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The Preventive Effect of Green Tea on the Gap Junction Intercellular Communication in Renal Epithelial Cells Treated with a Renal Carcinogen

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Abstract. *Objective: Clinical studies imply that (-)-epigallocatechin-3-gallate (EGCG), a main ingredient of green tea catechins, has a chemopreventive action against cancers and suppresses the proliferation of cancer cells. However, there is no report about its chemopreventive effect for renal cancer. We previously determined that renal carcinogens suppressed the gap junction intercellular communication (GJIC) of renal epithelial cells. In this study, we investigated the effect of EGCG on the GJIC of renal epithelial cells treated with a renal carcinogen. Materials and Methods: Mardin-Darby canine kidney (MDCK) cells were used to determine the protective effects of EGCG on dimethylnitrosamine-induced alteration of GJIC and connexin 43 (Cx 43). The maximum concentration of EGCG was determined by the lactate dehydrogenase assay method. The scrape-loading dye transfer method was used to assess the expression and cellular localization of Cx 43. The phosphorylation status of Cx 43 was determined by Western blot analysis. Results: The optimal noncytotoxic concentration of EGCG was determined to be 10 μg/ml. The levels of GJIC and Cx 43 expression were markedly decreased in MDCK cells exposed to dimethylnitrosamine. A 12-h pretreatment with EGCG greatly ameliorated the GJIC-inhibitory effects of dimethylnitrosamine. Conclusion: These results suggest that the preservation of GJIC may indicate the chemopreventive effect of green tea on renal epithelial cells treated with a renal carcinogen in vitro.*

Green tea is consumed primarily in China, Japan, India, parts of North Africa and the Middle East (1). Green tea contains a 15% mixture of catechins. Of the four major catechins, (-)-epigallocatechin-3-gallate (EGCG) is the most prevalent (2). Green tea is implicated as a chemopreventive agent against the development of various tumors (3-5). The protective activity of green tea is generally assumed to be due to its free radical scavenging or a protective effect against gap junction intercellular communication (GJIC) inhibition (2,6). However, little is known about the possible role of green tea in the development of renal cell carcinoma. Yoshioka *et al.* reported that green tea catechins played an active chemopreventive role in chemical renal carcinogenesis in rats (7). However, the mechanisms of the anticarcinogenic activities of green tea are still elusive.

Renal cell carcinoma is a malignant tumor of the kidney that accounts for about 3% of all malignancies. Acquired cystic kidney disease (ACKD) is commonly seen in patients who have undergone dialysis and its incidence increases with the duration of dialysis. Patients with ACKD have a propensity to develop adenocarcinomas. However, the etiology and prevention of ACKD transformation is still unclear (8).

Gap junctions are membrane channels that mediate direct cell-cell communication properties by exchanging ions and metabolites less than 1.2 kD (9). These channels are formed of two hemichannels from the neighboring cells, and each hemichannel is composed of six proteins called connexins. The connexins belong to a multigene family with fourteen members cloned in the murine genome and differentially expressed among tissues (10). Gap junctions have been found to play important roles in the completion of embryonic development, maintenance of tissue homeostasis and regulation of normal cell growth (10-12). The disruption of GJIC may be important in carcinogenesis (13). The results of our previous studies have indicated that GJIC may play a role in renal carcinogenesis (14, 15) and that renal tumor tissues are characterized by low or aberrant connexin expression (16). Loss of GJIC properties upon treatment with tumor-promoting agents like dimethylnitrosamine has supported this idea. In recent years, transfection of different connexin genes into tumor cell lines that exhibit little or no coupling has been shown to restore intercellular communication (17).

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We investigated whether the major component of green tea catechins may prevent the blockage of GJIC in tumor promoter-treated renal cells.

Materials and Methods

Cell culture reagents. Dulbecco's modified Eagle's medium (DMEM), Lucifer yellow dye and EGCG were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Fetal calf serum (FCS) was purchased from Life Tech Oriental Co. (Tokyo, Japan). Dimethylnitrosamine (DMN) was obtained from Wako Pure Chemical Industry (Osaka, Japan). The EGCG was dissolved in ethanol at a concentration of 10 mg/ml and stored at 4˚C, then diluted in ethanol further before use.

Cell culture. Mardin-Darby canine kidney (MDCK) cells, obtained from the Riken Cell Bank, were cultured in DMEM supplemented with 10% FCS and L-glutamine (0.2 mg/ml) at 37˚C in an atmosphere of 95% air and 5% carbon dioxide.

