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Effect of Urine on Clonal Growth of Human Bladder Cancer Cell Lines[1]

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ABSTRACT

Human urine contains growth factors; their physiological roles have not been established. The effect of normal human urine was examined *in vitro* on clonal growth of human bladder cancer cell lines. Clonal growth of HT-1376, HT-1197, and T24 was enhanced by five different fresh human urine samples from young men. Colony stimulating activity was detected in fractions with a molecular weight greater than 5000 by ultrafiltration. Sephadex G-50 gel chromatography identified two peaks of colony stimulative activity in HT-1376 with molecular weights of approximately 6000 and greater than 12,400, respectively; these two peaks also possessed immunoreactive epidermal growth factor (EGF) and NRK-49F transforming activities. The three bladder cancer cell lines possessed large quantities of EGF specific binding sites and exogenous EGF stimulated colony formation; EGF concentrations in human urine were found to be remarkably higher than those of exogenously added EGF which stimulated clonal growth of bladder cancer cell lines. Moreover, it was demonstrated that fresh urine samples (5%) incubated with anti-human EGF monoclonal antibody (KEM-10) neutralized completely the colony stimulating effects in HT-1376. These results indicate that fresh human urine stimulates clonal growth in human bladder cancer cell lines and that a major part of the activity is represented by urinary EGF. The data promote urinary EGF as a progressive agent of human bladder cancer.

INTRODUCTION

Recent progress in cancer research, especially on oncogenes and anticogenes, is increasing our understanding of the initial steps of cancer cell development as well as those of mechanisms related to tumor progression (1, 2). However, it is not yet clarified whether physiological growth factors modulating the growth of normal tissues play important roles in cancer cell development or not. In bladder cancer research, experimental studies suggest that urine stimulates the growth of urinary bladder cancer. Oyasu *et al.* (3, 4) reported the presence of a carcinogen in urine influencing the progression of preinvasive neoplastic lesions and urine itself acted as a cancer progressive agent. In a clinical study, Neal *et al.* (5, 6) reported that invasive bladder cancer possessed many more EGF[3] receptors than superficial bladder cancer and concluded that the presence of EGF receptors has a close relationship to clinical prognosis. The recent demonstration that human urine contains a number of peptide growth factors enhances the significance of these observations.

The present experiments demonstrate that human urine stimulates *in vitro* cellular growth of cultured human bladder cancer cell lines and characterize the activity.

MATERIALS AND METHODS

Reagents. Recombinant hEGF(1–53) and hTGF-α(1–50) (7) were kindly provided by Earth Chemical Co., Ltd. (Ako, Hyogo, Japan). Anti-human EGF MoAB (KEM-10) was kindly provided by Wakunaga Pharmaceutical Co., Ltd. (Kohda-cho, Hiroshima, Japan); the method of raising this MoAb and its characteristics were previously reported (8, 9). BSA (Cohn Fraction V) was purchased from Sigma Chemical Co. (St. Louis, MO); $Na^{125}I$ was from Du Pont New England Nuclear (Boston, MA); Bio-Gel P-4 was from Nippon Bio Rad Laboratories (Tokyo, Japan); Sephadex G-50 superfine was from Pharmacia Fine Chemicals AB (Uppsala, Sweden); and agar was from Difco Laboratories (Detroit, MI). Radioiodinated EGF and TGF-α were prepared by the chloramine-T method with $Na^{125}I$, purified by gel filtration on a Bio-Gel P-4, and eluted with 0.1 N acetic acid. Specific activities ranged from 0.64 to 2.14 MBq/μg (1 MBq = 27 μCi).

Cell Lines and Culture Conditions. Three human bladder cancer cell lines (HT-1376, HT-1197, and T24), a normal rat kidney fibroblast cell line (NRK-49F), and a human epidermoid carcinoma cell line (A-431) were obtained from the American Type Culture Collection (Rockville, MD). All three bladder cancer cell lines were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) supplemented with 10% fetal calf serum (Boehringer Mannheim GmbH, Mannheim, German), A-431 in RPMI 1640 with 10% fetal calf serum and NRK-49F in Dulbecco's modified Eagle's medium with 5% calf serum (Gibco Laboratories, Grand Island, NY). All cell lines were cultured at 37°C in a humidified CO_2 incubator.

