Expression of Transferrin Receptors in Human Erythroleukemic Lines: Regulation in the Plateau and Exponential Phase of Growth

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ABSTRACT

The present study was undertaken in an attempt to elucidate the mechanism(s) underlying transferrin (TRF) receptor expression in human erythroleukemic (K562 and HEL) lines during the exponential and the plateau phase of growth.

TRF receptor synthesis is enhanced when stationary cells are subcultured at low density in fresh medium. This rise occurs in either the presence or the absence of serum, which is associated with cell proliferation or quiescence, respectively. In the presence of serum, it is not inhibited by the addition of hydroxyurea (i.e., an agent blocking DNA synthesis). Thus, the receptor synthesis is enhanced not only in subcultures of actively proliferating cells (in the presence of serum), but also in subcultures of quiescent elements (in the absence of serum or upon the addition of serum plus hydroxyurea).

Conversely, the ferritin content is markedly decreased when stationary cells are subcultured at low density, in either the presence or the absence of serum.

These results suggest that stationary cells subcultured in fresh medium undergo a depletion of their intracellular iron pool, which in turn may represent the stimulus triggering TRF receptor synthesis. This hypothesis is supported by two observations: (a) both the depletion of this pool and the rise in TRF receptor synthesis are more marked in the absence than in the presence of serum; (b) addition of excess exogenous iron fully inhibits the rise of TRF receptor synthesis in cells subcultured with fresh medium and serum.

INTRODUCTION

Surface receptors for TRF were initially characterized on cells with an elevated iron requirement (e.g., hemoglobin-synthesizing erythroblasts) (1). However, these receptors are also present on cells requiring moderate amounts of iron, including non-hemoglobin-producing ones (2, 3). In this regard, TRF binding sites show a marked increase early after exposure to culture conditions stimulating cell proliferation (2-4). Both immunoassay and TRF binding studies indicate that this elevation results from a rise in the number of receptor molecules per cell, rather than a change in receptor affinity or availability (5, 6).

Other observations also suggest that TRF receptors are preferentially expressed on actively growing cells. Normal lymphocytes, stimulated to proliferate by mixed lymphocyte culture or mitogens, rapidly show a marked increase in the number of TRF binding sites (4). In normal fibroblast lines the number of TRF receptors is down-regulated during the transition from log growth to stationary phase (3, 6). Finally, a direct correlation between receptor expression and cell proliferation is observed in different types of human leukemias (cf. 7 Ref. 7). A more in-depth analysis has been carried out on activated T-lymphocytes, by simultaneous analysis of both DNA content and number of TRF receptors: although the receptor is expressed in G1, G2, and S, and M phases (8), its density peaks in the active phases of the cycle (9). Similarly, a human promyelocytic line (HL60) shows a maximum number of surface receptors just before cell division (9).

Conversely, α-interferon markedly inhibits the rise of TRF binding capacity of Daudi or erythroleukemic K562 cells subcultured in fresh medium at low density to stimulate their growth (10).

All these observations generated the concept that the expression of TRF receptors is dependent upon the rate of cell proliferation: accordingly, these receptors may represent a reliable marker for active cell growth. The present study was undertaken in an attempt to investigate the mechanism(s) responsible for the regulation of TRF receptor expression during the exponential and the plateau phase growth of human erythroleukemic lines.

MATERIALS AND METHODS

Cell Culture

K562 (11) and HEL (12) erythroleukemic lines were grown in RPMI-1640 medium containing 10% (v/v) FBS (Flow Lab.). Cultures were started at 10⁵ cells/ml in plastic culture flasks (Corning, Corning, NY), and the medium was changed every 4 days. In some experiments, high density cultures were washed 3 times in RPMI-1640 medium and then grown in the presence of BSA (1 mg/ml) (Boehringer-Mannheim, Mannheim, West Germany); under these conditions the cells did not grow but remained viable for at least 3 days. Cell viability was assessed by the trypan blue exclusion test.

125I-Labeled Transferrin Binding Assay

Purified human transferrin (>99%) was conjugated with 125I by the solid-phase lactoperoxidase system (New England Nuclear radioiodination system), as previously reported (13). 125I-Transferrin binding capacity was investigated on both intact cells and those dissolved in Triton X-100 (1%).

