Differential Proliferative Responses of Syrian Hamster Embryo Fibroblasts to Paraquat-generated Superoxide Radicals Depending on Tumor Suppressor Gene Function

Thomas M. Nicotera, Christopher Privalle, T. C. Wang, Mitsuo Oshimura, and J. Carl Barrett

Laboratory of Molecular Carcinogenesis, Environmental Carcinogenesis Program, National Institute of Environmental Sciences, NIH, Research Triangle Park, North Carolina 27709; [T. M. N., T. C. W., J. C. B.]; Biophysics Department, Roswell Park Cancer Institute, Buffalo, New York 14263 [T. M. N.]; Department of Biochemistry, Duke University, Duke University Medical Center, Durham, North Carolina 27710 [C. P.]; and Department of Molecular and Cellular Genetics, School of Life Sciences, Tottori University, Nishinomiya 60, Yonago-City, Tottori-ken, Japan [M. O.]

ABSTRACT

Oxygen radicals have been widely implicated in neoplastic transformation; however, little is known regarding their mode of action. In an attempt to delineate potential mechanisms of action, an analysis of superoxide effects on cell growth was studied in normal and tumor immortal cell lines derived from normal Syrian hamster embryo (SHE) fibroblasts. The two immortal cell lines differed in their ability to suppress tumorigenicity of tumor cells in cell hybrids. One cell line suppressed tumorigenicity (sup+), while a second clone was unable to suppress tumorigenicity (sup−). Paraquat was used to generate superoxide through its capacity to be reduced by NAD(P)H and to generate superoxide radicals. The growth response of the various cell types was measured by colony-forming ability as well as by tritiated thymidine incorporation using autoradiography. At low paraquat concentrations (25 μM), primary SHE cells and two sup+ clones showed up to a 40% enhancement in colony formation, while two sup− clones showed no increase. Toxicity was observed at high doses, starting at approximately 100 μM paraquat. Since oxygen radicals are also mutagenic, primary SHE cells were examined for chromosomal aberrations. Chromatid gaps and breaks were induced at all concentrations of paraquat used. Thus, superoxide not only causes cellular toxicity at high doses but at low doses enhances cell growth of certain cells (primary SHE cells and sup+ cells) but not others (sup− cells). Therefore, differing responses of cells at different stages of neoplastic progression must be considered in understanding oxygen radical effects in growth control and carcinogenesis.

INTRODUCTION

Oxygen radicals have been widely implicated in neoplastic transformation; however, little is known regarding their mode of action (1, 2). Data supporting oxygen radical carcinogenesis has been generated from studies using the mouse skin two-stage model of initiation and promotion and also from studies of radiation carcinogenesis. The oxidative burst immediately following application of the tumor promoter TPA2 has been suggested to play a role in tumor promotion (3). An oxidative burst by phorbol ester, as detected by chemiluminescence, is inhibited by the addition of exogenous SOD or the SOD mimic, copper(I)-3,5-(diisopropylsalicylate)2, indicating the presence of superoxide radical anion (3). This concept is further supported by the observations that SOD or copper(II)-3,5-(diisopropylsalicylate)2 inhibits tumor promotion in the mouse skin model (4), in JB6 mouse epidermal cell lines (5), in hamster embryo fibroblasts (6), and in C3H 10T½ fibroblasts following X-irradiation (7). Exposure of C3H 10T½ cells to either activated neutrophils (8) or to xanthine/xanthine oxidase (9) provides additional evidence that oxygen radicals can play a causative role in morphological transformation. Anchor- age-independent growth of JB6 cells is also inhibited by the addition of SOD as early as 1 h after exposure to xanthine/xanthine oxidase treatment (10). Although the preponderance of this data is indirect, it is highly suggestive that oxygen radicals play a role in carcinogenesis or tumor promotion.