Lactate dehydrogenase assays. Dimethylnitrosamine was used as an introducer for the chemical transformation of MDCK cells. The influence of EGCG on renal epithelial cells containing MDCK cells is unknown. Therefore, to determine the maximum noncytotoxic concentration of EGCG on MDCK cells, the activity of the lactate dehydrogenase released by those cells into the culture media was measured. The MDCK cells were seeded into 35-mm dishes at a density of 5.0×10^4/dish. When the cells were subconfluent, the medium was replaced with fresh DMEM containing 10% FCS and EGCG at concentrations between 10-4 mg/ml and 1 mg/ml. After 24 h, the lactate dehydrogenase concentration in the culture medium was measured using a commercial *in vitro* toxicology assay kit (Sigma Chemical Co.).

MDCK cell treatment with a renal carcinogen and EGCG. When the MDCK cells were subconfluent, the medium was replaced with fresh DMEM containing 10% FCS and the predetermined concentration of EGCG. Later, the medium was supplemented with DMN adjusted to a final concentration of 0.1%. When the cells had been cultured for 12 h in 35-mm dishes, GJIC assay and other studies were performed as described below (15).

GJIC assay. Cells were treated with EGCG for 1.5 h and 12 h and GJIC was determined. Lucifer yellow dye is transferred between neighboring cells *via* GJIC. To investigate the rates of GJIC in confluent MDCK cells, we used the scrape-loading dye transfer technique. Lucifer yellow dye and phosphate buffer saline (PBS) solutions were warmed to room temperature prior to use. The monolayer cells were rinsed with PBS three times and then the Lucifer dye solution (0.5 mg/ml PBS) was added to the cells and the dye mechanically loaded into the cells using surgical blade. After 3 min at room temperature, the extracellular dye solution was removed and then the cells were rinsed with PBS three times. The degree of dye transfer between cells from the scraped edge was determined with an epifluorescence microscope. Normal GJIC function results in the transfer of the dye loaded into the scraped cells to the adjacent rows of cells.

Immunofluorescent staining of Cx 43. Connexin 43 (Cx 43) is the major gap junction gene expressed in renal epithelial cells. MDCK cells were grown and treated in the wells of commercial slides (Permanox Lab-Tec Chamber slides; Nunc Inc., Naperville, IL, USA). After extensive washing with PBS, the cells were fixed in ice-cold acetone for 3 min, rinsed in PBS and incubated with PBS containing 0.2% (V/V) Tween-20 for 60 min. The cells were then incubated with 1:100 monoclonal anti-mouse Cx 43 antibody (Zymed Laboratories, San Francisco,CA, USA) for 90 min. The cells were washed with PBS and incubated with 1:1000 biotinylated F $(ab')_2$ fragment of goat anti-mouse IgG (H+L) (Zymed) for 90 min, then incubated with fluorescence-conjugated streptavidin (Dako Japan, Kyoto, Japan) for 60 min. After extensive washing, the cells were photographed using a fluorescence microscope. All procedures were carried out at room temperature.

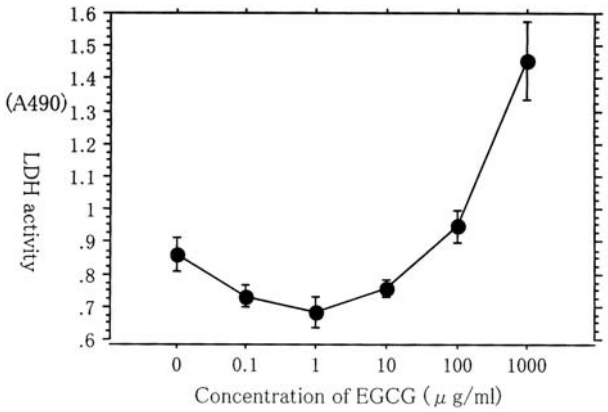

Figure 1. *Cytotoxicity of EGCG in cultured MDCK cells. Lactate dehydrogenase activity in the cultured medium was measured using a commercial kit. The assay indicated that 10 μg/ml EGCG in the culture media did not influence a cytotoxic effect on MDCK cells.*

