Selective Toxicity Induced by Picolinic Acid in Simian Virus 40-transformed Cells in Tissue Culture

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SUMMARY

When cultured normal and SV40-transformed normal rat kidney and BALB/3T3 cells were exposed to picolinic acid, cell proliferation ceased. Most of the normal cells remained in a quiescent G1 (G0) state and viable for prolonged periods of time. In contrast, SV40-transformed cells progressed to the S and G2 phases of the cell cycle and remained viable only up to 90 to 120 hr. Then, most of the cells began to die. However, a very small fraction of the cell population (approximately 0.01%) developed into variants resistant to picolinic acid. Prevention of development of variants, and therefore destruction of all transformed cells, was obtained by addition of glycerol to picolinic acid-treated cells. Untransformed cells were unaffected by the same treatment. These results suggest that differential tumor toxicity should be feasible.

INTRODUCTION

We have reported previously that short-term treatment with picolinic acid arrests normal cells in G1 (G0); however, the block in transformed cells was dependent upon the transforming virus and independent of species or origin of the cell line (8). Thus, the selective growth arrest induced by picolinic acid revealed a basic difference in growth control mechanism(s) between normal and transformed cells (8). Normal cells treated with picolinic acid showed no toxic effects (8). However, cytotoxicity was observed in all transformed cells whether they were blocked in G1, in G2, or at random (8).

The purpose of this investigation was to study the effects of prolonged incubation with picolinic acid on the growth of normal and SV40-transformed NRK and BALB/3T3 cells in tissue culture. Experiments presented demonstrate that selective destruction of SV40-transformed cells can be accomplished by pretreatment with picolinic acid followed by treatment with picolinic acid and glycerol. A preliminary report of this work has been presented (7).

MATERIALS AND METHODS

Materials. Nicotinamide, nicotinic acid, α-picolinic acid, EDTA, and diethylenetriaminepentaacetic acid were obtained from Sigma Chemical Co., St. Louis, Mo.; picolinamide was from Matheson, Coleman and Bell, Norwood, Ohio. Isonicotinamide, isonicotinic acid, quinaclic acid, 1,10-phenanthroline, and salicylhydroxamic acid were from Aldrich Chemical Co., Milwaukee, Wis. Glycerol was obtained from Fisher Scientific Co., Pittsburgh, Pa.

Cell Culture. Cells were grown in Dulbecco-Vogt modified Eagle's medium containing 10% (v/v) calf serum (Colorado Serum Co.) as previously described (8). NRK2 cells and BALB/3T3 clone C/3 cells were obtained from E. Scolnick (NCI). NRK clone 5W isolated by D. Wallach (NCI) was used in this study. SV40-transformed NRK (SV40-NRK) were obtained from R. Ting (Biotech. Research, Inc.). SV-NRK P3C1T7 was used in this study. SV40-transformed BALB/3T3 (SVT2) cells were obtained from G. Todaro (NCI).

In all experiments the cells were grown in 20-cm tissue culture dishes (Costar). Unless otherwise indicated, all cell types were plated at a density of 2.5 x 10^5 cells/dish. After 24 hr in normal medium, new media with (treated) or without (control) 3 mM picolinic acid were added. After 48 hr exposure to picolinic acid, media were replaced by new media with and without the experimental substances. Unless otherwise indicated, the medium was changed in treated and control cultures every 48 hr.

Viability. To evaluate cell survival after treatment with combination of substances, we measured the ability of the cells to form colonies. The colony-forming ability of SV40-NRK cells exposed to 10 to 100 mM glycerol and 3 mM picolinic acid was evaluated as described by Rozengurt and Chin Po (17).

Cytofluorographic Analysis. FMF was used to monitor the effects of picolinic acid on cell DNA content (8, 10). The frequency distribution of fluorescent emission per cell (proportional to DNA content) was measured in 10^5 cells with a cytofluorograph 4800A (Bio/Physics Systems, Inc., Baldwin Place, N. Y.) and analyzed with a Model 2102 multichannel analyzer (Bio/Physics Systems, Inc.).

Other Procedures. Cells were counted with a Coulter counter.

RESULTS

Effect of Picolinic Acid on Growth of NRK and SV40-NRK Cells. It has been previously shown that picolinic acid reversibly inhibits the growth of cultured cells in a dose-dependent manner (8). Chart 1 shows the growth of NRK

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1 A portion of this study was supported by the Veterans Administration, MRRS 657/2620-01.

2 Supported during part of this work by an Individual National Research Service Award 1F32 CA05166-01 from the National Cancer Institute. To whom requests for reprints should be addressed, at Nuclear Medicine Laboratory (315JC), Veterans Administration Hospital, Saint Louis University, St. Louis, Mo. 63125.

Received April 22, 1977; accepted August 25, 1977.