A key component of tumor promotion is cell proliferation which is enhanced by TPA and other tumor promoters (11, 15). It is speculated that many of the pleiotropic responses elicited in cells by TPA appeared to be mediated through its binding to the protein kinase C receptor (13), although the events subsequent to this interaction are not defined. A comparison of the oxidant responses to TPA show that the promotion-sensitive mouse strain SSIN generates a higher level as compared to the promotion-resistant strain C57BL/6J (14). A positive correlation is further observed between the oxidant response and hyperplasia (14). In primary rat hepatocytes, the growth stimulatory effects of TPA are abolished by the addition of exogenous SOD, indicating that the superoxide radical mediates at least part of the enhanced growth response (15). Inhibition of growth is reversed by the addition of the CuZnSOD inhibitor diethyldithiocarbamate in this system (15). Moreover, other tumor promoters, including saccharin, phenobarbital, and benzoyl peroxide, also stimulate DNA synthesis when added to serumless media, and the addition of SOD completely abolishes growth. Other antioxidant compounds such as vitamin E, retinoic acid, and 7,8-benzoflavone have also been shown to prevent stimulation of hepatocyte growth by TPA (15).

To understand further the nature of the prooxidant growth response, we asked whether paraquat-generated superoxide radicals can modulate the proliferation of Syrian hamster cells at specific stages of the transformation process. Paraquat (1,1'-dimethyl-4,4'-bipyridilium) was used to generate O2− through its capacity to be reduced by NAD(P)H followed by autooxidation by molecular oxygen. These studies were carried out using normal Syrian hamster embryo (SHE) cells and two immortal, non-tumorigenic cell lines derived from SHE cells following treatment with carcinogens. The two immortal cell lines differ in their ability to suppress tumorigenicity of benz-(a)pyrene-induced tumor cells in cell hybrids (16). Loss of tumor suppressor gene products in these cells occurs prior to complete neoplastic conversion. The cells have wild-type RB (17) and p53 genes2 and thus have lost a possibly new tumor suppressor gene. We examined whether oxygen radicals, generated by paraquat, would stimulate or inhibit cell growth. It has been suggested that diminished levels of MnSOD are associated with the transformed phenotype (18) and that increased expression of MnSOD suppresses the malignant phenotype in melanoma cells (19). Therefore, we measured endogenous SOD and catalase activities in the above-mentioned cell lines. In order to assess whether oxygen radicals can simultaneously cause

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1 To whom requests for reprints should be addressed, at Laboratory of Molecular Carcinogenesis, National Institute of Environmental Sciences, NIH, P.O. Box 12233, Research Triangle Park, NC 27709.

2 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; SOD, superoxide dismutase; DES, diethylstilbestrol; 13-HODE, 13-hydroxy-9(Z),11(E)-octadecanoic acid.

3 R. Whitehead, R. Wiseman, and J. C. Barrett, p53 Mutations in Syrian hamster cells, manuscript in preparation.
genetic damage at concentrations that affect cell growth, analyses of chromosomal damage by paraquat were also undertaken.

MATERIALS AND METHODS

Cell Culture. SHE cell cultures were established from 13-day gestation fetuses and grown as described previously (20). The immortal clones used in this investigation were isolated after treatment of primary SHE cells with either DES (DES-4 clone; Ref. 18) or with asbestos (10W clone; Ref. 21). Cell lines that either retain (sup +) or have lost (sup -) this tumor suppression gene function were subcloned from the same parental cells. Sup + and sup - variants from two independently derived carcinogen-induced cell lines (10W and DES-4) were examined in this study (16).

Colony-forming Efficiency. Cells were plated overnight in 10 ml of media (normal cells at 2000 cells/dish and 300 cells/dish for immortalized cells) in 100-mm plastic dishes and treated the following day with various concentrations of paraquat (Sigma) for 24 h. Following treatment, the paraquat was replaced with 10 ml of media. Cells were allowed to grow for 7 days with one refedding at midweek, after which the surviving colonies were fixed in methanol, stained in Giemsa (10% in water), and scored.

Autoradiography. Cells were plated at 50,000/well in 2.5-mm² wells on a multiwell slide in a total of 0.3 ml of media and allowed to become density-arrested. After 3 days growth, cells were washed two times in media (without fetal calf serum) and incubated for 24 h in media containing 5% platelet-poor plasma, 1.0 μCi of [3H]thymidine (Amersham) and paraquat. The plastic wells were removed from the slide, and the treated cells attached to the slide were washed two times with phosphate-buffered saline and fixed in methanol for 10 min. Slides were then washed in water, treated two times with 10% trichloroacetic acid for 5 min each to remove unincorporated radiolabel, and washed again in water and dried.

