Effects of Sonicated Eosinophils on the in Vitro Sensitivity of Human Lymphoma Cells to Glucose Oxidase

Michael K. Samoszuk, Vincent Nguyen, Cherry T. Thomas, and Dawn M. Jacobson
Pathology Department, University of California, Irvine, California 92717

ABSTRACT

We report here that cultured human lymphoma cells in the absence of sonicated eosinophils are sensitive to killing by glucose oxidase (β-D-glucose:oxygen-oxide reductase; EC 1.1.3.4) at concentrations as low as 0.025 μg/ml, a level that can be rapidly attained in s.c. tumor implants in mice that receive a single nonlethal injection of enzyme. Multiple clonogenic assays were used to measure the survival of human lymphoma cell lines (H9 and ARH-77) cultured for 14 days in complete RPMI 1640 supplemented with exogenous glucose oxidase (0.025-2.5 μg/ml) or an immunoconjugate containing glucose oxidase (0.25-25 μg/ml) in the presence or absence of catalase (10 μg/ml) or an equal number of sonicated human eosinophils with or without supplemental 100 μM Br−, I−, or SCN−. In addition, we used an immunoassay to measure the concentration of glucose oxidase in s.c. implants of the Sp 2/0 myeloma tumor at 0–30 min after an i.v. injection of 50 μg of enzyme into 21 BALB/c mice. Doses of glucose oxidase as small as 0.025 μg/ml killed more than 3 logs of tumor cells. Catalase completely inhibited, and sonicated human eosinophils partially inhibited, the killing by glucose oxidase or immunoconjugate, whereas supplemental halides had no effect. Glucose oxidase i.v. produced levels >0.04 μg/g of tumor for 30 min after injection with a peak concentration of 0.079 μg/g of tumor within 5 min of injection. These results are important because certain human lymphomas contain extensive extracellular deposits of eosinophil peroxidase, thereby making these tumors potentially less susceptible to killing by otherwise therapeutic doses of glucose oxidase.

INTRODUCTION

Glucose oxidase is a M, 160,000 flavoenzyme that is produced by Aspergillus niger. The enzyme is not present in mammalian cells. Under physiological conditions, it catalyzes the following reactions:

\[
\text{β-D-Glucose} + \text{enzyme-FAD} \rightarrow \text{enzyme-FADH}_2 + \text{D-glucono-δ-lactone}
\]

\[
\text{Enzyme-FADH}_2 + \text{O}_2 \rightarrow \text{enzyme-FAD} + \text{H}_2\text{O}_2
\]

Some lymphomas and carcinomas are sensitive to killing by H2O2 (1–9). Consequently, in vitro and in vivo studies have demonstrated that in the presence of adequate oxygen concentrations, glucose oxidase has potent tumoricidal activity (4–8) mediated in part by hydrogen peroxide. Lymphomas in particular have an intrinsic sensitivity to the toxic effects of hydrogen peroxide (5–8). The mechanisms of cytotoxicity of hydrogen peroxide are incompletely understood at this time but are believed to be mediated by free radical production.

In recent preclinical studies, we have demonstrated that glucose oxidase has relatively predictable toxicities and is probably safe for initiating Phase I human studies (10). Before such clinical trials can begin, however, it will be necessary to answer some important questions. For example, what effect does glucose oxidase have on the survival of cultured lymphoma cells as measured in a clonogenic assay? Does deglycosylated glucose oxidase have superior pharmacokinetic properties in vivo compared to native enzyme, as is the case for ricin A chain (11, 12)? What concentration of glucose oxidase can be attained within lymphoma implants in vivo?

Perhaps the most interesting biological issue that needs to be addressed was raised by our recent immunoscintigraphic finding that certain human lymphomas contain extensive extracellular deposits of eosinophil peroxidase (13). Because eosinophil peroxidase increases the cytotoxic activity of hydrogen peroxide in the presence of Br−, I−, or SCN− (14, 15), we hypothesized that sonicated human eosinophils could be used to increase the tumoricidal activity of glucose oxidase as measured in a clonogenic assay. This report describes the results of our experiments that were intended to explore these issues prior to the start of clinical safety studies using glucose oxidase in lymphoma patients.