Western blots of Cx43. The treated MDCK cells were washed twice with ice-cold PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF). The cells were lysed on ice in lysis buffer (25mM Tris-HCl, pH 7.4, 50mM NaCl, 2% sodium dodecyl sulphate (SDS), 1mM PMSF, 1mM Na_3VO_4, 2 μg/ml aprotinin), sonicated for 30 sec on ice, then clarified by centrifugation at 10,000g for 20 min at 4˚C. The protein concentration was determined in the supernatant using the Bio Rad Detergent Compatible Protein Assay (Bio Rad Laboratories, Tokyo, Japan). Ten micrograms of protein from lysates were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene diflouride membranes (Millipore Japan, Tokyo, Japan). WB-F344 rat liver epithelial cells served as the positive control. Proteins affixed to membranes were blocked with PBS containing 5%(W/V) skimmed milk, 0.2% Tween-20 and 0.1% sodium azide (blocking solution), then incubated with rabbit anti-Cx 43 polyclonal antibody (Zymed) diluted 2,500-fold with blocking solution at room temperature for 2 h. After washing with PBS containing 0.2% Tween-20, the membranes were incubated with peroxidase-conjugated anti-rabbit IgG (Amersham International, Bucks, UK) for 1 h at room temperature. Cx proteins were visualized using the ECL-chemiluminescence detection kit (Amersham).

