Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2085–2087, April 1984
Cell Biology

Active oxygen acts as a promoter of transformation in mouse embryo C3H/10T½/C18 fibroblasts

(tumor promotion/oxygen radicals/radical scavengers)

ROBERT ZIMMERMAN AND PETER CERUTTI

Department of Carcinogenesis, Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges s/Lausanne, Switzerland

Communicated by Bruce N. Ames, December 12, 1983

ABSTRACT **There is much evidence from *in vivo* and *in vitro* carcinogenesis studies that active oxygen species play a role in tumor promotion. We tested directly whether superoxide produced extracellularly by xanthine-xanthine oxidase (X-XO) has the capacity to promote initiated mouse embryo C3H/10T½ fibroblasts. Cell cultures initiated with either ^{137}Cs γ-rays or benzo[*a*]pyrene diol epoxide I were found to transform 3–30 times more effectively when subsequently treated daily for 3 weeks with nontoxic doses of X-XO. Scavengers of active oxygen radicals such as superoxide dismutase or superoxide dismutase in combination with catalase reduced the frequency of appearance of transformed foci by 3–25 times when compared to cultures receiving X-XO alone. These results show that active oxygen species such as superoxide and H_2O_2 can act in a promotional manner that mimics the effects of the mouse skin promoter phorbol 12-myristate 13-acetate in this system. X-XO also acted as a weak complete carcinogen.**

The observation that certain antioxidants are anticarcinogenic in experimental animals and in *in vitro* culture systems suggests a role for active oxygen (superoxide radicals $O_2^{\cdot-}$; hydroxyl radicals ·OH; singlet oxygen $^1O_2$; hydrogen peroxide H_2O_2) in the transformation process (1–5). For example, vitamins C and E, butylated hydroxytoluene, butylated hydroxyanisole, glutathione, bovine erythrocyte Cu–Zn superoxide dismutase (SOD), and the biomimetic analog Cu-diisopropylsalicilate were anticarcinogenic under certain conditions (5–10). It is interesting to note that most tumor cells are deficient in Mn^{2+} SOD, which represents an important defense system against superoxide radicals (1).

Carcinogenesis is a multistep process and the simplest models distinguish an initiation and promotion phase (11, 12). Antioxidants can inhibit initiation if they counteract the formation of DNA damage—e.g., by scavenging active oxygen formed by radiation (13, 14) or by decreasing metabolic activation of carcinogens to their ultimate form (15–17). Several observations support the notion that active oxygen plays a role in promotion. For example: (*i*) certain organic peroxides such as benzoylperoxide, lauroylperoxide (18), and H_2O_2 (19) possess promotional activity; (*ii*) Cu-diisopropylsalicilate inhibits tumor promotion by phorbol 12-myristate 13-acetate (PMA) in mouse epidermis (20); and (*iii*) membrane-active compounds—e.g., the classical mouse skin promoter PMA and the complete carcinogens aflatoxin B_1 and benzo[*a*]pyrene—may produce a prooxidant state and lipidperoxidation in the cell because they stimulate the arachidonic acid cascade, elicit an oxidative burst, and disturb the conformational integrity of the cellular membranes (21). Correspondingly, they induce chromosomal damage by indirect action and their clastogenicity for human lymphocytes is diminished by antioxidants (22, 23). PMA also induces the formation of a diffusible clastogenic factor (CF) in human leukocytes (24). Preliminary data suggest that the CF consists of free arachidonic acid and arachidonic acid hydroperoxides (21). (Pre-)neoplastic tissue is usually surrounded by a belt of inflammatory leukocytes. CF released by these cells in response to promoters may induce chromosomal damage and play a role in promotion or progression (21, 24, 25).

We tested directly whether active oxygen possesses a promotional effect for initiated mouse embryo fibroblasts. C3H/10T½/C18 cells were initiated either with low doses of γ-rays or of the ultimate metabolite benzo[*a*]pyrene diol epoxide I {7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE I)}. They were then treated repeatedly with xanthine-xanthine oxidase (X-XO). X-XO produces a burst of $O_2^{\cdot-}$ (26) extracellularly in the culture medium. After initiation with x-rays and BPDE I, X-XO treatment exerted a strong promotion effect. Repeated X-XO treatment alone also transformed 10T½ cells but with much lower efficiency.

