Inhibitory Effect of Testosterone on Gap Junctional Intercellular Communication of Human Transitional Cell Carcinoma Cell Lines

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ABSTRACT
A dye transfer method was applied to investigate the effect of testosterone on gap junctional intercellular communication (IC) of two kinds of human transitional cell carcinoma cell lines, JTC-30 and JTC-32. When JTC-30 cells were cultured with testosterone at nontoxic concentrations (17-69 \mu M), a dose and time dependent inhibition of dye transfer was observed. More than 90% inhibition occurred after exposure to 69 \mu M testosterone for 96 h. The inhibition was reversed rapidly after testosterone deprivation. Similar results were obtained with JTC-32 cells. 17\beta-Estradiol showed no inhibitory effect on IC of both transitional cell carcinoma cell lines even at toxic levels. Testosterone exhibited no inhibitory effect on IC of human fibroblasts. The inhibitory effect of 5\alpha-dihydrotestosterone was almost similar to that of testosterone. At concentrations examined, cyproterone acetate influenced neither dye transfer nor the inhibitory effect of testosterone, suggesting a mechanism of testosterone action different from that of the known receptor system. Since blockage of IC has been indicated as one reliable evidence for tumor promotion, current results suggest that testosterone is a possible endogenous promoter of the bladder carcinoma and may therefore possibly play a role on the sexually different incidence of bladder carcinoma.

INTRODUCTION
The interiors of adjacent cells in tissues are directly interconnected by special membrane channels of gap junctions. Cytoplasmic low molecular weight compounds are transferred directly from a cell to adjacent ones through gap junctions (1, 2). This mechanism is designated as gap junctional intercellular communication, which may play a crucial role in the transfer of information among cells and control harmonized cell proliferation and differentiation, leading to maintenance of tissue homeostasis (3). Recent studies have reported that there is a good association between the inhibition of gap junctional intercellular communication and tumor promotion (4-10). Many animal experiments have demonstrated tumor induction by hormones, particularly those produced by the gonads (11-15), and bladder carcinoma has been known to occur in higher incidence in males than in females. Accordingly, it was investigated whether testosterone and 17\beta-estradiol influenced gap junctional intercellular communication of human urothelial cell lines established from TCCs of the bladder.

MATERIALS AND METHODS

Chemicals. Testosterone, 17\beta-estradiol, 5\alpha-dihydrotestosterone, and fluorescent Lucifer yellow CH were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were obtained from following sources: synthetic medium DM-170, from Kyokuto Pharmaceutical Industrial Co. Ltd. (Tokyo, Japan); synthetic medium α-minimal essential medium and fetal bovine serum, from GIBCO Laboratories (Grand Island, NY); and cyproterone acetate, from Schering AG (Berlin, Federal Republic Germany).

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The abbreviation used is: TCC, transitional cell carcinoma.

Cells Culture. TCC cell lines JTC-30 and JTC-32 (16) were cultured in DM-170 medium supplemented with 10% fetal bovine serum under 5% CO\textsubscript{2} and 95% air at 37°C. Human fibroblasts were prepared from surgical specimens of the skin and prostate (17). Fibroblasts were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum and grown under an atmosphere of 5% CO\textsubscript{2} and 95% air at 37°C. The culture medium was changed every 3 days if not otherwise mentioned. Steroids dissolved in acetone were added to culture medium and the final concentration of acetone in the medium was adjusted to 0.125%. Acetone at this concentration showed neither cytotoxicity nor any effect on intercellular communication.

Cytotoxicity of Steroids. The toxicity of steroids to TCC cells was examined by the influence on their colony forming ability (18). Two exposure periods of 48 h and 7 days were used to establish short and long term cytotoxicity. Eight hundred TCC cells in exponentially growing culture were plated onto 60-mm plastic culture dishes. After 18 h culture, the medium was changed and either an appropriate amount of steroids dissolved in acetone or the solvent itself was added. After 2 days, the medium was removed from all dishes, and the dishes were rinsed twice with DM-170 medium. Steroid treated cells were then divided into two groups, one receiving only solvent and the other receiving the corresponding concentration of the same steroid again. After another 5 days, the medium was removed from all dishes and cells were rinsed twice with DM-170 medium and cultured further in DM-170 medium only. Seven days later, cells were fixed and stained with Giemsa. Colonies with more than 20 cells were scored as survival colony formation. The number of colonies formed in control cultures for JTC-30 and JTC-32 was 161 ± 6.4 (SEM) and 169 ± 6.5, respectively. Taking the control value as 100%, cytotoxicity was expressed as the mean of relative colony forming ability of 5 independent cultures.

