Tumorigenic Conversion of a Rat Urothelial Cell Line by Human Polymorphonuclear Leukocytes Activated by Lipopolysaccharide

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Chronic inflammation is a significant risk factor for the development of urinary bladder cancer. We have shown that inflammation induced by killed Escherichia coli and also by its lipopolysaccharide (LPS) strikingly enhances N-methyl-N-nitrosourea (MNU)-initiated rat bladder carcinogenesis. Aspirates from the bladder lumen contained a large quantity of hydrogen peroxide (H2O2) and several cytokines. In this study, we tested the hypothesis that reactive oxygen intermediates (ROI) released from activated polymorphonuclear leukocytes (PMN) are involved in inflammation-associated bladder carcinogenesis. Using an immortalized nontumorigenic rat urothelial cell line, MYP3, we examined the effect of LPS-activated PMN on malignant transformation. MYP3 cells pretreated with or without MNU were exposed daily to LPS-activated PMN for one week and were then tested for growth in soft agar. In contrast to no colony formation by the parental cells, a varying number of colonies developed from cells treated with LPS-activated PMN. Although combined treatment with MNU and PMN was most effective (P<<<<0.01), cells treated with LPS-activated PMN alone also formed a small number of colonies. Addition of catalase, which decomposes H2O2, and/or an antioxidant, α-tocopherol, reduced the number of colonies induced by LPS-activated PMN (P<<<<0.05). Cells derived from colonies were tumorigenic in athymic nude mice. However, tumorigenicity in mice was greater with cells treated with both MNU and PMN than with cells treated with PMN alone. Our results suggest that ROI released from LPS-activated PMN may be one of the mechanisms involved in the carcinogenesis associated with active urinary tract infection.

Key words: Transformation — PMN — H2O2 — LPS — Urothelial tumor

Chronic inflammation is associated with the development of urinary bladder cancer. Animal studies indicate that urinary tract infection is a significant risk factor for the development of bladder cancer. Although the risk is most significantly associated with chronic infection, it also increases with the number of episodes of acute cystitis and multiplies with tobacco smoking.11 Our investigation on the role of chronic inflammation in urinary bladder carcinogenesis was facilitated greatly by the availability of an in vivo model, the heterotopically transplanted rat urinary bladder in male Fischer 344 rats, which was developed in our laboratory for investigating tumor promotion by urine components.12 Using this model, we demonstrated that repeated instillation into the bladder lumen of either killed Escherichia coli or its endotoxin, lipopolysaccharide (LPS), strikingly enhanced bladder tumorigenesis initiated with a single dose of N-methyl-N-nitrosourea (MNU).11,12,15 The marked enhancement of tumorigenesis by killed E. coli treatment was closely associated with migration of polymorphonuclear leukocytes (PMN) into the urothelium and also into the lumen of the bladder. The inflammatory response was associated with an increase in the hydrogen peroxide (H2O2) concentration and several cytokines (IL-1α, IL-6, and tumor necrosis factor-α) in the bladder lumen.12 These observations in vivo suggested the possibility that reactive oxygen intermediates (ROI) and cytokines released during the inflammatory response might contribute to carcinogenesis. When stimulated by LPS, PMN generate a large quantity of H2O2 and ROI as a source of ROI causes DNA damage and induces formation of 8-hydroxydeoxyguanosine (8-OHdG), an indicator of oxidative DNA damage.18 8-OHdG has been shown to increase in the DNA of target tissue, and increased oxidative stress is directly related to increased tumor development.19,20 Furthermore, an immortalized rat bladder epithelial cell line, MYP3 was transformed with H2O2 of either an exogenous or an endogenous source.21,22 We have also shown

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Abbreviations used: PMN, polymorphonuclear leukocytes; LPS, lipopolysaccharide; MNU, N-methyl-N-nitrosourea; H2O2, hydrogen peroxide; 8-OHdG, 8-hydroxydeoxyguanosine; ROI, reactive oxygen intermediates; FBS, fetal bovine serum; dG, deoxyguanosine; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism.
that TNF-α released in inflammation can induce malignant transformation in MYP3 cells. The present investigation was designed to test the hypothesis that PMN, when stimulated by LPS, can generate ROI and induce malignant transformation in MYP3 cells. Formation of 8-OHdG was measured to assess DNA damage by activated PMN.

