A Bioactive Polyphenolic Constituent in the Bark of *Pterocarpus indicus*, Willd and Its Effects on Animal Cell Phenotypes *in Vitro*

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Earlier isolated plant polyphenol PI, which is carcinostatic on mice implanted with Ehrlich carcinoma [S. Takeuchi *et al.*, *Agric. Biol. Chem.*, **50**, 569 (1986)], showed transient, nonspecific, non-cytotoxic growth inhibition *in vitro* on both normal and malignant cells. Examination of its effects on alteration of native and TPA-induced *in vitro* phenotypes revealed that PI suppress both the plasmin and ODC activities of the cells that increase with spontaneous malignancy, and also the TPA-induced ODC activity increase. TPA-induced decrease of ^{125}I -mEGF-binding ability was reversed by PI, to larger extent in the HeLa cells than in the untransformed RME-5-3-1 cells.

A woody legume, *Pterocarpus indicus*, Willd, contains a bioactive polyphenolic substance, PI, isolated from the bark extract as a brownish powder.¹⁾ PI gave a single peak in HPLC and its homogeneity was confirmed by the single symmetrical sedimentation boundary in analytical ultracentrifugation. The molecular weight was approximately 20,400. Though PI seemed to be a polymeric flavanol, its homogeneity and its molecular weight larger than those of most of this group indicate some unusual characteristic. PI had a strong antiplasmin activity, and also a carcinostatic effect on mice bearing ascites Ehrlich carcinoma.¹⁾

This paper deals with later studies on the bioactivities of PI, especially those on *in vitro* phenotypes closely related to cell transformation, which are the suppressive effect on the cellular plasmin activity, the influence on the cellular ODC activity, native or TPA-induced, and the interaction between PI and TPA on the variation of ^{125}I -mEGF-binding ability of cells. Many malignant animal cells secrete a plasminogen activator^{2~4)} that activates

plasminogen to active plasmin.^{5,6)} The mode of induction of this activator appears to be closely related to malignant cell transformation of various origins. Ornithine decarboxylase (ODC) participates in the growth regulation of animal cells through polyamine biosynthesis,^{7,8)} and the variation of ODC activity is said to be closely related to the phenotypic malignancy of cells.^{9,10)} The epidermal growth factor (EGF) secreted from many varieties of animal cells, is said to control metabolic regulation by binding with a specific receptor (EGF-R) on cell surfaces.^{11,12)} Its amount in transformed cells is often abnormally lower or higher than that in normal cells.^{11~16,25)} In our previous work, for example, that untransformed RME-5-3-1 cells bind EGF strongly, but the transformed RME-5-1/TMT cells do not.¹⁶⁾ 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) is a potent tumor promotor that causes pronounced perturbations of cellular phenotypes,^{17~22)} with induction of plasminogen activator in cells, transient enhancement of the ODC activity, and reduction of EGF-binding

by cells.

MATERIALS AND METHODS

Isolation of PI.¹⁾ Five hundred grams of the powdered bark of *Pterocarpus indicus*, Willd was extracted with 70% aqueous methanol. The filtered extract was concentrated and adsorbed on a Sephadex LH20 column (4 × 70 cm), which was washed with water and eluted with 90% aqueous dioxane. The concentrated active fraction was dialyzed against distilled water, dried, and dissolved in a small volume of 90% aqueous methanol for preparative HPLC (column: Shodex Polymerpak D-2014, 2 × 55 cm, solvent: 90% MeOH, flow rate: 8 ml/min). The main peak at 14 min retention time in HPLC was lyophilized after dialysis. PI was obtained as a brownish powder (350 mg) and gave a single HPLC peak.

Cells and culture media. RME-5-3-1 cells: Untransformed diploid fibroblasts of RME-5-3-1 cells used in this study were established in our laboratory from C3H/He mouse embryos by long-term culture with a benzaldehyde-containing medium.¹⁶⁾ RME-5-1/TMT cells: Transformed malignant RME-5-1/TMT cells¹⁶⁾ were reestablished from the tumors in C3H/He mouse induced by implantation of spontaneously transformed RME-5-1 cells,¹⁶⁾ which were established from the same embryos as RME-5-3-1 cells in normal medium. Culb TC/R/TC cells: Malignant Culb TC/R/TC cells are rat liver cells transformed by treatment with 4-nitroquinoline-*N*-oxide, *in vitro*, back-transplanted into rats, and reestablished.²³⁾ This cell line was a gift from the late Prof. H. Katsuta of Dokkyo University. HeLa cells: This cell line derived from a human cervical carcinoma was a gift from Prof. M. Umeda of the Yokohama City University School of Medicine.