Collection of Urine. Morning void urine samples were collected from five healthy adult male volunteers (31–34 years old) who were taking no medication. Urine at each void was immediately centrifuged at 10,000 × *g* for 1 h at 4°C. The supernatant was filtered through a Millex GV filter (Millipore, Tokyo, Japan) and stored at −80°C. Ultrafiltration of 50 ml of urine through a Diaflo YM-5 membrane (nominal molecular weight cutoff, 5000), in a Diaflo Cell (type 8200) (Amicon Corp., Danvers, MA) pressurized with N_2 produced 5.0 ml of 10-fold concentrated urine. For the gel filtration study, a 40-fold concentrated urine was used that was prepared from one donor.

Gel Filtration Studies. The 40-fold concentrated urine was chromatographed on a Sephadex G-50 superfine column (1.6 x 88.0 cm) which was equilibrated and eluted with 1 N acetic acid at a flow rate of 12 ml. Successive 2.3-ml fractions were collected and lyophilized. The column was calibrated with cytochrome *c*, ^{125}I-BSA, ^{125}I-EGF, and $Na^{125}I$.

RIA for EGF. Human EGF RIA was performed using anti-EGF serum (TRK-102) as described previously (8). Recombinant hEGF was used as reference standard and tracer preferential.

Growth Study. Growth promoting effects on bladder cancer cell lines and NRK-49F of the fresh urine (5%), concentrated urine, chromatographed urine, EGF, and MoAb were examined in a soft agar clonogenic assay as described previously with slight modification (10). Cancer cells (50,000 cells/dish) and NRK-49F cells (20,000 cells/dish) were suspended in 0.9 ml of their growth medium containing 0.3% agar and spread over a prehardened feeder layer containing 0.9 ml of their medium supplemented with 0.5% agar in a 35-mm Petri dish. Test

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[3] The abbreviations used are: EGF, epidermal growth factor; TGF-α, transforming growth factor α; h, human; RIA, radioimmunoassay; BSA, bovine serum albumin; MoAb, monoclonal antibody.

samples dissolved in 0.2 ml culture medium were placed on the top of the prehardened agar layers. After 14 days of incubation at 37°C in a humidified CO_2 incubator, colonies with a diameter greater than 70 μm were counted using a colony counter (Oriental Instruments, Ltd., Tokyo, Japan).

EGF Binding to Human Bladder Cancer Cell Lines. Scatchard plot analyses were performed to examine the numbers and affinities of EGF binding sites in each cell line as reported previously (10). Approximately 150,000 cancer cells were seeded into individual wells of a 24-multiwell culture plate (Corning, NY). After 48 h incubation, the cells were washed with calcium-magnesium free phosphate buffered saline (pH 7.3). Each well received 0.5 ml culture medium with 0.1% (w/v) BSA containing various concentrations of ^{125}I-labeled EGF with or without 100-fold excess of unlabeled EGF and incubated for 3 h at 4°C. After the incubation, medium was removed and the wells were washed twice with 1 ml ice-cold PBS(−). The cells were dissolved in 0.5 ml 1 N NaOH and the cell-bound radioactivity was measured in a gamma spectrometer (Aloka, Tokyo, Japan). Specific binding of EGF was determined by subtracting the radioactivity measured in the presence of a 100-fold excess of each unlabeled EGF from those measured in the absence of the unlabeled EGF. Cell numbers for each well were determined by counting the number of cells in a separate well prepared under the same conditions as was used for the receptor assay.

Characterization of Anti-hEGF MoAb (KEM-10). To determine the effect of KEM-10 on EGF, a radioreceptor assay was performed using a crude membrane fraction of A-431 as described previously with slight modifications (8). An increasing concentration of KEM-10 was incubated either with 0.93 pmol of ^{125}I-EGF or with 0.42 pmol of ^{125}I-TGF-α for 30 min at 24°C and then they were incubated with the A-431 membrane fraction for 3 h at 4°C. Immediately after the incubation, the sample mixture was centrifuged at 10,000 × g for 15 min and the radioactivity in the pellet was measured in a gamma spectrometer.

