Effects of Transferrin-Indium on Cellular Proliferation of a Human Leukemia Cell Line

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ABSTRACT

In previous studies, we have demonstrated that transferrin-gallium inhibits cellular proliferation by a mechanism whereby cellular iron utilization is impaired. Since indium, a similar class 3A metal, has not been well studied, we examined its effects on cellular iron uptake and cellular proliferation. In these studies, we provide evidence that indium, when bound to transferrin, has a 50-fold higher effect on inhibition of cellular proliferation than indium added as indium salt. Cells exposed to relatively low concentrations of transferrin-indium exhibit markedly increased transferrin receptor expression best, as with transferrin-gallium, these cells incorporate an inappropriately low amount of iron, suggesting that there is a defect in the release of internalized iron from transferrin. In further studies, we utilize a monoclonal antibody against transferrin receptor that inhibits transferrin-mediated iron uptake. This antibody exhibits a dose-related inhibition of cellular proliferation, and when both transferrin-indium and monoclonal antibody are added to media, there is a more than additive effect on inhibition of cellular proliferation.

INTRODUCTION

Iron is transported in plasma by the M, 80,000 protein transferrin (1). Cellular iron uptake is facilitated by transferrin binding to its specific cell surface receptor (2–6). Many in vitro studies of cell lines grown in defined media (serum free) demonstrate that transferrin is required for cellular proliferation (7–9). Although it is likely that transferrin functions in maintaining cell growth by means of cellular iron transport (10–12), the exact nature of the iron requirement has not been defined. Under physiological conditions in vivo, approximately one-third of plasma transferrin is iron saturated, leaving the remainder of the protein free to bind other metals, including zinc, gallium, indium, and manganese (1). The ability of transferrin to bind metals other than iron appears to have medical diagnostic and therapeutic implications. For example, a number of studies have indicated that the tumor uptake of $^{67}$Ga, an isotope commonly used for tumor detection in vivo, is enhanced by binding to transferrin (13–15). Gallium nitrate has been shown to have therapeutic potential in vivo, particularly against high grade lymphomas resistant to other chemotherapeutic agents (16). Earlier studies have suggested that the inhibitory effects of gallium nitrate on cellular proliferation in vivo were enhanced when transferrin was added to the medium (17). Our more recent studies have shown that the cytotoxicity of gallium added as gallium salts in vitro is increased more than 10-fold when the gallium is added as transferrin-gallium (13, 18).

In these latter studies, we have better defined the action of transferrin-gallium by demonstrating that inhibition of cellular iron utilization is a prerequisite for gallium to exert its cytotoxic effect (18). These studies indicated that cells treated with transferrin-gallium are unable to release iron from internalized transferrin-iron, probably due to an inability to acidify the intracellular compartment that allows iron to be released from transferrin (18).

We have elected to investigate the properties of another class 3A metal, indium. Preliminary in vitro evidence has suggested that an indium salt has tumoricidal activity but is “less active” than a gallium salt (19). Also, in vivo evidence in both rats and mice has shown that indium salts may be more toxic than gallium salts, causing severe hepatic and renal toxicity (19–21). Thus, indium has not been studied extensively as a chemotherapeutic agent. However, $^{111}$In used in diagnostic studies has been shown to bind to transferrin in vivo (22). On the basis of the finding that gallium inhibition of tumor cell growth is markedly facilitated by transferrin binding, we wondered to what degree the binding of indium to transferrin would potentiate leukemic cell cytotoxicity in vitro. Our studies presented here demonstrate that indium-saturated transferrin results in a dose-related decrease in cellular proliferation of human HL-60 cells and is 50 times more potent than when indium is added as indium salt.

To better understand the mechanism of cytotoxicity by indium, we have performed studies that indicate that Tf-In results in increased HL-60 promyelocytic leukemia cell transferrin receptor expression, but it is associated with inappropriately low iron uptake. Additionally, we find that a monoclonal antibody that blocks transferrin-mediated iron uptake added to cells containing transferrin-indium causes a more than additive effect on inhibition of cellular proliferation.