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cells exposed to 3 mM picolinic acid. After 36 to 48 hr in picolinic acid, no mitotic cells (Fig. 1A) or further increase in cell number could be observed. FMF analysis after 48 hr of exposure to picolinic acid showed approximately 90% of the cell population in G1 (Chart 2B). Chart 2C shows the FMF profile of NRK cells treated with 3 mM picolinic acid for 6 days. The results show that approximately 95% of the cells are arrested in G1. Furthermore, when picolinic acid was removed, the cells remained spread on the substratum in normal manner without any visible mitosis for 1 additional week. They remained dormant during this period without loss of viability. Thus, NRK cells resisted the cytotoxic action of picolinic acid for prolonged periods of time.

Picolinic acid also inhibited SV40-NRK cell growth (Chart 1), and there was no further increase in cell number after 40 hr. FMF analysis showed that SV40-NRK cells were predominantly in S and G2 phases of the cell cycle after 48 hr of treatment (Chart 3). They were apparently progressing to a G2 block in a dose-dependent manner (Chart 3). At this time, no mitotic cells could be observed (Fig. 1D); when picolinic acid was removed, this inhibition was reversible within 24 hr (data not shown). The effects of prolonged incubation of SV40-NRK cells in medium containing 3 mM picolinic acid were tested. In contrast to the results with NRK cells, SV40-NRK cells began to die after 48 to 96 hr (Chart 1). Most of the cells became granular and rounded and floated off the substratum after 3 to 5 days. These floating cells were not viable. They did not adhere to the substratum and disintegrated after 2 to 3 days when resuspended in fresh medium without picolinic acid. Interestingly, a very small number of SV40-NRK cells (approximately 0.01%) remained elongated and attached to the substratum. These remaining cells grew into populations of variant cells resistant to picolinic acid.

Effect of Picolinic Acid on Growth of BALB/3T3 and SV40-transformed BALB 3T3 (SVT2) Cells. Untransformed BALB/3T3 cells treated with picolinic acid were predominantly arrested in G1, while SV40-transformed BALB/3T3 (SVT2) progressed through the cell cycle into S and G2 (8). With longer exposure to picolinic acid, different responses of normal and transformed BALB/3T3 cells were observed. Untransformed BALB/3T3 cells resisted the action of picolinic acid in a similar manner as NRK cells and remained in a G1 (G0) state for 2 weeks. In contrast, SVT2 cells began to die after 48 to 72 hr of treatment with picolinic acid. Then, in a similar fashion as with SV40-NRK cells, a population of cells resistant to 3 mM picolinic acid could be derived. Further details of the isolation procedure will be published elsewhere.

Survival of Untransformed NRK, BALB/3T3 and Transformed SV40-NRK and SVT2 Cells under Different Experimental Conditions Nonpermissive for Growth. The development of variants resistant to picolinic acid would prevent effective and selective cytotoxicity for SV40-NRK and SVT2 cells by picolinic acid. Therefore, in order to prevent such a possibility, it would be necessary rapidly and effectively to destroy all SV40-NRK and SVT2 cells. To accomplish the selective destruction of transformed cells with protection of normal cells, we utilized the observation that transformed cells treated with picolinic acid were more susceptible to a 2nd chemical agent than were untreated cells or their normal counterparts (7).

NRK cells synchronized by picolinic acid were unaffected by media changes or addition of new media containing 10 mM glycerol and 3 mM picolinic acid (Chart 1; and Fig. 1B). The cell number remained constant, and no mitotic activity...
was observed for 48 to 72 hr. Furthermore, after 72 hr the agents were removed and the cells doubled in number within 24 hr (data not shown). Thus, the effects of picolinic acid were reversible and were not affected by the presence of glycerol.

In contrast to NRK, SV40-NRK cells treated with picolinic acid died within 24 to 48 hr after addition of new media containing 3 mM picolinic acid and 10 mM glycerol (Chart 1; Fig. 1e). Glycerol was equally effective at 10 to 100 mM. About 35% of the SV40-NRK cells pretreated with picolinic acid were destroyed within 12 hr by addition of new media containing 10 mM glycerol (Chart 1). With an additional 12 hr of exposure to glycerol alone, the cell number remained constant (Chart 1), and most of these cells survived. Thus, these results showed that no cells survived the effects of picolinic acid plus glycerol and that these effects were probably rapid since no colonies developed from cells treated for only 12 hr with both agents. The experiments depicted in Chart 1 and Fig. 1 have been repeated 5 times with similar results.

Similarly, when SVT2 cells were exposed to picolinic acid and glycerol, the cells were destroyed; untransformed BALB/3T3 cells were unaffected by the same treatment (data not shown).

