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Effects of Arsenic Compounds on Proliferation and Nitric Oxide Synthesis in C3H 10T 1/2 Murine Fibroblasts

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Exposure to arsenic, either through chronic consumption of contaminated water or inhalation, is associated with increased risk of cancer, yet the mechanism by which arsenicals promote neoplastic change remains undefined. The carcinogenic process involves the formation of heritable genetic changes in the DNA of normal cells and this process may be enhanced by environmental agents that increase cellular proliferation, increase DNA damage and decrease the ability to repair damage or cause immunosuppression. We describe the inhibition of cellular proliferation of C3H 10T1/2 murine fibroblasts in the presence of 1.0 μM arsenate or arsenite; yet cacodylic acid had no significant effect on cell growth in culture at this concentration. Both arsenate and cacodylate, at micromolar concentrations, slightly stimulated cell growth and cell density when cells were treated with interferon-γ/lipopolysaccharide (IFN-γ/LPS). At 1 μM, arsenate and cacodylate also slightly increased IFN-γ/LPS-induced nitric oxide (NO) synthesis in this cell line, consistent with the increase in cell number observed, whereas 1 μM arsenite significantly increased NO production on a per-cell basis. In contrast, arsenite significantly inhibited NO synthesis at concentrations above 10 μM arsenite as, to a lesser extent, did arsenate and cacodylate. These results suggest that ingestion of arsenicals could alter cellular generation of NO and interfere with its associated physiological functions. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Exposure to inorganic arsenic has been implicated in the etiology of various forms of human cancer, including that in lung, skin, bladder, liver and other tissue sites (reviewed in Refs 1–3), as well as in the etiology of Blackfoot Disease, a cardiovascular disease characterized by inadequate peripheral circulation.[4] More recently Yamamoto *et al*. demonstrated the potential co-carcinogenicity of an organic arsenic compound, cacodylic acid, in rats treated with mutagenic chemicals.[5] Although chromosomal damage and mutations can occur in cells treated with arsenicals, the direct action of arsenic on DNA to cause a mutation has not been proven.[2] As a consequence, much interest is focused on elucidating the mechanism(s) by which arsenic exerts its apparent carcinogenic effects.

The process of carcinogenesis is characterized by the sequential modification of various key cellular genes through mutation, ultimately leading to proliferative disregulation.[6] The creation of a heritable mutation in a gene can be influenced by a number of cellular and extracellular events, including cellular proliferation,

amounts and types of mutagenic species and rates of DNA repair. Although many carcinogens exist in the environment, of both natural and synthetic origin,[7] their mechanisms of action vary. Some agents such as radiation, light or alkylating agents can interact directly with DNA to create a DNA lesion, which, if not repaired prior to DNA replication in a dividing cell, will cause the formation of a mutated gene in the daughter cell. It is for this reason that agents which increase proliferation rate may also be carcinogenic, by decreasing the opportunity for repair and increasing the likelihood of permanently incorporating the mutation in the genome.[8] An example of this synergistic action between cellular proliferation and exposure to a mutagenic agent is seen in the mutagenicity of nitric oxide (NO) in the Ames assay, in which exposure to NO must occur during active bacterial replication if increased mutation frequencies are to be observed.[9] As a consequence, in many cases neither DNA damage nor proliferation independently is sufficient to cause a permanent mutation leading to a neoplastic cell; however, the combination may be particularly effective.

The endogenous production of potentially mutagenic species, such as nitrogen oxides, hydroxyl radical and superoxide anion, through normal cellular processes has dramatically changed the way in which we think about the carcinogenic process. It is no longer necessary to invoke exposure to environmental mutagens to explain the etiology of many aging-related cancers, as the potential for mutation exists within the cellular machinery itself. It has been estimated that a normal human cell accumulates 10^4 DNA hits per day per cell from endogenous oxidative radicals, while a rat experiences 10^5 damaged DNA bases per cell per day, as a result of higher metabolic rates.[10] As a consequence, carcinogenic agents either may act by stimulating proliferation in the face of constant endogenous radical production and/or may act to increase the production of endogenous oxidants. Consistent with this theory of carcinogenesis, associations are observed for infection/inflammation with increased cancer incidence, and there is a strong association between agents that increase proliferation and cancer.[11, 12]