MATERIALS AND METHODS

Cell and cell culture MYP3 is an immortalized urothelial cell line established in our laboratory from a rat bladder treated with MNU. It maintains the characteristics of epithelial cells in culture, is anchorage-dependent for growth, and is nontumorigenic in athymic nude mice. MYP3 cells were grown in Ham’s F-12 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10 μM non-essential amino acids (GIBCO), 2.7 mg/ml of dextrose (Sigma Chemical Co., St. Louis, MO), 1 μg/ml of hydrocortisone (Sigma), 5 μg/ml of transferrin (GIBCO), 10 μg/ml of insulin (GIBCO), 10 ng/ml of epidermal growth factor (GIBCO), 100 μg/ml of streptomycin (GIBCO), and 100 U/ml of penicillin (GIBCO). When supplemented with 10% fetal bovine serum (FBS) (GIBCO), the medium was designated as complete medium. Cells were incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Preparation of human PMN PMN were isolated from heparinized peripheral blood collected by venipuncture from healthy volunteers. Blood was diluted 1:1 (v:v) in Dulbecco’s phosphate-buffered saline (GIBCO) and layered on Ficol-Paque PLUS solution (Amersham Pharmacia Biotech, Piscataway, NJ). After centrifugation at 400 g for 30 min at room temperature, the cell pellet was collected, and PMN were recovered from the cell pellet by further sedimentation with 3% dextran (Sigma). Erythrocytes were removed from the pellet by hypotonic lysis. PMN were shown to be more than 98% pure by morphologic examination after Giemsa staining.

Measurement of 8-OHdG MYP3 cells were seeded in the outer well of a 6-well plate containing the complete medium at a density of a 5.0×104 cells/well. Twenty-four hours after plating, the medium was replaced with serum-free F-12 medium containing MNU (50 μg/ml, Sigma). After 1 h of exposure to MNU or vehicle (DMSO) only, the cells were washed twice with plain medium. After 24 h culture in the complete medium, PMN (4.0×106 cells/well) alone, LPS (50 μg/ml) alone, or PMN and LPS in combination suspended in the complete medium were added to the inner well (Fig. 1). The content of the inner well was changed daily. After 7 days of treatment (Fig. 2A), cells derived from each well were seeded separately at 5.0×104 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% Noble agar. The cells were incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C. On days 9, and 18, 0.5 ml of 0.3% agar in the complete medium was added. After 28 days, colonies consisting of more than 20 cells were counted.

In an attempt to neutralize H2O2 produced by LPS-treated PMN, MYP3 cells were cultured in the presence of LPS and PMN with or without 50 μM α-tocopherol and/or 250 U/ml of catalase added to the inner well (Fig. 1). Cells were then grown in soft agar as described above, and colonies were counted after 28 days.

Measurement of H2O2 We modified the colorimetric method described by Pick and Keisari. PMN were seeded on 24-well plates (Becton Dickinson, Franklin Lakes, NJ) containing phenol red solution (Hanks’ balanced salt solution, 100 μg/ml of phenol red (Sigma), 50 μg/ml of horseradish peroxidase (Sigma)) at a density of 1.0×106 cells/well with or without 0.1, 1, 10, 50, or 100 μg/ml of E. coli-derived LPS (Sigma), 5 or 50 μM α-tocopherol (Sigma) in DMSO (Sigma), and 100, 250, or 500 U/ml of catalase (Sigma). After 1 and 6 h, the medium was centrifuged at 14,000 rpm, and 1 ml of supernatant was mixed with 10 ml of 1 N NaOH. Absorbance was read at 610 nm in a spectrophotometer.

In vitro transformation of MYP3 cells An in vitro transformation assay was performed in a double chamber (Falcon) as illustrated in Fig. 1. MYP3 cells were seeded in the outer well of a 6-well plate containing the complete medium at a density of a 5.0×104 cells/well. Twenty-four hours after plating, the medium was replaced with serum-free F-12 medium containing MNU (50 μg/ml, Sigma). After 1 h of exposure to MNU or vehicle (DMSO) only, the cells were washed twice with plain medium. After 24 h culture in the complete medium, PMN (4.0×106 cells/well) alone, LPS (50 μg/ml) alone, or PMN and LPS in combination suspended in the complete medium were added to the inner well (Fig. 1). The content of the inner well was changed daily. After 7 days of treatment (Fig. 2A), cells derived from each well were seeded separately at 5.0×104 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% Noble agar. The cells were incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C. On days 9, and 18, 0.5 ml of 0.3% agar in the complete medium was added. After 28 days, colonies consisting of more than 20 cells were counted.