MATERIALS AND METHODS

Materials. Human apo-Tf, zinc chloride, and ferric chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Indium nitrate hydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI). TR3A7 anti-Tf receptor antibody was obtained through the courtesy of Dr. York Miller. Goat anti-rabbit fluorescein isothiocyanate-conjugated monoclonal antibody was obtained from Coulter Corp. (Hialeah, FL). Sodium $^{111}$Iiodide and $^{99m}$FeCl$_3$ were obtained from Du Pont New England Nuclear (Boston, MA) and Amersham Corp. (Arlington Heights, IL), respectively. Saturation of apo-Tf with $^{99m}$Fe and iodination of $^{99m}$Fe-Tf were performed as described previously (4, 23).

Preparation of Various Tf Forms. Tf-Fe (1, 18), Tf-Zn (18, 24), and Tf-In (18) were prepared by using modifications of previously described methods. For the respective preparations, 3 mol of metal as ferric chloride, zinc chloride, or indium nitrate were added to each mol of apo-Tf dissolved in 20 nM acetic acid-150 mM sodium chloride, pH 3.5. The pH of the solution was slowly raised in gradual increments to 7.4 with 1 M NaHCO$_3$, resulting in a final concentration of 30 mM NaHCO$_3$. Saturation of Tf by the different metals was confirmed spectrophotometrically using a DU-40 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) by measuring the change in absorbance at wavelengths of 242 nm (zinc and indium) and 654 nm (iron). For each metal Tf form, saturation of both metal-binding sites was confirmed by noting the change in absorbance that occurred progressively with the addition of increasing molar concentrations of metal until a 3 M excess of metal to Tf had been reached. Subsequently, for...
TRANSFERRIN-INDIUM EFFECTS ON CELLULAR PROLIFERATION

Inhibition of Cellular Proliferation by Indium Is Enhanced When Bound to Transferrin. As shown in Fig. 1, HL-60 cells grown in RPMI 1640 supplemented with insulin show a progressive decrease in proliferation with the addition of increasing doses of transferrin added as Tf-In. This inhibition of proliferation is similar to the dose effect seen in our previous studies utilizing Tf-Ga (18, 28). When indium is added as indium salt, no inhibition of growth is noted at concentrations of 10 and 100 μg/ml (Fig. 1), although when 300 μM indium is added, there is an 18 ± 5% inhibition of cellular proliferation. Thus, inhibition of cellular proliferation is enhanced markedly when the indium is bound to transferrin. Therefore, much higher concentrations of indium are necessary to inhibit proliferation when added as indium salt. It should also be noted that the experiments with indium salts, as with our studies with gallium salts, are performed in media containing 10% fetal calf serum in order to maintain cellular proliferation, so it is possible that the high concentrations of indium allow for some of the metal to bind to the calf transferrin.

This dose-related effect of Tf-In on inhibition of cellular proliferation is almost identical to that seen with gallium, and as with Tf-Ga, the addition of Tf-Fe to serum-free media containing Tf-In reverses the effects of Tf-In in a dose-related fashion (Table 1). Similar to the results shown in Fig. 1, Table 1 also shows that in this serum-free system, the addition of indium as indium salts to media containing Tf-Fe has a negligible effect on inhibition of cellular proliferation as compared to the same amount of indium added as Tf-In.

Measurement of Cellular 125I-Transferrin Binding during Cellular Proliferation in Media Containing Different Transferrin Forms. Cells plated in defined media containing 50 μg/ml of different transferrin forms were harvested, washed by centrifugation, and preincubated at 37°C to remove bound nonradioactive transferrin as described in “Materials and Methods.” As shown in Table 2, 24 h after subculture, cells treated with Tf-Zn show a significant increase in transferrin binding when compared to cells treated with Tf-Fe. This increase persists at

RESULTS

Inhibition of Cellular Proliferation by Indium Is Enhanced When Bound to Transferrin. As shown in Fig. 1, HL-60 cells grown in RPMI 1640 supplemented with insulin show a progressive decrease in proliferation with the addition of increasing

Fig. 1. Inhibition of cellular proliferation when either Tf-In or indium nitrate is added to media. Points, mean of two experiments. Control cells represent cell counts obtained at 4 days when 50 μg/ml of Tf-Fe are added to the media. The abscissa is in μM indium so as to compare with previous results using Tf-Ga and gallium salts (18). A Tf-In concentration of about 210 μM indium causes 50% inhibition of growth.

