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Modifiers of free radicals inhibit *in vitro* the oncogenic actions of x-rays, bleomycin, and the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate

(cell transformation/catalase/superoxide dismutase/chemoprevention/promotion)

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ABSTRACT Using short-term cultures of hamster embryo cells, we have examined the effects of the free-radical scavenger superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) and the enzyme catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) on x-ray- and bleomycin-induced transformation and on the enhancement of radiogenic transformation by the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA). We find that superoxide dismutase inhibits (*i*) transformation induced by x-ray and bleomycin and (*ii*) promotional action of TPA *in vitro*. The results suggest that the oncogenic action of x-rays and bleomycin and the enhancement of oncogenic transformation by TPA are mediated in part by free radicals. The findings also suggest that superoxide dismutase can serve as an inhibitor of oncogenesis and that its actions, as seen in this *in vitro* system, are most predominantly on inhibiting late events in the progression of cellular transformation—those associated with *promotion*.

The generation of reactive oxygen species in living systems exposed to radiation and the protective action by cysteine, cysteamine, and a variety of radical scavengers are well recognized (1–6). More recently, other compounds have been shown to produce free radicals. These include the tumor promoters 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (7, 8) and teleocidin (9) and the widely used chemotherapy agent bleomycin (10).

Utilizing freshly explanted cultures of hamster embryo cells (11–13), we evaluated in this investigation the effects of enzymes that catalytically scavenge the intermediates of oxygen reduction (*i*) on x-ray- and bleomycin-induced transformation and (*ii*) on the enhancement of radiogenic transformation by TPA. We find that superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) significantly inhibits the oncogenic effects of both x-rays and bleomycin and prevents the enhancement of transformation by TPA. The inhibitory effects of catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) were insignificant in this cell system.

Our data suggests that x-ray, bleomycin, and TPA may be inducing their oncogenic effects in part through free-radical mechanisms. Our finding that the cellular content of catalase and SOD varies from one cell type to another suggests that these factors may contribute to variations in cell competence to transformation (14).

MATERIALS AND METHODS

Hamster Embryo Cell Culture. Minced midterm whole embryos from golden hamsters (Lakeview, Wilmington, MA) were used as a source of normal cells (11–14). Primary cultures were established by progressive dissociation of minced fresh tissue. For transformation experiments, the cells of 3-day-old primary cultures were suspended by trypsinization, and 10^3 cells were cloned onto 60-mm Petri dishes on x-irradiated (4,000 rad; 1 rad = 0.01 J/kg = 0.01 Gy) syngeneic feeder cells (15) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) (GIBCO).

X-Irradiation. Cells were irradiated at room temperature 24 hr after plating with 3 Gy (300 rad) of x-rays at a dose rate of 0.322 Gy/min and incubated at 37°C in 5% CO_2/95% air with weekly medium changes. The source of x-rays was a Siemens 300-kVP constant-potential generator with an additional filter of 0.2-mm of copper. Controls consisting of untreated cultures were maintained under the same conditions as were experimental cultures. After the 10-day incubation period, cells were fixed and stained with Giemsa and assayed for cell survival, cloning efficiency, and transformation (11–14).

Bleomycin Treatment. Bleomycin (Bristol, Syracuse, NY) was added (1 μg/ml of medium) to hamster cell cultures 24 hr after cloning. The compound was left on the cultures throughout the experiment, after which cultures were fixed and stained and assayed for transformation.

TPA Treatment. A single lot (no. 025) of TPA (Consolidated Midland, Brewster, NY) was used throughout and prepared in dimethyl sulfoxide prior to each experiment. The final concentrations of TPA and dimethyl sulfoxide in the growth medium were 0.16 μM (0.1 μg/ml) and 0.01%, respectively.

SOD. Obtained from Diagnostic Data (Mountain View, CA), SOD was added to the cells at 10 units/ml (2.7 μg/ml) at plating time or after irradiation as described. This concentration of SOD produced no observable toxicity. SOD was removed 24 hr later or at the termination of the experiment as described.

Catalase. Obtained from Sigma, catalase was added at 10 μg/ml at plating time and retained throughout the experiment. No toxicity was observed at this concentration.

