Enhancement of Transformation in Vitro of a Nontumorigenic Rat Urothelial Cell Line by Interleukin 6

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ABSTRACT

Chronic inflammation of the urinary tract is a significant risk factor for the development of bladder cancer. We have shown that acute and chronic inflammation induced by intravesical instillations of killed Escherichia coli strikingly enhances N-methyl-N-nitrosourea (MNU)-initiated rat bladder carcinogenesis. To test the hypothesis that cytokines released during inflammation may be involved in the enhancement of bladder carcinogenesis, we conducted an in vitro experiment. Using soft agar growth as an index of transformation, we examined the effect of inflammation-associated cytokines on the enhancement of MNU-initiated transformation of MYP3 cells, an anchorage-dependent nontumorigenic rat bladder epithelial cell line. In the first experiment, after 1-h exposure to MNU (50 μg/ml), cells (5 × 10^6) were grown in soft agar in the presence of interleukin (IL)-1α, IL-6, IL-8, or tumor necrosis factor-α (10 to 100 ng/ml). Colonies consisting of more than 20 cells were counted 4 weeks later. Among the cytokines tested, IL-6 (100 ng/ml) significantly increased colony counts over those for the untreated controls (P < 0.001). In the second experiment, the cells treated with MNU similarly as in the first experiment were cultured with or without IL-6 (100 ng/ml) for 1 week before the cells (5 × 10^6) were grown in soft agar in the presence or absence of IL-6. IL-6 pretreatment increased colony counts irrespective of subsequent IL-6 treatment (P < 0.05). Moreover, IL-6-stimulated anchorage-dependent growth of MNU transformants far exceeded that of the parental MYP3. However, among the transformants, there was no parallel relationship in response to IL-6 between anchorage-dependent and -independent growth. Our results suggest that IL-6 may provide a selective growth advantage to MNU-initiated bladder epithelial cells in vitro and that it may be a factor accounting for the marked enhancement of inflammation-associated rat bladder carcinogenesis.

INTRODUCTION

Chronic inflammation has been implicated in the pathogenesis of several forms of cancer, including gastric carcinoma after atrophic gastritis (1), colon carcinoma after ulcerative colitis (2), and squamous carcinoma in the draining sinus of chronic osteomyelitis (3). Both human 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 and multiples with tobacco smoking (4,5). Patients who are paraplegic secondary to spinal cord injury (9) or are infected with Schistosoma haematobium (10) are particularly vulnerable to the development of bladder cancer because of a close association with chronic urinary tract infection.

Our investigation on the role of chronic inflammation in urinary bladder carcinogenesis was facilitated greatly by the availability of an in vivo model, HTB, which was developed in our laboratory (11). We repeatedly instilled either KEC or its endotoxin, lipopolysaccharide, into HTBs in which carcinogenesis had been initiated by a single small dose of MNU (12–14). The Fischer 344 rats treated with KEC or lipopolysaccharide had a striking increase in the number of tumors, whereas only a few tumors developed in the controls receiving MNU or KEC alone. The marked enhancement by KEC treatment was closely associated with migration of polymorphonuclear leukocytes into the urothelium and of other inflammatory cells into the lamina propria. Additionally, we demonstrated the presence of several cytokines, including IL-1, IL-6, and TNF, in aspirates from KEC-treated HTBs. These observations suggested the possibility that cytokines released during the inflammatory process were implicated in enhancement of bladder carcinogenesis. We conducted the present experiment to determine whether exposure in vitro to several types of cytokines can enhance or induce anchorage-independent growth as an index of transformation in MNU-initiated MYP3 cells, a nontumorigenic rat urothelial cell line with a near-normal chromosomal constitution.

MATERIALS AND METHODS

Cells and Cell Culture. MYP3 is a hyperdiploid (44, XY, +1, +7) cell line in which maintains characteristics of epithelial cells in culture; the cells express a keratin (K5) mRNA by Northern blot analysis, do not form tumors in nude mice, nor colonies in soft agar (15). The cells were grown in Ham’s F-12 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10 μM nonessential amino acids (GIBCO), 2.7 mg/ml dextrose (Sigma Chemical Co., St. Louis, MO), 1 μg/ml hydrocortisone (Sigma), 5 μg/ml transferrin (GIBCO), 10 μg/ml insulin (GIBCO), 10 ng/ml epidermal growth factor (GIBCO), 100 μg/ml streptomycin, and 100 units/ml penicillin (GIBCO) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. When supplemented with 10% FCS (GIBCO), this medium was designated as complete medium.