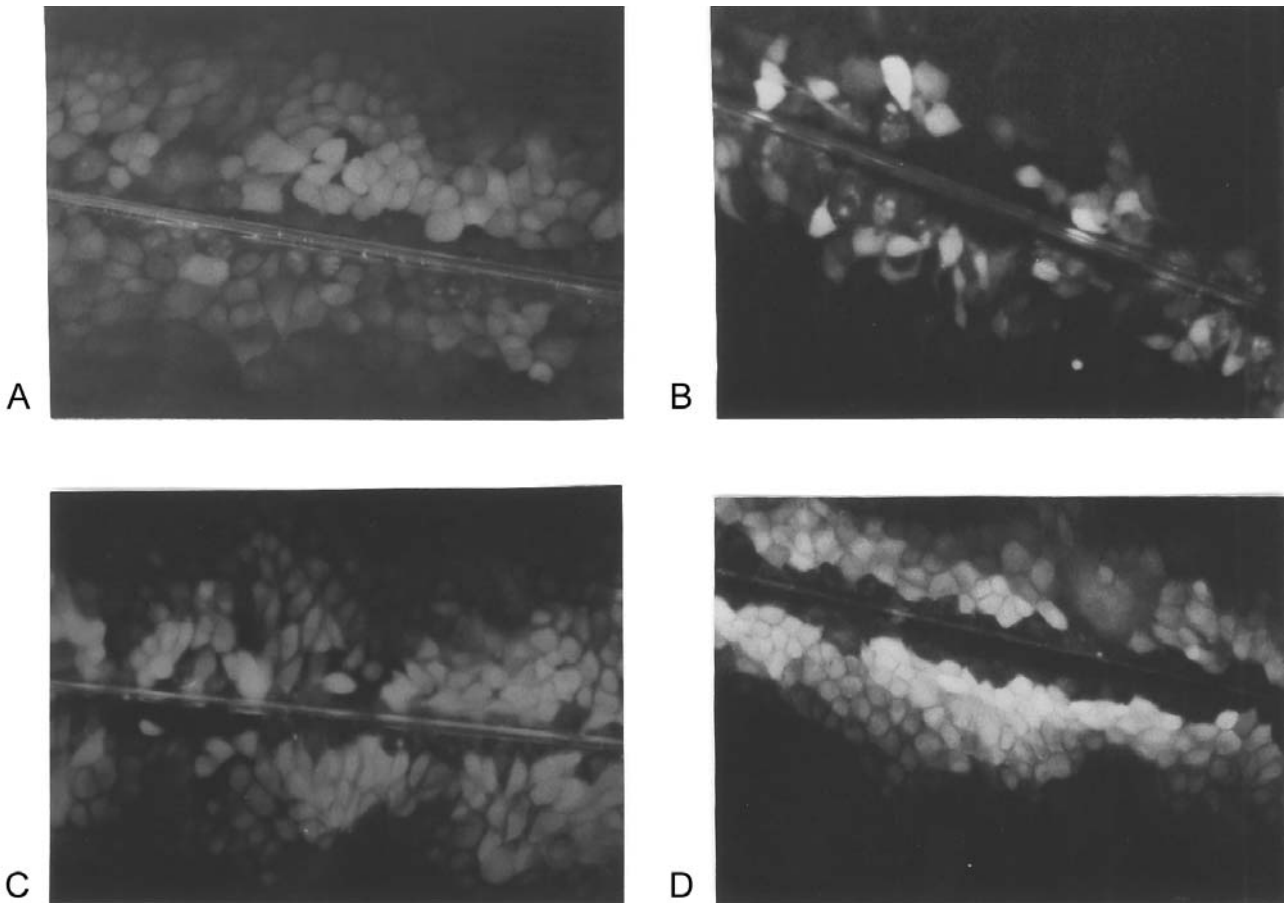

Figure 2. *EGCG preserved GJIC function of MDCK cells treated with dimethylnitrosamine. The GJIC function was observed using the scrape-loading dye transfer technique as described in Materials and Methods. (A) MDCK cells without treatment; (B) MDCK cells exposed to 0.1% DMN; (C) MDCK cells treated with 10 μg/ml EGCG; (D) MDCK cells pretreated with 10 μg/ml EGCG and then exposed to 0.1% DMN. (Original magnification x200)*

Results

Determination of a nontoxic concentration of EGCG on MDCK cells. Figure 1 indicates that 10 μg/ml EGCG in the culture media did not exert a cytotoxic effect on MDCK cells.

EGCG preserved the GJIC function of MDCK cells exposed to DMN. Figure 2C shows that the level of GJIC in MDCK cells pretreated with EGCG was not different from that in untreated MDCK cells (Figure 2A). Figure 2B indicates that the level of GJIC was markedly decreased in MDCK cells exposed to DMN, and the exposure of MDCK cells to EGCG for 1.5 h showed no significant effect on the level of GJIC in MDCK cells exposed to DMN(data not shown). However, Figure 2D shows that the level of GJIC in MDCK cells exposed to DMN was preserved when the cells were pretreated with EGCG for 12 h.

Immunofluorescent staining of Cx 43. We examined the effect of EGCG on the Cx 43 protein expression in MDCK cells using immunofluorescent staining. Linear fluorescent plaques were localized at the plasma membrane of untreated MDCK cells (Figure 3A). The level of Cx 43 protein expression in MDCK cells pretreated with EGCG was not different from that in untreated MDCK cells (Figure 3B). The Cx 43 membranous plaques were less evident in MDCK cells exposed to DMN than in unexposed cells, and some plaques were detected in the perinuclear cytoplasmic compartment (Figure 3C). However, the level of Cx 43 protein expression in MDCK cells exposed to DMN was maintained when the cells were pretreated with EGCG (Figure 3D).

Western blots of Cx 43. We investigated the effect of EGCG on the expression of Cx 43 protein by Western blotting. MDCK cells exhibited three clearly distinct immunoreactive

