The Effects of Dexamethasone and Tetradecanoyl Phorbol Acetate on Plasminogen Activator Release by Human Acute Myeloid Leukemia Cells

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This investigation was undertaken to examine the extent to which leukemic cell functions are susceptible to regulation in vitro and to investigate their heterogeneity in this regard. Since plasminogen activator release is known to be a modulatable cellular function that can be influenced by antiinflammatory steroids and tetradecanoyl phorbol acetate (TPA), the effect of these two compounds on the secretion of urokinase- or tissue-type enzymes by leukemic cells was studied. The release of both enzyme species could be stimulated or suppressed by these substances by mechanisms that were inhibitable by actinomycin-D and hence required transcription of new mRNA. Plasminogen activator release by cells from 41/45 patients with AML was either stimulated or inhibited by 10⁻¹⁰M dexamethasone, implying that most AML cells possess glucocorticoid receptors. In 26/45 cases, the enzyme was inhibited by this steroid to less than 25% of control values. Pronounced inhibition of this degree was not encountered with normal polymorphonuclear leukocytes. Plasminogen activator secretion by AML cells was profoundly inhibited in 20/41 cases by 1 ng/ml TPA and stimulated in 8/41 cases. Leukemic blasts varied considerably in their response to dexamethasone and TPA. Plasminogen activator release should prove a sensitive means of monitoring the responses of AML cells to biologically active compounds.

Peripheral blood leukocytes from patients with myeloid leukemia release serine proteases that function as activators of the plasma zymogen, plasminogen. In a recent study of this phenomenon, we have observed that leukemic cells derived from different patients were dissimilar with respect to plasminogen activator release, both quantitatively in terms of the rate of enzyme secreted per cell, and qualitatively in terms of the molecular species of plasminogen activator that was secreted. Leukemic cells secreted either the urokinase-type or the tissue-type plasminogen activator. In acute myeloid leukemia (AML), cellular tissue-type plasminogen activator release was associated with an unfavorable prognosis for induction of remission.

In most other systems that have been studied, synthesis and release of plasminogen activators have been found to be inducible cellular functions that can be modulated by hormones, retinoids, and other compounds that affect expression of the malignant phenotype. It therefore seemed possible that an in vitro study of the effects of pharmacologically active substances on the synthesis of plasminogen activators by leukemic cells would provide a quantitative means of estimating the susceptibility of these cells to genetic modulation. Such information would contribute to the knowledge of the biology of the leukemic cell and might provide a rational basis for the classification and treatment of leukemias.

In this article we record our observations on the effects of the antiinflammatory steroid, dexamethasone, and the tumor promoter, tetradecanoyl phorbol acetate (TPA), on plasminogen activator release by AML cells incubated in vitro. These two compounds were chosen for their ability to influence plasminogen activator release in other systems. Furthermore, TPA has been shown to induce differentiation in the human promyelocytic cell line HL-60 and in cells from patients with AML.

MATERIALS AND METHODS

Subjects

Blood samples were obtained from 15 healthy laboratory workers and from 45 patients with AML. All diagnoses of leukemia were based on histologic and histochemical examination of peripheral blood and bone marrow specimens. Romanowsky-stained preparations, cytochemical stains, and ultrastructural features were used to classify specimens according to the French-American-British recommendations.

Cells

Blood was taken by sterile venipuncture into tubes containing preservative-free heparin (Thromboliquine, Organon Teknika, Holland) to give a final concentration of 5 U/ml. Cells were fractionated by dilution with RPMI 1640 medium and centrifugation on a layer of Ficoll-Hypaque. Leukemic blast cells were harvested from the plasma–ficol interface and freed of contaminating erythrocytes by incubation for 5 min in 0.83% ammonium chloride (pH 7.4). Polymorphonuclear leukocytes (PMN) were isolated from the blood of normal individuals as described by Granelli-Piperno et al.

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Supported in part by grants from the South African Mutual Life Assurance Society, the National Cancer Association, the South African Medical Research Council, and the University of Cape Town Leukemia Centre and Staff Research Fund.