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Measurement of 8-OHdG MYP3 cells were seeded in the outer well of a 6-well plate containing the complete medium at a density of a 2.0×104 cells/well. Twenty-four hours after plating, the cells were cultured for 1 h in the presence of LPS (50 μg/ml) alone, or PMN (4.0×106 cells/well) and LPS in combination with or without 50 μM of α-tocopherol and/or 250 U/ml of catalase, which were added to the inner chamber containing Dulbecco’s phosphate-buffered saline (Fig. 1). After 1 h of treatment, DNA was extracted from MYP3 cells with the use of DNA extractor WB kits (Wako Chemical, Richmond, VA). DNA was dissolved in 20 mM sodium acetate (pH 5.0) under argon gas, digested to nucleotides by nuclease P1 (Sigma)
LPS-activated PMN on Urothelial Cells

at 65°C for 10 min, and treated with calf intestinal alkaline phosphatase (Promega, Madison, WI) at 37°C for 60 min. The content of 8-OHdG in the digested DNA and the total amount of deoxyguanosine (dG) were measured by an electrochemical detector coupled with reverse-phase high-performance liquid chromatography (ESA, Inc., Chelmsford, MA) and a UV detector (ESA). The content of 8-OHdG was expressed as the ratio of 8-OHdG × 10^5 to total dG.

**Tumorigenicity in athymic nude mice** Cells (5.0×10^5) were injected s.c. in each dorsal flank of male athymic BALB/c nude mice (Harlan Sprague-Dawley, Indianapolis, IN) suspended in 0.2 ml of 50% Matrigel (Collaborative Research, Bedford, MA) diluted with serum-free medium. The mice were monitored twice a week for the development of tumors and were killed 36 weeks after inoculation. Tumors were excised and submitted for histologic examination.

**Analysis of H-ras, K-ras, and p53 gene mutation**

Because of a recent report of activation of H- and K-ras genes by PMN, possible mutations of the H-ras, K-ras, and p53 genes were examined by the polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) method. The oligonucleotide primers up- and downstream, respectively, are shown below (bp of amplified fragments):

### H-ras:

- exon 1: 5'-ATGACAGAATACAAGCTTGT-3' and 5'-CTCACCTCTATAGGGATC-3' (116 bp)
- exon 2: 5'-AGGACCTTAAAGCTGTTC-3' and 5'-GACTTGTTGTTGATGCGC-3' (184 bp)

### K-ras:

- exon 1: 5'-CTCTGCTGAAAAATGACTGAGT-3' and 5'-GCAGCATTTACCTCTAC-3' (125 bp)
- exon 2: 5'-TCCTACAGGAAAACAGTAG-3' and 5'-ATGGCAATACACAAAGAGCC-3' (137 bp)


**RESULTS**

**H₂O₂ production by LPS-activated PMN** For determination of the minimal dose of LPS that maximally stimulates H₂O₂ production, PMN (1.0×10⁶ cells/well) were treated with LPS at various concentrations for 1, 2, and 6 h, and H₂O₂ released into the medium was measured. When PMN were treated with 50 and 100 µg/ml of LPS, the release of H₂O₂ was maximal after 2 h of incubation (Fig. 3). Because 100 µg/ml of LPS was markedly cytotoxic to MYP3 cells, we used 50 µg/ml of LPS in the subsequent experiment.

**Measurement of 8-OHdG by LPS-activated PMN** The content of 8-OHdG in cells untreated and cells treated with LPS alone was 0.59 and 0.49 (8-OHdG/10⁵ dG), respectively. On the other hand, that in cells treated with LPS-activated PMN was 1.47 (8-OHdG/10⁵ dG), which was a 2.5-fold increase over the untreated control. The content of 8-OHdG enhanced by the treatment with LPS-activated PMN was reduced to 75, 71 and 81% by addition of α-tocopherol, catalase, or their combination, respectively.

**Effect of LPS-activated PMN on colony formation by MYP3 cells** We examined the effect of LPS-activated PMN on the transformation of MYP3 cells. Combination treatment with MNU, LPS, and PMN was most effective in inducing colonies, as compared with other treatments (P<0.01) (Fig. 2B). It is to be noted that colonies were also induced by treatment with PMN and LPS and without carcinogen. After completion of the soft-agar assay, clonal growth was established from 2 to 3 randomly selected colonies from each of groups 1, 4, and 5 shown in Fig. 2B. Cell lines designated as PLM-1 and -2 were established from group 1 (exposed to MNU, PMN, and LPS); M-1 and M-2 from group 4 (exposed only to MNU); and PL-1 and -2 from group 5 (exposed to PMN and LPS). These clones were used in the following experiments.

