Beaucage, M. H. Caruthers, *J. Am. Chem. Soc.* **108**, 2040 (1986); R. T. Pon, M. J. Damha, K. K. Ogilvie, *Tetrahedron Lett.* **26**, 2525 (1985); X. Gau, B. L. Gaffney, M. Senior, R. R. Riddle, R. A. Jones, *Nucleic Acids Res.* **13**, 573 (1984).
17. M. Yarus, *Arch. Biochem. Biophys.* **174**, 350 (1976); G. J. Quigley, M. Teeter, A. Rich, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 64 (1978).
18. M. Steinmetz-Kayne, R. Benigno, N. R. Kallenbach, *Biochemistry* **16**, 2064 (1977).
19. M. Bina-Stein and D. M. Crothers, *ibid.* **14**, 4185 (1975).
20. S. S. M. Lam and P. R. Shimmel, *ibid.*, p. 2775.
21. P. R. Schimmel and D. Soll, *Annu. Rev. Biochem.* **48**, 601 (1979).
22. P. Schimmel, *ibid.* **56**, 125 (1987); B. Roe, M. Sirover, B. Dudock, *Biochemistry* **12**, 4146 (1973).
23. M. Sundaralingman and S. T. Rao, *Int. J. Quantum Chem.* **10**, 301 (1983); R. E. Dickerson *et al.*, *Science* **216**, 475 (1982).
24. S. H. Kim *et al.*, *Science* **185**, 435 (1974); A. Jack, J. E. Ladner, A. Klug, *J. Mol. Biol.* **108**, 619 (1976); N. H. Woo *et al.*, *Nature* **286**, 364 (1980).
25. D. A. Zarling, C. J. Calhoun, C. C. Hardin, A. H. Zarling, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6117 (1987).
26. E. Goldman, W. M. Holmes, G. W. Hatfield, *J. Mol. Biol.* **129**, 567 (1979).
27. B. A. Roe, *Nucleic Acids Res.* **3**, 21 (1975).
28. G. L. Igloi, F. von der Haar, F. Cramer, *Biochemistry* **16**, 1696 (1977).
29. We thank M. Lyttle, Biosearch Division of New Brunswick Scientific Co. for the 5′ DMT-riboA^{bz}-3′-CPG columns used to synthesize the tDNA^{Phe} containing a 3′ riboadenosine. This work was supported by NIH grant GM-30400 and a grant from the Graduate College of the University of Oklahoma.

7 January 1988; accepted 26 April 1988

Induction of Gene Amplification by Arsenic

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Arsenic is a well-established carcinogen in humans, but there is little evidence for its carcinogenicity in animals and it is inactive as an initiator or tumor promoter in two-stage models of carcinogenicity in mice. Two arsenic salts (sodium arsenite and sodium arsenate) induced a high frequency of methotrexate-resistant 3T6 cells, which were shown to have amplified copies of the dihydrofolate reductase gene. The ability of arsenic to induce gene amplification may relate to its carcinogenic effects in humans since amplification of oncogenes is observed in many human tumors. The inability of arsenic to induce gene mutations may relate to the negative results of arsenic in long-term animal studies and suggests that these experiments may not detect some environmental agents that act late in the carcinogenic process in humans.

ARSENIC IS AN UNUSUAL ENVIRONMENTAL substance in terms of its carcinogenic response in humans and animal models. Exposure of humans to inorganic arsenic compounds in drugs, drinking water, and occupational environments is associated with increased risks of skin cancer, lung cancer, and possibly liver cancer (*1, 2*). However, little evidence exists for the carcinogenicity of arsenic to animals (*2–5*). Yet, sodium arsenite and sodium arsenate reproducibly induce morphological transformation of rodent cells in culture (*6, 7*). A distinction does exist, however, between arsenic and most chemicals that induce cell transformation in that arsenic is inactive in inducing gene mutations at specific genetic loci (*7*). An elucidation of the mechanism of arsenic-induced cellular changes may help in better understanding human carcinogenesis and the relationship between carcinogen-induced events in humans and in rodents. The use of long-term rodent carcinogenicity studies as assays for detecting potential human carcinogens requires an understanding of the apparent false negative results in the animal cancer studies.