Dye Transfer Assay. Gap junctional intercellular communication was measured by the dye transfer method (6). In brief, 5 × 10\textsuperscript{4} cells (TCC cells) and 3 × 10\textsuperscript{5} cells (fibroblasts) were plated onto different dishes, cultured for 7 to 9 days and 6 to 8 days, respectively, until forming a confluent area and then exposed to additives for scheduled periods. These assays were done in growing phase of cultured cells. A cell was impaled with glass capillaries close to the nucleus and fluorescent Lucifer yellow CH (M, 437.3; 10% in 0.33 M lithium chloride solution) was microinjected under a stable pressure for 2 to 4 s at room temperature under a microinjectoscope (Olympus injectoscope). The capillary was prepared using an automatic magnetic puller (Narishige Co., Tokyo, Japan). The extent of dye transfer was measured at room temperature under a fluorescence phase-contrast microscope by scoring the number of fluorescent cells. Dye was transferred rapidly to adjacent cells in control cultures as shown in the photomicrograph of Fig. 3B. Transfer reached plateau approximately 15 min after injection and was almost unchanged for at least another 15 min. Measurement of dye transfer was, therefore, carried out 20 min after injection. The level of dye transfer of TCC cells did not depend on the cell density after culture for 6 days. Cultures were performed in triplicate for each experiment. At least 28 independent injections were performed to determine the mean number of dye coupled cells for each culture and gap junctional intercellular communication of cells exposed to additives was expressed as the percentage of the mean fluorescent cell number of control cultures. Each experiment was repeated at least 2 times and the result was expressed as the mean ± SEM. The averaged number of dye coupled cells in the control cultures of JTC-30 cells, JTC-32 cells, and fibroblasts ranged from 41 to 53, from 32 to 41, and from 78 to 97, respectively, in each experimental series performed.

Recovery of Inhibited Gap Junctional Intercellular Communication. TCC cells (5 × 10\textsuperscript{4}) were seeded in 60-mm plastic culture dishes and cultured in DM-170 medium. Seven days after seeding, the medium was changed and testosterone was added at a concentration of 69 \mu M.
Ninety-six h later, dishes were rinsed twice with fresh DM-170 medium and then divided into two groups: one receiving only acetone and the other receiving 69 μM testosterone again. After an appropriate culture time, dye transfers of these two groups were examined.

Effect of Cyproterone Acetate on the Inhibition of Gap Junctional Intercellular Communication by Testosterone. JTC-30 cells (5 × 10⁵) were seeded in 60-mm plastic culture dishes and cultured in DM-170 medium. Ten days later, the medium was changed and culture was continued in the presence of 0, 24, and 48 μM cyproterone acetate. After 24 h, the medium was removed from all dishes. Cells were rinsed twice with DM-170 medium and then cultured further in a fresh DM-170 medium containing corresponding concentrations of cyproterone acetate and testosterone at concentrations of 17 to 69 μM. Ninety-six h later, dye transfer was examined.

RESULTS

Effect of Testosterone on the Cloning Efficiency and Dye Transfer of JTC-30 Cells. When JTC-30 cells were incubated for 48 h with several concentrations of testosterone (from 0.35 to 173 μM), no inhibition of the colony forming ability was recognized at 104 μM or lower (Fig. 1A) and inhibition observed at 173 μM was less than 30%. Incubation of JTC-30 cells for 7 days with testosterone caused no inhibition of colony formation at 35 μM or lower, below 20% inhibition at 69 μM and more than 30% inhibition at 104 μM or higher. Dye transfer was measured after exposure to testosterone for 6, 24, 48, and 96 h at concentrations indicated in Fig. 1B. Testosterone inhibited gap junctional intercellular communication at noncytotoxic concentrations (17 to 69 μM). Inhibition rates increased according to the exposure time to testosterone at concentrations exhibiting inhibition.

Effect of Testosterone on Cloning Efficiency and Dye Transfer of JTC-32 Cells. Testosterone showed almost the same toxicity to JTC-32 cells as to JTC-30 cells (Fig. 2A). Forty-eight h incubation with testosterone induced no inhibition of colony forming ability at 69 μM or lower and below 30% inhibition at 104 μM or higher. Seven days of incubation with testosterone caused no inhibition at 35 μM or lower, below 30% inhibition at 69 μM and more than 30% inhibition at 104 μM or higher. Dye transfer experiments were performed after exposure to testosterone from 0.35 to 173 μM (Fig. 2B). Inhibition of gap junctional intercellular communication was observed at nontoxic concentrations. Inhibition correlated with exposure time to testosterone.

Reversibility of Inhibitory Effect of Testosterone on Gap Junctional Intercellular Communication. The inhibition of gap junctional intercellular communication of both cell line cells increased with the time of incubation with testosterone and was reversed quickly by removal of testosterone as shown in Figs. 3 and 4.

