Selective Killing of Transformed Cells by Exploitation of Their Defective Cell Cycle Control by Polyamines

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ABSTRACT

The effects of methylglyoxal bis(quanylhydrazone) (MGBG) upon the proliferation of normal, nontransformed 3T3 fibroblasts and their SV40 virus-transformed counterparts (SV-3T3) were studied. MGBG is a potent inhibitor of the synthesis of the polyamines spermidine and spermine; in addition, it interferes with the intracellular functioning of polyamines by virtue of its being a structural analog of spermidine. Treatment of proliferating 3T3 and SV-3T3 fibroblasts with MGBG resulted in fundamentally different responses in these model normal and tumorigenic cell lines. Proliferating 3T3 cells were reversibly arrested at some point in G1 by MGBG treatment; in contrast, the proliferation of SV-3T3 cells was little affected by MGBG, such that these tumor cells continued to progress through the cell cycle at an appreciable rate. Neoplastic transformability therefore appears to be associated with the loss of a polyamine-sensitive growth regulatory or "restriction" point in G1. The loss of this control process is considered to be analogous to the loss of other growth regulatory mechanisms observed upon transformation. By pretreating proliferating cultures of 3T3 and SV-3T3 cells with MGBG and subsequently treating such cultures with hydroxyurea, a cytotoxic drug that kills cells only in the S phase of the cell cycle, a highly selective killing of the still cycling tumorigenic SV-3T3 cells was obtained. In contrast, normal 3T3 cells were protected from the cytotoxic effects of hydroxyurea by virtue of their MGBG-induced growth arrest in the insensitive G1 phase. These studies demonstrate the feasibility of selectively killing transformed cells by exploiting the biochemical basis underlying a difference in the regulation of growth between normal and transformed cells.

INTRODUCTION

The polyamines putrescine, spermidine, and spermine have long been implicated in the regulation of cellular proliferation (41). Thus, the onset of cell proliferation is associated with increased polyamine synthesis in tissues in vivo (33, 38), and in cells in tissue culture (12, 15, 24). Studies using inhibitors of polyamine synthesis have demonstrated that polyamines are intimately involved in the proliferative process and not simply associated with it (11, 20, 33, 35). In particular, MGBG, a potent inhibitor of the putrescine-activated S-adenosylmethionine decarboxylase (44), can specifically block the synthesis of spermidine and spermine both in vivo (30) and in vitro in tissue-cultured cells (11, 35). This study describes the extension of our original studies with normal rat fibroblasts (35) to a detailed comparison of the effects of MGBG on a normal mouse fibroblast cell line and its tumorigenic counterpart. The difference in response of such normal and tumor cells to treatment with MGBG can be exploited to selectively kill the tumor cell population.

The selective killing of tumor cells with relative sparing of the normal cell population is the much-sought-after goal of the cancer chemotherapist. One particular class of agent used in cancer chemotherapy comprises drugs that kill proliferating cells in only a specific portion of the cell cycle, e.g., hydroxyurea and arabinosylcytosine in S phase. However, such drugs do not differentiate between normal and tumor cells, and their use therefore results in considerable destruction of rapidly proliferating normal tissues in vivo such as the bone marrow and gut epithelium. The feasibility of selectively protecting normal cells but not tumor cells from the cytotoxic effects of such chemotherapeutic agents was first demonstrated by the studies of Pardee and James (28). These workers showed that inhibitors of protein synthesis and cyclic adenosine 3':5'-monophosphate metabolism could arrest proliferating normal cells in the G1 phase without significantly affecting the proliferation of transformed cells. Such pretreated transformed cells could be selectively killed by subsequent treatment with drugs cytotoxic for cells in S phase or mitosis. Our studies demonstrate that another class of inhibitor, i.e., one that interferes with the role played by polyamines, can similarly be used to selectively kill tumor cells.

MATERIALS AND METHODS

Cell culture. Swiss 3T3 fibroblasts and their SV40 virus-transformed counterpart (SV-3T3) were a generous gift of Dr. M. Vogt of the Salk Institute. The normal 3T3 cell line exhibited good density-dependent inhibition of growth and was highly serum dependent for proliferation. Both cell lines were cultured under identical conditions in a humidified 10% CO2-90% air incubator at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cell cultures were routinely tested for Mycoplasma contamination and found to be free as judged by orcein staining and agar subculture procedures.

Growth curves were derived from cell cultures initiated by seeding cells at low density in 5-cm-diameter Corning plastic tissue culture plates (2 x 10⁴ cells/dish). Cell cultures were treated with MGBG after they had entered into a logarithmic phase of growth. Cell numbers were determined using a Coulter Counter.
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Cell Cycle Analysis. Flow microfluorometric analyses of cell populations were performed following staining by the mithramycin procedure (8). The stained cells were analyzed in a Los Alamos design microfluorometer (42) with an argon laser at 457 nm, and the data displayed on a storage oscilloscope were photographed.