Table 1 Effects of different transferrin forms on proliferation of HL-60 cells

<table>
<thead>
<tr>
<th>Transferrin form (μg/ml)</th>
<th>% of inhibition of proliferation* (%) of control cells at 4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tf-In</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>Tf-Ga</td>
<td>0 ± 12</td>
</tr>
<tr>
<td>Tf-In (50 + 7.5 μM indium)</td>
<td>300 4 ± 4</td>
</tr>
<tr>
<td>Tf-In (7.5 μM indium)</td>
<td>300 150 4 ± 4</td>
</tr>
<tr>
<td>Tf-In (300 μM indium)</td>
<td>300 300 40 ± 8</td>
</tr>
</tbody>
</table>

* Values represent mean of two experiments ± range; if cell count mean is greater than control, a 0% value is assigned.

Control represents cell counts at 4 days for cells grown in 50 μg/ml Tf-Fe.

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Table 2. Addition of monoclonal antibody to HL-60 cells

<table>
<thead>
<tr>
<th>TR3A7(^a) µg/ml</th>
<th>Inhibition of cell growth(^b) (%)</th>
<th>Significance(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>44</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>30</td>
<td>54</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

\(^a\) Cells have been plated for 2 passages in specific antibody concentrations.

\(^b\) Cell counts are compared 5 days after second plating and percentage of inhibition represents differences in counts obtained with control cells grown for 2 passages in no antibody. Mean of 3 experiments is represented.

\(^c\) Whether and to what extent value is significant as compared to control (NS, not significant).

Reduction in iron uptake, was added to HL-60 cells in order to determine its effects on cellular proliferation. In these experiments, the monoclonal antibody in various concentrations was incubated with HL-60 cells in serum-free media containing Tf-Fe and insulin. Cells plated with 60 µg/ml of the purified monoclonal antibody showed a 25% reduction in growth as compared to control cells (\(^P\) < 0.05) and continued exposure to this concentration of antibody resulted in significant cell death within an additional 48 h. With lower concentrations of antibody during the first passage, cells plated at \(8 \times 10^5\) cells/ml showed no change in cell growth as measured by increases in cell count 5 days postculture. After cells are replated at \(8 \times 10^5\) cells/ml in the same concentration of purified monoclonal antibody (Table 3), it can be seen that with repeated exposure there is a significant decrease in proliferation of cells plated with 10 and 30 µg/ml of antibody.

Using \(^{125}\)I-Tf binding experiments, we found that cells at 2 days postculture in 10 µg/ml of antibody specifically bind 85% less transferrin as compared to control cells grown for 2 days with no addition. These data agree with our previously published data utilizing a different preparation of this antibody added to cells grown in fetal calf serum. These studies (28) indicated that, besides initial inhibition of \(^{125}\)I-transferrin binding,\(^4\) continued exposure to the antibody resulted in “downregulation” of surface transferrin receptor with an associated decrease in cellular proliferation. Our studies indicate that it takes longer than 24 h for the antibody to have its greatest effect on inhibition of \(^{125}\)I-transferrin binding as well as “downregulation” receptor. Thus, cells exposed to lower concentrations of the monoclonal antibody during the first passage may grow normally, since during the first 24 h the cells may be able to utilize exogenous Tf-Fe as well as some of the remaining endogenous iron to allow for normal proliferation. Thus, taken together with more recently published studies utilizing another cell line grown in serum-free media (27), the decrease of proliferation seen with the second passage utilizing the monoclonal antibody points to the face that this monoclonal antibody specifically causes inhibition of cell-mediated iron uptake resulting in inhibition of growth.

We hypothesized that the combination of Tf-In, which causes decreased iron uptake associated with high surface transferrin receptors, with the monoclonal antibody that causes decreased iron uptake associated with low surface transferrin receptors might have an additive or more than additive effect on growth inhibition. As shown in Fig. 3, cells grown with 50 µg/ml Tf-In, but no monoclonal antibody showed the expected increase in cell growth that is seen with control cells (grown in Tf-Fe). Monoclonal antibodies added to the Tf-In caused a highly significant and dose-related inhibition of proliferation. Comparing the above data with that shown in Table 3 suggests the addition of both Tf-In and monoclonal antibody TR3A7 to

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\(^4\) Unpublished data.
given as a constant infusion in vivo, substantial binding of Tf-Ga. These in vitro studies are important since indium cellular proliferation at doses almost identical to those seen The cells treated with Tf-In show a dose-dependent decrease in cellular proliferation. Both of these values are significantly lower (P < 0.002).