**Effect of α-tocopherol and catalase on H₂O₂ release by LPS-activated PMN and on transformation of MYP3 cells** We examined the effect of α-tocopherol and catalase on H₂O₂ release into the medium by LPS-activated PMN. The level was reduced by the addition of α-tocopherol, catalase, and their combination in a dose-dependent manner after both 1 and 6 h of treatment (groups 3–9, P<0.005; group 10, P<0.05; respectively, as compared with group 2, Fig. 4). The inhibitory effects were, however, maximal after 1 h, and further incubation had little additional effect (only the 1 h data are shown) (Fig. 4). Because catalase was cytotoxic when tested at the highest concentration, we chose 50 µM α-tocopherol and 250 U/ml of catalase in the subsequent experiment.

The number of colonies induced by LPS-activated PMN (group 2) was reduced by addition of α-tocopherol, catalase, or their combination (groups 3, 4, and 5) (P<0.05,
The colony formation initiated by MNU alone (group 1) was not affected by addition of \( \alpha \)-tocopherol, catalase, or their combination (groups 6, 7, and 8).

**Tumorigenicity of transformants in athymic nude mice**

The tumor yield was highest in the group receiving the PLM clones (Table I). Both clones induced tumors in 4 of 6 mice, and the tumors ranged in size from 3×2 to 10×5 mm. Microscopically, they were squamous cell carcinomas with desmoplastic stroma (Fig. 6A). Although the tumors were in general poorly differentiated, keratin cyst formation was observed in 2 of 4 tumors derived from each clone. The mitotic frequency was 1 to 2 per high-power field. Both PL-1 and PL-2 clones yielded a small tumor in one of 6 recipients. The tumor cells were confined within Matrigel, arranged in small strands or nests, and were bounded by dense desmoplastic stroma (Fig. 6B). Mitotic figures were absent, and apoptotic bodies were present. One of the tumors showed pseudoglandular architecture focally. One of the M clones (M-1) gave rise to a small tumor in 2 of 6 mice. These tumors resembled those in the PL tumors in that their growth was confined within Matrigel. Small, uniform-sized tumor cells were arranged in single file (Fig. 6C). Pseudoglandular or microcystic architecture and mitotic figures were absent. The tumors of the M-1, PL-1, and PL-2 clones appeared far less aggressive in their growth potential than did tumors of PML clones.

**Analysis of H-ras, K-ras, and p53 gene mutation**

Mutation involving exons 1 and 2 of the H-ras and K-ras genes, and exons 5–8 of the p53 gene, was not detected in
any of the cell clones used for the in vivo tumorigenicity assay.

**DISCUSSION**

We previously showed that continuous inflammatory stimuli induced by KEC (killed *E. coli*) or LPS treatment strikingly accelerated MNU-initiated rat urinary bladder carcinogenesis. Because PMN were found in the bladder mucosa and because aspirates from the bladder lumen contained H$_2$O$_2$ and several cytokines at high concentrations, we hypothesized that ROI including H$_2$O$_2$ and several cytokines were involved in tumor formation. Two inflammation-associated cytokines and H$_2$O$_2$ were tested: IL-6 was found to be a potent growth factor in stimulating the growth in vitro of MYP3-derived tumor cell lines, but not of the parental cell line MYP3. Tumor necrosis factor-$\alpha$, on the other hand, was found to be a potent mutagen by generating H$_2$O$_2$ in vitro. H$_2$O$_2$, when given exogenously as well as when generated intracellularly by transfection of cells with an H$_2$O$_2$-generating expression vector in vitro, was found to be a potent inducer of malignant phenotype, yielding poorly differentiated transitional to squamous carcinoma in athymic nude mice.

The present investigation was an extension of our previous work and was designed to test the hypothesis that PMN are indeed a source of ROI involved in tumor formation. To assess the generation of ROI and DNA damage, we measured H$_2$O$_2$ released by activated PMN and the content of 8-OHdG in MYP3 cells. We observed that a) H$_2$O$_2$ release was stimulated strikingly by LPS, as was reported by other investigators; b) LPS-activated PMN enhanced the level of 8-OHdG and accelerated colony formation by MNU; c) PMN alone were likewise mutagenic; and d) catalase, which decomposes H$_2$O$_2$, and $\alpha$-tocopherol, an antioxidant, reduced H$_2$O$_2$ release, the level of 8-OHdG, and colony formation by LPS-activated PMN.

The present investigation clearly supports the contention that acute inflammation in the bladder can generate ROI in the presence of *E. coli* (or its LPS) and can induce DNA damage and malignant transformation in urothelial cells. ROI have been reported to induce single-strand breaks in cellular DNA, oxidation of DNA bases, chromosomal aberration, and DNA-protein cross-links. Because of the report that stimulated human leukocytes cause activating mutations in the *K-ras* proto-oncogene, we examined our transformed cells for the presence of mutation in the *H-ras*, *K-ras*, and *p53* genes. None was found. It remains to be determined which gene(s) is the target of transformation by ROI generated by PMN.