Materials. MGBG (Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in isotonic phosphate-buffered saline immediately before use. Mithramycin was a generous gift of Dr. Nathan Belcher (Charles Pfizer and Co., Inc., New York, N.Y.).

RESULTS

Effects of MGBG on Cell Proliferation. When exponentially expanding populations of 3T3 cells are treated with MGBG, the cell population approximately doubles in number and subsequently enters a plateau phase where cell number remains essentially constant over a period of several days (Chart 1). Following incubation for 4 days in MGBG, cell proliferation returns to approximately normal rates upon removal of the drug, demonstrating the essentially reversible nature of this treatment (Chart 1). In contrast, MGBG has a lesser effect upon the proliferation of SV-3T3 cells but does slow down the growth rate considerably (Chart 1). However, this largely appears to reflect a balance between proliferation and cell death, since such cultures contain considerable numbers of dead, floating cells.

Treatment of proliferating 3T3 cells with MGBG leads to a progressive accumulation of cells predominantly in the G1 phase of the cell cycle (Chart 2). Thus, the growth arrest of 3T3 cells by MGBG appears to be the result of these cells entering a quiescent state at some point in G1. In contrast, SV-3T3 cells continue to cycle in the presence of MGBG, showing little perturbation of their cell cycle distribution and in particular little change in the size of their S-phase population (Chart 2).

Effects of Hydroxyurea on 3T3 and SV-3T3 Cells in the Presence and Absence of MGBG. Treatment of proliferating normal 3T3 cells with 2 mM hydroxyurea, a drug specifically cytotoxic to cells in S phase (40), leads to considerable cell kill, resulting in cell loss from tissue culture plates (Chart 3). Removal of hydroxyurea after 5 days of incubation in its presence results in continuing cell detachment with no detectable recovery of proliferative capability.5 While a proportion of proliferating 3T3 cells treated with hydroxyurea remains attached to dishes and is viable as judged by exclusion of the vital dye trypan blue, such cells have a very distorted morphology and have lost their proliferative potential.5 In contrast, 3T3 cells treated for 2 days with MGBG and then treated for 5 days with MGBG and hydroxyurea demonstrate little cell killing and exhibit a rapid resumption of proliferation following removal of the 2 drugs (Chart 3) similar to the reversibility of treatment with MGBG alone (Chart 1). Thus, pretreatment of 3T3 cells with MGBG results in their protection from the cytotoxic effects of subsequent treatment with hydroxyurea.

In contrast, proliferating SV-3T3 cells and MGBG-pretreated SV-3T3 cells demonstrate rather similar cell kill kinetics following hydroxyurea treatment (Chart 3). MGBG pretreatment of SV-3T3 cells therefore affords little protection against the cytotoxic effects of hydroxyurea in accordance with the small effect of MGBG upon the size of the S-phase population (Chart 2). Further, recovery of cellular proliferation following removal of the 2 drugs is much delayed compared to the rapid resumption of proliferation of 3T3 cells under identical conditions (Chart 3). The delayed recovery observed with SV-3T3 cells appears to be the result of a balance between the proliferation of the few cells surviving this combined treatment and the continuing detachment of cells that have been killed. Ultimately,

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5 H. T. Rupniak and D. Paul, unpublished observations.
the few cells surviving combined treatment with MGBG and hydroxyurea proliferate into colonies that repopulate the tissue culture plate.

Under all conditions where cells detach from tissue culture plates following drug treatment, such cells as were present in the floating debris were unable to exclude the vital dye trypan blue and were incapable of reattachment when plated into new dishes in fresh medium in the absence of drugs. All cells that detach from tissue culture plates under such conditions are therefore nonviable, and cell counts of attached cells can be considered to be an accurate indicator of the proportion of cells remaining viable (though not necessarily capable of proliferation) at a given time after drug treatment.

If one compares the 3T3 and SV-3T3 cell populations at 4 days after the removal of the combination of MGBG and hydroxyurea, then one sees that the 3T3 cell population has increased some 8-fold following the initiation of drug treatment (Day 1), while in contrast the SV-3T3 cell population has been reduced by about a factor of 5. This represents a considerable differential gain in cell number by the normal cell population over the tumor cell population and demonstrates a highly selective means of killing tumor cells by protecting normal cells from drug cytotoxicity. In principle, this procedure could be repeated following recovery of the normal cell population to selectively produce successive and cumulative reductions in the size of the tumor cell population.

