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Dehydroascorbate Uptake as an *in Vitro* Biochemical Marker of Granulocyte Differentiation[1]

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ABSTRACT

We tested the hypothesis that the rate of cellular uptake of dehydroascorbate in cultures of developing granulocyte-macrophage progenitors *in vitro* would serve as a biochemical marker of neutrophil maturation. Suspension cultures of low-density, nonadherent, T-lymphocyte-depleted bone marrow cells from eight normal volunteers were cultured in medium containing 10% human placental conditioned medium and were harvested at intervals over 14 days. The harvested cells were tested for their ability to take up dehydroascorbate. Mean cellular uptake rate increased 12-fold by Day 10, at which time the cells had differentiated to neutrophils. Uptake increased by less than 2-fold in cells which had been induced to differentiate to mature mononuclear phagocytes with 12-*O*-tetradecanoylphorbol-13-acetate. Additional studies using HL-60 cells induced to differentiate with dimethyl sulfoxide or 12-*O*-tetradecanoylphorbol-13-acetate support the view that a major increase in dehydroascorbate uptake in cultured granulopoietic progenitors is a manifestation of a neutrophil differentiation.

INTRODUCTION

Human leukocytes take up and reduce dehydroascorbate from culture media (6). Reduced ascorbate may serve as part of the cellular mechanisms which inactivate potentially injurious oxidants and free radicals (5). A previously reported study showed that dehydroascorbate uptake and reduction was impaired in peripheral blood neutrophils from patients with chronic granulocytic leukemia and that these cells exhibited decreased resistance to acute radiation injury (19). The cellular uptake of dehydroascorbate is dependent upon time, concentration of dehydroascorbate in the medium, and the ability of the cell to reduce dehydroascorbate. Enzymatic reduction is effected by the free cytosol enzyme dehydroascorbate reductase (EC 1.8.5.1) in plant (7) and animal tissues (2, 9, 10) and in human cells (5). Dehydroascorbate is more avidly taken up into mature neutrophils than monocytes, lymphocytes, erythrocytes, or fibroblasts (5). The stage of differentiation at which granulocyte precursors begin to increase dehydroascorbate uptake has not been heretofore defined.

We hypothesized that a progressively increasing rate of dehydroascorbate uptake would occur as granulocyte progenitors differentiated to neutrophils but that the rate of change would be less as progenitor cells differentiated to mononuclear phagocytes. To test this hypothesis, rates of cellular dehydroascorbate uptake were measured (*a*) in cells from sequentially harvested normal bone marrow cells enriched for progenitor cells which had been induced to undergo neutrophilic differentiation with colony-stimulating activity and to undergo macrophage differentiation with TPA[4] (1, 8), and (*b*) in a human progranulocytic leukemia cell line (HL-60) induced to differentiate to neutrophils with dimethyl sulfoxide or to monocytes with TPA.

MATERIALS AND METHODS

Technique for Suspension Cultures. After obtaining informed consent, bone marrow cells were aspirated from 8 paid normal volunteers by posterior iliac crest puncture and processed as described previously (3). Cells were directly aspirated into heparinized syringes. Briefly, low-density bone marrow cells were obtained by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N. J.) density cut. Cell suspensions were depleted of monocytes using a carbonyl iron-magnet technique. Adherent cells were removed by using nylon-fiber columns. Suspensions were depleted of T-lymphocytes by placing cell suspensions incubated with washed sheep erythrocytes (Prepared Media Laboratories, Tualatin, Oregon) on Ficoll-Paque (3). The colony-forming units granulocyte-macrophage-enriched suspensions were plated in 35-mm plastic Petri dishes at 3 to 5 × 10^5/ml in McCoys Medium 5A with 15% fetal calf serum, amino acids, and antibiotics (Grand Island Biological Co., Grand Island N. Y.). Human placental conditioned medium was used as a source of colony-stimulating activity (17). For some experiments, TPA (Sigma Chemical Co., St. Louis, Mo.) at 10^{-7} M was added to induce monocyte-macrophage differentiation. Proliferating cells were incubated at 37.5° in a fully humidified atmosphere of 7.5% CO_2 in air. Cells were harvested on Days 3, 7, 10, and 14 of culture, washed, and resuspended in phosphate-buffered saline (0.9%) for analysis of dehydroascorbate uptake. Morphological analysis of harvested cells was assessed after staining with Wright's stains and α-naphthyl butyrate esterase.

HL-60 Cells. HL-60 cells were a generous gift from Dr. Robert C. Gallo (NIH). Cells were plated at 3 × 10^5 cells/ml in Roswell Park Memorial Institute Medium 1640 as above. Neutrophil maturation was induced by adding dimethyl sulfoxide at a final concentration of 1.2%. Cells harvested at Day 5 were predominantly maturing neutrophils (60 to 70%). Alternatively, TPA (10^{-7} M) induced predominantly monocytes-macrophages (80 to 90%).