Transformation Assay. After 10 days of incubation, the medium was removed and cells were fixed and stained with Giemsa (11–14). A differential count was made of normal and transformed colonies, the latter being identified by their morphological appearance among the surviving colonies. Both normal and transformed colonies were scored so that both transformation frequency and cell survival could be assessed within the same experiment. Transformed colonies were identified by their irregular growth pattern and their tendency to form multilayers as compared to controls (11–14). The relationship between this

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; SOD, superoxide dismutase.

morphology and the malignant nature of the cells has been documented (11–13).

Cellular Assays for Catalase and SOD. Assays for catalase and SOD followed standard procedures and were carried out on logarithmic-phase primary and secondary cultures of hamster embryo utilized in the transformation experiments. SOD was assayed by measuring inhibition of the reduction of nitrobluetetrazolium (16). Catalase was assayed by a spectrophotometric method that monitors the absorption of H_2O_2 at 240 nm; the rate of decrease in absorption at 240 nm is a function of catalase activity in the sample (17).

RESULTS

Fig. 1 summarizes the data on cell transformation of hamster embryo cells after exposure to 300-rad x-rays with and without the addition of SOD at 10 units/ml. SOD present at the time of irradiation but removed immediately afterwards reduced the incidence of transformation by a factor of 1.5. This reduction is on the borderline of statistical significance. A marked increase in the effectiveness of SOD was seen when it was present throughout the incubation period, resulting in a significant reduction in the incidence of induced transformation.

The effects of SOD on transformation induced by bleomycin is illustrated in Fig. 2. SOD present on the cells throughout the experiment significantly inhibited bleomycin-induced transformation.

We found that catalase did not have a significant inhibitory effect on transformation induced by either x-rays (Table 1) or bleomycin (not shown) when present throughout the experiment. When added concomitantly with SOD, inhibition of transformation was enhanced, but the effect was not significant.

The actions of SOD and catalase on TPA enhancement of radiogenic transformation are presented in Table 1. TPA enhanced cell transformation by x-rays. Its effect was reduced by SOD and to a lesser extent by catalase. The inhibitory action of SOD on TPA prevailed even when SOD was added along with TPA 24 hr after irradiation.

Table 2 describes the cellular content of SOD and catalase in the hamster cells. A lower cellular content of catalase was found (not shown) in mouse C3H $10T^1/_2$ cells, a line widely used in transformation experiments (18).

DISCUSSION

We showed that (*i*) oncogenic transformation induced *in vitro* by x-rays and bleomycin and (*ii*) the enhancement of this transformation by the tumor promotor TPA, a producer of free radicals (8), can be inhibited by SOD, an agent that modifies the availability of free oxygen radicals to the cells. SOD catalyzes the conversion of the superoxide radical (O_2^-) to H_2O_2 plus O_2, and H_2O_2 is removed by catalase, which converts it to H_2O + O_2 (6). The efficient elimination of O_2^- and H_2O_2 prevents the formation of toxic substances, such as the highly reactive hydroxyl radical, OH· (6). The observed insignificant action of catalase in modulating cell transformation may reside in the high cellular content of this enzyme in the hamster cells, which may suffice for converting the available H_2O_2 into H_2O + O_2.

Both x-rays and bleomycin interact with DNA (19–21); bleomycin induces single- and double-stranded DNA breaks, a fragmentation that requires molecular oxygen (21). Whereas the superoxide molecule enhances bleomycin-induced DNA damage, SOD inhibits the process (22). SOD has been shown *in vivo* to reduce x-ray-induced toxicity (4) and in cell culture to inhibit the