Effect of 17β-Estradiol on Cloning Efficiency and Dye Transfer of JTC-30 and JTC-32 Cells. When JTC-30 or JTC-32 cells were incubated for 7 days with 17β-estradiol, more than 30% inhibition of cloning efficiency was observed at 37 μM or higher (Fig. 5A). 17β-Estradiol showed no apparent inhibition of gap junctional intercellular communication on both TCC cell lines even after exposure to a toxic dose of 74 μM for 120 h (Fig. 5B).

Effect of Testosterone on Dye Transfer of Human Fibroblasts. The results of experiments using three kinds of fibroblasts, two from human skins and one from a human prostate, were identical. Testosterone had no effect on the gap junctional intercellular communication within 96 h exposure up to a concentration of 173 μM.

Effects of 5α-Dihydrotestosterone on Cloning Efficiency and Dye Transfer of JTC-30 Cells. Toxicity of 5α-dihydrotestosterone was not stronger than that of testosterone for JTC-30 cells as shown in Fig. 6A. After incubation of cells with 5α-dihydro-
Fig. 3. Patterns of dye transfer in control, testosterone treated and testosterone deprived JTC-30 cells. Cells was injected with Lucifer yellow CH (*) and the picture was taken 20 min after the injection, × 300. A, phase-contrast micrograph of cells cultured in the presence of 0.175% acetone for 96 h (control); B, fluorescence photomicrograph of A; C, phase-contrast micrograph of cells cultured with testosterone (69 μM) for 96 h; D, fluorescence photomicrograph of C; E, phase-contrast micrograph of cells 8 h after removal of testosterone (69 μM) with which cells had been incubated for 96 h; F, fluorescence photomicrograph of E.

Testosterone for 48 h, no apparent inhibition of colony formation was observed at 103 μM or lower. Inhibition below 30% was observed at 172 μM. When cells were incubated for 7 days, inhibition of colony formation was more than 30% in the presence of 5α-dihydrotestosterone at 103 μM or higher. Inhibition of gap junctional intercellular communication by 5α-dihydrotestosterone was observed at noncytotoxic concentrations (34–69 μM). The inhibition by 5α-dihydrotestosterone as a function of concentration was rather less than that of testosterone (Fig. 6B).

Effect of Cyproterone Acetate on Inhibition of Gap Junctional Intercellular Communication of JTC-30 Cells by Testosterone. Cyproterone acetate at concentrations of 24 and 48 μM neither showed apparent influence on gap junctional intercellular communication of JTC-30 cells nor antagonized the inhibitory effect of testosterone (17 to 69 μM).

DISCUSSION

Testosterone is found to inhibit gap junctional intercellular communication of two human TCC cell line cells at noncytotoxic levels. Since cytotoxicity could contribute to the destruction of gap junctional intercellular communication (19), testosterone levels which cause inhibition in the current study were confirmed to be noncytotoxic by testing the reversibility of the inhibition after testosterone deprivation as well as the influence on colony forming ability. Inhibition is not caused by 17β-estradiol at levels similar to those for testosterone. Furthermore, testosterone does not manifest inhibitory effects on gap junctional intercellular communication of human fibroblasts examined. Therefore, it appears likely that inhibition of gap junctional intercellular communication is a specific function of testosterone to TCC cell line cells.

The cytosol receptor system and reduction of testosterone to 5α-dihydrotestosterone are usually required for physiological action of testosterone at androgen target cells (20). Cyproterone acetate, one of the potent antiandrogens, however, has no effects on the inhibition of gap junctional intercellular communication by testosterone, and further, 5α-dihydrotestosterone is found not to be more effective in inhibiting gap junctional intercellular communication than testosterone. The results suggest that a mechanism different from the known receptor system for androgen action might be involved in the inhibition of gap junctional intercellular communication of TCC cell lines by androgens.

The present results were obtained by short term exposure of
cells to testosterone at concentrations much higher than the physiological levels (0.01–0.04 μM). Inhibition of gap junctional intercellular communication by testosterone, however, is found to be time and dose dependent. Therefore, it is possible that transitional cells which are continuously exposed to sex hormones after sexual maturation are affected similarly by testosterone. The tumorigenic effect of testosterone is still controversial. In a few studies tumors were induced by testosterone in various organs of several species (11–15) including bladder of the rat (21), while sex hormones have been reported not to induce gene mutation (22). In the meantime, the blockage of gap junctional intercellular communication has been indicated as one reliable piece of evidence for tumor promotion (4–10). Since serum levels of testosterone in adult males are approximately 10 to 20 times those in females, the current result that testosterone inhibits gap junctional intercellular communication of TCC cell line cells suggests the compound as an endogenous promoter of the bladder carcinoma in addition to environmental risk factors such as occupations and smoking habits and therefore may offer a way to clarify the mechanism of sexually different incidence of human bladder carcinoma.

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REFERENCES

2. Loewenstein, W. R. Junctional intercellular communication. The cell-to-cell


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