NTB-2 nuclear track photographic emulsion (Kodak) was prepared to a 1:1 (v/v) slurry with deionized water, mixed gently, and warmed to 42°C in complete darkness. Slides were briefly and gently dipped in this emulsion so that an even film coated the slide. The slides were allowed to dry for 2 h and then refrigerated in a light-proof slide box for 72 h. At this time, the emulsion on the slides was developed, fixed, and then stained first in eosin yellow and then in Giemsa. The percentage of cells with incorporated radiolabeled thymidine were scored by light microscopy.

SOD Assay. Cells were collected, washed two times in phosphate-buffered saline, and disrupted by sonication for 10 s at 50 W with a Branson sonifier. Triton X-100 was added to sonicates to a 0.05% final concentration and centrifuged at 40,000 rpm for 30 min. The protein concentration was determined densitometrically and the negatives were used to determine the relative peak size by densitometric scanning. Activity was also determined spectrophotometrically using a cytochrome C reduction method (25).

Catalase. Catalase activity was measured spectrophotometrically at 240 nm by the decrease in H₂O₂ following the addition of sonicate (12). The assay solution consisted of 1.0 ml of 0.059 M H₂O₂ in 50 mM Na₂HPO₄, pH 7.0, and sonicate in a 3.0 ml total reaction volume. One unit of activity is defined as 1.0 µmol of H₂O₂ decomposed/min at 25°C.

Chromosome Aberrations. Normal SHE cells were incubated with paraquat for 24 h and arrested in metaphase with Colcemid (0.4 µg/ml). Cells were then harvested, treated for 10 min in hypotonic KCl (0.075 M), fixed in acetic acid: methanol (1:3), and washed two times in the same solution. Chromosomes were prepared on slides and then stained in Giemsa (3% in 0.05 phosphate buffer, pH 6.8, for 15 min). One hundred cells were analyzed per point.

RESULTS

Sup + but not Sup - Cells Are Growth-stimulated by Paraquat.

The colony-forming efficiency of normal SHE cells and the sup + clones of both the DES-4 and 10W cell lines were enhanced up to 40% at a low paraquat concentration (25 μM), whereas the sup - clones were not (Fig. 1, A and B). High doses of paraquat were toxic to all of the cell lines. Toxicity responses of both DES-4 clones to paraquat concentrations >50 μM were similar to those of the SHE cell controls with an LD₅₀ of 200 μM paraquat. In contrast, the 10W clones varied considerably, demonstrating LD₅₀ of 85 and 125 μM paraquat for the sup - and sup + cells, respectively. A similar result was obtained with the alkylating agent mitomycin C (0.5 μg/ml), which also generates O₂ - upon activation and demonstrates 20 to 30% increase in colony-forming efficiency in both 10W and DES-4 sup + clones but not in their respective sup - clones (data not shown).

Autoradiographic analysis of DNA synthesis indicated enhanced growth in the primary SHE cells and in both sup + clones as compared to the sup - clones. Upon addition of paraquat, the primary SHE cells...
and the sup" clones demonstrated an additional 80 to 100% greater level of [3H]thymidine incorporation with respect to the untreated cells at 24 h, whereas the sup" clones demonstrated less than a 20% increase (Fig. 2). DNA synthesis was not observed until 24 h following paraquat treatment (data not shown).

**Variations in Catalase and SOD Activities Do Not Correlate with Growth Stimulation.** Densitometric quantitation of MnSOD activity indicated marginally elevation in the 10Wsup" clone and 50% elevation in the 10Wsup" clone compared to primary SHE cells. The 10W-SA clone showed dramatically higher (5-fold) MnSOD activity, which is attributable to increases in the major MnSOD electromorph and in a secondary electromorph not readily observable in the other cell lines. Two electromorphs of both CuZnSOD and of MnSOD were evident in the polyacrylamide gels (Fig. 3B). CuZnSOD was identified as the more rapidly migrating form through inhibition with 2.0 mM cyanide and consequent elimination of its achromatic band (Fig. 3A). The gels revealed similar levels of CuZnSOD activity in all of the cell lines examined (Fig. 4).

Catalase activity also showed considerable variability among these clones. In general, the 10W clones showed parallel changes in MnSOD and catalase activity, although there was no quantitative correlation. The 10Wsup" and SA clones demonstrated a 3-fold increase in catalase activity compared to primary SHE cells. In contrast to the 10W clones, the DES-4 sup" cells showed a reversed trend of lower MnSOD activity and elevated catalase activity, while the DES sup" clone demonstrated a concomitant decrease in MnSOD and catalase activities.