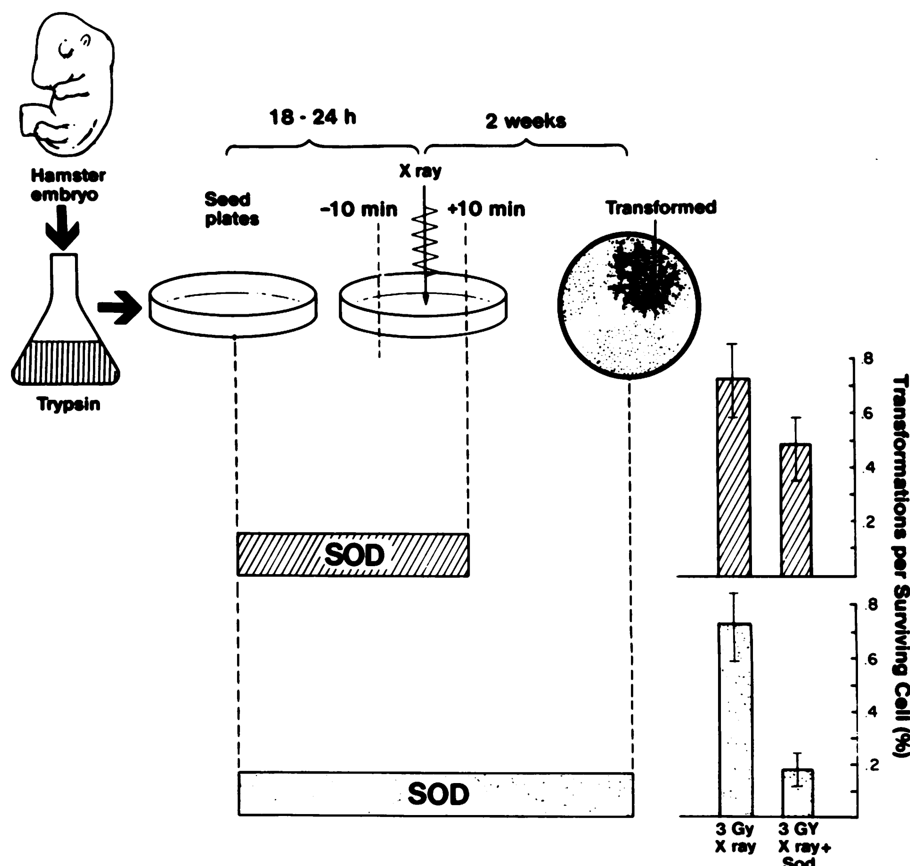

FIG. 1. The effect of SOD on the incidence of oncogenic transformation in hamster embryo cells produced by 3-Gy x-rays. The SOD (10 units/ml) was added in two different protocols as illustrated. h, Hour.

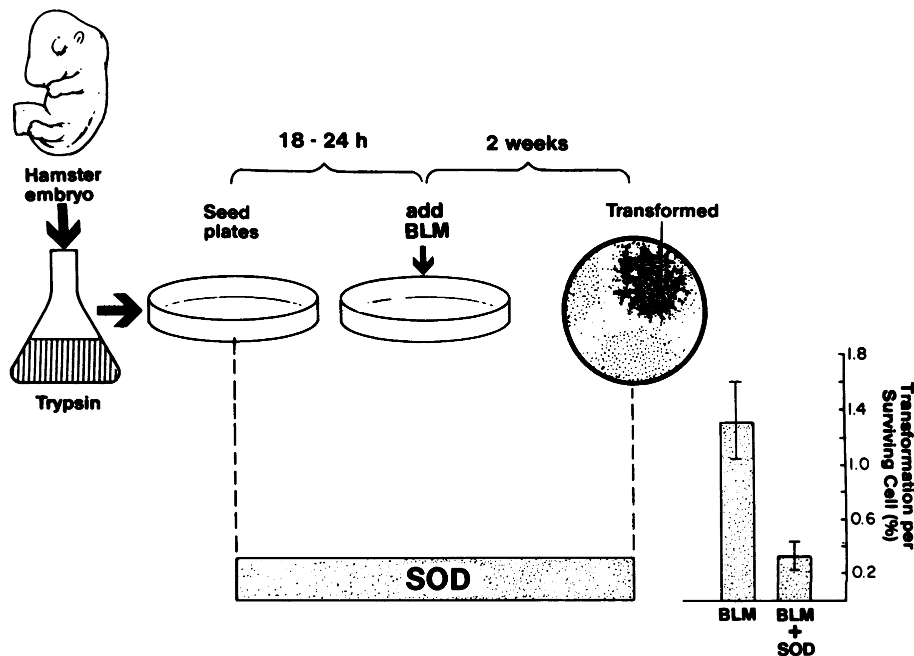

FIG. 2. The effect of SOD (10 units/ml) on the incidence of oncogenic transformation in hamster embryo cells produced by the chemotherapy agent bleomycin (BLM; 1.0 μg/ml). h, Hour.

clastogenic effects of TPA (23), a free-radical producer (8).