The enzymic production from arginine of NO, a free-radical gas, as a physiological messenger and as a defensive agent against invading pathogens has been implicated in the pathogenesis of neoplasia.[13–15] NO, in the presence of oxygen or oxygen metabolites, can form more reactive species such as NO_2, peroxynitrite and peroxynitrite radical which cause DNA damage through various mechanisms, including deamination, single strand breaks and the formation of oxidized DNA bases.[14] Consequently agents that influence cellular formation of NO could affect the level of mutation and/or various physiological functions mediated by NO. On the other hand NO, produced in response to tumor necrosis factor, has also been shown to be instrumental in destroying tumor cells, as part of the body's normal immune surveillance system.[16, 17] Consequently, the endogenous production of NO may have both positive and negative influences on tumor development, depending on the tissue and stage in the carcinogenic process.

Although NO is potentially mutagenic under certain circumstances, it also has significant antioxidant properties[18, 19] and its production *in vitro* may be protective against DNA damage by other radical species. Although inhibition of carcinogenesis is observed when 10T1/2 cells are treated with NO synthase inhibitors,[15] recent work in our laboratory with antisense deoxyoligonucleotides specifically targeting the inducible NO synthase enzyme mRNA has shown that when NO synthase is selectively inhibited, neoplastic transformation is enhanced.[20] *Lubec et al*.[21] also recently reported that low-dose arginine supplementation decreases tumor incidence and increases survival in mice, and that low-dose arginine stimulates the immune system and blocks lipid peroxidation, effects they attribute to the enhanced production of NO. Consequently NO may serve to reduce the damaging effects of other cellular oxidants, outweighing the negative effects of NO-mediated mutation.

Because of the potential importance of NO in carcinogenesis, we sought to determine the effects of arsenicals (arsenite, arsenate and cacodylate) on both the endogenous production of NO and proliferation of C3H 10T1/2 murine fibroblasts. The murine cell line is well characterized, synthesizes NO in response to IFN-γ/LPS treatment and is widely used in a standard assay for neoplastic transformation; in it, effects on neoplastic transformation by NO synthase inhibitors have been demonstrated.[15, 20] It therefore offers an ideal model for investigating the biochemical and cellular effects of arsenic related to endogenous NO production and carcinogenesis.

MATERIALS AND METHODS

Chemicals

Lipopolysaccharide (LPS, *Escherichia coli*, serotype 0127:B8), aminoguanidine, sodium arsenate and cacodylic acid were obtained from Sigma Chemical Co., St Louis, MO, USA. Sodium arsenite was from J. T. Baker Chemical Co., Phillipsburg, NJ, USA. Mouse interferon-γ (IFN-γ) was from Gibco/BRL, Gaithersburg, MD, USA.

Cell culture

C3H 10T1/2 murine fibroblasts (ATCC No. CCL226), between passages 9 and 13, were used for all experiments. Cells were initially seeded at a density of 20 000 cells per 35 mm culture dish (5000 per dish for growth-curve experiments) and allowed to grow to confluence in Eagle's basal medium supplemented with 5% bovine calf serum (Hyclone Laboratories, Inc.) and gentamicin sulfate (25 μg ml^{-1}). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Unless otherwise noted, treatments were carried out on confluent monolayers of cells. Cell number was determined by trypsinizing cells for 20 s (4 mg trypsin in 1 ml PBS plus 0·2 g l^{-1} EDTA), washing with phosphate-buffered saline (PBS) and counting a dilution of the cell suspension with a Coulter counter.

Nitrite analysis

Nitrite concentration in cell culture medium was determined by adding 40 μl of medium to 960 μl of absorbing reagent (0.5% sulfanilic acid, 0.002% *N*-(1-naphthyl)ethylenediamine dihydrochloride, 14% glacial acetic acid) and incubated at room temperature for 15 min. Absorbance of the solution at 553 nm was measured on a Shimadzu UV160 spectrophotometer and compared against standard solutions of aqueous sodium nitrite.

