents that selectively induce either monocytic or granulocytic differentiation. Our data imply that inducers of monocytic differentiation produce a significant increase in expression of the insulin receptor. Treatment with either Vit D or PMA produces a greater than 2-fold increase in binding after 72 h of treatment. Scatchard analysis of HL-60 cells treated with Vit D indicates that the increase in insulin binding is due to an increase in receptor number with little alteration of high affinity Kd. In contrast, a decrease in insulin receptor expression is associated with granulocytic differentiation. Chemotactic receptor expression has been similarly studied during HL-60 differentiation (19, 23). HL-60 cells increased formyl-peptide chemotactic receptor expression when cells were transformed to granulocytes and monocytes.

The relationship between insulin receptor expression and differentiation of HL-60 cells has been previously reported. Palumbo et al. (14) reported a decrease in insulin receptor binding when HL-60 cells were induced to granulocytes with DMSO and retinoic acid. Inducers of monocytic differentiation were not studied. Yamanouchi et al. (13) reported an increase in insulin receptor binding in HL-60 cells induced to monocytes with PMA and Vit D. Protein synthesis inhibition by cycloheximide correlated with a decrease in insulin receptor expression after Vit D treatment. In contrast to our study, they found an increase in insulin receptor binding with greater than 1 μM retinoic acid. We propose that an increase in insulin receptor expression is associated only with monocytoid differentiation, while decreased insulin binding occurs during myeloid differentiation.

Commitment to increase in insulin receptor expression with Vit D was studied as a sensitive marker of control of cellular differentiation. Commitment is defined operationally as the capacity of cells that have been exposed to inducer to express the new markers in the absence of inducer. Exposure to Vit D for 1 h was the shortest time tested and demonstrated commitment to an increase in insulin receptor expression in a portion of cells. With longer intervals of exposure to Vit D, a greater percentage of cells was committed to expression of this marker of differentiation. However, increase in receptor expression did not correlate with an increase in NBT reduction. For example, cells exposed to Vit D for only 12 h induced 37% differentiation as determined by NBT and esterase staining, but an 80% increase in insulin binding as compared to continuously exposed cells. This may indicate that an increase in insulin binding is a more sensitive indicator of differentiation than cytochemical stains or that cells committed to insulin receptor expression depend on a critical period of exposure to Vit D. With agents that increase intracellular cyclic AMP, complete commitment to chemotactic receptor expression or the ability to generate superoxide requires elevation of intracellular cyclic AMP for greater than 48 h (19). Phorbol esters maintain early change in cellular differentiation only when the phorbol ester is bound to the membrane receptor for greater than 16 h (24).

The relationship between insulin receptors in hematopoiesis and tumor cells has been studied (25-29). Leukemias associated...
with immature myeloblasts and monoblasts possess higher insulin receptor expression compared to chronic myelocytic leukemias and more differentiated lymphomas (30). A decrease in receptor expression during differentiation of the Friend erythro-leukemia cell line induced by DMSO has been reported by Ginsberg et al. (7). Thomopoulos et al. have demonstrated a decrease in insulin receptor expression as marrow erythroid precursors mature to the reticulocyte (31). U-937 cells, a monocytic cell line with insulin binding characteristics similar to the HL-60 line, demonstrated a decrease in insulin receptor expression after exposure to retinoic acid (17). In the U-937 system, Rouis et al. (32) demonstrated similar findings to our cell line in that insulin receptor expression was selectively altered with monocytic and myelocytic inducing agents. Transferin receptor expression was reduced by each inducing agent. Robert et al. (15) have demonstrated that the specific insulin binding in the U-937 cell line could be increased with prednisolone. The early (60 min) effect of treatment with 50 nm PMA was a decrease in high affinity binding of insulin by U-937 cells. Thomopoulos et al. (33) have demonstrated a similar early increase in insulin receptor expression in U-937 and HL-60 cells with 160 nm PMA when the binding assay was performed at 37°C. The early effect of PMA on these cells may modulate insulin processing.

We have confirmed the difference in insulin receptor expression on granulocytes and monocytes. We have extended this observation to the HL-60 cell line as the cells specifically differentiated to either granulocyte or monocyte. An increase in insulin receptor binding serves as an early and sensitive marker of monocytic differentiation. We demonstrated that HL-60 prolifer and differentiation requires further study. The HL-60 cell line may serve as a model for the intracellular events for insulin receptor function, structure, and regulation.

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REFERENCES


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