In this report, we present evidence that sodium arsenite and sodium arsenate are potent enhancers of amplification of the dihydrofolate reductase (DHFR) gene in mouse 3T6 cells. Amplification of the DHFR gene was measured in mouse 3T6 cells by selecting cells that form colonies in the presence of methotrexate. Cells with an increased copy number of the DHFR gene have increased levels of DHFR enzyme and are resistant to methotrexate (MTX^R) (*8–10*). Treatment of mouse 3T6 cells with sodium arsenite or sodium arsenate induced dose-dependent increases in the number of MTX^R colonies (Fig. 1). Sodium arsenite was active at a lower concentration than sodium arsenate. This is the same relative potency of the two compounds in cell transformation assays (*7*), and the concentration ranges for induction of gene amplification and cell transformation are the same. Sodium arsenite has been noted to be more active than sodium arsenate in other biological assays, and it has been suggested that this is due to differences in the uptake of trivalent versus pentavalent arsenic (*11*). Sodium arsenite and sodium arsenate induced MTX^R colonies at MTX concentrations of 150 to 300 n*M* (Fig. 2). To test for a possible interaction between methotrexate and arsenic, cells were grown in lower doses of methotrexate and arsenic and no increased viability was observed.

The surviving MTX^R 3T6 colonies were isolated, grown in culture and shown to be resistant to MTX and to have amplified DHFR genes. The copy number of the DHFR gene was estimated by comparing the extent of hybridization by dot blot analysis of radiolabeled cloned DHFR cDNA or actin gene to DNA isolated from parental 3T6 cells and MTX^R clones (*8, 10*). Approximately 50% (9/17) of the MTX^R clones induced by arsenic (0.2 to 0.8 μg/ml) had amplified copy numbers of the DHFR gene ranging from 2- to 11-fold, which is consistent with the findings of others (*9, 10*).

Table 1. Effect of sodium arsenate and sodium arsenite on MTX^R colonies.

Treatment*	Relative survival (%)	MTX^R colonies/ 5×10^5 cells†	MTX^R colonies/ 5×10^5 surviving cells
None	100	0.7 ± 0.6	0.7
Sodium arsenite (μ*M*)			
0.2	100	2 ± 1.5	2
0.4	100	5 ± 2	5
0.8	115	7 ± 3	6
1.6	45	17 ± 3	38
3.1	15	33 ± 5	226
6.2	1	82 ± 14	8200
Sodium arsenate (μ*M*)			
1	100	2 ± 1	2
2	100	4 ± 1	4
4	100	13 ± 6	13
8	40	23 ± 9	57
16	11	62 ± 13	569
32	1	161 ± 27	16000

*0.2 μ*M* sodium arsenate is equivalent to 0.025 μg/ml; 1 μ*M* sodium arsenate is equivalent to 0.313 μg/ml. †Colonies selected in 200 n*M* MTX for 21 days (±SD).

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The data in Figs. 1 and 2 are expressed as MTX^R colonies per cell treated and show that arsenic induces a large increase in the absolute number of MTX^R cells. The toxicity of arsenic to mammalian cells was measured by a reduction in the colony-forming efficiency of the treated cells (Table 1). Arsenic induced MTX^R cells at doses that were nontoxic as well as at toxic doses. When the number of MTX^R cells per surviving cell was calculated, the frequency of MTX^R cells was observed to be $>10^{-2}$ after treatment with the highest doses of sodium arsenite or sodium arsenate.

To determine whether the ability to induce gene amplification was a general property of carcinogenic metals, nickel chloride and nickel sulfate were tested over the concentration ranges (1 to 20 μg/ml) that induce cell transformation of Syrian hamster embryo cells (*12*). Neither increased the number of MTX^R colonies at any of the doses tested in several assays.

The results of this study clearly indicate that arsenic is an effective enhancer of DHFR gene amplification. The mechanism by which arsenic induces gene amplification remains unknown. Mutagens and carcinogens, such as ultraviolet light and alkylating agents, have been shown to induce gene amplification in rodent cells (*10, 13, 14*). Because arsenic is not mutagenic at specific genetic loci (*7*), its ability to induce gene amplification is unlikely to be due to direct DNA base damage. Gene amplification has been hypothesized to arise by either a recombinational mechanism or by inhibition of DNA synthesis and overreplication (*14, 15*). Arsenic treatment of mammalian cells induces chromosome aberrations, endoreduplication and sister chromatid exchanges (*7*). Similarly, hydroxyurea increases chromosome aberrations and induces DHFR gene amplification in cells in culture (*16*). These effects may relate to the mechanism of induction of gene amplification by these chemicals.