Submitted June 15, 1982; accepted September 10, 1982.

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Experimental Protocol

Cells were washed once by centrifugation and suspension in RPMI 1640 and were resuspended in RPMI 1640 containing 3% fetal calf serum (FCS) to give 4 x 10^6 cells/ml. One-milliliter samples of this suspension were added to 35-mm Falcon plastic Petri dishes, and dexamethasone or TPA were added to give concentrations covering the ranges 0-10 M and 0-10 ng/ml, respectively. Dexamethasone (Sigma Chemical Co., St. Louis, Mo.) and TPA (Dr. P. Borchert, Minn.) were kept as stock solutions in absolute ethanol at 10 M and 100 ng/ml, respectively. These were diluted in RPMI 1640 containing 3% FCS so that the addition of 10 μl to each 1 ml of medium gave the desired final concentration. The dishes were incubated for 24 hr at 37°C in a humid atmosphere of 5% CO2 in air. At the end of this period, the medium was harvested by centrifugation and replaced with fresh medium containing compounds at the same concentration as before. At the end of this second period, the medium was again collected by centrifugation. The medium samples, referred to as harvest fluids, were stored at -80°C for analysis of plasminogen activator activity.

Cells were inspected under phase contrast microscopy after 24 and 48 hr of in vitro incubation. Cellular viability was assessed by Trypan blue exclusion.

Plasminogen Activator Assay

Plasminogen activator activity in harvest fluids was assayed by measuring plasminogen-dependent release of soluble radioactive fibrin degradation products from insoluble 125I-fibrin-coated multiwell tissue culture plates (Linbro, Cat. no. FB-16-24, Flow Labs). This was done as previously described,22 save for the addition of 4 μg of plasminogen per well instead of 2 μg. Results were calculated in terms of urokinase units by reference to urokinase standards assayed simultaneously.

Immunochemical Analysis of Plasminogen Activators

Molecular species of plasminogen activators present in harvest fluids released by normal and leukemic cells were identified as urokinase-type or tissue-type using specific inhibitory antibodies to these enzymes. Harvest fluid samples were incubated for 1 hr at 4°C with serial twofold dilutions of purified rabbit antibody and assayed for residual activity using the 125I-fibrin assay. All procedures have previously been described in detail.23

Effect of Compounds on DNA Synthesis

Cells (4 x 10^6) were suspended in 1 ml of medium containing dexamethasone or TPA at the required concentration and incubated for 18 hr before adding 3H-thymidine to give a final concentration of 5 μCi (1 μg)/ml. After a further 8 hr of incubation, 1 ml of ice-cold 10% trichloracetic acid was added to each culture dish and dishes were set at 4°C for 30 min. The precipitate was then collected on a Whatman GFC filter and washed with 30 ml of 5% ice-cold trichloracetic acid. The filter was dried and counted in Liquifluor-toluene in a Packard liquid scintillation spectrometer.

RESULTS

Normal granulocytes and cells from patients with AML differed markedly in their ability to survive in culture. At the end of 48 hr, at least 85% of leukemic cells were still viable, whereas only approximately 60% of normal granulocytes survived for 24 hr, and by 48 hr, this figure dropped to 30%. The limited capacity for in vitro survival shown by granulocytes has necessitated our confining our observations on these cells to the first 24-hr period of incubation. Since maximal effects of TPA and dexamethasone on plasminogen activator release by AML cells were observed during the second 24-hr period of exposure to these compounds, the results obtained during this period are presented.

We present the effects of TPA or dexamethasone on plasminogen activator release in terms of suppression or stimulation relative to control values observed in the absence of these compounds. For the sake of brevity and descriptive convenience, we have arbitrarily graded these effects as follows: pronounced suppression—less than 25% of control value; moderate suppression—25%-80% of control value; insignificant effect—80%-140% of control value; stimulation—greater than 140% of control.