MYU3L, a rat bladder carcinoma cell line which is tumorigenic and highly invasive, but not metastatic, and LMC19, another rat bladder carcinoma cell line which is tumorigenic and highly invasive and also metastatic (15), were grown in Ham’s F-12 medium supplemented with 5% FCS, 10 μM nonessential amino acids, 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Both of these cells form numerous colonies in soft agar (15).

Cell Growth Assay. Cells were seeded on a 96-well plate containing complete medium for 24 h. Then cells were cultured in a test medium containing 0.1% FCS with IL-1, IL-6, IL-8, or TNF (0 to 100 ng/ml) in flat-bottomed 96-well plates at 1 × 10^4 or 2.5 × 10^5 cells/well. After 1 to 4 days of culture, cell proliferation was assessed by the addition of 20 μg of the vital dye MTT (1 mg/ml Sigma) for 4 h. The blue dye taken up by the cells was dissolved in DMSO (100 ml/well), and its absorbance at 490 nm was read on an Automated Microplate reader (Bio-Tec, Winooski, VT; Ref. 16). A preliminary study with the MTT assay showed that there was no quantitative difference in absorbance between MYP3 and its MNU-induced transformants. Thus, we were able to use absorbance for estimating the number of cells without having to apply a specific correction.

Transformation of MYP3 Cells with MNU in Vitro. MYP3 cells were seeded on 24-well plates containing complete medium at a density of 5 × 10^4
cells/plate. Twenty-four h after plating, the medium was replaced with serum-free Ham’s F-12 medium containing MNU (Sigma) at three different concentrations (50 to 200 μg/ml). After 1-h exposure to MNU with rocking at 10 cycles/min, the cells were washed twice with the medium without serum and cultured with complete medium. MNU treatment once a week was repeated up to three times. Twenty-four h after the last MNU treatment, cells were seeded at a density of 5 × 10^5 cells/35-mm dish in 2 ml of 0.3% Noble agar (Difco, Detroit, MI) in complete medium. This suspension was layered over 2 ml of 0.6% agar in complete medium in 35-mm dishes. The cells were incubated in a humidified atmosphere of 95% air and 5% CO\textsubscript{2} at 37°C. One ml of 0.3% agar in complete medium was added at days 9 and 18. After 28 days, colonies of more than 20 cells were counted.

Experiment 1. Twenty-four h after 1-h exposure to MNU (50 μg/ml) once, MYP3 cells (5 × 10^5) were subjected to soft agar growth in the presence of recombinant mouse IL-1α (10 ng/ml; Genzyme, Boston, MA), recombinant mouse IL-6 (100 ng/ml; Genzyme), recombinant human IL-8 (10 ng/ml; Genzyme), or recombinant human TNF-α (100 ng/ml; Genzyme). These doses were selected based on the data derived from the growth assay with MYP3 cells (Fig. 1). On days 9 and 18, 1 ml of 0.3% agar in complete medium containing a test cytokine was added. After 28 days, colonies were counted.

Experiment 2. Twenty-four h after a 1-h exposure to MNU (50 μg/ml) once, MYP3 cells were cultured in complete medium with or without IL-6 (100 ng/ml) for 1 week, and then the cells (5 × 10^5) were grown in soft agar in the presence or absence of IL-6 (100 ng/ml). On days 9 and 18, 1 ml of the 0.3% agar medium containing IL-6 (100 ng/ml) was added. After 28 days, colonies were counted.