Our findings suggest that the carcinogenicity of arsenic may be related to its ability to enhance the amplification of genes important in cancer development. Recent studies indicating that cellular oncogenes are amplified in human and animal tumors (*17–19*) support the hypothesis that this is an important step in neoplasia. Oncogene amplification in some cancers correlates with the degree of progression to malignancy (*20–22*). The importance of gene amplification in carcinogenesis is also supported by the finding that many tumors have karyological indications of gene amplification, such as double minute chromosomes and homogeneous staining regions, which are cytological evidence of gene amplification (*18, 19*).

Arsenic is not an initiator or tumor promoter in two-stage models of animal carcinogenesis (*23, 24*). The difficulty in detecting arsenic carcinogenicity in animal models may be related to its ability to cause gene amplification but not gene mutations. An analysis based on the multistep model of carcinogenesis of humans occupationally exposed to arsenic has indicated that exposure to arsenic appears to act at a late stage in the carcinogenic process (*25*). The human data were postulated not to be consistent with the hypothesis that arsenic acts during the prmotion phase of the carcinogenic process because the epidemiological data do not show reversibility of the excess lung cancer mortality after exposure ceased (*25*). Amplification of an altered or activated oncogene may be a late stage in neoplastic progression, and induction of this process would increase the incidence of tumors in an exposed population. Thus, we propose that arsenic acts specifically in the progression phase of carcinogenicity.

This hypothesis would explain why arsenic is not an effective complete carcinogen, initiator, or tumor promoter. The endpoints of most models of tumor promotion are benign or preneoplastic lesions (*26–28*), and the progression of these lesions to the malignant state may represent a distinct phase in the carcinogenic process. O'Connell *et al.* (*26*) and Hennings *et al.* (*27*) have shown that certain chemicals can enhance cancer development when applied after initiator and tumor promoter treatments. No chemical has been shown to act specifically in this progression phase (as a "tumor progressor") (*28*). Since oncogene amplification has been shown in some tumors to correlate with the degree of progression of the cancers, the demonstration that arsenic induces gene amplification in cells in culture and acts in a late stage in human carcinogenesis supports the hypothesis that this human carcinogen acts in the progression phase. These findings suggest (*26, 28*) that new in vitro and in

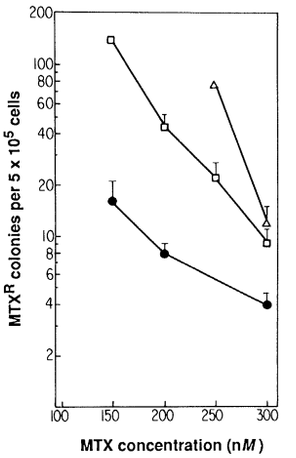

Fig. 2. Effect of varying MTX concentration in sodium arsenate– or sodium arsenite–induced MTX^R cells. Cells were treated as described in Fig. 1 except that the MTX concentration in the culture was varied. The cells were either untreated (●), exposed to 16 μM sodium arsenate (△), or exposed to 1.6 μM sodium arsenite (□).