Statistics. One-way analysis of variance followed by Duncan's multiple range test were performed to analyze the effect of EGF on the colony formation by the cells. The effect of urine and anti-hEGF MoAb were compared using two-way analysis of variance followed by Student's two-tailed unpaired t test. $P < 0.05$ was considered significant.

RESULTS

Effect of Human Urine on the Clonal Growth of Human Bladder Cancer Cell Lines. With two human bladder cancer cell lines (HT-1376, HT-1197), all five fresh void urine samples (5%) induced significant increases in colony formation. With T24, four of five fresh urine samples induced statistically significant activity; with regard to the remaining one, it induced a large number of colonies but this increase was not statistically significant (Table 1). To identify colony stimulating activity, fresh and fractionated urine samples using ultrafiltration were examined at the same condition (5%) in one human bladder cancer cell line (HT-1376). Fractionated urine samples (M_r > 5000) indicated the most potent activities followed by fresh and filtered urine samples (Table 2).

Effect of Gel Chromatographed Urine Fractions on the Clonal Growth of a Human Bladder Cancer Cell Line (HT-1376). Profiles of colony formation activity in Sephadex G-50 superfine gel chromatography are shown in Fig. 1. Colony stimulating activity was detected in two peaks; one eluted in a position between the void volume and cytochrome c (M_r, 12,400), and the other in a position smaller than cytochrome c corresponding to authentic hEGF.

Study of EGF on Gel Chromatographed Urine. Sephadex G-50 profiles of EGF-RIA and transforming activity are also shown in Fig. 1. Immunoreactive EGF and NRK-49F transforming activity were present in two peaks: one in position between the void volume and cytochrome c; and the other at the elution position of authentic hEGF. These two peaks coincided exactly with the colony stimulating activity on HT-1376.

Table 1 *Effect of human fresh urine on growth of three human bladder cancer cell lines*

Sample	No. of colonies[a]		
	HT-1376	HT-1197	T24
Control[b]	99 ± 19	317 ± 15	115 ± 18
A	440 ± 53[c]	608 ± 9[c]	200 ± 22[c]
B	409 ± 17[c]	495 ± 5[c]	167 ± 20
C	391 ± 48[c]	447 ± 11[c]	199 ± 21[c]
D	529 ± 8[c]	509 ± 12[c]	192 ± 12[c]
E	600 ± 46[c]	583 ± 35[c]	233 ± 12[c]

[a] Mean ± SEM.
[b] Cultured medium.
[c] $P < 0.05$.

Table 2 *Effect of human fresh and fractionated urine on growth of human bladder cancer cell line (HT-1376)*

Sample	No. of colonies[a]		
	Fresh	Fractionated (M_r >5000)	Filtered
Control[b]	99 ± 19	99 ± 19	99 ± 19
A	440 ± 53[c]	587 ± 27[c]	82 ± 8
B	409 ± 17[c]	515 ± 36[c]	55 ± 13
C	391 ± 48[c]	579 ± 14[c]	84 ± 8
D	529 ± 8[c]	608 ± 75[c]	71 ± 7
E	600 ± 46[c]	600 ± 40[c]	106 ± 8

[a] Mean ± SEM.
[b] Cultured medium.
[c] $P < 0.05$.