**Paraquat Is Clastogenic at Toxic and Growth-stimulating Doses.** Cells analyzed for chromosomal damage following paraquat treatment at doses and duration (24 h) similar to that used for analysis of colony-forming efficiency demonstrated that the aberrations observed were primarily chromatid gaps and breaks (Table 1). These data correspond with previously reported patterns of paraquat-derived chromosomal damage in other cell lines (26). Chromosome-type damage was also observed but at a low frequency. Chromosomal aberrations occurred at both toxic and growth stimulatory doses.

**DISCUSSION**

Tumor promotion by oxygen radicals has been extensively studied in mouse epidermal cells and fibroblast cell lines (3–11). Despite the increasing evidence for the involvement of oxidative stress in tumor promotion and carcinogenesis, little is known concerning the mechanism(s) of action of these reactive intermediates. It is unclear whether prooxidant tumor promoters operate by means of a genetic or epigenetic mechanism or in combination (27). Furthermore, antioxidant enzymes potentially capable of modulating toxicity or mitotic responses have not been sufficiently characterized throughout the neoplastic progression in any individual cell type. In an attempt to address some of these questions, we have used the herbicide paraquat to specifically generate endogenous \( \text{O}_2^- \) without the added complications elicited by the pleiotropic tumor promoter TPA. More specifically, we asked what changes in growth can be affected by endogenous \( \text{O}_2^- \) radicals in Syrian hamster embryo cell lines at various stages of neoplastic progression.

A consistent, differential growth response was observed between early and late passage SHE-derived cell lines following treatment with paraquat-generated superoxide radicals. One primary and two early passage carcinogen-immortalized (10W and DES-4 sup") clones demonstrated up to a 40% enhancement in the colony-forming effi-
ciency, while late passage (10W and DES-4 sup- ) clones exhibited no such growth enhancement when compared to their respective control cells. The maximum stimulatory concentration (25 μM) occurred significantly below that required for the onset of cellular toxicity, which was generally greater than 100 μM paraquat. These results were substantiated through the measurement of paraquat-stimulated DNA synthesis as observed by autoradiography. Similarly, only the primary SHE cells and the two sup+ clones showed enhanced DNA synthesis with 25 μM paraquat concentration. These results clearly delineate differing responses to oxygen radicals of cells at differing stages of neoplastic progression. Understanding these interactions between oxygen radicals with specific genotypes may be critical in understanding oxygen radical effects in carcinogenesis.

Measurement of antioxidant enzyme activities in the DES cells revealed decreased MnSOD activity and variable catalase activities, whereas the 10W cell lines exhibited increased MnSOD and catalase activities. Stepwise progression to the tumorigenic phenotype (normal SHE → sup+ → sup-) appears to amplify the degree of altered antioxidant enzyme activities. These results differ from the observation that MnSOD is generally reduced in transformed cells (18) and in initiated cells following TPA treatment (28–30). Changes in antioxidant enzyme levels may, therefore, reflect the particular means of chemical transformation as well as the cell type used. What is apparent is that all transformed cell lines examined demonstrate some form of altered antioxidant enzyme levels whether increased or decreased, which is a reflection of an altered redox status that may consequently affect both the mitotic response and the capacity to modulate intracellular toxicity. An important question that remains to be addressed is whether paraquat treatment alters the levels of antioxidant activities in these cells.

Cell growth stimulated by paraquat correlated only with cell type and was independent of antioxidant enzyme levels. On the other hand, toxicity in response to paraquat treatment inversely correlated with antioxidant enzyme levels. The 10W cell lines possessing increasing levels of MnSOD and catalase activities with neoplastic progression also demonstrated increasing toxicity in response to paraquat treatment. In contrast, the DES-4 clones possessing diminished MnSOD activity along with either increased (sup+) or decreased catalase activity (sup-) did not exhibit changes in toxicity as measured by clonal growth in response to paraquat treatment when compared to primary SHE cells. Our results are in general agreement with other reports that elevated SOD activity (whether CuZn or MnSOD) correlates poorly with resistance to either paraquat or xanthine/xanthine oxidase treatment in mammalian cells (31–33). One study found a significant correlation between increased glutathione peroxidase activity and paraquat resistance (32), while a second report observed a better correlation between increased catalase activity and resistance to active oxygen generation (33). Thus, it is as yet unclear which factors are the most significant in attenuating cellular toxicity resulting from oxidative stress.