MATERIALS AND METHODS

Cell Culture and Transformation Assays. Mouse embryo C3H/10T½ fibroblasts were cultured in basal Eagle's medium containing 10% fetal bovine serum (GIBCO). We followed the experimental protocol and criteria for transformation that have been established in previous experiments for two-stage carcinogenesis in the 10T½ system (27–29). Further experimental information is contained in the tables.

Post-Initiation Treatments. Xanthine oxidase was obtained from Boehringer, Rotkreuz, catalase was from Serva, Basel, and SOD was prepared by a modification of the method of McCord and Fridovich (30) and possessed a specific activity of 4400 units/mg of protein. X-XO reaction mixtures were prepared by adding the required amount of a sterile xanthine solution to sterile phosphate-buffered saline, to which the appropriate amount of the stock xanthine oxidase solution was added immediately prior to use. In the experiments involving the addition of SOD or catalase or both, these enzymes were then added to the reaction mix. Reaction mixtures were kept on ice and prepared such that the addition of 100 μl per dish resulted in the required final concentrations in the medium. The exact concentrations used are detailed in the legends to the tables. Cells were treated daily for 3 weeks, beginning 48 hr after carcinogen treatment or 48 hr after plating in cultures that did not receive carcinogen.

RESULTS AND DISCUSSION

Superoxide Generated by X-XO Is a Weak Complete Carcinogen. The 10T½ *in vitro* transformation system has been used in our study. It has been well characterized and is particularly useful because it can be applied to two-step carcino-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: X-XO, xanthine-xanthine oxidase; SOD, superoxide dismutase; CAT, catalase; PMA, phorbol 12-myristate 13-acetate; CF, clastogenic factor; BPDE I, 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

Table 1. Transformation of (noninitiated) 10T½ cells by daily treatment with X-XO

Experiment	Treatment	Viable cells, no. per dish	Dishes, total no.	Dishes with transformed foci, no.	Transformed foci, no.			Total foci/ total dishes	Dishes with foci/ total dishes
					Type II	Type III	Total		
A	None	138	47	7	5	2	7	0.15	0.15
	X-XO*	138†	44	16	17	0	17	0.39	0.36
B	None	135	47	0	0	0	0	—	—
	X-XO*	135†	37	4	6	0	6	0.16	0.11
C	None	150	29	0	0	0	0	—	—
	X-XO‡	150†	33	12	11	1	12	0.36	0.36
D	None	138	47	1	1	0	1	0.02	0.02
	X-XO*	138†	35	0	0	0	0	—	—

*2 μg of xanthine/0.2 μg of xanthine oxidase daily for 3 weeks.
†The number of viable cells was the same as in untreated samples because the X-XO treatment was nontoxic and nonstimulatory (see text).
‡20 μg of xanthine/2 μg of xanthine oxidase daily for 3 weeks.

genesis experiments. For example, a promotional effect of PMA could be demonstrated for cells that had been initiated with 3-methylcholanthrene, benzo[*a*]pyrene (27), x-rays (28), and ultraviolet light (29).

The conditions for the X-XO treatment in complete tissue culture medium were first established. The formation of the primary product of the X-XO reaction, uric acid, was followed spectrophotometrically by measuring the change in the absorbance at 293 nm (26). Using 20 μg of xanthine oxidase per ml, an increase in absorbance was observed for 30–40 min at 37°C. After this time the production of uric acid slowed down and its breakdown became faster than its formation. Doubling the enzyme concentration resulted in a 2-fold increase in the rate of the change of absorbance at 293 nm. The effect of X-XO treatment on cell survival was measured both as colony-forming ability and growth rate (determined by daily cell counts for 8 days—i.e., 4 days in exponential growth and 4 days in confluency) and established the nontoxic conditions used in the transformation experiments.