Acute Leukemia Cells. Bone marrow cells from 5 patients with acute myeloblastic leukemia (M1 according to the FAB classification scheme) were aspirated from informed consenting adults and processed in the same way as were the marrow cells from normal volunteers. Three patients were studied at the time of original diagnosis and 2 were studied at the time of first relapse. In every case, the patient presented with bone marrow failure, circulating blast counts in excess of 5.0 × 10^9/liter, and a marrow aspirate or biopsy which contained more than 70% myeloblasts. None of the patients had had preexisting myeloproliferative

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[4] The abbreviation used is: TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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disorders. These cells were held in culture for no more than 12 hr. The dehydroascorbate uptake assay was performed on freshly obtained low-density marrow cells, more than 80% of which were myeloblasts.

Measurement of Dehydroascorbate Uptake. Dehydroascorbate uptake was measured according to our previously described techniques (5, 6, 18). Harvested cells were incubated for 20 min at 37° in 1 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4, containing 5.5 mM glucose and 100 μg of [^{14}C]dehydroascorbate, 0.02 μCi/μg, freshly prepared from L-[^{14}C]ascorbic acid (New England Nuclear, Boston, Mass.), and L-ascorbic acid (Sigma). Dehydroascorbate was prepared by bubbling Br_2 vapor through a freshly prepared solution of reduced ascorbate in 0.154 M NaCl; Br_2 was washed out with water-saturated N_2. Dehydroascorbate concentrations in suspending buffer were measured colorimetrically, using the dinitrophenylhydrazine method of Roe *et al.* (15). Uptake was stopped by placing the reaction tubes in ice water. Cells were immediately washed 3 times with 4° 0.154 M NaCl and then counted using a hemocytometer. Aquasol (New England Nuclear) was added to centrifuged cells, and radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. Dehydroascorbate uptake was expressed as nmol/10^8 cells/20 min.

RESULTS

The capacity of normal developing granulocytic cells to incorporate dehydroascorbate increased linearly with time in culture to Day 10 (Chart 1*a*). Mean base-line uptake was 43 ± 26 (S.D.) nmol/10^8 cells/20 min, and mean Day 10 uptake was 500 ± 97. The increase represented a 12-fold rise in cellular uptake. Mean cell numbers increased only slightly with time in culture until Day 10 (Chart 1*b*) and then began to decline. Examination of cytocentrifuge preparations of serially harvested cells revealed that initially plated cells were predominantly small mononuclear cells with lymphoid morphological characteristics. Cells showed progressive evidence of neutrophil maturation to Day 10 (60 to 90% neutrophils); thereafter, macrophages were the predominant cell type.

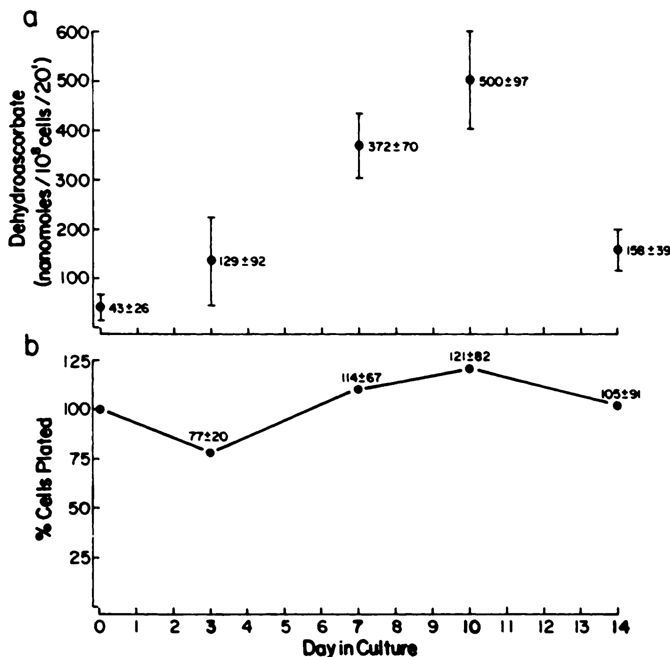

Chart 1. Cellular uptake of dehydroascorbate increased progressively with time in culture until Day 10 (*a*). Mean Day 10 values were 12-fold higher than those obtained from initially plated Day zero cells. This correlated with maximal degree of neutrophilic maturation from morphological studies. At the same time, mean cell numbers increased only slightly by Day 10 and then began to decline (*b*). *Bars*, S.D.

