ABSTRACT

Chronic infection/inflammation of the urinary tract is a significant risk factor for the development of bladder cancer. The present study examined the hypothesis that hydrogen peroxide (H2O2) and cytokines released during inflammation are involved in the enhancement of bladder carcinogenesis. Using growth in soft agar and tumorigenicity in athymic nude mice as indices of transformation, we examined the effect of H2O2 and cytokines on the enhancement of N-methyl-N-nitrosourea (MNU)-initiated transformation of MYP3 cells, an anchorage-dependent nontumorigenic rat bladder epithelial cell line. MYP3 cells pretreated with or without MNU were exposed to H2O2 (0.001 to 0.1 mM) daily for 1 week in monolayer culture and were then tested for growth in soft agar. A marked increase in colony numbers was observed in the cells that were MNU-initiated and exposed to H2O2 (P < 0.01). Furthermore, H2O2 exposure alone at 0.01 mM or 0.1 mM caused colony formation in soft agar. The transformants induced by MNU plus H2O2 or H2O2 alone formed high-grade transitional cell carcinomas when injected into nude mice. The growth of these transformants was stimulated by several cytokines (interleukin 1α, interleukin 6, and tumor necrosis factor-α) better than the parental cells both on a plastic surface and in soft agar. Our results indicate that H2O2 causes genetic change(s) to induce tumorigenic conversion in urothelial cells and that the transformants are stimulated to grow because of their selective response to several cytokines. We suggest that these mechanisms may be involved in the in vivo carcinogenesis associated with chronic urinary tract infection.

INTRODUCTION

Chronic infection/inflammation has been implicated in the pathogenesis of several forms of cancer, including gastric carcinoma associated with atrophic gastritis (1) and colon carcinoma associated with ulcerative colitis (2). Squamous cell carcinomas developing in the draining sinuses of chronic osteomyelitis are also attributed to chronic inflammation (3). Both epidemiological (4, 5) and animal studies (6-8) indicate that urinary tract infection is a significant risk factor for the development of bladder cancer. Although the risk is associated most significantly with chronic infection, it also increases with the number of episodes of acute cystitis andmultiples with tobacco smoking (4, 5). Patients who are paraplegic secondary to spinal cord injury (9) and individuals who are chronically infected or LPS-treated HTBs (15). These observations suggested the possibility that reactive oxygen intermediates and cytokines released during the inflammatory process were implicated in the enhancement by KEC or LPS-treated HTBs (15). These observations suggested the possibility that reactive oxygen intermediates and cytokines released during the inflammatory process were implicated in the enhancement by KEC or LPS of MNU-initiated rat bladder carcinogenesis. Reactive oxygen intermediates including H2O2 are known to act as carcinogens, cause mutations, and induce the expression of proto-oncogenes (16-19).

Recently, we reported that IL-6 provides a selective growth advantage to MNU-exposed rat urothelial cells in vitro (15). We now report that exposure in vitro to H2O2 and several types of cytokines enhances transformation of a nontumorigenic rat urothelial cell line.

MATERIALS AND METHODS

Cells and Cell Culture. MYP3 was obtained from a small nodule that developed in an HTB treated with MNU. It is a hyperdiploid (44, XY, +1, +7) cell line that maintains characteristics of epithelial cells in culture. The cells express keratin 5 mRNA and form neither tumors in nude mice nor colonies in soft agar (20). The cells were grown in Ham's F-12 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10 μM nonessential amino acids (Life Technologies, Inc.), 2.7 mg/ml dextrose (Sigma Chemical Co., St. Louis, MO), 1 μg/ml hydrocortisone (Sigma), 5 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml epidermal growth factor, 100 μg/ml streptomycin, and 100 units/ml penicillin (Life Technologies, Inc.) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. When supplemented with 10% FCS (Life Technologies, Inc.), this medium was designated as complete medium.