It has been shown previously that the growth inhibition was specific for picolinic acid (8). After 48 hr of pretreatment with picolinic acid, we tested other chelating agents and analogs of picolinic acid to determine specificity. Nicotinic acid, nicotinamide, isonicotinic acid, isonicotinamide, and picolinamide at 1 to 3 mM did not have significant effects on both NRK and SV40-NRK cells; EDTA, diethylenetriaminepentaacetic acid, quinaldic acid, and 1,10-phenanthroline at 1 to 2 mM caused extensive cell death in both cell lines. Therefore, all of these agents either were toxic to or did not have any effect on both NRK and SV40-NRK cells. However, we found that the iron chelator (1) salicylhydroxamic acid (1 mM) alone and, more effectively, in combination with glycerol (10 to 100 mM) rapidly (within 8 hr) killed all SV40-NRK cells pretreated with picolinic acid for 48 hr. The same treatment, however, was cytotoxic for 20% of the NRK cell population at 24 hr. Nevertheless, the remaining 80% of the NRK cells survived the treatment, although the reversibility was slow and the cells divided asynchronously.

**DISCUSSION**

The results presented here clearly show a differential cytotoxicity between normal and transformed cells exposed to picolinic acid for a prolonged period of time. Additionally, only the SV40-transformed cells pretreated with picolinic acid became sensitive to glycerol (Chart 1; Fig. 1). This second agent, therefore, prevented the emergence of variants resistant to picolinic acid. These findings may reflect a general inability of SV40-transformed cells to accumulate in a quiescent G1 (G0) state after treatment with picolinic acid (8).

Our present results are consistent with previous studies that showed that under conditions nonpermissive for growth, normal cells remain viable whereas transformed cells die (3, 14, 16–18). It has been reported that, when cell growth is inhibited by nutritional deprivation, transformed cells die whereas untransformed cells survive and proliferate if appropriate nutrient conditions are restored. Thus, according to the report of Pardee (14), suboptimal nutritional conditions induce normal cells to stop in G0 and survive, whereas transformed cells die. Certain growth conditions, which arrest normal cells in G1, induce growth arrest and cell death in virally transformed cells (3). Differences in survival between 3T3 cells and SV3T3 cells under nutritional deprivation have also been observed (16). Similarly, Schiaffonati and Baserga (18), using human diploid fibroblasts (WI-38) and their SV40-transformed counterparts, found that under conditions nonpermissive for growth normal cells remained attached to the surface whereas transformed cells died.

In the present studies, cell death in SV40-transformed cells was associated with progression of these cells to the S and G2 phases of the cell cycle (Chart 3). This suggests the possibility of a relationship between cytotoxicity and position of the cell in the cycle (9, 12). However, cell death was observed in all transformed cell lines whether they were blocked in G1, in G2, or at random (8). For example, Kirsten sarcoma virus-transformed NRK cells showed cytotoxicity during prolonged incubation with picolinic acid, but they were blocked in G2. In this case it is not known whether the G2 arrest induced by picolinic acid is at the same point as are normal cells. In the present model, we have shown that the cytotoxic actions of picolinic acid are potentiated by a second agent (e.g., glycerol) in cells apparently progressing to a G2 block. The relative sensitivities of cells progressing to G2 versus G1-arrested cells to a second agent are presently being investigated.

The possible mechanism(s) of action of picolinic acid, which may involve interference with NAD+ metabolism, cyclic 3':5'-AMP or ions, have been discussed in Ref. 8. It has been shown recently that picolinic acid appears to induce the growth inhibition in NRK cells by specifically withholding iron from the cells (5–7). Therefore, the differential effects of picolinic acid on normal and transformed cells may be due to different iron or other trace metal ion requirements by normal and transformed cells (5–7). Supporting this concept is the demonstration that picolinic acid interacts with transferrin and that salicylhydroxamic acid, an iron-chelating agent, partially mimicked picolinic acid effects in the present studies. Salicylhydroxamic acid and glycerol have been used recently as chemotherapeutic agents for trypanosomiasis (4). Both aerobic and anaerobic pathways of ATP production were blocked by simultaneous administration of glycerol and salicylhydroxamic acid, and the parasite was rapidly destroyed. Therefore, the possibility should be considered that picolinic acid, salicylhydroxamic acid, and glycerol might act in the cells by a similar mechanism as in trypanosomes.

The findings presented here, if applicable to neoplastic cells in vivo, could have important practical implications since they indicate a new experimental approach to cancer therapy. We have shown selective destruction of transformed cells with concomitant protection of normal cells. These results, considered in conjunction with published reports of differential protection (15, 17), suggest that a
similar degree of selective cytotoxicity might be achieved in vivo in some type of malignant cells by using picolinic acid, its analogues (2,13,11), or iron chelators plus a second chemical agent.

REFERENCES


![Fig. 1. Phase microscopy of NRK (A) and SV40-NRK (D) cells treated with 3 mM picolinic acid for 48 hr. Then, media were changed with new media containing 10 mM glycerol and 3 mM picolinic acid; this treatment destroys most of SV40-NRK cells (E) without appreciable effects on NRK cells (B) by 24 hr. At that time, media were changed by new media without the experimental substances. Within 24 hr of media change, mitotic figures were observed in NRK cells (C), while only debris of SV40-NRK cells (F) could be observed (for details, see text). x 125.](image-url)
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