DM-160 basal medium (Kyokuto Pharm., Tokyo) containing 10% fetal bovine serum (FBS, Gibco), 50 units/ml penicillin G and 50 µg/ml streptomycin was used in all of the experiments except for the ¹²⁵I-mEGF binding assay, in which Dulbecco's modified Eagle's media (DMEM, Gibco) containing 10% calf serum (Gibco) and antibiotics as above were used.

Growth curves. Growth curves of cells at 37°C, in a humidified atmosphere of 5% CO₂, were obtained by counting the viable cell numbers at intervals by staining with Crystal Violet in a 60 mm dish (Corning) with 4 ml of medium, which was renewed every other day. All experiments were done in triplicate.

Plasmin assay by fibrin overlay method. Cellular plasmin activity was examined by the fibrin overlay method of Jones *et al.*⁵⁾ RME-5-1/TMT cells were implanted into 60 mm dishes, to a density of about 10⁵ cells/dish and cultured in a 5% CO₂ incubator at 37°C for 4 days. Each culture to be overlaid was washed twice with PBS (–). The

cells were then partially removed with a rubber policeman at the center of the plate in a criss-cross 5 mm in width, for easy observation. Then the fibrin-agarose mixed medium, which contained an appropriate amount of a test sample, was overlaid on the cells, and the dish was further incubated at 37°C for 48 hr. The cloudy fibrin-agarose overlay on the cells was cleared by plasmin activated by malignant cells.

Ornithine decarboxylase (ODC) assay. The ODC activity of the cells was measured by the release of ¹⁴CO₂ from DL-[1-¹⁴C]-ornithine (5.4 mCi/mM, New England Nuclear), essentially by the method by Russel and Snyder⁸⁾ and O'Brien *et al.*²⁴⁾ To assay by measurement of ¹⁴CO₂ release, quiescent RME-5-3-1 cells trypsinized from the confluent culture were suspended in DM-160 medium containing 10% FBS (approximately 3 × 10⁶ cells/100 mm dish) and cultured in a 5% CO₂ incubator at 37°C, with or without addition of PI (12.5 µg/ml), TPA (500 µg/ml) or both. The cells were collected at intervals with a rubber policeman and homogenized in 1 ml of extracting solution.¹⁶⁾ To measure ¹⁴CO₂ release, solution containing 0.4 µmol ornithine, 100 µmol sodium phosphate, 0.4 ml cell extract, and added PI (0 to 50 µg/ml) was preincubated at 37°C for 10 min, then 0.25 µCi of labeled ornithine was added to give a final volume of 2.0 ml in a 30-ml Erlenmeyer flask. After 30 min of incubation, the reaction was stopped by addition of 1 ml of 2 M citric acid. CO₂ released during incubation was trapped in 0.2 ml of ethanolamine contained in the polyethylene center well attached to the rubber stopper of the flask. The center well was transferred to scintillation vial containing 2 ml of ethanol and 10 ml of toluene scintillant, and radioactivity was measured in a Beckman LS-150 counter. The results were expressed as nmoles of CO₂ released per 10⁶ cells.

¹²⁵I-mEGF binding assay. Radiolabeling of mouse epidermal growth factor (mEGF, Collaborative Research, receptor grade) with Na ¹²⁵I (New England Nuclear, carrier free) was done using the method of DeLarco *et al.*,¹³⁾ and the binding assay was done by the method of Todaro *et al.*¹⁴⁾ RME-5-3-1 cells or HeLa cells were cultured (2 × 10⁴ cells/16 mm well, in 1 ml of 10% calf serum containing DMEM) at 37°C for 48 hr, and the medium was then renewed. After 24 hr of cultivation, the medium was again replaced by the same new medium, or by a medium containing an appropriate amount of PI, TPA (500 ng/ml), or both the cells were cultured 5 hr more, and the cells were washed twice with 1 ml of the binding buffer.¹⁴⁾ The binding assay was done for 60 min at 20°C by the addition of 3 ng of ¹²⁵I-mEGF (containing approximately 167,000 cpm) in 0.2 ml of binding buffer. The ¹²⁵I-mEGF-treated cells were washed four times with 0.5 ml of binding buffer and were solubilized in 0.5 ml of the lysing buffer,¹⁴⁾ and the contents were transferred into counting tubes. The radioactivity was measured with a gamma counter (Aloka TDC-501).