In Vitro Transformation of MYP3 Cells. MYP3 cells were seeded on 24-well plates containing the complete medium at a density of 5 × 10^4 cells/well (Fig. 1A). Twenty-four hours after plating, the medium was replaced with serum-free Ham's F-12 medium containing MNU (Sigma) at the concentration of 50 μg/ml (15). After 1-h exposure to MNU with rocking at 10 cycles/min, the cells were washed twice with the medium without serum. After a 24-h culture in the complete medium, cells were exposed to H2O2 (0 to 0.1 mM) with daily exchange of the H2O2-containing complete medium. This dose range was selected because the concentrations above this range were found to be highly cytotoxic (Fig. 2). Once a week, cells were harvested from individual wells, and the same number of cells (5 × 10^5/well) were passaged individually up to three times. After 1–4 weeks exposure to H2O2, cells derived from each well were seeded separately at 5 × 10^5 cells/35-mm dish in 2 ml of 0.3% Noble agar (Difco, Detroit, MI) in the complete medium. This suspension was layered over 2 ml of 0.6% agar in the complete medium in 35-mm dishes. The cells were incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C. The doses of H2O2 used were based on a preliminary study. On days 9 and 18,
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37 days taken up by cells during 4 h culture was dissolved in DMSO (100 ml/well), and its absorbance at 495 nm was read on an automated microplate reader (Bio-Tec, Winooski, VT; Ref. 21). A preliminary study with the MTT assay showed that absorbance was directly proportional to the number of cells.

Tumorigenicity in Athymic Nude Mice. After trypsinization, cells were washed twice with Ham’s F-12 medium without serum and resuspended in 0.2 ml of the medium without serum or in 0.2 ml of 50% Matrigel (Collaborative Research, Bedford, MA) diluted with the same medium. Groups of six male athymic BALB/c nude mice (Harlan-Sprague-Dawley, Inc., Indianapolis IN) received 5 × 10⁶ cells injected s.c. at each dorsal flank. The mice were monitored twice a week for the development of tumors and were killed after 11–13 weeks. Tumors at the inoculation sites were removed and examined microscopically.

Statistical Analysis. Statistical analysis was performed with a one-way ANOVA test; P < 0.05 was considered significant.

RESULTS

Cytotoxicity of H₂O₂ to MYP3 Cells. MYP3 cells (2.5 × 10⁶ cells/well) were plated on a 96-well plate in the complete medium, and 24 h later, H₂O₂ was added at seven different concentrations (10⁻³ to 10² mM). Forty-eight h later, viable cells were counted by MTT assay. A small but significant decrease in cell count was observed with concentrations up to 0.1 mM (P < 0.05). Concentrations above 1 mM caused a drastic decrease in viable cell count (Fig. 2). Thus, the concentrations up to 0.1 mM were used in the subsequent experiments.

Effect of H₂O₂ on Colony Formation in Soft Agar by MYP3 Cells Pretreated with or without MNU. A marked increase in colony numbers was observed with MYP3 cells that were pretreated with MNU and were exposed to H₂O₂ for 1 week (309% of control at the H₂O₂ concentration of 0.01 mM, and 355% of control at 0.1 mM; Fig. 1B). Furthermore, MYP3 control cells treated with higher concentrations of H₂O₂ alone (0.01 mM or 0.1 mM) formed colonies in soft agar (Fig. 1B).

Next, we examined the effect of the duration of H₂O₂ exposure on colony formation in soft agar. MNU-initiated cells were exposed to 0.1 mM of H₂O₂ daily for 1–4 weeks, and then the cells were cultured in soft agar. Significantly increased colony counts were observed in proportion to the length of H₂O₂ exposure as compared with the group not exposed to H₂O₂ (P < 0.001; Fig. 3, group 2 versus group 4). MYP3 cells treated with H₂O₂ alone formed an increased number of colonies in proportion to the duration of H₂O₂ treatment (Fig. 3, group...
Table I Tumorigenicity in athymic nude mice of MYP3 and its transformants

<table>
<thead>
<tr>
<th>Cells</th>
<th>Matrigel(—)</th>
<th>Matrigel(+)</th>
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<tbody>
<tr>
<td>MYP3</td>
<td>0/6</td>
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<tr>
<td>P3MH-1</td>
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<td>P3M-3</td>
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*a Cells designated as P3MH-1, -2, and -3 were treated with MNU and H2O2; P3H-1, -2, and -3, with H2O2 only; and P3M-1, -2, and -3, with MNU only.

*b Cells (5 x 10⁶) were injected s.c. in dorsal flanks of nude mice with or without 50% Matrigel. The mice were killed after 11-13 weeks.