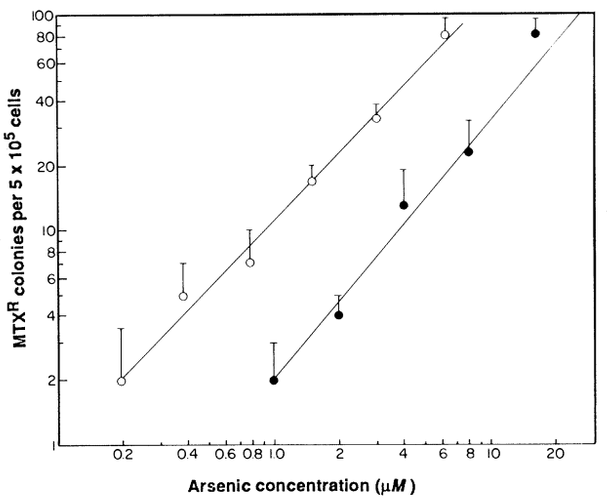

Fig. 1. Induction of MTX^R colonies of 3T6 cells by sodium arsenite (●) or sodium arsenate (●). Mouse 3T6 cells, obtained from the American Type Culture Collection (Rockville, Maryland), were grown in Dulbecco's modified Eagle's reinforced medium (Biolabs, Northbrook, Illinois) supplemented with 10% dialyzed calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Dialyzed serum was obtained from Gibco and additionally dialyzed at 4°C against 40 volumes of 0.15M NaCl, 1 mM Na_2HPO_4 (*p*H 7.0) for 3 days. All gene amplification assays were initiated with cells at near confluence and 24 hours after the last medium change. Cells were transferred by gentle trypsinization with 0.1% trypsin (1:250, Gibco) for 5 min at 37°C. Cells (5×10^5) were plated into 20 100-mm dishes in complete medium and treated either at the time of plating or 24 hours later with 250 mM MTX obtained from Sigma (St. Louis, Missouri) and sodium arsenite or sodium arsenate. Stock solutions of 1 mM MTX were prepared by dissolving the chemical in 1M $NaHCO_3$, diluted 1:10, and stored at 4°C for <2 weeks after filter sterilization. Sodium arsenite and sodium arsenate were dissolved in water, filter-sterilized, and diluted into complete medium immediately before use. The plates were incubated at 37°C for 21 days with no further change or with weekly refeedings of medium and then fixed with absolute methanol and stained with 10% aqueous Giemsa (Fisher). MTX^R colonies of cells were counted with a stereomicroscope. In the absence of arsenic, an average of 0.67 ± 0.58 MTX^R colonies per 5×10^5 cells were observed. All experiments were repeated at least three times.

vivo assays are required to detect chemical carcinogens which act specifically in late stages of carcinogenesis but are not tumor promoters or are weak tumor promoters.

REFERENCES AND NOTES

1. *IARC Monogr. Eval. Carcinog. Risk Chem. Hum.* **23**, 37 (1980).
2. *IARC Monogr. Eval. Carcinog. Risk Chem. Hum. Suppl.* **4**, 50 (1982).
3. N. Ishinishi, A. Yamamoto, A. Hisanaga, T. Inamasu, *Cancer Lett.* **21**, 141 (1983).
4. P. Rudnay and M. Borzsonyi, *Magyar Onkologia* **25**, 73 (1981).
5. G. Pershagen, G. Nordberg, N. Bjorklund, *Environ. Res.* **34**, 227 (1984).
6. J. A. DiPaolo and B. C. Casto, *Cancer Res.* **39**, 108 (1979).
7. T. C. Lee, M. Oshimura, J. C. Barrett, *Carcinogenesis* **6**, 1421 (1985).
8. A. Varshavsky, *Cell* **25**, 561 (1981).
9. P. C. Brown, T. D. Tlsty, R. T. Schimke, *Mol. Cell. Biol.* **3**, 1097 (1983).
10. T. D. Tlsty, P. C. Brown, R. T. Schimke, *ibid.* **4**, 1050 (1984).
11. S. A. Lerman, T. W. Clarkson, R. J. Gerson, *chem.-Biol. Interactions* **45**, 401 (1983).
12. Z. Qiao, and J. C. Barrett, *Toxic. in Vitro*, in press.
13. S. Lavi, *J. Cell Biochem.* **18**, 149 (1982).
14. R. T. Schimke, *Cell* **37**, 705 (1984).
15. A. Varshavsky, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3673 (1981).
16. A. B. Hill and R. T. Schimke, *Cancer Res.* **45**, 5050 (1985).
17. H. E. Varmus, *Annu. Rev. Genet.* **18**, 553 (1984).
18. G. Klein and E. Klein, *Nature* **315**, 190 (1985).
19. D. L. George, *Cancer Surveys* **3**, 497 (1984).
20. C. D. Little *et al.*, *Nature* **306**, 194 (1983).
21. M. Schwab *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4940 (1984).
22. G. M. Brodeur, R. C. Seeger, M. Schwab, H. E. Varmus, J. M. Bishop, *Science* **224**, 1121 (1984).
23. R. K. Boutwell, *J. Agric. Food Chem.* **11**, 381 (1963).
24. C. Baroni, G. J. van Esch, V. Saffiotti, *Arch. Environ. Health* **7**, 668 (1963).
25. C. C. Brown and K. C. Chu, *J. Natl. Cancer Inst.* **70**, 455 (1983).
26. J. F. O'Connell, A. J. P. Klein-Szanto, D. M. DiGiovanni, J. W. Fries, T. J. Slaga, *Cancer Res.* **46**, 2863 (1986).
27. H. Hennings *et al.*, *Nature* **304**, 67 (1983).
28. J. C. Barrett, *Carcinogenesis*, M. J. Mass *et al.*, Eds. (Raven, New York, 1985), vol. 8, p. 423.