RESULTS

Effects of PI on the proliferation of cultured cells

Both of the RME-5-3-1 and Culb TC/R/TC cells showed almost no proliferation during 72 hr of cultivation with 100 $\mu\text{g/ml}$ of PI in the medium, with medium renewals at 24 hr intervals. At the PI concentrations below 50 $\mu\text{g/ml}$, however, the growth curves of the both cell lines ran nearly parallel with but slightly lower than that of the control. As seen in Fig. 1, the proliferation rates recovered upon the exclusion of PI from the medium at 24 hr of the cultivation of RME-5-3-1 cells in PI-containing medium (100 $\mu\text{g/ml}$). It is suggested that the effect of PI on the cell proliferation is temporary and reversible, without functional damage to the cells.

Effects of PI on plasmin activation by RME-5-1/TMT cells

The production of plasminogen activator¹⁵⁾ by malignant RME-5-1/TMT cells was confirmed by the clearing of the cloudy fibrin-agarose overlay on the cultured cells, in the fibrin-agarose overlay method.⁵⁾ With more than added PI 12.5 $\mu\text{g/ml}$, however, no clarification of the overlay was observed, indicating the suppression of the plasmin activity. The concentration required for the suppression seems higher than that was found in a previous experiment for the direct antiplasmin activity ($\text{ED}_{50} = 2.5 \mu\text{g/ml}$) using the esterolytic activity of plasmin as a marker, on tosyl-arginine methyl ester.¹⁾

Effects of PI on ODC activities

The extract of RME-5-3-1 cells ($10^6/100 \text{ mm}$ dish), cultured for 6 hr from the quiescent state in normal medium, was incubated in the presence of PI, and released $^{14}\text{CO}_2$ was measured. PI inhibited ODC activity directly by $\text{ED}_{50} = 20 \mu\text{g/ml}$. Also, the effect of PI on the cellular ODC activity was examined by cultivation of RME-5-3-1 cells in the medium containing PI at various concentrations. At times the treated cells were collected and ODC activity

FIG. 1. Recovery from Growth Inhibition after Removal of PI from a Culture of RME-5-3-1 Cells.

RME-5-3-1 cells were grown at 37°C in PI-free medium (○), PI-containing medium (100 $\mu\text{g/ml}$; ●), or with medium exchange at 24 hr intervals from PI-containing to PI-free medium (△). The numbers of viable cells were counted at intervals with Crystal Violet staining. Whiskers indicate maximum and minimum counts.

FIG. 2. Inhibition by PI of Cellular ODC Activity.

Quiescent RME-5-3-1 cells were cultured at 37°C in media containing various concentration of PI, and the ODC activities were measured at 4 hr (closed column) and 8 hr (open column) of cultivation. The radioactivities of released $^{14}\text{CO}_2$ from DL-[1- ^{14}C]-ornithine by inoculation with the cell extracts were measured by trapping with ethanolamine.

of their extracts was measured. As seen in Fig. 2, cellular ODC activities in the treated cultures were dose-dependently reduced in the media containing PI at concentrations between 12.5 $\mu\text{g/ml}$ to 50 $\mu\text{g/ml}$, after both 4 and 8 hr.

FIG. 3. Suppression by PI of the Increase of ODC Activity Induced by TPA.

Quiescent RME-5-3-1 cells were cultured with media containing PI (12.5 μ g/ml) and/or TPA (500 ng/ml). (A) control, (B) in PI-containing medium, (C) in TPA-containing medium, and (D) in (PI+TPA)-containing medium, and ODC activities were measured, by the same methods in the Fig. 2, at 6 hr of cultivation.