Fig. 4. Growth potential on plastic surface of MYP3 transformants induced by MNU and/or H2O2. Cell lines designated as P3MH-1, -2, and -3 were derived from group 2 in Fig. 3, which was exposed to both MNU once and H2O2 for 4 weeks; P3H-1, -2, and -3 from group 3, which was exposed only to H2O2 for 4 weeks; and P3M-1, -2, and -3 from group 4, which was exposed only to MNU once. Cell (1 x 10⁶) were seeded onto 96-well plates in the complete medium. Twenty-four h later, cells were cultured in F-12 medium containing 0.1% FCS. After 1-4 days of culture, cell proliferation was assessed by the MTT assay. Only the results after 96 h culture are shown. Bars, SD of triplicate samples.

3. Untreated MYP3 cells formed no colonies (Fig. 3, group I). After completion of the 4-week study, clonal growth was established from three randomly selected colonies from each of groups 2-4. Cells designated as P3MH-1, -2, and -3 were derived from group 2 (exposed to both MNU once and H2O2 for 4 weeks); P3H-1, -2, and -3 from group 3 (exposed only to H2O2 for 4 weeks); and P3M-1, -2, and -3 from group 4 (exposed only to MNU once). These clones were used in the following experiments.

Growth Potential of MYP3 Transformants Induced by MNU and/or H2O2. The growth potential of the nine MYP3 transformants on a plastic surface was examined. Cells (1 x 10⁶) were seeded onto 96-well plates in the complete medium. After 24 h, test medium containing 0.1% FCS was added. When examined by the MTT assay 96 h later, all of the transformants demonstrated higher cell counts than did the parental cells (119 to 278% of the parental cells; P < 0.05-0.001; Fig. 4). When the transformants were examined at 24, 48, and 72 h, similar responses were observed (data not shown).

Tumorigenicity of MYP3 Transformants In Athymic Nude Mice. The nine MYP3 transformants (5 x 10⁶ cells) induced by MNU and/or H2O2 as described above were injected s.c. in the dorsal flanks of nude mice with or without 50% Matrigel. Neither the parental MYP3 cells nor cell lines treated with MNU alone formed tumors. (In a study conducted recently, neither type of cells formed tumors under similar experimental conditions when observation was extended to 30 weeks.) In contrast, all clones exposed to H2O2, irrespective of MNU treatment, formed tumors with or without Matrigel (Table I). They were high-grade transitional cell carcinomas with foci of squamous differentiation.

Effect of Cytokines on Growth of MYP3 Transformants on Plastic Surface and in Soft Agar Culture. The effect of IL-1α, IL-6, IL-8, and TNF-α on the growth of MYP3 and its transformants was examined. Individual cytokines were added to wells at a final concentration of 0.01, 0.1, 1, 10, or 100 ng/ml. Forty-eight h later, the number of cells was assessed by the MTT assay. In all cell lines tested, IL-1α and IL-8 demonstrated no significant effect either on stimulation or inhibition of cell growth (data not shown). All transformants responded to IL-6 better (183 to 240% of the respective untreated controls; P < 0.001) than did the parental cells (157% of

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untreated controls; \( P < 0.05 \) when 100 ng/ml of IL-6 were added (Fig. 5A). All of the transformants except for P3M-2 also responded to TNF-\( \alpha \) (100 ng/ml) better (182 to 213% of the respective untreated controls; \( P < 0.01 \) to 0.001) than did the parental MYP3 cells (131% of untreated controls; \( P < 0.05 \); Fig. 5B). In the soft agar culture (Fig. 6), all transformants responded to IL-6 (100 ng/ml) with a significant increase in colony number (198–315% of the respective untreated controls; \( P < 0.001 \)) and size. IL-1a slightly stimulated the growth of all transformants (117–149% of the respective untreated controls; \( P < 0.01 \) to 0.001) than did the parental MYP3 cells (131% of untreated controls). IL-8 was ineffective with all transformants. The P3H-1) responded (117–167% of the respective untreated controls; \( P < 0.05 \); Fig. 5B). In the soft agar culture (Fig. 6), all transformants responded to IL-6 (100 ng/ml) with a significant increase in colony number (198–315% of the respective untreated controls; \( P < 0.001 \)) and size. IL-1a slightly stimulated the growth of all transformants (117–149% of the respective untreated controls; \( P < 0.01 \) to 0.001). Interestingly, the response to TNF-\( \alpha \) differed among the transformants; only cells treated with H\(_2\)O\(_2\) (P3MH-1 and P3H-1) responded (117–167% of the respective untreated controls; \( P < 0.05 \)–0.001). IL-8 was ineffective with all transformants. The parental cells did not form colonies, whether or not cytokines were present.