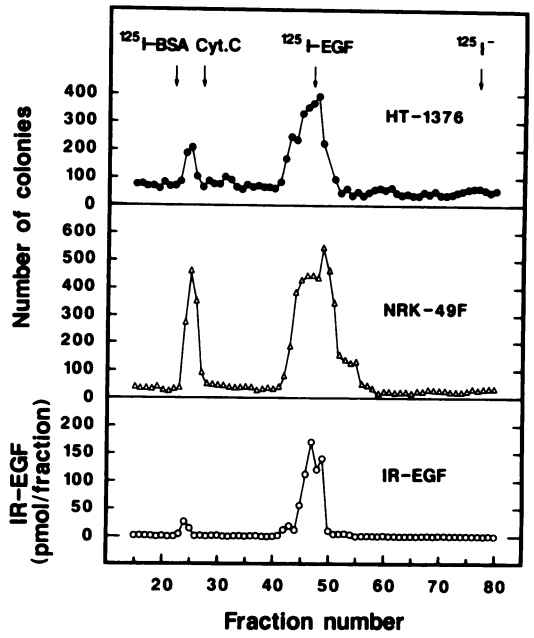

Fig. 1. Sephadex G-50 superfine gel exclusion chromatography of one concentrated human urine. The concentrated urine was applied in 750 μl of 1 N acetic acid and eluted as described. Lyophilized 50-μl aliquots were also assayed for RIA of EGF. Aliquots (400 μl) were assayed for clonogenic assay of a human bladder cancer cell line, HT-1376 and NRK-49F. Markers: BSA, M_r 68,000; cytochrome c, M_r 12,400; human recombinant EGF, M_r 5,900. *IR*, immunoreactive.

Effect of hEGF on the Clonal Growth of Human Bladder Cancer Cell Lines. Clonal growth was enhanced by EGF at the concentrations greater than 0.1 nM in all cell lines tested (Fig. 2) and reached maximum colony numbers at 1.0 nM.

Binding of Labeled EGF to Human Bladder Cancer Cell Lines. Scatchard plot analyses revealed that there were specific binding sites with a single affinity for labeled EGF in all three bladder cancer cell lines (Table 3).

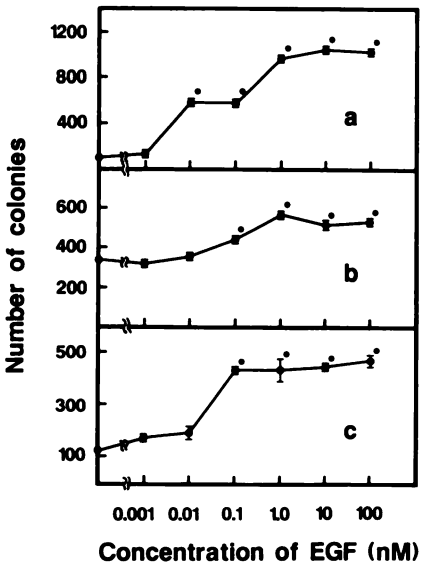

Fig. 2. Effect of hEGF on the clonal growth of three human bladder cancer cell lines (*a*, HT-1376; *b*, HT-1197; *c*, T24). Each *point* represents means of triplicates; *bars*, SEM. *, $P < 0.05$.

Table 3 *EGF receptors in human bladder cancer cell lines*

Cell lines	K_d (nM)	^{125}I-EGF binding sites/cell ($\times 10^5$)
HT-1376	2.31	9.3
HT-1197	1.49	5.3
T24	1.83	3.3

Amount of EGF in Urine and Its Concentrate. IR-EGF ranged from 12 to 55 nM in the five fresh urine samples. The concentrated urine used in the gel chromatography study contained 660 nM immunoreactive EGF which was approximately 40-fold higher than the amount in the raw sample.

Inhibitory Effect of Anti-hEGF MoAb (KEM-10) on the Growth Stimulatory Activity in Urine. Binding of radioiodinated EGF to the A-431 membrane fraction was inhibited by KEM-10 in a dose dependent manner. The binding was completely inhibited by addition of 100 μg/ml KEM-10 (Fig. 3). In contrast, this MoAb did not interfere with the binding of TGF-α to the A-431 membrane fraction. The effect of KEM-10 was examined on EGF stimulated colony formation by the NRK-49F cell lne. EGF (1 nM) markedly enhanced colony formation (Fig. 4). KEM-10 inhibited EGF stimulated colony formation in a dose dependent manner and the effect reached its maximum at concentrations greater than 50 μg/ml. This inhibitory effect of KEM-10 (50 μg/ml) on colony formation was partially reversed by addition of 100 nM EGF. KEM-10 inhibited urinary colony stimulating effects on HT-1376 significantly (Fig. 5).