We are relatively ignorant of the mechanisms by which free radicals may influence neoplastic transformation and to what extent they influence initiation and promotion (13). One could speculate that their actions involve membrane lipid peroxidation (24), which may result in a cascade of extranuclear and nuclear events (23, 25), with cell transformation being one of the consequences. Thus, cellular protective systems or externally added protective agents may serve as inhibitors of oncogenesis.

In earlier studies we have shown that selenium, which is involved in the selenium-dependent glutathione peroxidases (24, 25) inhibits radiation and chemically induced transformation *in vitro* (26). Intracellular depletion of glutathione has been shown recently to enhance cellular radiosensitization (27). Recent work in hepatic systems is of interest in this connection. The administration of reduced glutathione to rats bearing tumors induced by alflatoxin B_1, (28), a powerful hepatocarcinogen (29), resulted in a regression of tumor growth (30).

The reduction of liver glutathione in mice resulted in an increased lipid peroxidation by benzo(*a*)pyrene, methylcholantrene, and phenobarbital (24), and adriamycin-induced toxicity in isolated hepatocytes was reduced by the glutathione redox cycle (30).

More recently, adriamycin has been shown to be actively cytotoxic without entering cells, thus implicating the cell membrane as the initial target (31).

SOD penetrates poorly through the cell membrane (32); thus, its inhibitory action on transformation probably resides in events originating at the level of the cell surface. Although there was some inhibition of transformation when SOD was present for a short period of time, the more dramatic inhibitory action on x-ray-induced transformation required the presence of SOD for the prolonged postirradiation incubation. In addition, the inhibitory effects of SOD on the enhancement of transformation by TPA were observed even when SOD and TPA were added 24 hr after irradiation.

The present results suggest that SOD plays an inhibitory role in oncogenic transformation by inhibiting promotion. The events in promotion that are affected by SOD could be mediated in part through multiple free radical interactions with cellular components (25) and through oxygen radicals generated by inter-

Table 1. Inhibition of cell transformation by SOD and catalase after exposure to x-rays and TPA

Treatment	Surviving fraction* ± SEM	% transformation ± SEM
Control	(4.6 ± 0.12)	<0.001
SOD	0.95 ± 0.04	<0.001
Catalase	0.97 ± 0.03	<0.001
TPA	0.92 ± 0.05	<0.001
Me_2SO	0.95 ± 0.04	<0.001
3 Gy	0.75 ± 0.12	0.74 ± 0.14
3 Gy + SOD	0.91 ± 0.09	0.17 ± 0.06
3 Gy + catalase	0.80 ± 0.11	0.66 ± 0.09
3 Gy + SOD + catalase	0.87 ± 0.12	0.13 ± 0.10
3 Gy + TPA	0.69 ± 0.14	1.62 ± 0.12
3 Gy + SOD + TPA	0.89 ± 0.10	0.55 ± 0.08
3 Gy + catalase + TPA	0.82 ± 0.13	1.32 ± 0.15
3 Gy + catalase + SOD + TPA	0.93 ± 0.12	0.41 ± 0.09
3 Gy + SOD†	0.80 ± 0.10	0.21 ± 0.07
3 Gy + TPA†	0.64 ± 0.12	1.56 ± 0.08
3 Gy + SOD† + TPA†	0.82 ± 0.13	0.61 ± 0.11

Me_2SO, dimethyl sulfoxide.

* Number of colonies counted/(number of cells plated × plating efficiency). Plating efficiency (shown ± SEM in parentheses) = number of colonies counted/number of cells plated × 100.

† Compound(s) added 24 hr after irradiation and left throughout the experiment.

Table 2. Cellular content of catalase and SOD* in hamster embryo cells (He) per μg of protein

HE cultures	Catalase	SOD
Primary	6 units/96 μg ± 2%	37 ng/72 μg ± 2%
Secondary	7.5 units/130 μg ± 2%	37 ng/97.5 μg ± 2%

* No SOD or catalase were detected in fetal calf serum, within the sensitivity of the assay (100 ng of SOD; 1 unit of catalase).

action with thiols in the growth medium. The inhibition of promotion *in vitro* by SOD may be of a similar nature to the reported inhibition of promotion *in vivo* by free-radical modifiers (33).

Although further work is essential for full evaluation of these results, the present report suggests that SOD may serve as an effective inhibitor of carcinogenesis, at least as judged by this *in vitro* system, and that SOD is best effective in inhibiting later events in neoplastic progression—those associated with promotion.

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