In the first series of experiments we studied the transformation of (noninitiated) 10T½ cells by daily treatment with 2 μg of xanthine/0.2 μg of xanthine oxidase per ml or 20 μg of xanthine/2 μg of xanthine oxidase per ml for 3 weeks. Different lots of heat-inactivated fetal calf serum were used for each experiment to avoid a specific serum effect. The data are summarized in Table 1, which lists (*i*) the number and type of foci scored, (*ii*) the total number of foci per dish, and (*iii*) the number of dishes with transformed foci divided by the total number of dishes. The number of cells plated was adjusted so that each 60-mm dish contained ≈135–150 viable cells. Our data indicate that X-XO treatment caused low levels of transformation in three of four experiments. We attribute the fact that no transformation was observed in experiment D and some spontaneous transformation in experiment A to differences in the serum lots. We conclude that active oxygen produced by X-XO can act as a complete, albeit weak, carcinogen.

The induction of DNA damage by X-XO may be necessary for its initiating activity as a complete carcinogen. Indeed, it has been found previously that X-XO treatment induces chromosomal aberrations in human lymphocytes (31). Extracellular $O_2^{\cdot-}$ does not readily penetrate the cellular envelope, but uncharged related molecules such as the perhydroxy radical $HO_2^{\cdot}$, H_2O_2, and ·OH radical do. These active oxygen species induce DNA damage of the type usually associated with ionizing radiation—e.g., DNA strand breakage and base damage of the 5,6-dihydroxy-dihydrothymine type (32). H_2O_2 has been shown to be mutagenic (33, 34) and carcinogenic (35) and it is interesting to note that nonmutagenic drugs that increase the peroxisome population and consequently H_2O_2 formation are carcinogenic (36).

Superoxide Generated by X-XO Is a Potent Promoter. Table 2 contains the data of the two-step carcinogenesis experiments. In these experiments cells were initiated with γ-rays and 48 hr later the daily treatment with X-XO was started and continued for 3 weeks. X-XO treatment after an initiating dose of 100 rads (1 rad = 0.01 gray) increased the transformation frequency by a factor of 6 ($P < 0.01$) [experiment A, P values were determined by using the test to calculate a difference between two sample proportions of a binomial population (37)]. After an initiating dose of 400 rads, which caused significant transformation by itself, the promotional effect of X-XO was reduced to a factor of 1.5 ($P < 0.08$) (experiment B). The promoting effect of X-XO strongly sur-

Table 2. Two-stage carcinogenesis of 10T½ cells by initiation with γ-rays or BPDE I and later treatment with X-XO

Experiment	Post-initiation treatment*	Viable cells, no. per dish	Dishes, total no.	Dishes with transformed foci, no.	Transformed foci, no.			Total foci/ total dishes	Dishes with foci/ total dishes
					Type II	Type III	Total		
A	None†	123	44	4	5	0	5	0.11	0.09
	X-XO†	123‡	44	24	27	2	29	0.66	0.54
	X-XO/SOD + CAT†	123‡	35	1	1	0	1	0.03	0.03
B	None§	117	47	19	19	2	21	0.45	0.40
	X-XO§	117‡	32	19	36	4	40	1.25	0.60
C	None§	140	47	8	5	3	8	0.17	0.17
	X-XO/SOD§	140‡	43	2	1	1	2	0.05	0.05
D	None¶	560	48	1	1	0	1	0.02	0.02
	X-XO¶	560‡	49	24	25	6	31	0.63	0.49
	X-XO/SOD¶	560‡	43	9	9	1	10	0.23	0.21

*2 μg of xanthine/0.2 μg of xanthine oxidase at 48 hr after initiation daily for 3 weeks; where indicated, 10 units of SOD per ml or 10 units of SOD and 195 units of CAT per ml were added concomitantly.
†Initiation with γ-rays (100 rads).
‡See footnote † to Table 1.
§Initiation with γ-rays (400 rads).
¶Initiation with BPDE I (0.5 μM).