MATERIALS AND METHODS

Glucose Oxidase and Immun conjugate. Glucose oxidase was purified, processed, and analyzed for enzymatic activity as described previously (10). An immun conjugate directed against human eosinophil peroxidase was created by cross-linking glucose oxidase with glutaraldehyde to a murine monoclonal antibody called EOS (10, 13). In order to quantify the immunoreactivity of the immun conjugate, we used a Lineweaver-Burke approach as described by Schuhmacher et al. (16). In brief, serial 2-fold dilutions of sonicated eosinophils (starting at 2 × 107 cells/ml) or a negative control cell line were incubated for 60 min with a fixed concentration (30 μg/ml) of immun conjugate (approximately 50 units/mg).

After the cells were washed, the bound conjugate was measured spectrophotometrically by its glucose oxidase activity (10). The immunoreactivity data were then analyzed using a double inverse plot of immun conjugate binding to target cells over the range of cell concentrations. The immunoreactive fraction at infinite antigen excess was then extrapolated by calculating the reciprocal of the intercept on the total/bound (Y) axis.

Pharmacokinetics of Deglycosylated Glucose Oxidase. We attempted to reduce the clearance of glucose oxidase by carbohydrate receptors in the mouse liver by deglycosylating the enzyme with α-mannosidase and endo-β-galactosidase H (Boehringer-Mannheim, Indianapolis, IN) using a method described by Kalisz et al. (17). Following overnight digestion of the glucose oxidase with the deglycosylating enzymes, the deglycosylated glucose oxidase was purified by low pressure liquid chromatography over a hydroxylapatite column (Bio-Rad Laboratories, Richmond, CA) using a linear gradient of phosphate buffer from 10 to 400 mM at pH 6.8. The deglycosylated glucose oxidase eluted as a single sharp peak at a phosphate concentration of approximately 20 to 40 mM.

After a buffer exchange into phosphate-buffered saline (pH 7.4) using a Sephadex PD-10 column (Pharmacia, Piscataway, NJ), the enzyme was analyzed for purity by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad), which showed a single broad band at approximately M, 75,000. Purified enzyme was then filter sterilized and stored refrigerated up to 14 days before use. The enzymatic activity of the deglycosylated enzyme averaged 105 units/mg of protein (compared to approximately 200 units/mg for the native enzyme).

In order to measure the blood half-life of the deglycosylated enzyme, 18 adult BALB/c mice (20–22 g body weight) were given i.v. injections of 100 μg of enzyme in a volume of 200 μl of phosphate-buffered saline. At intervals of 0, 15, 30, 45, 60, and 120 min after injection, groups of three mice were phlebotomized, and the serum concentration of glucose oxidase was measured with a capture enzyme-linked immunosorbent assay (10). The serum half-life was extrapolated from a plot of mean serum concentration versus min after...
sonication procedure was intended to mimic the in vivo degranulation of eosinophils. Pilot clonogenic assays performed with glucose oxidase and intact eosinophils demonstrated no significant effect on survival. Catalase was tested because of its ability to neutralize hydrogen peroxide.

Sonication was performed for 10 s at medium power (setting, 50) using a Vibra Cell ultrasonicator (Sonics and Materials, Inc., Danbury, CT) and 3.6 × 10^6 eosinophils suspended in 1.5 ml of ice-cold complete cell culture medium. Microscopic examination of the sonicate revealed amorphous cellular debris and intact granules which could be precipitated by centrifugation. When measured with a colorimetric assay, greater than 90% of the peroxidase activity could be removed by centrifugation, suggesting that less than 10% of the peroxidase was liberated by sonication into the fluid phase.

In a subsequent set of experiments, the lymphoma cells were cocultured with glucose oxidase plus sonicated eosinophils in the presence of supplement 1", Br", and SCN" (100 μM each). For negative control purposes, we also performed clonogenic assays consisting of untreated lymphoma cells only or lymphoma cells plus sonicated eosinophils only.