DISCUSSION

The present study demonstrates that five urine samples from healthy male volunteers stimulate the clonal growth of three human bladder cancer cell lines. Clonal growth is well known to correlate with the tumorigenicity of cancer cells (11, 12). Several reports indicate that clonal growth correlates with clinical behavior of bladder cancer and might have prognostic value (13–16). Accordingly, it is reasonable to postulate that fresh human urine possesses the capacity, under the appropriate circumstances, to promote growth of human bladder cancer tissues. In this context, we characterized the growth promotive properties of human urine from young men upon human bladder cancer cell lines.

Based on the result of an ultrafiltration, a major activity stimulating clonal growth of bladder cancer cell lines existed in a molecular size greater than 5000. Gel filtration studies of a concentrated urine identified biological activity in two peaks; one eluted at a position between the void volume and cytochrome *c* and the other at a position smaller than cytochrome-*c*. By using a rat model, Yura *et al.* (17) and Messing *et al.* (18) claimed that urinary EGF may play an important role in the progression of bladder cancers. The smaller peak eluted at the position of human EGF. By using human EGF RIA, we measured the concentration of immunoreactive EGF in each fraction and found that not only the smaller peak but also the larger one possesed EGF-like immunoreactivity. We proposed that the smaller peak was EGF and the high molecular weight form was the precursor of EGF reported previously (19–21). This observation was further supported by the EGF bioassay demonstrating that both of these activities stimulated colony formation of NRK-49F cells.

It is well established that human urine contains a large amount of EGF (22, 23). The present study revealed that all five urines examined contained immunoreactive EGF at concentrations greater than 12 nM. Also, all bladder cancer cell lines possessed a large number of EGF receptors and exogenously added EGF at concentrations greater than 0.1 nM stimulated the clonal growth of these cell lines. Since EGF concen-

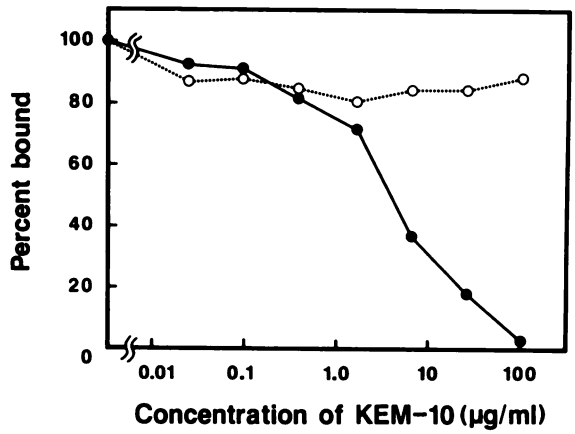

Fig. 3. Effect of anti-human EGF MoAb (KEM-10) on binding of ^{125}I-labeled EGF and TGF-α to membrane fractions of A-431 cells. ^{125}I-Labeled EGF (●) and ^{125}I-labeled TGF-α (○) were used as labeled antigen. Binding is expressed as percentage of maximal binding after background subtraction. Data represent means of duplicate determinations.

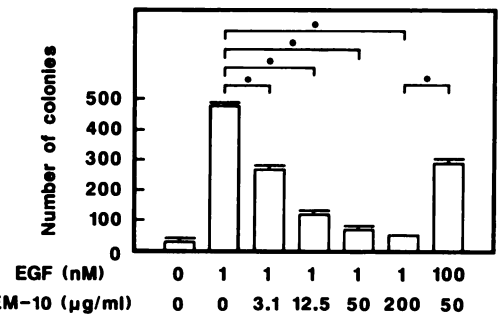

Fig. 4. Effect of anti-human EGF MoAb (KEM-10) on the EGF stimulated clonal growth of NRK-49F cells. NRK-49F cells were stimulated by EGF (1.0 nM). KEM-10 was preincubated with EGF for 30 min at 37°C. As excess ligand, 100 nM EGF was added to the dishes. Each *point* represents means of triplicates; *bars*, SEM. *, $P < 0.05$.