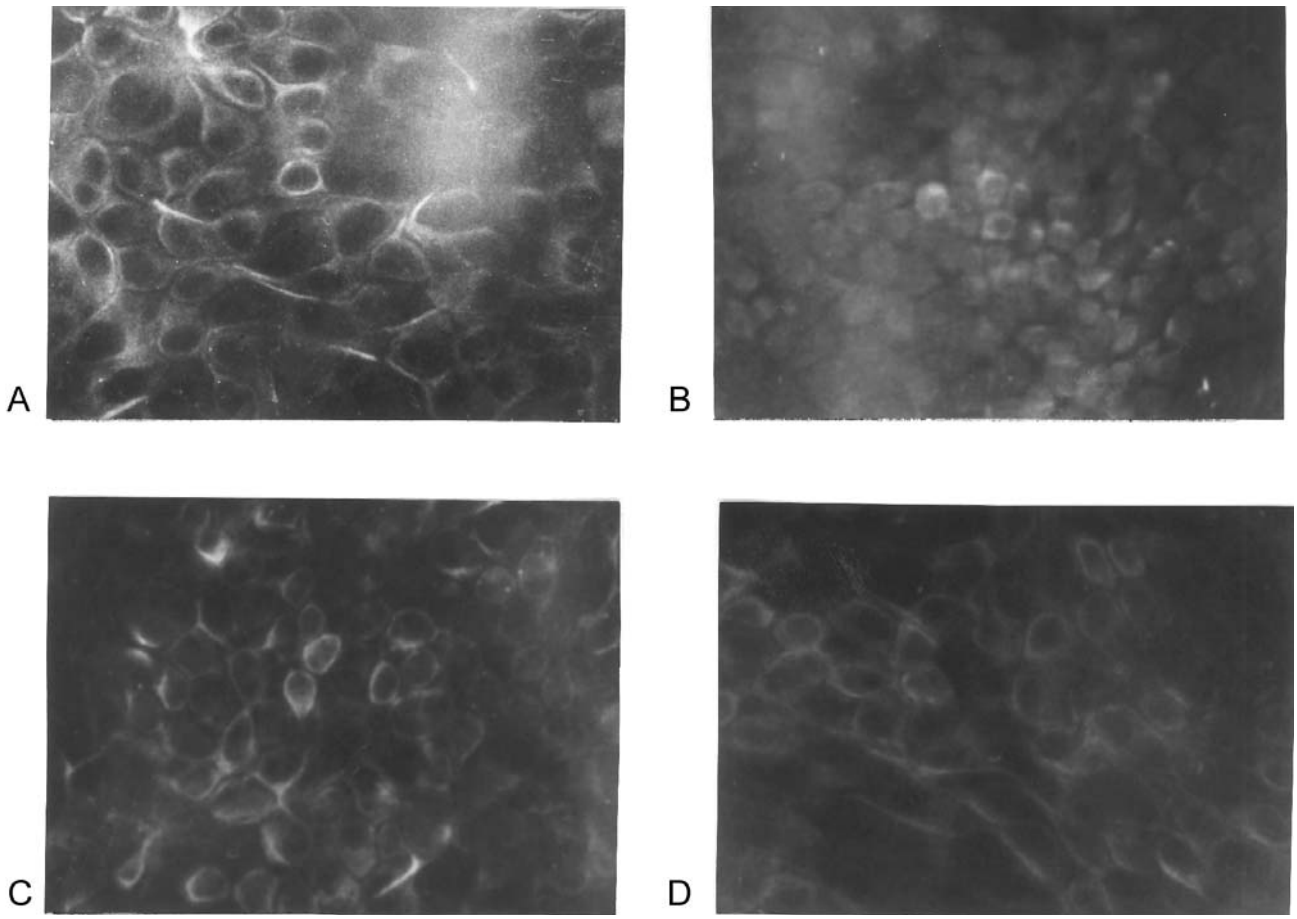

Figure 3. *Localization of Cx 43 protein in MDCK cells altered by DMN exposure with or without EGCG. MDCK cells grown in wells of Lab-Tex chamber slides were fixed and stained with monoclonal anti-Cx 43 antibody using an indirect immunofluorescent method. (A) MDCK cells without treatment; (B) MDCK cells exposed to 0.1% DMN; (C) MDCK cells treated with 10 μg/ml EGCG; (D) MDCK cells pretreated with 10 μg/ml EGCG and then exposed to 0.1% DMN. (Original magnification x200)*

bands (43, 45 and 47 kDa). DMN inhibited the phosphorylation of Cx 43. EGCG pretreatment increased the phosphorylation of Cx 43 protein (Figure 4).

Discussion

Our results demonstrated that the preservation of GJIC might be a potential mechanism of the preventive effect from green tea on renal epithelial cells treated with a renal carcinogen *in vitro*. Dimethylnitrosamine decreased cell-membrane-associated Cx 43 levels, thereby increasing the cytoplasmic Cx 43 concentration (15,18). EGCG enhanced the expression of Cx 43 protein at the plasma membrane. EGCG up-regulated the expression of Cx 43 protein by affecting the phosphorylation state in MDCK cells exposed to DMN. These results indicate that one mechanism of the chemopreventive effect of EGCG in renal epithelial cells is the recovery of GJIC function by the normalized distribution of Cx 43 protein. This is in accordance with data from literature when similar effects of epicatechin on rat liver cells were described (19).

Few epidemiological studies have examined the association between green tea and renal cell carcinoma. Nonetheless, there is suggestive evidence that a Western diet high in meat and low in vegetables and fruits promotes renal cell carcinoma development (20). Yuan *et al.* reported that they did not find any association of renal cell carcinoma risk with consumption of coffee, black tea, or alcoholic beverages (21). A typical black tea beverage contains 3-10% catechins.