Isolation of Cytoplasmic RNA and Northern Blot Analysis. Cells grown in monolayers were harvested at early confluency. RNA was prepared by lysing of cells in hypotonic buffer containing NP40 (Sigma), followed by removal of nuclei (17). Cytoplasmic RNA (10 μg) was electrophoresed onto a formaldehyde/1.0% agarose gel and blotted onto a nylon filter (ICN Biomedicals, Irvine, CA; Ref. 18). The nylon filter was hybridized with \textsuperscript{32}P-labeled cDNA probe in 50% formamide, 5× saline-sodium phosphate-EDTA, 0.1% SDS, 5× Denhardt’s solution, and 100 μg/ml salmon sperm DNA at 42°C for 15 to 20 h. Extensive washing was done, twice in 1× SSC-0.1% SDS at room temperature for 10 min and once in 0.5× SSC-0.1% SDS at 65°C for 40 min. Subsequently, the filter was exposed to X-ray film with an intensifying screen at −70°C for autoradiography.

Preparation of a Probe for Rat IL-6 Receptor. Rat IL-6 receptor cDNA was prepared from mRNA of MYP3 cells by the reverse transcriptase-PCR method. The two nucleotide bases used were 5'-tggtgcagccggatccacctgcca-3' as an upstream primer, and 5'-cctggaagggcagctgctgt-3' as a downstream primer (19). One μg of total RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (GIBCO) at 42°C for 60 min in a 10-μl mixture with the downstream primer. One μl of reverse-transcribed mixture was subjected to the PCR in a 20-μl mixture [10 mm Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.01% gelatin, 20 mM each deoxynucleotide triphosphate (A, G, T, and C), 0.5 unit Taq polymerase (Cetus Perkin-Elmer, Norwalk, CT), and 0.25 pmol primer] (20). Forty cycles of reaction at 94°C, 55°C, and 72°C for 60, 90, and 150 s, respectively, were run in a DNA Thermal Cycler (Cetus Perkin-Elmer). The PCR products (434 bp) were subcloned into the pCRTM II vector with the use of a TA Cloning Kit (Invitrogen, San Diego, CA). The insert as a rat IL-6 receptor cDNA fragment was confirmed by nucleotide sequencing. The 453-bp EcoRI-EcoRI fragment containing rat IL-6 receptor cDNA was excised from the vector and was used as a probe.

**RESULTS**

Effect of IL-1, IL-6, IL-8, and TNF on Growth of MYP3 Cells in Monolayer Culture. IL-1, IL-6, IL-8, or TNF was added into individual wells at a final concentration of 1 × 10^−2, 1 × 10^−1, 1, 10, or 100 ng/ml. Forty-eight h later, the number of cells was assessed by MTT assay. IL-1 and IL-8 demonstrated neither an inhibitory nor a stimulatory effect at any concentration. TNF and IL-6 clearly promoted growth at 100 ng/ml (P < 0.01) and marginally at 10 ng/ml (P < 0.05). The cytokines at other concentrations demonstrated no significant effect (Fig. 2).

Transformation In Vitro of MYP3 Cells by MNU Treatment. The number of transformants increased in parallel with the frequency of MNU treatment (Table 1) but did not correlate with the concentration of MNU. The single treatment with 50 μg/ml of MNU induced 5 to 10 transformants/dish. Therefore, we used this concentration of MNU in the subsequent experiments.

Effect of Cytokines on Transformation of MYP3 Cells Pre-treated With or Without MNU. In experiment 1, after 1 h exposure to MNU (50 μg/ml) and subsequent 24 h culture in complete medium, cells (5 × 10^5) were grown in soft agar in the presence of IL-1, IL-6, IL-8, or TNF. IL-6 (100 ng/ml) significantly increased colony counts as compared to those for the PBS-treated controls (435% of control; P < 0.001; Fig. 3A). IL-1 (10 ng/ml) stimulated the growth slightly (147% of control; P < 0.05). In contrast, IL-8 (10 ng/ml) and TNF (100 ng/ml) were inhibitory. Because IL-6 was the most effective among the cytokines tested, we examined, in experiment 2, the possibility that MNU-induced DNA damage might be “fixed” by mitotic stimuli induced by IL-6. Twenty-four h after 1-h exposure to MNU, MYP3 cells were cultured in complete medium with or without IL-6 (100 ng/ml) for 1 week. Then the cells (5 × 10^5) were grown in soft agar in complete medium with or without IL-6 (100 ng/ml). As was demonstrated in experiment 1, IL-6 treatment during soft agar growth (Fig. 3B, Group 3) increased colony counts significantly (Fig. 3B,