cells results in a more than additive effect on inhibition of proliferation. In other experiments by using selected concentrations of Tf-In and TR3A7, it is possible to show that both agents added to cells together result in a far greater percentage of inhibition than would be expected if each agent acted independently. For example, when 100 µg/ml of Tf-In is added to cells, there is a 11% decrease in cellular proliferation, whereas 60 µg/ml of monoclonal antibody results in a 26% inhibition of proliferation. Both of these values are significantly lower (P < 0.05) than when there is no addition. More importantly, when both of these agents at the above concentrations are added together, there is a 70% inhibition of cellular proliferation, indicating again a more than additive effect on inhibition of cellular proliferation.

DISCUSSION

In this paper, we present data that measure the effects of a class 3A metal, indium, on cellular proliferation. Earlier studies had suggested that these metals including gallium had cytotoxic potential in vitro (91). We have shown that gallium bound to transferrin in vitro results in markedly increased inhibition of cellular proliferation (18, 28). It has been hypothesized that gallium, when given in vivo as a constant infusion, allows for substantial "protein binding" (29) of the metal to transferrin, resulting in enhanced cytotoxicity and decreased renal toxicity. In the current paper, we show that indium, added as Tf-In to HL-60 cells in vitro, results in greater than 50-fold enhanced cytotoxicity when compared to indium added as indium nitrate. The cells treated with Tf-In show a dose-dependent decrease in cellular proliferation at doses almost identical to those seen with Tf-Ga. These in vitro studies are important since indium salts had been thought to be less cytotoxic in vitro than gallium salts (19). These studies also suggest that if indium were to be given as a constant infusion in vivo, substantial binding of indium to transferrin (22, 30) may result in increased tumoricidal effect, particularly when it is recognized that previous studies utilizing indium in vivo (20, 21) did not give the drug as a constant infusion. Infusion of indium should allow for substantial binding of the metal to transferrin since our own unpublished data as well as the data of others (30) indicate that transferrin metal binding sites have a higher affinity for indium than gallium.

In the experiments shown in this paper comparing different transferrin forms, Tf-In causes a marked relative increase in transferrin receptor expression on the cell surface. In spite of this increase in transferrin receptor expression, cells treated with Tf-In as compared to Tf-Zn showed an inappropriately low incorporation of iron when a trace dose of 59Fe transferrin was added to the media. These findings are almost identical to those with Tf-Ga (18). Further studies will be performed with Tf-In, but it might be predicted that similar to observations with Tf-Ga (18), Tf-In-treated cells will show an inability to acidify the intracellular compartment that allows iron to be released from transferrin.

In further experiments shown in this paper, we have utilized a monoclonal antibody (TR3A7) directed against the transferrin receptor. The anti-human transferrin receptor monoclonal antibody has been utilized previously to inhibit the growth of HL-60 cells grown in fetal calf serum-supplemented media (28). As with other transferrin monoclonals (31), the antibody appears to specifically inhibit transferrin-mediated iron uptake by both blocking transferrin binding and decreasing surface transferrin-binding sites as a result of a "down-regulation" of surface transferrin receptor. These studies as well as our recent published studies of another cell line grown in serum-free media (27) indicate that relatively low concentrations of the antibody allow cells to proliferate normally during the first passage. However, when the cells are replated at lower density in the same concentration of antibody, growth is inhibited.

Based on the above information, we examined the additive effects of: (a) monoclonal antibody, an agent that inhibits cellular mediated iron uptake by inhibition of transferrin-iron binding to the cell surface; and (b) Tf-In, an agent that interferes with intracellular iron utilization. The data presented demonstrate that the addition of both agents to HL-60 cells in vitro results in a more than additive effect on inhibition of cellular proliferation.

Recent data utilizing gallium in vitro (32) strongly suggest that agents that interfere with iron utilization are potent inhibitors of DNA synthesis, at least in part by specific inhibition of ribonucleotide reductase (32). Thus, further studies of indium both in vitro and bound to transferrin in vivo should be performed in order to better understand indium effects on tumor growth as well as toxicity. Also, treatment strategies that utilize combinations of anti-transferrin receptor monoclonal antibodies, agents including indium or gallium bound to transferrin, and inhibitors of ribonucleotide reductase such as hydroxyurea should be considered in the treatment of certain rapidly proliferating neoplasms.

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REFERENCES


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