Effects of TPA and Dexamethasone on Cellular Morphology and Viability

During the first 48 hr of in vitro culture, TPA at 0.1 ng/ml, 1 ng/ml, or 10 ng/ml had no adverse effects on
the viability of AML cells. In contrast, TPA at 1 ng/ml was moderately toxic to normal granulocytes, and at 10 ng/ml it was uniformly lethal within 24 hr.

As reported by Pegoraro et al., TPA induced a striking change in the morphology and adherence of AML cells in culture. At 10 ng/ml, TPA caused the cells to adhere to the plastic surface as clusters or as spread macrophage-like cells. Adherence was also frequently observed at 1 ng/ml but was usually not noted at 0.1 ng/ml.

Dexamethasone had no effect on the morphology of normal or leukemic cells. At 10^6 M, this hormone occasionally caused viability to decrease during the first 48 hr of in vitro culture; at 10^7 M this effect was not seen.

**Plasminogen Activator Release by Normal or Leukemic Cells**

Enzyme secretion by leukemic cells of the 45 patients included in this study ranged from 0.001 to 22.1 urokinase units/10^7 cells/24 hr. Of these cells, 32 secreted plasminogen activator of the urokinase-type and 11 secreted plasminogen activator of the tissue-type. In two cases, both enzymes could be detected.

Neutrophils from normal subjects released from 0.006 to 0.3 urokinase units/10^7 cells/24 hr. In all cases this enzyme was of the urokinase-type. These results have previously been reported.

**The Effects of Dexamethasone on Cellular Plasminogen Activator Release**

Dexamethasone was added to cultures of normal granulocytes and AML cells to give final concentrations ranging from 0 to 10^6 M, and harvest fluids were assayed for plasminogen activator content. The results observed when cultures were treated with 10^7 M dexamethasone are presented in Fig. 1, where each point represents the average of the results for duplicate cultures expressed as a percentage of the average of results from triplicate control cultures to which steroid was not added.

In 12/13 cultures of normal PMN, 10^7 M dexamethasone caused moderate suppression of plasminogen activator release. In the remaining case, the steroid had little effect.

The effects of dexamethasone on AML cells were more complex and in many cases more striking. Dexamethasone at 10^7 M either stimulated, had relatively little effect, or inhibited plasminogen activator release. Typical examples of these 3 responses are shown in Fig. 2.

In most cases, the effects of dexamethasone were time-dependent (being more obvious after 48 hr than they were after 24 hr) and concentration-dependent, being maximal or very nearly so at 10^7 M. These
relationships are illustrated in Fig. 3. Two unusual responses were observed in which the steroid caused pronounced inhibition of plasminogen activator release at $10^{-8}M$, whereas it stimulated at $10^{-7}M$.

There was a tendency for the release of the tissue-type plasminogen activator to be inhibited to a greater extent than release of urokinase but this was not statistically insignificant. In 6/45 cases, $10^{-7}M$ dexamethasone stimulated plasminogen activator release, and in 35/45 cases, significant suppression was observed. Dexamethasone had an insignificant effect on cells from the remaining 4 cases (Fig. 1). In 26/45 cases, plasminogen activator release by leukemic cells was inhibited to less than 25% of control values. Pronounced inhibition of this degree was not encountered with any of the preparations of normal PMN studied at any of the concentrations of dexamethasone.

Results obtained with $10^{-6}$ and $10^{-8}M$ dexamethasone were similar to those obtained with $10^{-7}M$, whereas $10^{-9}M$ dexamethasone had significantly less effect on plasminogen activator release.

The effect of dexamethasone on plasminogen activator secretion could be prevented if actinomycin-D (1 $\mu$g/ml) was added to the cultures together with the dexamethasone, indicating that transcription of new mRNA was required for its inhibitory or stimulatory effect to manifest itself.