A low level of chromosomal damage was observed at concentrations of paraquat that also caused growth enhancement and continued to increase in a dose-dependent fashion. The types of chromosomal damage observed were primarily chromatid gaps and breaks, which is consistent with what has been observed previously following paraquat treatment (26). Some chromosome-type aberrations were also observed but appeared to be formed in a random process. Therefore, it is possible that oxygen radicals may contribute to the transformation process through their capacity to act as clastogens and mutagens as well as contributing to the growth enhancement of selected clones of cells.

The biphasic response to paraquat challenge would suggest that multiple modes of action are taking place, and the type of response elicited depends on the concentration used. Our results show that paraquat-generated O2- can directly or indirectly affect complex biological functions such as cell growth at low concentrations and superoxide dismutation. One of the major consequences of O2- generation in cells is the peroxidation of membrane lipid components (34). It has been suggested that lipid peroxidation activates phospholipase A2, to preferentially release peroxidized fatty acids from membranes (35, 36) through the hydrolysis of the sn-2 acyl bond of glycerophospholipids. In so doing, arachidonic acid, which is covalently linked to the C-2 position of the glycerol backbone, is also released (37). Free arachidonate release initiates the biosynthesis of eicosanoids necessary for cell proliferation (38). Recently, it has been shown that release of the linoleic acid metabolite, 13-HODE, rather than arachidonic acid metabolites serves a role in modulating intracellular functions.

Table 1 Paraquat-induced chromosome aberrations in primary Syrian hamster embryo cells

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Fig. 4. Summary of antioxidant enzyme activities for MnSOD, CuZnSOD, and catalase. Each of these enzymes was assayed as described in “Materials and Methods” in primary SHE cells and SHE cell-derived clones. Statistical analysis was performed as in Fig. 2 and differences were noted for catalase and MnSOD activities when compared to primary SHE cells. * P < 0.05 and ** P < 0.01.
regulatory function in epidermal growth factor signal transduction of SHE cell fibroblasts (39). The lipoxigenase inhibitor nordihydroguaiaretic acid at 1.0 μM concentration effectively inhibited epidermal growth factor-stimulated DNA synthesis, while the cyclooxygenase inhibitor was effective only at high concentrations. It is possible that 13-HODE synthesis is also stimulated by pararquant treatment through the same processes that stimulate the release of eicosanoids. This assumption is given credence by the observation that the addition of 13-HODE stimulates DNA synthesis in supB cells but not in supB cells (39). Therefore, generation of oxygen radicals can apparently bypass the hormone receptor-mediated signal that triggers the biosynthesis of a mitogenic intermediate, which is then capable of eliciting differential growth responses in cell populations at different stages of carcinogenesis.

A redox control mechanism has been demonstrated for the binding of the Fos-Jun heterodimer to the DNA regulatory elements known as the protein activator-1 (AP-1) binding site (40). In vitro DNA binding is mediated by a specific and highly conserved cysteine residue in close proximity to DNA. In addition, a protein factor that reduces Fos and Jun and stimulates Fos-Jun DNA-binding activity in vitro was identified. A similar redox control mechanism has been demonstrated in Escherichia coli for the H2O2-dependent oxyR regulon genes (41) and is also likely for the superoxide-dependent regulon controlled by soxR (42), both of which are necessary for the maintenance of a stable redox state. The oxyR gene product is a transcriptional activator of pertinent genes and is activated by direct oxidation. Consequently, the oxyR protein acts as both a sensor and transducer of an oxidative stress signal (41). Redox regulation of transcription factors may represent a common mechanism that affects numerous biological functions, including that of cell growth in prokaryotes (43) and eukaryotes (44). Therefore, it is possible that the undefined sup+ suppressor gene is involved in the control of cell growth and is regulated by a similar redox mechanism. In light of this, the sup+ cells may represent cells that have become resistant to regulation of cell growth by redox mechanisms. Although the precise nature of the relationship between redox regulation and suppressor gene function is presently unclear, the SHE cell sup+/sup system offers a unique model to study these possibilities.

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