passes the transforming effect of the X-XO treatment *per se* (see Table 1). These data are comparable to those reported by Kennedy *et al.* (28) who used PMA as a promoter with x-ray-initiated 10T½ cells. Analogous results were obtained when cells were initiated with the ultimate benzo[*a*]pyrene metabolite, BPDE I (experiment D). A concentration of 0.5 μM BPDE I was used, which resulted in a total initial DNA adduct concentration of 6.8 μmol/mol of DNA phosphate. BPDE I was only a very weak complete carcinogen, but the transformation frequency increased by as much as 25-fold when the BPDE I-initiated cells were treated subsequently with X-XO ($P < 0.01$). X-XO treatment of initiated cells resulted mostly in an increase in the number of type II foci.

To further elucidate the mechanism of X-XO promotion we studied the effect of the simultaneous addition of SOD or SOD and catalase (CAT) at the time of the X-XO treatments. As shown in Table 2, SOD addition reduced the transformation frequency by >50% after initiation with BPDE I ($P < 0.01$) (experiment D) and SOD with CAT, by nearly 94% after initiation with 100 rads of x-rays ($P < 0.01$) (experiment A). The observation that SOD with CAT possessed maximal anticarcinogenic effect is not unexpected. SOD dismutases $O_2^{\cdot-}$ to H_2O_2, which is a weak tumor promoter in its own right (19). CAT destroys H_2O_2 under formation of O_2 and H_2O.

Recent evidence suggests that DNA damage induced by active oxygen species plays a role in tumor promotion. Tumor promoters elicit complex pleiotropic responses in most cells and at least some of them involve the modulation of gene expression (11, 12). For example, the induction of ornithine decarboxylase is intimately associated with the promoting action of PMA (12, 38, 39). We have recently found that SOD and CAT, and most efficiently a combination of the two enzymes, strongly inhibited the induction of ornithine decarboxylase in mouse mammary tumor cells Mm5mt/Cl, implicating $O_2^{\cdot-}$ and H_2O_2 as intermediates in the induction process (40). Similarly, butylated hydroxytoluene and other antioxidants inhibited ornithine decarboxylase induction by PMA in mouse skin (41). The intermediacy of H_2O_2 in genomic induction by PMA was also demonstrated for exogenous copies of mouse mammary tumor virus in Mm5mt/Cl cells. CAT strongly inhibited the induction, whereas SOD had only a small effect (unpublished data).

Our present results demonstrate that active oxygen can exert a promotional effect. The relationship between DNA damage induced by active oxygen, modulation of gene expression by this mechanism, and tumor promotion remains to be established, however.

We thank Dr. P. Amstad for the BPDE I-DNA adduct concentrations and Miss I. Zbinden for her excellent technical assistance. This work was supported by Grant 3'627.80 of the Swiss National Science Foundation and a grant from the Swiss Association of Cigarette Manufacturers.