The Effects of TPA on Plasminogen Activator Secretion

Leukemic and normal cells were treated with TPA at concentrations of 0.1, 1, and 10 ng/ml and harvest
Although, as observed by Granelli-Piperno et al., dexamethasone suppressed and TPA tended to stimulate plasminogen activator release by normal granulocytes, these effects were generally less marked than those observed when AML cells were treated with these compounds at the same concentrations. Differences between AML cells and normal PMN in these respects may have been due to differences in their neoplastic status or in the extent to which they had differentiated. Normal bone marrow myeloblasts would have been a preferable control cell population to have used and such studies are currently in progress. Furthermore, PMN cells survived poorly in culture, so that it was difficult to design experimental protocols that could be used to draw valid comparisons between AML cells and normal granulocytes. For these reasons we do not wish to suggest that differences between normal granulocytes and AML cells that we have observed necessarily identify differences between normal and leukemic cells.

By most clinical and laboratory criteria, the leuke-
mias comprise a group of well defined disorders. Within each diagnostic category, however, one frequently observes patients whose disease differs strikingly in its presentation, progress, and response to therapy. The results in this article provide further examples of this phenotypic diversity.

Plasminogen activator release by AML cells incubated in vitro differed between patients both in terms of the type and the amount of enzyme secreted and in terms of the extent to which this secretion could be modulated by dexamethasone or TPA. The regulation of plasminogen activator synthesis and release has proved to be a genetically controlled cellular function in all cells studied to date. Our data provide good reason to believe that the same holds true for AML cells and that the phenotype of each myeloid leukemic cell clone is uniquely governed by the action of genes that vary in the degree to which their expression can be modulated. Sufficient data are not yet available to allow significant correlations to be drawn between modulation of plasminogen activator release, other criteria of cellular differentiation and clinical features of this disease.

Since cells from 41/45 patients with AML showed a significant change in the rate of plasminogen activator secretion in response to 10^{-7}M or 10^{-6}M dexametha-
sone, one might infer that the great majority of AML cells possess glucocorticoid receptors. Using techniques that measure binding of radioactive steroid, Lippman et al. and Gailani et al. found that 20%–30% of disrupted AML cell specimens possessed glucocorticoid receptors. However, intact cells were used for receptor measurement, and leukemic blasts from all patients were found to contain glucocorticoid...
receptors. Our results tend to confirm the need for intact cells for receptor measurements. The most sensitive and informative assay may be the indirect enzymatic one that we have used, in which the effects of steroid-receptor engagement are amplified many-fold by effects on cellular plasminogen activator release.

Most studies of the effects of dexamethasone on plasminogen activator synthesis have reported inhibition of enzyme release by this steroid. Roblin has recently suggested that the synthesis of the urokinase-type enzyme is inhibitable by dexamethasone, whereas that of tissue plasminogen activator is not. Our observations with leukemic cells show that this is not a general rule. The release of both tissue-type and urokinase-type plasminogen activators were susceptible to the effects of dexamethasone, and in these cells, both enzyme species could be inhibited or stimulated by this steroid (Figs. I and 2). Therefore, it is the cell type rather than the species of enzyme that is the final determinant of dexamethasone responsiveness and, unlike other cell types studied, AML cells may show stimulation of plasminogen activator secretion in response to this glucocorticoid.

Although TPA is best known as a promoter of carcinogenesis and for its ability to enhance expression of the transformed phenotype, it has been shown to induce differentiation in leukemic cells. Similarly, TPA usually induces the synthesis of plasminogen activator when added to other cells cultured in vitro, yet as shown by our studies, this compound usually inhibits release of this enzyme when applied to leukemic cells (Fig. 4). In these two respects, therefore, AML cells respond to TPA in an anomalous fashion. The reason for this and its significance must await further study.

In conclusion, we feel it appropriate to emphasize the usefulness of the measurement of plasminogen activator synthesis as a sensitive means of monitoring cellular responses to biologically active compounds. The implications of this conclusion for rational approaches to the management of AML are obvious. It is hoped that further studies will justify this conclusion and will establish the characterization of plasminogen activator release as a useful procedure for predicting prognosis and optimal therapeutic options in this disease.

ACKNOWLEDGMENT

We thank L. Wood, J. Meadows, J. Dark, C. Feurns, J. Morris, D. Stuart, and S. Milton for expert technical, administrative, or nursing assistance.

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