RESULTS

The effect of various arsenicals on the log-phase growth of C3H 10T1/2 cells is shown in Fig. 1. Cells were plated at a density of approximately 5000 per 35 mm dish and counted daily for one week, at which time control cells were nearly confluent. At a concentration of 1.0 μM arsenite, the rate of cell growth was significantly inhibited (80% inhibition relative to control cultures). Arsenate (1.0 μM) reduced the rate of growth by about one-third, while the same concentration of cacodylate had no significant effect on log-phase growth of this cell line. Previously Gonsebatt *et al.*[22] reported that 100 nM arsenite or arsenate inhibited lymphocyte proliferation rates by approximately 50%; however, both species of arsenic were reported to be equipotent in their effectiveness. In contrast we observed a significantly greater effect for arsenite in C3H 10T1/2 cells, consistent with its reported higher toxicity for other cell lines.[23]

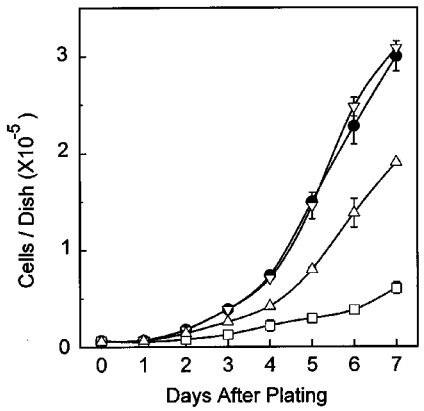

Figure 1 Effect of arsenicals on the log-phase growth of C3H 10T1/2 murine fibroblasts. Approximately 5000 C3H 10T1/2 cells were plated on P-35 Falcon tissue culture dishes. Twenty-four hours later (Day 0) cultures were treated with either sodium arsenite (□), sodium arsenate (△), cacodylate (▽) or control buffer solution (●). The final concentration of arsenical in the cell culture medium was 1.0 μM and each was administered in 25 μl of PBS (pH 7.4). Values reported represent the means ± SEM for four determinations of cell number using a Coulter counter as described in the Materials and methods section.

Treatment of confluent C3H 10T1/2 cells with IFN-γ/LPS causes the induction of the inducible form of the NO synthase enzyme,[15] resulting in the cellular generation of NO which can be measured in the cell culture medium as nitrite, as described in the Materials and methods section, above. IFN-γ/LPS treatment also causes slightly increased saturation density of cells in the culture dish (approximately 50% more cells), as long as the level of NO produced is not excessive. Addition of an inhibitor of NO synthase, aminoguanidine, blocked NO synthesis and slightly increased the saturation density of cells observed

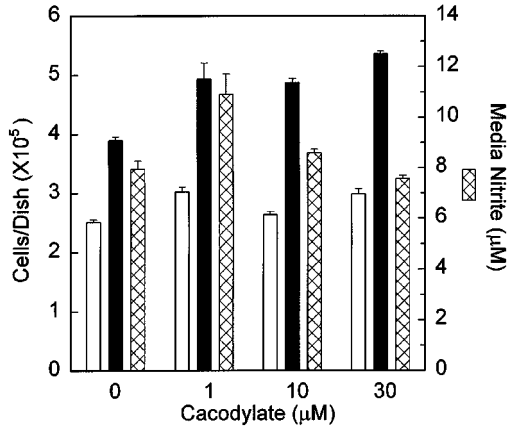

Figure 2 Effect of cacodylic acid on cell number and NO production in IFN-γ/LPS-stimulated C3H 10T1/2 cells. C3H 10T1/2 cells were grown to confluence on 35 mm Falcon dishes. At the time of media change, cells were treated with 25 μl cacodylic acid in PBS to yield the indicated concentration. After seven days cells were counted for control (□) and IFN-γ/LPS-treated (■) dishes and nitrite was measured in the medium of all IFN-γ/LPS-treated cells (▩) as described in the Materials and methods section. Values represent the means of triplicate dishes ±SEM.