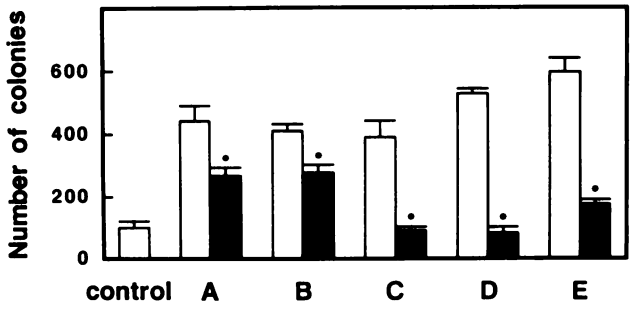

Fig. 5. Effect of 5% human fresh urine (□) and anti-human EGF MoAb, KEM-10 (■), on the growth of the human bladder cancer cell line, HT-1376. Cells (5×10^4) are plated as described in "Materials and Methods." KEM-10 (50 μg/ml) was preincubated with each urine sample (A–E) for 30 min at 37°C. The colonies were counted 14 days after addition of samples. Each *point* represents means of triplicates; *bars*, SEM. *, $P < 0.05$.

trations in the 5% urine were approximately 10-fold higher than those of exogenously added EGF which stimulated cellular growth, urinary EGF may be responsible for the stimulated clonal growth of three bladder cancer cell lines. To examine this possibility, we determined the effect of an exogenously added MoAb against EGF on the urine stimulated clonal growth of bladder cancer cell line. This MoAb blocked the binding of labeled EGF to its receptors and inhibited the effect of exogenously added EGF on colony formation by NRK-49F cells. Thus, the anti-EGF MoAb used in this study neutralized the biological activity of EGF by blocking the binding of EGF to its receptors. Since this MoAb inhibited the clonal growth of bladder cancer cell line stimulated with fresh urine, it is reasonable to postulate that EGF in urine can serve as a major growth factor for this cancer cell line.

The presence of EGF in urine was demonstrated in various animals including mouse, rat, and human. In the mouse, it was revealed that EGF is synthesized in the epithelial cells of the distal tubuli of nephrons (24); however, the physiological implication of urinary EGF has not been clearly elucidated. Normal epithelial cells of the urinary tract possess specific receptors for EGF acting to maintain the normal integrity of the epithelial lining. When a cancer cell arises from the epithelium of the urinary tract, urinary EGF may stimulate its growth. In animal models, several studies reported the importance of urinary EGF in the progression of bladder cancers. Yura *et al.* (17) identified in a rat urinary bladder cancer model a tumor enhancing urinary fraction as EGF. Messing and Reznekoff (25) demonstrated that exogenous EGF stimulated *in vitro* growth of normal and malignant human urothelium. Moreover, several clinical observations also suggested that urine stimulates the growth of urinary bladder cancer: Davis (26) reported two cases in which carcinomatous ulceration of bladder disappeared following ureterosigmoidostomy; Abeshouse and Scherlis (27) reviewed six cases in which urinary diversion resulted in spontaneous disappearance of bladder tumor or retrogression. Taken together these observations support the involvement of urinary EGF in the progression of bladder cancers.

Some bladder cancer cell lines synthesize and secrete TGF-α (28); this growth factor has a structural homology to EGF and the ability to compete for EGF receptor binding (29, 30). Since the amount of TGF-α produced by cancer cells was very low compared to urinary EGF, it is likely that urinary EGF plays a major role in the early development of bladder cancers. However, when cancer cells spread to the extrabladder wall and acquire the capacity to produce a large amount of TGF-α, we speculated that TGF-α may enhance bladder cancer growth as an autocrine growth factor.

In the present study, we demonstrated that human fresh urine has the capacity to promote the clonal growth of human bladder cancer cell lines and that the major factor responsible for this effect is likely to be urinary EGF. These observations support the concept that physiologically present and bioactive peptides in human beings, such as growth factors and hormones, partly regulate the progression of cancer cells.

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