It is to be noted that, in the present investigation, LPS-activated PMN alone were only weakly tumorigenic in MYP3 cells and that the tumorigenic potential of the trans-
formed cells was far less than that of transformants induced by the combination of MNNU and LPS-activated PMN. The finding indicates that the tumorigenicity associated with acute and chronic urinary tract infection is the sum of various causative factors involved in the inflammation process.

REFERENCES

1) Cerutti, P. A. Oxidant stress and carcinogenesis. *Eur. J. Clin. Invest.* **21**, 1–5 (1991).

2) Correa, P. Human gastric carcinogenesis: a multistep and multifactorial process-first American Cancer Society award lecture on cancer epidemiology and prevention. *Cancer Res.* **52**, 6735–6740 (1992).

3) Di Biscaglia, A. M., Order, S. E., Klein, J. L., Waggoner, J. G., Sjogren, M. H., Kuol, G., Houghton, M., Choo, Q. L., and Hoofnagle, J. H. The role of chronic viral hepatitis in hepatocellular carcinoma in the United States. *Am. J. Gastroenterol.* **86**, 335–338 (1991).

4) Feig, D. I., Reid, T. M., and Hopp, M. L. *Oxidant stress and carcinogenesis.*

5) Preston-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A., and Oyasu, R. Persistence of carcinogen-altered cell population in rat urothelium which can be promoted to tumors by chronic inflammatory stimulus. *Cancer Res.* **54**, 2630–2632 (1994).

6) Klein, J. B., Payne, V., Schepers, T. M., and McLeish, K. R. Bacterial lipopolysaccharide enhances polymorphonuclear leukocyte function independent of changes in intracellular calcium. *Inflammation* **14**, 599–611 (1990).

7) Nathan, C. F. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *J. Clin. Invest.* **80**, 1550–1560 (1987).

8) Abu-Shakra, A. and Zeiger, E. Formation of 8-hydroxy-2-deoxyguanosine following treatment of 2-deoxyguanosine or DNA by hydrogen peroxide or glutathione. *Mutat. Res.* **390**, 45–50 (1997).

9) Ichinose, T., Yajima, Y., Nagashima, M., Takenoshita, S., Nagamachi, Y. and Sagai, M. Lung carcinogenesis and formation of 8-hydroxy-deoxyguanosine in mice by diesel exhaust particles. *Carcinogenesis* **18**, 185–192 (1997).

10) Shimoda, R., Nagashima, M., Sakamoto, M., Yamaguchi, N., Hirohashi, S., Yokota, J. and Kasai, H. Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. *Cancer Res.* **54**, 3171–3172 (1994).

11) Okamoto, M., Kawai, K., Reznikoff, C. A. and Oyasu, R. Transformation *in vitro* of a nontumorigenic rat urothelial cell line by hydrogen peroxide. *Cancer Res.* **56**, 4649–4653 (1996).

12) Okamoto, M., Reddy, J. K. and Oyasu, R. Tumorigenic conversion of a non-tumorigenic rat urothelial cell line by overexpression of H$_2$O$_2$ generating peroxisomal fatty acyl-CoA oxidase. *Int. J. Cancer* **70**, 716–721 (1997).

13) Okamoto, M. and Oyasu, R. Transformation *in vitro* of a nontumorigenic rat urothelial cell line by tumor necrosis factor-$

14) Jackson, J. H., Vollenweider, M., Hill, J., Rodriguez, H.,
Schwabacher, A. W., Mitra, G. and Kuo, C. Y. Stimulated human leukocytes cause activating mutations in the K-ras proto-oncogene. *Oncogene*, 14, 2803–2808 (1997).

27) Wang, D., Weghorst, C. M., Calvert, R. J. and Stoner, G. D. Mutation in the p53 tumor suppressor gene in rat esophageal papillomas induced by N-nitrosomethylbenzylamine. *Carcinogenesis*, 17, 625–630 (1996).

28) Chu, S., Huang, Q., Alvares, K., Yeldandi, A. V., Rao, M. S. and Reddy, J. K. Transformation of mammalian cells by overexpressing H$_2$O$_2$ generating peroxisomal fatty acyl-CoA oxidase. *Proc. Natl. Acad. Sci. USA*, 92, 7080–7084 (1995).

29) Nguyen, T., Brunson, D., Crespi, C. L., Penman, B. W., Wishnok, J. S. and Tannenbaum, S. R. DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. *Proc. Natl. Acad. Sci. USA*, 89, 3030–3034 (1992).