Fig. 1. Experimental design for testing the effect of IL-1, IL-6, IL-8, and TNF on the growth of MYP3 cells. A, Experiment 1. MYP3 cells (5 × 10^5 cells/well) were seeded on 24-well plates ( ). Twenty-four h after plating, cells were exposed to MNU (50 μg/ml) for 1 h. Twenty-four h later, cells (5 × 10^5) were cultured in soft agar containing IL-1α (10 ng/ml), IL-6 (100 ng/ml), IL-8 (10 ng/ml), or TNF-α (100 ng/ml). After 28 days, colonies of more than 20 cells were counted (X). B, Experiment 2. 24 h after 1-h exposure to MNU ( ) or PBS ( ), cells were cultured in medium with or without IL-6 (100 ng/ml) for 1 week, and then the cells (5 × 10^5) were grown in soft agar in the presence or absence of IL-6 (100 ng/ml). After 28 days, colonies were counted. Clonal growth was established from representative colonies and was used in the experiment illustrated in Fig. 4.
Twenty-four h later, the medium was changed to a test medium containing 0.1% FCS to which IL-1, IL-6, IL-8, or TNF was added individually at a final concentration of (2.5 x 10^4/well) were seeded on a 96-well plate containing F-12 complete medium.

After completion of colony counting, clonal growth was established from three randomly selected colonies each of Fig. 3B, Groups 1—4, and one from Fig. 3B, Group 6. Cells designated as P3M6—1, -2, and -3 were derived from group 1; P3M6—4, -5, and -6 from group 2; P3M6—7, -8, and -9 from group 3; P3M6—10, -11, and -12 from group 4; and P3M6—13 from group 6. These clones were used in the following experiments.

Growth Potential and Response to IL-6 of MYP3 Transformants Induced by MNU and Subsequent IL-6 Treatment (P3M6s). The growth potential of the MYP3 transformants (P3M6s) on a plastic surface and in soft agar culture was examined. Cells (1 x 10^6) were seeded onto 96-well plates in complete medium. Forty-eight h later, cells were induced in the groups treated with IL-6 alone without prior MNU treatment (Fig. 3B, Groups 1—3). For example, most of P3M6—7 cells did not respond well to IL-6 in monolayer culture but responded very well in soft agar, whereas the response of P3M6—10 cells was opposite; the cells responded well to IL-6 in monolayer culture but marginally in soft agar. P3M6—12 cells responded to IL-6 equally well in both monolayer and soft agar cultures. The parental cells did not form colonies, whether or not IL-6 was present (Fig. 4C).

**DISCUSSION**

In previous reports, we showed that a continuous inflammatory stimulus induced by KEC or lipopolysaccharide strikingly accelerated colony number and size (Fig. 4, B and C). Interestingly, the degree of response to IL-6 in anchorage-independent growth was not parallel to that in anchorage-dependent growth (Fig. 4C). For example, P3M6—7 cells did not respond well to IL-6 in monolayer culture but responded very well in soft agar, whereas the response of P3M6—10 cells was opposite; the cells responded well to IL-6 in monolayer culture but marginally in soft agar. P3M6—12 cells responded to IL-6 equally well in both monolayer and soft agar cultures. The parental cells did not form colonies, whether or not IL-6 was present (Fig. 4C).

**Expression of IL-6 Receptor mRNA.** On Northern blot analysis, expression of IL-6 receptor mRNA was detected in all types of cells tested. The hybridization signal was most intense in MYP3 and MYP3 transformants (P3M6—7 and -12), moderate in MYU3L cells, and weak in LMC19 cells (Fig. 5). There was no difference in the expression level between MYP3 and its transformants (Fig. 5).

**Table 1 Colony-forming efficiency of MYP3 cells after MNU treatment**

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<th>Concentration of MNU (μg/ml)</th>
<th>Frequency of MNU treatment</th>
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<td>100</td>
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Group 3 versus Group 4, P < 0.001). IL-6 treatment for 1 week before soft agar growth strikingly increased colony numbers, irrespective of the subsequent IL-6 treatment (Fig. 3B, Group 1 versus Group 3, P < 0.05; Fig. 3B, Group 2 versus Group 4, P < 0.01). A few colonies were induced in the groups treated with IL-6 alone without prior MNU treatment (Fig. 3B, Groups 5, 6, and 7).