Recent studies have shown that green tea extract exerts strong anticarcinogenic effects in the skin, lungs and liver (22). Yoshioka *et al.* provided evidence for the cancer-protective effects of green tea against N-ethyl-N

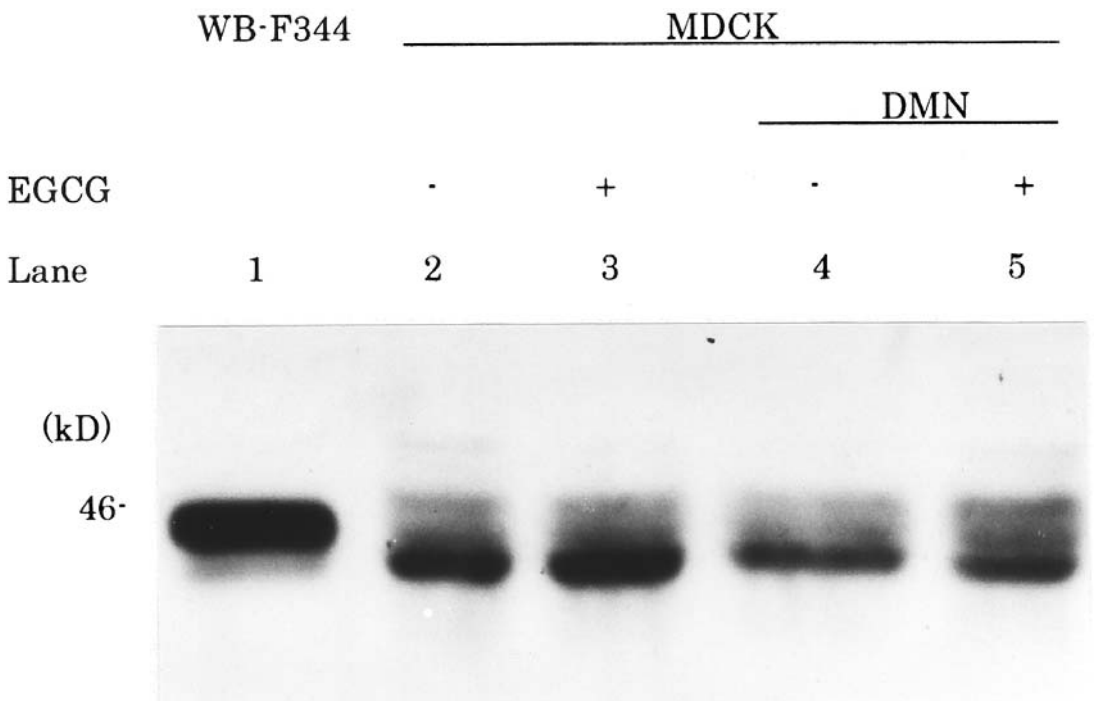

Figure 4. *Western blots of Cx 43 protein expresstion. Changes of Cx 43 protein expression in MDCK cells was altered by DMN-exposure with or without EGCG. Lane 1, WB cells as positive control; lane 2, MDCK cells treated with ethanol; lane 3, MDCK cells treated with EGCG; lane 3, MDCK cells exposed to DMN; lane 4, MDCK cells treated with EGCG and exposed to DMN.*

hydroxyethylnitrosamine-induced renal carcinogenesis (23). Green tea components have been shown to inhibit carcinogenesis by several mechanisms (24). On the other hand, Aminah *et al.* found that green tea performs limited antineoplastic activities (25). Little information is available about renal cancer protection, so we examined the preventive effect of EGCG on renal epithelial cells exposed to a tumor carcinogen.

Cx 43 is a major gap junction protein in the human kidney expressed in glomerular epithelial, renal tubular and surrounding cells (26). Cx 43 expression in a human renal cancer cell line is significantly decreased as compared to a normal, noncarcinogenic, renal cell line (27). The present study showed that EGCG prevented DMN-induced inhibition of GJIC in normal renal epithelial cells by the normalized distribution of Cx 43 protein.

It is widely known that the incidence of end-stage renal disease and chronic renal failure has increased in advanced countries because of the escalating incidence of diabetes and cardiovascular disease. Patients with chronic renal failure often experience acquired cystic kidney disease, which may subsequently lead to the development of renal cell carcinoma. One strategy for reducing the prevalence of renal cancer may be administering ECGC to patients with chronic renal dysfunction.

Conclusion

These results suggest that the preservation of GJIC may indicate the preventive effect of green tea on renal epithelial cells treated with a renal carcinogen *in vitro*.

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