TRF Receptor Assay

Assay on Intact Cells. The binding reactions were performed in 12×75-mm polypropylene tubes in RPMI 1640 medium containing 0.1% BSA (Fraction V; Sigma Chemical Co., St. Louis, MO). Cell concentration was 5×10⁶ cells/ml, labeled TRF, 500 ng/ml, and unlabeled TRF, 0.1 mg/ml. Unbound ligand was removed by passing cells through a density cushion, as described (13). After incubation, 200-μl aliquots of the cell suspension, layered over 150 μl of a mixture of dibutyl phthalate and dinonyl phthalate (Merck, Darmstadt, West Germany) up to a final density of 1.025 in 400-μl plastic microfuge tubes, were centrifuged in a Hettich microfuge (10,000 g, 5 min). The resulting supernatant and most of the dibutyl phthalate cushion were aspirated. The tips of the vials containing the cell pellet were then severed with a scalpel and transferred into plastic vials, and the radioactivity was measured in a gamma counter. Total binding corresponded to the radioactivity in the cell pellet. Non-specific binding, represented by the radioactivity bound to the cells in the presence of cold TRF (1 mg/ml), was less than 5% of the total radioactivity bound per 10⁶ cells.

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Specific binding was represented by the difference between total and nonspecific values.

Assay on Cells Dissolved in Triton X-100. A simple assay was used to measure TRF receptor activity according to a method previously reported by Ecarot-Charrion et al. (14) and slightly modified by us (15). This method is based on the difference in solubility of free and TRF-bound receptors in polyethylene glycol (14). Cell samples, dissolved in PBS containing 1% Triton X-100 (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma), were centrifuged for 30 min at 20,000 x g for 30 min. Dissolved receptors were incubated in a total volume of 0.2 ml for 30 min at 37°C in a 0.1 M citrate-Tris buffer solution (pH 5.0) containing 0.1% BSA, 0.1% Triton X-100, and 200 ng of [125I]-TRF. The receptor-TRF complex was precipitated with 0.8 ml of polyethylene glycol solution (12%, w/v) in 0.1 M citrate-Tris buffer (pH 5.0) containing the carrier human γ-globulin (0.1%). The tubes were placed in an ice bath for 30 min and then centrifuged at 13,000 x g for 15 min at 4°C. The supernatant and the precipitate were tested for radioactivity. Coprecipitation of free TRF was measured by omitting the receptor from the tubes, while nonspecific TRF binding was determined by preincubating the samples with 1 mg of nonradioactive TRF before adding radioactive TRF.

[^H]Thymidine Incorporation

Tritiated thymidine (2 μCi; New England Nuclear, Inc., Boston, MA; specific activity, 20 Ci/mmol) was added to the culture medium 2 h prior to termination of incubation. After labeling, the cells were processed as described (15).

Ferritin Concentration

The intracellular concentration of ferritin was evaluated by a radioimmunoassay, using a kit from Hoechst (Behring Institute, Frankfurt am Main, West Germany). Samples for ferritin determination were lysed in distilled H2O, freeze-thawed 3 times, and centrifuged at 13,000 x g, 30 min, at 4°C; the supernatant was used for radioimmunodetermination of ferritin.

Protein concentration was determined by the dye binding method (Bio-Rad Laboratories, Richmond, CA).

TRF Receptor Biosynthesis

In order to investigate the biosynthesis of TRF receptors, 10^7 cells grown under different conditions were incubated for 1 h at 37°C in methionine-free minimal essential medium (Eurobio, Paris, France), containing 5% dialyzed FBS and 100 μCi of [35S]methionine. After 3 washings in PBS, cells were lysed in PBS containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 20,000 x g for 30 min at 4°C; the supernatant was mixed for 30 μl of Sepharose-TRF obtained by fixing human TRF to cyanide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) (2 mg of TRF bound per ml of gel). After 1 h of incubation at 20°C, Sepharose-TRF was washed 3 times with 1.5 ml of PBS-0.1% Triton X-100-0.1% Nonidet P-40-1 mM phenylmethylsulfonyl fluoride and 3 times with the same buffer containing also 0.5 mM sodium chloride. TRF receptors were recovered from Sepharose-TRF by adding 50 μl of 2% SDS-5% β-mercaptoethanol. Samples were then boiled for 2 min at 100°C and finally analyzed by SDS-polyacrylamide gel electrophoresis as in Ref. 16. Gels were then stained with Coomassie blue and treated for autoradiography as reported (17).