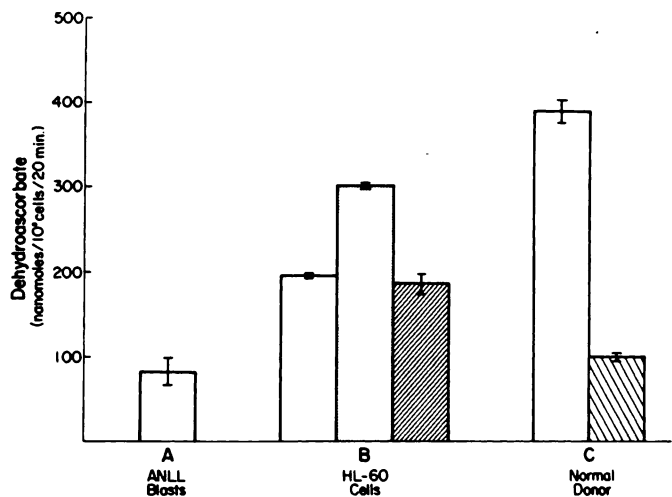

Chart 2. Mean cellular dehydroascorbate uptake from blast cells from 5 patients with acute nonlymphocytic leukemia (*A*) and unstimulated HL-60 cells (□), HL-60 cells induced to neutrophils with dimethyl sulfoxide (□), and HL-60 cells treated with TPA to induce monocyte-macrophage maturation (▨). (*B*). Cells were harvested at Day 5. Results are from 2 separate experiments; *bars*, S.D. *C*, normal colony-forming unit granulocyte-macrophage (n = 2) stimulated by human placental conditioned medium (□) or human placental conditioned medium plus TPA (▧). Cells were harvested at Day 7.

Futher experiments support the hypothesis that the capacity for dehydroascorbate uptake increases with maturation of granulocyte-monocyte precursors. Leukemic blast cells from 5 patients with acute nonlymphocytic leukemia (Chart 2*A*) had a mean cellular uptake of 81 ± 16 nmol/10^8 cells/20 min. All 5 patients had overt acute myeloblastic leukemia, and none had an excess number of progranulocytes. Noninduced HL-60 leukemic progranulocytes, as shown in Chart 2*B*, had a mean dehydroascorbate uptake of 194 ± 2 after 10 days in culture. Simultaneously cultured HL-60 cells which were induced to undergo neutrophil maturation exhibited substantially increased uptake to 306 ± 2 at Day 10. When monocyte-macrophage differentiation was induced with TPA, no change was noted relative to uninduced cells. The most noticeable differences in dehydroascorbate uptake were noted in studies on progenitor-enriched cell suspensions from normal donors. These cells were induced along 2 pathways of differentiation: to neutrophils by human placental conditioned medium; and to monocytes-macrophages by addition of TPA. As shown in Chart 2*C*, Day 7 dehydroascorbate incorporation was significantly different in the 2 populations, 386 ± 14 in the predominantly neutrophilic cells and only 101 ± 3 in the monocytes-macrophages. [These values are comparable to those reported previously for mature blood granulocytes (420 ± 40) and for normal blood monocytes (161 ± 36) (5).]

DISCUSSION

Significant advances have been made in understanding genetic regulation of hemopoietic cellular differentiation using the classic model of Friend virus-induced murine erythroleukemia (13). These advances have derived, in part, from the development of quantitative assays for gene products which appear as erythroid cells and undergo terminal differentiation, such as catalase, spectrin, RBC antigens, globin, and globin mRNA (13). In contrast, gene regulation of granulocyte-monocyte differentiation is

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poorly defined. Assessment of differentiation in cultured granulocytic cells or myeloid leukemic cells has been conventionally estimated using qualitative techniques such as microscopic morphology and determination of the presence of surface membrane markers (4, 11, 16). Only more recently, biochemical measurements of the synthesis of lysozyme, arginase, and acid phosphatase in humans (14) and lactoferrin and lysozyme in the mouse (11, 12) have been correlated with cellular maturation in suspension cultures.

Our study provides evidence which suggests that another cellular activity, the rate of uptake of dehydroascorbate, also correlates with maturation of granulocyte progenitors *in vitro*. These observations are probably best explained by an increase in the synthetic rate of the cytosol enzyme dehydroascorbate reductase during maturation, since other studies on human nonmyeloid cells have shown that dehydroascorbate uptake is proportional to reductase activity (5). Unfortunately, dehydroascorbate reductase cannot routinely be measured directly in the number of cells generated in culture. We did note that mature neutrophils which developed from progenitors *in vitro* take up dehydroascorbate at the same rate as do freshly acquired normal peripheral blood neutrophils and, conversely, marrow cells induced to differentiate to monocyte-macrophages take up dehydroascorbate at rates similar to those of freshly isolated peripheral blood monocytes (5, 6, 18). These observations indicate that this *in vitro* system permits full expression of dehydroascorbate activity. Whether the increased rate of change with neutrophilic maturation is a result of the more rapid achievement of a postmitotic state by neutrophils relative to monocytes or whether the rate of change derives from a neutrophil-specific gene program remains to be determined. Nevertheless, our results suggest that quantitative analysis of dehydroascorbate uptake and reduction may prove to be a useful addition to currently measurable parameters of neutrophilic differentiation.

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