**DISCUSSION**

In our previous studies, we showed that continuous inflammatory stimuli induced by KEC or LPS strikingly accelerated MNU-initiated rat urinary bladder carcinogenesis (12–14). The tumors induced were different from those in the control group; a varying number of acute and chronic inflammatory cells were present within tumors (12). Moreover, we demonstrated an increased amount of H\(_2\)O\(_2\) (13) and several cytokines in the bladder aspirates (15). We hypothesized that reactive oxygen intermediates including H\(_2\)O\(_2\), which are known to cause DNA damage and tumorigenic conversion of a variety of cells (16–19, 22–25), and several cytokines released by inflammatory cells are responsible for the tumor formation.

To test the hypothesis, we conducted a series of experiments with MYP3, a nontumorigenic, anchorage-dependent rat urothelial cell line. The pertinent findings were that: (a) exogenous H\(_2\)O\(_2\) was a potent transformation-inducing agent for MYP3 cells. The transformants have acquired the potential of anchorage-independent growth and tumorigenicity in nude mice. MNU alone, at the dose level tested, was sufficient to induce anchorage-independent growth potential but was insufficient to produce tumors in vivo; (b) H\(_2\)O\(_2\) treatment did accelerate the colony formation by MNU in a dose-dependent manner; (c) the response to several cytokines has been altered in transformants. All transformants responded to IL-6 with an accelerated growth rate on a plastic surface and in soft agar cultures. The findings are consistent with our earlier observations (15). Of considerable interest and importance is that cells transformed by H\(_2\)O\(_2\) treatment (Fig. 4 and 5) exhibit an altered response to two additional cytokines; TNF-\( \alpha \), which only marginally stimulated parental cells in plastic surface culture, exhibited a strong stimulatory effect on transformants treated with H\(_2\)O\(_2\) alone or combined MNU and H\(_2\)O\(_2\). A stimulatory effect by TNF-\( \alpha \) was also demonstrated in soft agar assay with the cells transformed by H\(_2\)O\(_2\) (P3MH-1 and P3H-1). Although small, growth in soft agar of these two cells were stimulated by IL-1a. Such an altered response to cytokines has also been reported in other organ systems (26). It is clear that it may provide a selective growth advantage for transformed cells in vivo.

Extrapolating the present in vitro observation to the in vivo results we reported earlier (12–14), we suggest that: (a) the enhancement of bladder tumorigenesis by KEC or LPS is mediated by reactive oxygen intermediates and several cytokines, both of which are generated in inflammatory reaction; (b) H\(_2\)O\(_2\) by itself is a potent transformation-inducing agent; (c) H\(_2\)O\(_2\) can accelerate the action of a chemical carcinogen; and (d) transformed cells have acquired growth advantage over the nonneoplastic cells because of their positive response to stimulatory action by several cytokines including IL-1a, IL-6 and TNF-\( \alpha \) (Fig. 7).

The present study clearly demonstrates that H\(_2\)O\(_2\) acts as a carcinogen. Reactive oxygen intermediates have been reported to induce single-strand breaks in cellular DNA, oxidation of DNA bases, chromosomal aberrations, and DNA-protein cross-links (24, 27, 28). Furthermore, it was demonstrated that oxidative stress induced proto-oncogenes including c-fos, c-myc and c-jun (17, 29). Nevertheless, it is currently unclear as to which gene(s) is a target(s) for transformation of nontumorigenic urothelial cells induced by reactive oxygen intermediates including H\(_2\)O\(_2\). Our future investigation will attempt to detect the gene(s) involved in tumorigenic conversion of MYP3 cells.

**REFERENCES**

TRANSFORMATION BY H$_2$O$_2$ IN VITRO


Transformation *in Vitro* of a Nontumorigenic Rat Urothelial Cell Line by Hydrogen Peroxide

Masato Okamoto, Koji Kawai, Catherine A. Reznikoff, et al.


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