(data not shown). This effect of IFN-γ/LPS on cell saturation density was potentiated by all concentrations of arsenate and cacodylate tested

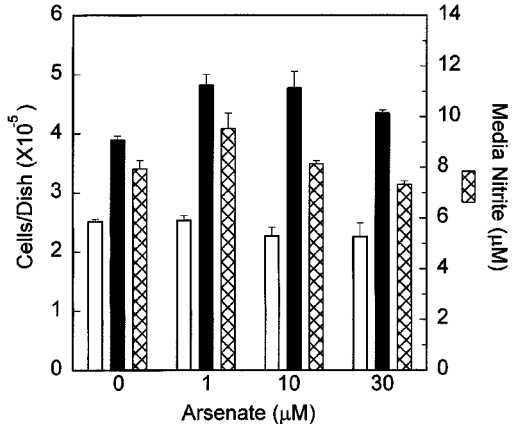

Figure 3 Effect of arsenate on cell number and NO production in IFN-γ/LPS-stimulated C3H10T1/2 cells. C3H 10T1/2 cells were grown to confluence on 35 mm Falcon dishes. At the time of media change, cells were treated with 25 μl arsenate in PBS to yield the indicated concentration. After seven days cells were counted for control (□) and IFN-γ/LPS-treated (■) dishes and nitrite was measured in the medium of all IFN-γ/LPS-treated cells (▩) as described in the Materials and methods section. Values represent the means of triplicate dishes ± SEM.

Table 1 Effects of arsenicals on cellular nitrite generation

Arsenical concn	NO_2^--produced[a] (fmol/cell)		
(μM)	Cacodylate	Arsenate	Arsenite
0	40.9±1.9	40.9±1.9	40.9±1.9
1	45.2±6.0	39.7±2.9	77.4±6.2**
10	35.3±0.4*	33.7±2.5	7.5±1.5**
30	28.3±0.7*	33.8±0.9*	11±1.2**

* $P<0.05$ relative to control using Student's *t*-test.
** $P<0.01$ relative to control using Student's *t*-test.

(Figs 2 and 3), resulting in a doubling of the cell number relative to untreated controls. Arsenite showed no such potentiating effect on cell number; rather, in most cases it blocked the IFN-γ/LPS-induced increase in saturation density normally observed. Such an effect would be consistent with the strong inhibition of log-phase growth of these cells observed with arsenite treatment.

Nitrite levels in the medium were significantly higher for cultures treated with 1.0 μM cacodylate (37% increase; $P=0.001$) and arsenate (20% increase; $P=0.06$), as shown in Figs 2 and 3; however, when adjusted for the increased cell number caused by the combination of IFN-γ/LPS and arsenical treatment, no significant increase in NO production on a per-cell basis was observed (Table 1). Higher concentrations of arsenate and cacodylate decreased NO generation back to the level of the IFN-γ/LPS-treated control. On a per-cell basis, all arsenicals demonstrated a significant reduction in NO produced at the highest dose of arsenical used (Table 1). Arsenite significantly inhibited NO synthesis (>80% inhibition; $P=0.001$) in this cell line at concentrations of 10 μM or higher without apparent cellular toxicity (Fig. 4), while 1 μM arsenite significantly elevated NO production (Table 1).

DISCUSSION

Nitric oxide is reported to be essential for the action of tumor necrosis factor[16, 17] in the elimination of tumor cells *in vivo* and it is also important in fighting infection associated with a variety of pathogenic organisms.[24, 25] Consequently, agents which suppress the normal expression of NO synthase and subsequent

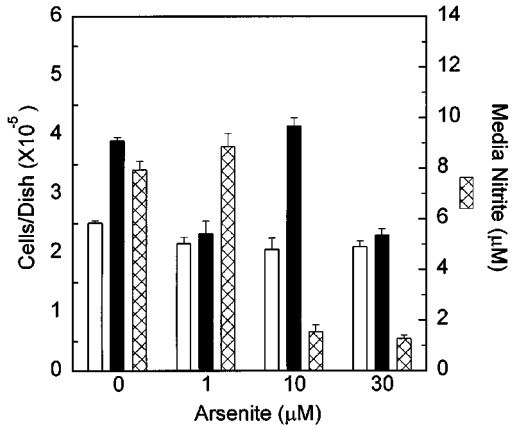

Figure 4 Effect of arsenite on cell number and NO production in IFN-γ/LPS-stimulated C3H 10T1/2 cells. C3H 10T1/2 cells were grown to confluence on 35 mm Falcon dishes. At the time of media change, cells were treated with 25 μl arsenite in PBS to yield the indicated concentration. After seven days cells were counted for control (□) and IFN-γ/LPS-treated (■) dishes and nitrite was measured in the medium of all IFN-γ/LPS-treated cells (▩) as described in the Materials and methods section. Values represent the means of triplicate dishes ± SEM.