**DISCUSSION**

The precise mode of action of the drug MGBG is as yet poorly defined. MGBG is a potent inhibitor of the putrescine-activated S-adenosylmethionine decarboxylase (44) and can consequently block the intracellular synthesis of the polyamines spermidine and spermine (12, 30, 35). However, the effects resulting from MGBG treatment cannot always be attributed simply to an inhibition of polyamine synthesis. Thus, MGBG can arrest mouse 3T3 and human WI-38 fibroblasts in G1 under conditions where no increase in polyamine levels is observed in untreated cultures (2). Similarly, doses of MGBG too low to elicit detectable inhibition of polyamine synthesis can nevertheless hold up to 3T3 cells in Go and thus reduce subsequent DNA synthesis. In addition, MGBG is a poorer inhibitor of polyamine synthesis in 3T3 and SV-3T3 cells than in rat embryo fibroblasts (35, 36). Similarly, studies with L1210 leukemic cells (26) and human embryo fibroblasts (19) have led to the conclusion that MGBG treatment blocked subsequent DNA synthesis through a mechanism other than an alteration of polyamine levels.

Because MGBG is structurally very similar to the naturally occurring polyamine spermidine (13), it is very probable that MGBG can act as a nonfunctional structural analog of spermidine within the cell and so interfere with the normal functions performed by spermidine, particularly since the natural polya mine can reverse the effects of MGBG (2, 11, 25, 35). This then probably represents the actual mechanism whereby MGBG exerts its antiproliferative effects. The close structural similarity between MGBG and spermidine is exemplified by the fact that both are transported into the cell by the same biological carrier system (6, 9). MGBG affects the ultrastructure (29) and functions (4, 31) of the mitochondrion, such that this may be the basis for the growth-inhibitor properties of MGBG.

Recent evidence for a possible role of polyamines in the regulation of mitochondrial metabolism (5) or in the organization of tRNA structure (7) lends further support to this proposal. Such a role could be reflected in an inhibition of protein synthesis as observed following MGBG treatment of cells in tissue culture (18, 19, 21), again possibly forming the basis for the antiproliferative effects of MGBG.

The present studies demonstrate that a normal mouse fibroblast cell line can be reversibly arrested at some point in G1 by treatment with MGBG. Thus, normal fibroblasts of mouse, rat (35), and human (2) origin all arrest in the G1 phase following MGBG treatment. In contrast, transformation of 3T3 cells by SV40 virus to produce a tumorigenic cell line is accompanied by the loss of regulation of cell proliferation by MGBG. The loss, either partial or complete, of this particular aspect of growth control may be a general property of tumor cells, since HeLa cells, a cell line derived from a human carcinoma, respond in similar manner to SV-3T3 cells following MGBG treatments. Similarly, Ehrlich ascites tumor cells (1) and rat brain tumor cells (15) exhibit either no or only small increases in their G1 populations following MGBG treatment, although these results cannot be taken to represent the behavior of all transformed cell types (10). These data substantiate our original proposal (37) that normal cells retain a polyamine-sensitive growth regulatory or "restriction" point in G1 at which it is decided whether the "polyamine status" of the cell is sufficiently favorable to enable it to enter into and complete a new cell cycle. In contrast, this polyamine-dependent regulatory mechanism is largely lost in tumorigenic cells.

The arrest of normal cells in G1 by interference with polyamine functioning appears to represent a situation analogous to the effects of serum deprivation or the limitation of certain key nutrients (17, 22, 27). All these adverse treatments shift normal cells into a quiescent or resting state of low metabolic activity characterized by the accumulation of cells at some point in the G1 phase of the cell cycle, often referred to as G0. The mechanisms responsible for this control have been proposed to increase the probability of survival of such cells under suboptimal or adverse conditions (17, 27). In contrast, transformed cells have either partially or completely lost this growth-regu-
The difference in growth-regulatory controls operating in normal and transformed cells is further exemplified by their different responses to treatment with certain metabolic inhibitors. Thus, treatment of cell cultures with inhibitors of protein synthesis or agents that interfere with cyclic nucleotide metabolism shift normal cells into a quiescent G0 state but have little effect upon the cell cycle progression of transformed cells (3, 28, 34, 43). Because transformed cells treated in this way continue to progress through S phase and mitosis at a considerable rate, they are rendered selectively sensitive to the cytotoxic effects of subsequent treatment with antioxidant agents that are cell cycle phase specific and so kill cells only in S phase or mitosis (3, 28, 34, 43). In contrast, under identical conditions, normal cells are spared from the cytotoxicity of such phase-specific agents by virtue of their having been shifted into an insensitive, quiescent state in G0. In this study, we demonstrate that the same principle of selective killing of transformed cells applies following treatment with another type of metabolic inhibitor that interferes with polyamine function.

The possibility of applying such a protocol to selectively kill tumor cells in vivo, while protecting normal tissues from the cytotoxic effects of this class of antitumor drugs, would clearly represent a fundamental advance in cancer chemotherapy. Consequently, we are currently studying the feasibility of using agents that affect polyamine function in order to determine whether the foregoing principle we have described is applicable in an in vivo setting.

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