Inhibitory Effect of Green Tea on Injury to a Cultured Renal Epithelial Cell Line, LLC-PK₁

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When cells from a cultured renal epithelial cell line, LLC-PK₁, were cultured under hypoxic conditions (oxygen concentration of 2% or less) before reoxygenation was applied (95% air, 5% CO₂), the leakage of lactate dehydrogenase (LDH) into the medium increased. This phenomenon was induced in the presence of dimethyl sulfoxide, a hydroxyl radical scavenger, suggesting the involvement of free radicals. Such oxidative stress was significantly inhibited by a green tea extract, and more potently by a tannin mixture. On the other hand, under ordinary culture conditions (95% air, 5% CO₂), there was cell injury, although the LDH leakage was less than that under hypoxia/reoxygenation, and such injury was induced by the green tea extract and the tannin mixture.

Key words: green tea; renal epithelial cell; LLC-PK₁; culture; lactate dehydrogenase

It is known that acute renal failure occurs as a result of reperfusion after a certain period of blood flow blockage. Histopathological studies of such ischemic acute renal failure, have revealed that casts were formed in unirnferous tubules, and that exfoliation and necrosis of epithelial cells occurred in proximal tubules, suggesting injury to tubular epithelial cells under this condition. According to this study, the kidney is an organ comprising cells of various types, and it is therefore difficult to investigate biochemical changes in tubular epithelial cells in a whole kidney. Recent years have seen technical advances in cell culture and the establishment of various kidney-derived cell culture lines which have the characteristic features peculiar to cells of the proximal tubule, distal tubule, or collecting tubule. In the present study, we investigated the effects of green tea, which is known to lessen oxidative stress in rats with renal failure, by using cells of a porcine kidney-derived cultured epithelial cell line, LLC-PK₁. This line had the nature of a proximal unirnferous tubule known to be severely injured in ischemic acute renal failure, and is thus established as an in vitro system for assessing ischemic-reperfusion renal injury.

Fetal calf serum (FCS) and Dulbecco's modified Eagle medium/ nutrient mixture F-12 (D-MEM/F-12) were purchased from Cell Culture Laboratories (Cleveland, OH, U.S.A.) and Life Technologies (Grand Island, NY, U.S.A.), respectively.

Fifty grams of dry green tea leaves, which had been produced in the Haibara district (Shizuoka, Japan), were added to 1 liter of hot distilled water (70°C) and shaken for 5 min. The resulting supernatant was freeze-dried to obtain a green tea extract. The green tea tannin mixture used in this study was Sunphenon® (Taiyo Kagaku Co., Yokkaichi, Japan), which had been prepared from a hot-water extract of green tea, as reported previously. It was composed mainly of (-)-epigallocatechin 3-O-gallate (18.0%), (-)-gallocatechin 3-O-gallate (11.6%), (-)-epicatechin 3-O-gallate (4.6%), (-)-epigallocatechin (15.0%), (+)-gallocatechin (14.8%), (-)-epicatechin (7.0%), and (+)-catechin (3.5%). Caffeine was purchased from Sigma Chemical Co. and theanine was obtained from Wako Pure Chemical Ind.

LLC-PK₁ cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air in 96-well culture plates (Corning Glass Works, Corning, NY, U.S.A.) with a 5% FCS-supplemented D-MEM/F-12 medium. After confluence had been reached, the cells were seeded in culture plates at 10⁵ cells per well. With three plates, the green tea extract was added, and the plates were incubated for 41 h. The cells were cultured for a further 6 h under a hypoxic condition (oxygen concentration of 2% or less) in a BB1 Gas Pak Pouch™ and then reoxygenized for 1 h under ordinary culture conditions (95% air, 5% CO₂) in a CO₂ incubator. In another experiment, the cells were cultured under ordinary culture conditions for 48 h. Any leakage of lactate dehydrogenase (LDH) into the culture medium was assayed as an index of cytotoxicity by a commercial kit from Wako Pure Chemical Ind. Ltd.

The results are each presented as the mean ± S.E. of 5 determinations. The data were analyzed for statistical significance by Dunnett's method, differences at p<0.05 being considered statistically significant.