RESULTS

Confluent K562 or HEL cells (1 to 1.2 x 10^6 cells/ml) were subcultured at low density (10^5 cells/ml) in the presence or absence of FBS, and TRF binding capacity was measured thereafter. In the presence of 10% FBS, this parameter showed an increase at 12 h, peaked at 24 to 48 h, and returned to baseline levels at 72 to 96 h, when cells became confluent (Fig. 1). In the absence of FBS, a marked increase in TRF binding capacity was observed; indeed, the rise was more marked than in subcultures supplemented with FBS (Fig. 1).

It is emphasized that stationary K562 or HEL cells subcultured in RPMI-1640 medium without additional serum (replaced by BSA, 1 mg/ml) remained viable for at least 72 h, but they did not proliferate (Fig. 1). In this regard, cells originally maintained in serum-free medium subcultured in the presence of 10% FBS exhibited a marked increase of [3H]thymidine incorporation, while those subcultured without serum (BSA, 1 mg/ml) did not display this phenomenon (Fig. 2).

These binding experiments were carried out on whole cells at 20°C, thus allowing the evaluation of surface receptors. Since most TRF receptors are on intracellular membranes, we have also evaluated [125I]-TRF binding on cells dissolved in 1% Triton X-100, in order to investigate both surface-bound and intracellular TRF receptors. Using this technique, we confirmed the results obtained on the TRF binding capacity of intact cells. Density arrested cells exhibited a marked increase of their TRF binding capacity when subcultured at low density in either the absence or presence of FBS (Table 1).

We have then investigated whether the changes in TRF binding capacity in subcultures of stationary K562 or HEL cells reflect variations in the rate of receptor biosynthesis. This parameter was evaluated by incubating cells in the presence of
Cells subcultured for 48 h at low density
Cells subcultured for 24 h at low density
High-density cells

Viable cells were counted daily by the trypan blue exclusion test. All points represent mean values from three separate experiments.

We have also analyzed whether the rise of TRF binding capacity is accompanied by opposite changes of the number of TRF receptors (18). Furthermore, we have measured the ferritin content of both stationary K562 and HEL cells and their subcultures at low density in either the presence or the absence of serum. In the presence of FBS the ferritin content shows a marked decrease, which is more pronounced in subcultures not supplemented with serum (Fig. 1; Table 3).

**DISCUSSION**

The experiments reported here may shed light on mechanisms underlying modulation of TRF receptor expression in the exponential and the plateau phase of growth of leukemic lines. The rise in TRF receptor synthesis occurring when stationary K562 and HEL cells are subcultured at low density occurs in both the presence and the absence of serum. Furthermore, addition of a drug blocking DNA synthesis (hydroxyurea) to subcultures supplemented with FBS did not inhibit the rise in the expression of TRF receptors. This rise may hence occur in subcultures of either actively dividing cells (i.e., FBS present) or quiescent elements (i.e., FBS absent or fetal calf serum plus hydroxyurea added). This clearly suggests that TRF receptor expression is not dependent upon the proliferative state of the cells.

Our observations on the correlation between TRF receptor expression and cell proliferation are in line with a recent study by Musgrove et al. (19). These authors examined the expression of TRF receptors in individual human tumor cells during transition from the exponential to the stationary phase of growth. A uniform decrease in receptor level was observed in the whole cell population, thus indicating that cells remaining in cycle also down-grade their TRF receptors. These results may suggest that TRF receptors are not a marker for proliferation per se, as previously suggested (8, 20), but they reflect the proliferative status of the cell population as a whole.

We have also observed that the level of ferritin is progressively increased when cells move from the log phase to the stationary phase of growth. Inversely, the ferritin content is sharply decreased when stationary cells are subcultured in fresh medium containing 10% fetal calf serum. This decline is more marked in cells subcultured without serum. Since the level of ferritin synthesis depends on both the amount of iron available and the level of iron utilization (21), we suggest the following interpretation. The amount of ferritin is more elevated in stationary cells, since they do not utilize a large part of iron entering into them. However, cells subcultured in fresh medium start utilizing iron, thus leading to a decrease of ferritin level more pronounced in cells subcultured without serum.