production of NO could adversely affect the body's ability to fight not only infectious diseases, but also developing tumors. Because carcinogenesis is a normal manifestation of the aging process, exposure to external mutagens is presumably not essential to the initiation of a tumor (although obviously carcinogen exposure can accelerate the process). However, exposure to chemicals which alter the normal production of endogenous free radicals could affect the incidence and progression of tumors. The decrease in proliferation rate observed in our study, as well as by others,[22] would suggest that increased proliferation rate is not the mechanism by which these compounds act either. It is possible, however, that—at higher toxic levels of arsenic—cell death could result, causing enhanced proliferation *in vivo*, as new cells are required to replace those killed by exposure to arsenic. Also, other cell types may be more sensitive to the toxic effects of arsenicals.

The biphasic effects of arsenicals on cellular NO production observed in this study suggest that, depending on the level of arsenical exposure, NO synthesis could be either enhanced or reduced. As discussed above, cellular NO may contribute to cellular mutation, or be protective, depending upon the level of other oxidative species present within cells. If arsenicals serve to reduce the overall level of oxidative free radicals, this may reduce the number of potentially initiating mutations leading to a tumor cell, yet it may hamper the ability of the organism to destroy developing tumors. We have also observed biphasic effects on NO generation in cells treated with the known cancer-preventive agent tamoxifen, of which low doses inhibit NO synthesis and higher doses, which inhibit neoplastic transformation, also increase NO generation.[26]

Our demonstration that arsenicals, particularly arsenite, can interfere both positively and negatively with the production of an important cellular free radical such as NO, suggests that interference with the normal physiological functions of NO could explain some of the carcinogenic activity of inorganic and organic arsenicals. Immunosuppression, induced by arsenic, could impair the ability of the organism to destroy aberrant cells, circumventing normal defense processes and allowing progression of pre-neoplastic cells to develop into a more life-threatening tumor. Indeed, the significant inhibition of NO synthesis caused by arsenite could potentially impair both the tumor-fighting and anti-infection apparatus of cells. We have observed previously that cells treated with inhibitors of NO synthase are more likely to become infected growing in tissue culture, while it is reported that mice lacking the gene coding for the inducible form of NO synthase are also more susceptible to infection.[27] Previously it was shown that arsenic impairs antibacterial defenses in mice.[28] Future studies addressing the effects of arsenicals on normal cellular processes, as well as the progression and metastasis of tumor cells in animal models, are needed to answer these questions. It is also possible that reduced cellular NO may reduce the antioxidant capacity of cells, particularly if arsenicals do not simultaneously cause a reduction in the level of oxygen radicals generated by cells.

The increase in saturation density observed when cells are treated with IFN-γ/LPS and either arsenate or cacodylate is interesting; however, the significance and meaning are not clear. Some chemopreventive agents, such as the retinoids, cause decreased saturation density and this property is thought to be associated with their anticarcinogenic properties.[29] Others, such as the tocopherols, appear to increase saturation density and are also effective inhibitors of neoplastic transformation.[30] The mechanism by which these

various agents, including the arsenicals, cause changes in confluent cell saturation density is unknown.

Finally, the effect of arsenicals on the endothelial NO synthase enzyme needs to be explored. NO is produced by endothelial cells as an intercellular messenger, which causes relaxation in smooth-muscle cells surrounding the endothelium of arteries through the activation of guanylate cyclase by NO, leading to vasodilation and lowered blood pressure.[31] Decreased NO synthesis by endothelial cells in arteries caused by arsenicals could lead to vasoconstriction and reduced peripheral circulation, either causing or exacerbating the cardiovascular effects of Blackfoot Disease associated with arsenic exposure. Ultimately, through an understanding of the precise biochemical effects of arsenic, we will be better able to assess the risks associated with its environmental exposure.

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