As the result of reoxygenation after 6 h of hypoxic culture, the leakage of LDH from the LLC-PK₁ cells into the culture medium markedly increased to about the level of 150 μIU/ml. LDH leakage from the cells decreased after dimethyl sulfoxide had been added to the medium. This suppression became more marked as the concentration of dimethyl sulfoxide increased, the LDH activity (118.9 μIU/ml) in the presence of 0.004 μm dimethyl sulfoxide being comparable to that in the absence of dimethyl sulfoxide (146.2 μIU/ml). On the other hand, when the green tea extract was added to the medium to give a final concentration of 1.25 μg/ml, leakage of the enzyme due to reoxygenation was significantly reduced to 130.1±4.8 μIU/ml, as shown in Fig. 1. This suppressive effect increased with increasing concentration of the green tea extract. A similar effect was also obtained with the tea tannin mixture, this effect being more potent than that of the green tea extract at higher concentrations. However, caffeine, which has an arousal action and is proper to tea leaves, was found to be devoid of such an effect, while theanine, a component contributory to the "tastefulness" of tea, significantly inhibited the leakage of LDH at concentrations of 25 and 50 μg. Similar changes produced by hypoxia/reoxygenation was observed under the ordinary culture conditions. As shown in Fig. 2, LDH leakage from the cells into the culture medium was found to be suppressed in a dose-dependent manner when the effect of the tea tannin mixture was examined. Similar to the effects of the tea tannin mixture, the extract had a suppressive effect at a low concentration.

Yonehara and Gembaf have demonstrated with LLC-PK₁ cells that the intracellular antioxidant, glutathione, was significantly decreased under hypoxic conditions. Snowdowne et al. and Kribben et al. have also reported that hypoxia caused the intracellular Ca²⁺ concentration to increase in another kidney-
derived culture cell line, LLC-MK₂, or in isolated uriniferous tubules. On the other hand, with regard to cell injury due to reoxygenation, Paller et al.⁴ proposed in 1984 the new theory that active oxygen is involved in the pathogenesis of ischemic-reperfusion renal injury, which had considerable influence in this field of science. Since then, a close relationship between ischemic-reperfusion injury in various organs and their diseases has become gradually apparent. In the proximal tubule-like LLC-PK₁ cells used in the present study, the leakage of LDH was suppressed when dimethyl sulfoxide, a hydroxyl radical scavenger, was added to the medium prior to initiating the culture, suggesting that free radicals produced by renal epithelial cells through hypoxia and reoxygenation were responsible for the cell injury. This finding is consistent with the observation reported by Paller and Neumann⁵ that renal epithelial cells produced free radicals in the primary renal cell culture. The fact that this type of cell injury was suppressed by the green tea extract, and more potently by tea tannin, a component of the green tea extract, is the in vitro evidence showing that green tea tannin directly acts on renal cells, in corroborarion of our previous finding that the oral administration of green tea tannin improved renal failure in rats under oxidative stress.⁶

On the other hand, when LLC-PK₁ cells were cultured only under ordinary conditions (95% air, 5% CO₂), the amount of
LDH that leaked into the medium was smaller (100–120 mIU/ml) than that under hypoxia/reoxygenation, demonstrating great oxidative stress due to hypoxia/reoxygenation at the cellular level. The green tea extract added to the medium at a concentration of 2.5 µg just before the initiation of culture resulted in significant inhibition of LDH leakage. The tea tannin mixture proved to be the most potent, showing significant inhibition at a concentration of 1.25 µg and at a 34% lower level at a concentration of 50 µg when compared with the value in the absence of the tannin mixture. However, similar to the results under hypoxia/reoxygenation, caffeine showed no such inhibitory effect as that observed with the tannin mixture. Theanine caused significant inhibition of LDH leakage only at a concentration of 50 µg.

The present study, which was designed for determining the effects of green tea on LLC-PK1 cells, a cell line having characteristic features similar to those of the proximal tubule, which is known to be injured in ischemic renal failure, provided additional evidence of the direct action of green tea on the kidney in parallel with its effects on mesangial cells.12)

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