22 April 1988; accepted 25 April 1988

Estrogen Binding, Receptor mRNA, and Biologic Response in Osteoblast-Like Osteosarcoma Cells

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High specific activity estradiol labeled with iodine-125 was used to detect approximately 200 saturable, high-affinity (dissociation constant ≅ 1.0 n*M*) nuclear binding sites in rat (ROS 17/2.8) and human (HOS TE85) clonal osteoblast-like osteosarcoma cells. Of the steroids tested, only testosterone exhibited significant cross-reactivity with estrogen binding. RNA blot analysis with a complementary DNA probe to the human estrogen receptor revealed putative receptor transcripts of 6 to 6.2 kilobases in both rat and human osteosarcoma cells. Type I procollagen and transforming growth factor–β messenger RNA levels were enhanced in cultured human osteoblast-like cells treated with 1 n*M* estradiol. Thus, estrogen can act directly on osteoblasts by a receptor-mediated mechanism and thereby modulate the extracellular matrix and other proteins involved in the maintenance of skeletal mineralization and remodeling.

OSTEOPOROSIS IS A BONE DISEASE characterized by a significant reduction in bone mass leading to increased susceptibility to fractures. The greatest loss is seen in trabecular bone, the cross-linking structure crucial in maintaining the integrity of the vertebral and hip bones. Bone mass is homeostatically maintained by a process described as coupling (*1*), which involves the interaction and activities of two bone cell types participating in formation and resorption, namely, osteoblasts and osteoclasts, respectively. The balance between bone formation and resorption is regulated by the action of hormones and growth factors on these cells. Approximately 25% of postmenopausal women suffer from an accelerated form of osteoporosis probably caused by a reduction in circulating estrogens. Estrogen is presumably necessary to maintain normal osteoblast function or to attenuate the activity of osteoclasts, or both (*2*). Administration of exogenous estrogens to postmenopausal women retards further reduction of bone mass, although it does not stimulate a net accumulation (*3*).

For a steroid hormone such as estrogen to have a direct effect on a cell, the presence of a high-affinity receptor is required. Steroid hormone–receptor complexes interact with specific genes and regulate their transcriptional activity (*4*). However, the presence of estrogen receptors (ERs) in bone or bone-related cells has not been demonstrated (*5*).

We have increased the sensitivity of the receptor assay by utilizing radiolabeled estradiol with a higher specific activity (*6*). Homogeneous populations of the well-characterized rat (ROS 17/2.8) and human (HOS TE85) osteosarcoma cell lines, both of which are phenotypically osteoblast-like, were used to reevaluate the existence of nuclear ERs in bone cells. A binding site that appears to saturate at an estradiol concentration of approximately 1.0 n*M* was identified in both cell lines (Fig. 1, A and B). This high-affinity binding site in the ROS (Fig. 1A, inset) and HOS (Fig. 1B, inset) cells is characterized by a dissociation constant (K_d) of 0.5 n*M* and 1.1 n*M*, respectively, consistent with the calculated value for the ER in typical estrogen target tissues, such as the uterus or human breast tumor cells (*7*). Unlike the uterus, which contains several thousand high-affinity ERs per cell, the osteosarcoma cells possess ~200 detectable high-affinity binding sites per nucleus (corresponding to approximately 10 fmol of receptor per milligram of protein). This value is not significantly altered when ERs are assayed under exchange conditions (1 hour at 37°C) and does not depend on estrogen treatment of the osteosarcoma cells. Our result suggests that the detection of ERs in bone cells was previously limited by less sensitive assay procedures (*5*). The higher capacity specific binding site seen in Fig. 1, A and B, at estradiol concentrations above 2.5 n*M* has been reported as a type II binding site in estrogen target tissues, although the function of this lower affinity site has not been clearly defined (*8*).

With the exception of testosterone, other steroid hormones such as dexamethasone (a glucocorticoid), progesterone, and dihydrotestosterone do not compete with labeled estradiol for binding to the receptor-like sites (Fig. 1, C and D). The synthetic estrogen, diethylstilbestrol, is the most effective competitor, which is consistent with the occurrence of a traditional ER in these osteoblast-like cells. Significant competition by testosterone could result from either the presence of a second sex-steroid binding protein or the existence of a unique ER in bone that also binds certain androgens (*9*). Alternatively, the presence of aromatase in the cell extracts could account for the testosterone competition by conversion of testosterone to estradiol.

Although the low ER number in bone cells precludes immunodetection, a human ER cDNA (*4, 10*) was used as an independent method for indirectly documenting the existence of the estrogen-binding protein.

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