1. Oberley, L. W. & Buettner, G. R. (1979) *Cancer Res.* **39,** 1141–1149.
2. Totter, J. R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1763–1767.
3. Ames, B. N., Cathcart, R., Schwiers, E. & Hochstein, P. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6858–6862.
4. Ames, B. N. (1983) *Science* **221,** 1256–1264.
5. Borek, C. & Troll, W. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1304–1307.
6. Benedict, W. F., Wheatley, W. L. & Jones, P. A. (1982) *Cancer Res.* **40,** 2796–2801.
7. Prasad, K. & Edwards-Prasad, J. (1982) *Cancer Res.* **42,** 550–555.
8. Fernandez-Pol, J., Hamilton, P. & Klos, D. (1982) *Cancer Res.* **42,** 609–617.
9. Novi, A. M. (1981) *Science* **212,** 541–542.
10. Leuthauser, S., Oberly, L., Oberly, T., Sorenson, J. & Ramakrishna, J. (1981) *Natl. Cancer Inst.* **66,** 1077–1081.
11. Hecker, E., Fusenig, N., Kunz, W., Marks, F., Thielman, H., eds. (1982) *Cocarcinogenesis and Biological Effects of Tumor Promotors,* (Raven, New York).
12. Diamond, L., O'Brien, T. & Baird, W. (1980) *Adv. Cancer Res.* **32,** 1–74.
13. RotiRoti, J. & Cerutti, P. (1974) *Int. J. Radiat. Biol.* **25,** 413–417.
14. Nygaard, O. & Simic, M., eds. (1983) *Radioprotectors and Anticarcinogens* (Academic, New York).
15. Wattenberg, L. (1980) in *Cancer 1980: Achievement, Challenges and Prospects,* eds. Burchenal, J. & Oettgen, H. (Grune & Stratton, New York), pp. 517–539.
16. Delong, M., Prochaska, H. & Talalay, P. (1983) in *Protective Agents in Cancer,* eds. McBrien, D. & Slater, T. (Academic, New York), pp. 175–196.
17. Amstad, P. & Cerutti, P. (1983) *Biochem. Biophys. Res. Commun.* **112,** 1034–1040.
18. Slaga, T., Triplett, L., Jotti, L. & Trosko, J. (1981) *Science* **213,** 1023–1025.
19. Slaga, T. & Nelson, K. (1983) in *Genes and Proteins in Oncogenesis,* eds. Weinstein, I. B. & Vogel, H. J. (Academic, New York), pp. 125–142.
20. Kensler, W., Bush, D. & Kozumbo, W. (1983) *Science* **221,** 75–77.
21. Cerutti, P., Emerit, I. & Amstad, P. (1983) in *Genes and Proteins in Oncogenesis,* eds. Weinstein, I. B. & Vogel, H. J. (Academic, New York), pp. 55–67.
22. Emerit, I. & Cerutti, P. A. (1981) *Nature (London)* **293,** 144–146.
23. Emerit, I., Levy, A. & Cerutti, P. A. (1983) *Mutat. Res.* **110,** 327–335.
24. Emerit, I. & Cerutti, P. A. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7509–7513.
25. Weitberg, A., Weitzman, S., Destrempes, M., Latt, S. & Stossel, T. (1983) *N. Engl. J. Med.* **308,** 26–30.
26. Kellogg, E. W. & Fridovich, I. (1975) *J. Biol. Chem.* **250,** 8812–8817.
27. Mondal, S., Brankow, D. W. & Heidelberger, C. (1976) *Cancer Res.* **36,** 2254–2260.
28. Kennedy, A. R., Mondal, S., Heidelberger, C. & Little, J. B. (1978) *Cancer Res.* **38,** 439–443.
29. Mondal, S. & Heidelberger, C. (1976) *Nature (London)* **260,** 710–711.
30. McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244,** 6049–6055.
31. Emerit, I., Keck, M., Levy, A., Feingold, J. & Michelson, A. M. (1982) *Mutat. Res.* **103,** 165–170.
32. Cerutti, P. (1974) *Naturwissenschaften* **61,** 51–59.
33. Levin, D. E., Hollstein, M., Christman, M., Schwiers, E. & Ames, B. N. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7445–7449.
34. Moody, C. S. & Hassan, H. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2855–2859.
35. Ito, A., Watanabe, H., Naito, M. & Naito, Y. (1981) *Gann* **72,** 124–175.
36. Reddy, J. K., Azarnoff, D. L. & Hignite, C. E. (1980) *Nature (London)* **823,** 397–398.
37. Goldstein, A. (1964) *Biostatistics* (MacMillan, New York), p. 101.
38. Weeks, C., Herrmann, A., Nelson, F. & Slaga, T. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6028–6032.
39. Takigawa, M., Verma, A. K., Simisman, R. C., Boutwell, R. K. (1982) *Biochem. Biophys. Res. Commun.* **105,** 969–976.
40. Friedman, J. & Cerutti, P. (1983) *Carcinogenesis* **4,** 1425–1427.
41. Kozumbo, W., Seed, J. & Kensler, T. (1983) *Cancer Res.* **43,** 2555–2559.