We suggest that the depletion of an iron intracellular pool occurring when stationary cells are subcultured in fresh medium may represent the biochemical trigger stimulating TRF receptor synthesis. In line with this hypothesis, the depletion of this pool...
EXPRESSION OF TRANSFERRIN RECEPTORS IN HUMAN ERYTHROLEUKEMIC LINES

Fig. 3. TRF receptor biosynthesis in HEL cells grown under different conditions. Cells were labeled with [35S]methionine, and TRF receptors were purified by affinity chromatography on Sepharose-TRF and then analyzed by SDS-polyacrylamide gel electrophoresis. The lower and upper bands correspond, respectively, to unglycosylated and glycosylated TRF receptors. Lane A, high-density cells; Lane B, cells subcultured for 24 h with fresh medium containing 1 mg of BSA per ml; Lane C, cells subcultured for 24 h with fresh medium containing 10% FBS.

Fig. 4. Effect of iron salts, actinomycin D, and hydroxyurea on TRF binding capacity of high-density HEL cell subcultured at low density with fresh medium containing 10% FBS. Both actinomycin D and hydroxyurea completely inhibited cell growth, while iron salts did not affect it. All points represent mean values from three separate experiments. •, control; ○, hydroxyurea; △, ferric ammonium citrate; ▲, actinomycin D.

Table 2 Ferritin concentration in K562 cells at different days of culture

<table>
<thead>
<tr>
<th>Day of culture</th>
<th>Cell no. (10^6 cells/ml)</th>
<th>Ferritin concentration (ng/mg protein)*</th>
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<tbody>
<tr>
<td>1</td>
<td>2.1</td>
<td>62 ± 8</td>
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<tr>
<td>2</td>
<td>3.5</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>6.2</td>
<td>124 ± 18</td>
</tr>
<tr>
<td>5</td>
<td>6.3</td>
<td>192 ± 25</td>
</tr>
</tbody>
</table>

* K562 cells, initially grown at 10^6 cells/ml, were harvested every day. The ferritin content was evaluated as described in “Materials and Methods.”

Table 3 Ferritin concentration in HEL and K562 cells grown under different conditions

<table>
<thead>
<tr>
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<th>Ferritin concentration (ng/10^6 cells)*</th>
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<tbody>
<tr>
<td>High-density cells</td>
<td></td>
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<tr>
<td>Cell subcultured for 24 h at low density in the presence of 10% FBS</td>
<td>11 ± 1.5</td>
</tr>
<tr>
<td>Cells subcultured for 48 h as above</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Cells subcultured for 24 h in the presence of BSA (1 mg/ml)</td>
<td>5 ± 1.2</td>
</tr>
<tr>
<td>Cells subcultured for 48 h as above</td>
<td>1.3 ± 0.5</td>
</tr>
</tbody>
</table>

* Ferritin concentration was evaluated as described in “Materials and Methods.”

Table 3 Ferritin concentration in HEL and K562 cells grown under different conditions

<table>
<thead>
<tr>
<th></th>
<th>HEL</th>
<th>K562</th>
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<tr>
<td>Cell subcultured for 24 h at low density in the presence of 10% FBS</td>
<td>20.3 ± 4.1</td>
<td>24 ± 5.5</td>
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</tr>
<tr>
<td>Cells subcultured for 48 h as above</td>
<td>11 ± 1.5</td>
<td>11 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Cells subcultured for 24 h in the presence of BSA (1 mg/ml)</td>
<td>10 ± 2</td>
<td>6.5 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Cells subcultured for 48 h as above</td>
<td>5 ± 1.2</td>
<td>5 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Cells subcultured for 48 h as above</td>
<td>1.3 ± 0.5</td>
<td>0.5 ± 0.3</td>
<td></td>
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</table>

* Mean ± SD from 3 separate experiments.

is more marked in the absence than in the presence of serum, while the rise in TRF receptor synthesis is more pronounced in cells subcultured without serum than in those grown with FBS. Furthermore, addition of excess exogenous iron completely inhibited the increase of TRF receptor synthesis observed when stationary cells are subcultured with fresh medium and serum. This interpretation is consistent with other studies, showing that expression of TRF receptors may be modulated by a feedback mechanism dependent upon the level of intracellular iron (7, 18, 22–27).

In conclusion, this study provides a clear interpretation of the differential expression of TRF receptors during the exponential and plateau phase of cell growth, based on fluctuations of the intracellular iron pool, rather than the rate of cell proliferation per se.

REFERENCES

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