Effect of IL-6 on Rat Urinary Bladder Carcinoma Cell Lines MYU3L and LMC-19. To test whether high-grade aggressive rat bladder carcinoma cell lines also respond to IL-6, we exposed MYU3L and LMC-19 (1 x 10^6) seeded on 35-mm dishes to IL-6 (10 ng/ml) during soft agar growth. A statistically significant response was exhibited only by LMC19 cells (P < 0.01; data not shown).

In previous reports, we showed that a continuous inflammatory stimulus induced by KEC or lipopolysaccharide strikingly accelerated...
MNU-initiated rat urinary bladder carcinogenesis (12–14). The tumors induced were quite different from those seen in the control group; markedly dilated capillaries were abundant in the tumor stroma, and aggregates of polymorphonuclear leukocytes were frequently found within neoplastic epithelium and in association with focal necrosis. A varying number of chronic inflammatory cells were present in the tumor stroma (12). We hypothesized that the release of various cytokines was causally related to the enhancement of tumor formation. To elucidate the relationship, we tested several cytokines in an in vitro transformation system. A nontumorigenic, anchorage-dependent cell line of rat urothelial cells, MYP3, was used. A single brief exposure to MNU readily converted MYP3 cells from an anchorage-dependent to -independent phenotype, and IL-6 significantly increased the number and size of the colonies (P < 0.001). Other cytokines were either marginally effective (IL-1) or inhibitory (IL-8 and TNF). IL-6 was far more effective in stimulating the growth of transformants than that of the parental cells in the anchorage-dependent culture.

IL-6 is a multifunctional cytokine, as are most cytokines. It was originally identified as a T-cell-derived cytokine that induces final maturation of B cells into antibody-producing cells (21, 22). It exhibits multiple biological activities that differ widely among various types of tissues and cells. IL-6 can enhance the proliferation of carcinoma cells (23–25), or it can inhibit the proliferation of several mammalian carcinoma cell lines (26, 27). A variety of malignant tumors, including squamous carcinomas and adenocarcinomas, have been shown to contain or synthesize IL-6, and autocrine growth stimulation is suggested as its possible function (24, 28, 29). Despite the wealth of information on IL-6, there have been only a few reports implicating IL-6 in tumorigenesis (30–33). Lu et al. (32, 33) have shown that IL-6 inhibits the growth of early-stage melanoma cells (which are metastatically incompetent), whereas it stimulates the growth of advanced-stage melanoma cells (which are metastatically competent), and that the latter type of cells synthesizes and uses IL-6 as an intracellular autocrine growth factor. Differential response to IL-6-mediated proliferation was also demonstrated between normal uterine cervical cells and human papilloma virus-immortalized and carcinoma-derived cervical cells in vitro (34). It is not known whether IL-6 is involved in urinary bladder carcinogenesis.

In this report, we have demonstrated a positive effect of IL-6 in an in vitro transformation model. We suggest the following as the possible mechanisms to account for this IL-6 action: (a) IL-6 selectively allows the growth of MNU-initiated MYP3 cells over the parental cells. Culturing MNU-treated MYP3 cells for 1 week in the presence of IL-6 enhanced the proportion of transformants because of their better response to IL-6. Thereby, more transformed cells were available during soft agar growth; (b) the fact that the response of tested cells to IL-6 differs between soft agar and monolayer culture suggests that IL-6 stimulates growth by different mechanisms. Because cells in
soft agar are stimulated adequately by various growth factors provided by 10% FCS and other supplements. IL-6, aside from acting as a growth factor, may provide an additional function, for example, acting as a modifier of cytoskeleton structure in a reversible manner. This alteration may favor anchorage-independent growth.

In summary, IL-6 markedly enhanced colony formation by MNUTransformed rat bladder cells, MYP3. We consider this observation to be novel. This might be one mechanism by which KEC enhances carcinogenesis in vivo. Although we observed no significant differences in IL-6-receptor mRNA expression between parental cells and transformants, it is possible that the transformants have a better binding affinity to IL-6 than do the parental cells, or that there are differences in the subsequent signal-transducing mechanism. These possibilities are under investigation in our laboratory.

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REFERENCES

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