The lymphoma cells from each experiment were plated at serial 10-fold dilutions (beginning at 2500 cells/ml) into 24 wells of 96-well microtiter plates. After 14 days of growth in a humidified CO₂ incubator, the number of wells showing growth at each concentration was counted by microscopic examination, and the following formula (Spearman estimator) was used to calculate the number of surviving clonogenic units (θ) at each dose of glucose oxidase (18):

\[
\theta = \left( e^{-}\frac{e^{-\mu}}{\theta} \right)
\]

where \( \gamma = 0.57722 \) (Euler's constant) and \( \mu \) is calculated as:

\[
\mu = -\frac{x_0 + d}{2 - 2 \sum p_i}
\]

In the latter formula, \( x_0 = \ln z \), where \( z \) is the highest concentration of the original suspension used; \( d = \ln a \), where \( a = 10 \) is the serial dilution factor; and \( p_i \) is the proportion of wells showing growth at dilution \( z_i \) (where \( i = \) the number of serial dilutions tested). Under the conditions used in these experiments (four serial 10-fold dilutions into 24 wells at each dilution), the maximum number of clonogenic units that could be estimated if growth were observed in all wells at all dilutions was 1760 units/ml.

We also attempted to study a model of tumor cell killing in which the lymphoma cells were first pelleted with sonicated eosinophils and then incubated for 1 h in the presence of varying concentrations of glucose oxidase immunoconjugate directed against eosinophil peroxidase. This model was intended to mimic an in vivo situation in which the lymphoma cells are intimately admixed with degranulated eosinophils that can bind the peroxidase-generating immunoconjugate. Following the incubation with immunoconjugate, the lymphoma cells were washed, and the clonogenic assay was performed as described above.

Accumulation of Glucose Oxidase in Tumor Implants. Adult BALB/c mice received s.c. inoculations into the flank of approximately 10^6 Sp 2/0 murine myeloma cells obtained from ascites passage. When the tumors reached approximately 10 mm in diameter, the mice received a single i.v. injection of 50 μg of native glucose oxidase. At 5-min intervals from 0–30 min, groups of three mice were sacrificed, the tumors were removed, and the concentrations of glucose oxidase were measured in the tumor homogenates by a capture enzyme-linked immunosorbent assay (10).

RESULTS

Glucose Oxidase Immunoconjugate. The double inverse Lineeweaver-Burke plot of the binding of the immunoconjugate to sonicated eosinophils is illustrated in Fig. 2. On the basis of the Y-axis intercept, the immunoreactivity of the immunoconjugate was extrapolated to be 65%. By contrast, intact eosinophils as the target yielded an immunoreactivity of less than 8%.

Pharmacokinetics of Deglycosylated Glucose Oxidase. The serum half-life of deglycosylated glucose oxidase was determined to be approximately 48 min, compared to a serum half-life of 45 min for the native enzyme. Because there was no substantial difference in the
half-life between native and deglycosylated forms of glucose oxidase, we elected to use only native enzyme in the subsequent studies.

Clonogenic Assays. The two cell lines that were tested yielded essentially identical results in duplicate assays, and representative data from a clonogenic assay of the ARH-77 cell line plus glucose oxidase are presented in Fig. 3. In the absence of eosinophils, glucose oxidase at concentrations as low as 0.25 μg/ml completely sterilized the cell cultures (no detectable growth). Eosinophils consistently produced partial neutralization of the killing by glucose oxidase, and supplemental I-, Br-, and SCN- (not plotted) did not reverse the neutralization. The Spearman estimate of the number of clonogenic units in the negative controls (tumor cells only and tumor cells plus sonicated eosinophils only) was 1760 clonogenic units/ml. Catalase (10 μg/ml) completely neutralized the killing by glucose oxidase, also yielding an estimate of 1760 clonogenic units/ml at all concentrations of glucose oxidase tested.

Representative data from the studies with the immunoconjugate and the H9 cell line pelleted with sonicated eosinophils are shown in Fig. 4. In this model, the sonicated eosinophils again seemed to provide protection from killing by the immunoconjugate because tumoricidal activity was observed only in the absence of eosinophils. Studies with the ARH-77 cell line produced similar results and are not shown.

Accumulation of Glucose Oxidase in Tumor Implants. The time course of glucose oxidase accumulation in s.c. tumor implants is tabulated in Table 1. Notably, there was significant accumulation of glucose oxidase (0.16% of the injected dose/g of tumor) only 5 min after injection, and levels in excess of 0.04 μg/g of tumor persisted for at least 30 min after injection.

DISCUSSION

Various forms of glucose oxidase (native enzyme, enzyme conjugated to monoclonal antibodies, and enzyme adsorbed to polystyrene microspheres) have been proposed as potential antineoplastic agents (1–8). The data from our studies indicate that relatively low concentrations of native glucose oxidase (0.025–0.25 μg/ml) are highly tumoricidal to cultured lymphoma cells in a clonogenic assay, provided that sonicated (degranulated) eosinophils are not present. This finding is significant because many human lymphomas (particularly Hodgkin’s disease) contain extensive deposits of eosinophil peroxidase. In our experiments, the neutralization of the tumoricidal activity of glucose oxidase by degranulated eosinophils was somewhat unexpected, particularly in view of the numerous previous reports that hydrogen peroxide augments the cytotoxicity of eosinophil peroxidase (8, 14, 15, 19–21). We speculate that the reason for this apparent
discrepancy is that eosinophil peroxidase seems to be cytotoxic only when directly adherent to target cell membranes. In the previous studies, purified eosinophil peroxidase was used to coat target cells directly prior to administration of hydrogen peroxide in short-term cytotoxicity assays, whereas the current study used sonicated (de-granulated) eosinophils mixed with tumor cells in a much more sensitive clonogenic assay.

Thus, in our clonogenic assay, the peroxidase in the sonicated eosinophils probably functioned in essentially the same manner as the exogenous catalase to eliminate hydrogen peroxide rapidly before it could kill the uncoated tumor cells. Accordingly, the highly reactive cytotoxicity assays, whereas the current study used sonicated (de-granulated) eosinophils mixed with tumor cells in a much more sensitive clonogenic assay.

It is important to note, however, that it is still uncertain whether our clonogenic assay model truly approximates the in vivo situation in human lymphomas that are characterized by degranulation of eosinophils. If, for example, eosinophil peroxidase from degranulated eosinophils directly coated tumor cells or vasculature in vivo, then exogenous glucose oxidase could still be expected to have potent tumoricidal activity because the peroxidase-coated cells would be exceptionally sensitive to killing by hydrogen peroxide.

Indeed, our study clearly demonstrated that tumoricidal levels of glucose oxidase can accumulate rapidly in s.c. tumor implants even in the absence of eosinophils. Specifically, a single nonlethal (10) dose of glucose oxidase (50 µg) rapidly produced levels of enzyme within the tumor (0.079 µg/g of tumor) that greatly exceeded the concentration (0.025 µg/ml) needed to kill more than 99% of the cells in a clonogenic assay (Fig. 3).

Moreover, it is particularly significant that a relatively high proportion of the injected glucose oxidase accumulated in the tumor implants only 5 min after injection (0.16% of the injected dose/g of tumor). By contrast, most monoclonal antibodies require many h to achieve tumor concentrations of only 0.01–0.03% of the injected dose (22). One possible explanation for this difference is that the hydrogen peroxide produced by glucose oxidase is known to initiate hydrolysis of endothelial cell phospholipids, thereby leading to reversible increases in vascular permeability (23). Thus, our data suggest that it is theoretically possible that intravascular glucose oxidase can promote its own passage across endothelial barriers into tumor tissue.

In addition to improving our understanding of the potential use of glucose oxidase as an antineoplastic therapeutic agent, this study also generated some other interesting findings. On the basis of our data, for example, it appears that there is no significant difference in the sensitivity of a B-cell line (ARH-77) and a T-cell line (H9) to killing by glucose oxidase. Moreover, there does not appear to be any pharmacokinetic advantage to using deglycosylated glucose oxidase, and there was no detectable increase in tumoricidal activity when using a peroxide-generating immunoconjugate targeted against human eosinophil peroxidase. Finally, our study documented the use of a novel method for rapidly and efficiently purifying human eosinophils from peripheral blood specimens. This simple method will undoubtedly aid other investigators who wish to explore the biology of eosinophils and human cancers in the future.

REFERENCES

Effects of Sonicated Eosinophils on the \textit{in Vitro} Sensitivity of Human Lymphoma Cells to Glucose Oxidase


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/10/2650

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/54/10/2650.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.