Effects of PI on TPA-induced ODC enhancement

We did an experiment on counteraction of TPA and PI, in which RME-5-3-1 cells were cultured in the media containing PI (12.5 μ g/ml) and/or TPA (500 ng/ml), in the same manner as above. As seen in Fig. 3, the ODC activity was decreased by treating the cells with medium containing PI alone, but was greatly increased by cultivation in TPA medium without PI, both after 6 hr of cultivation. When PI was present along with TPA, the TPA-induced increase of ODC were significantly suppressed, nearing the control level. These results were in accord with assumption of the counteraction between TPA and PI.

Influence of PI and/or TPA on 125 I-mEGF binding ability of cells

To examine the reciprocal action between PI and TPA on the variation of EGF-binding ability of cells, we first examined the effects of TPA and/or PI on the binding ability of RME-5-3-1 cells by precultivation for 3 days in normal medium and treatment with media containing TPA and/or PI for 5 hr. No influence of PI on the binding ability was observed

FIG. 4. Reversal by PI of the TPA-induced Decrease of 125 I-mEGF-binding Ability of RME-5-3-1 Cells.

RME-5-3-1 cells were cultured for 5 hr at 37°C in (A) blank medium, or in media containing (B) 500 ng/ml TPA (C) 500 ng/ml TPA and 12.5 μ g/ml PI, and (D) 500 ng/ml TPA and 25.0 μ g/ml PI. These cultured cells were treated with 0.2 ml of 125 I-mEGF containing buffer (3 ng/0.2 ml, about 167,000 cpm) at 20°C for 60 min, and the radioactivities of the treated cells were counted with a gamma counter.

FIG. 5. Reversal by PI of the TPA-induced Decrease of 125 I-mEGF-binding Ability of HeLa Cells.

HeLa cells were cultured for 5 hr at 37°C in (A) plain medium, or in media containing (B) PI, (C) TPA, and (D) TPA and PI. Concentrations: TPA 500 ng/ml, PI 12.5 μ g/ml. The EGF-binding assay was the same as in Fig. 4.

with media containing 12.5 μ g/ml or 25.0 μ g/ml PI. TPA in the medium (500 ng/ml) decreased the 125 I-mEGF-binding ability (Fig. 4B). With cells treated with the medium containing both TPA (500 ng/ml) and PI (12.5 μ g/ml or 25.0 μ g/ml), the decrease of EGF-binding ability by TPA was partially reversed by PI (Fig. 4C, D).

In another set of experiments, HeLa cells were used instead of RME-5-3-1 cells, as a representative example of a malignant cell line having high EGF-binding ability. As seen in Fig. 5, the results were very different from those obtained for RME-5-3-1 cells; both TPA and PI decreased the EGF-binding ability (Fig. 5B, C), but their simultaneous presence in the medium seemed to cancel each other's activity (Fig. 5D).

DISCUSSION

Our preliminary examination of the effects of PI on *in vitro* growth of animal cells found inhibition at concentrations above 50 $\mu\text{g/ml}$. However, this inhibition was not specific to malignant cells and the growth had recovered to similar to the normal rate 24 hr after the replacement of PI-containing medium by normal medium, which suggested that the activity of PI is essentially not cytotoxic. We accordingly examined the influence of PI on the *in vitro* phenotypic expression of animal cells, native or TPA-induced. PI suppressed the native plasmin- and ODC activities in the cells, the TPA-induced increase of ODC activity, and also the TPA-induced decrease of ^{125}I -mEGF-binding ability of the cells.

As has been described, the observed restorative effect of PI on the TPA-induced suppression of EGF-binding ability of HeLa cells was much greater than on RME-5-3-1 cells. It is very probable that this case is an additional example of the cell strain-dependent behavior of the EGF receptors that have been frequently reported and that have been attributed to the quantitative and qualitative differences of the receptor from cell to cell.^{11-16,25)}

It is unlikely that the apparently interactive action between TPA and PI observed is due to their chemical interactions, considering their chemical properties. As polyphenols show high affinities to proteins and are often bioactive due to these affinities, it is more reasonable to consider that the apparent interaction is physiological.

In this connection, Nishizuka²⁶⁾ has sug-

gested that TPA binds with and activates protein kinase C on the cell membrane. More recently other workers also have suggested that activated kinase C is involved in ODC induction and the decrease of EGF-binding ability.²⁷⁻²⁹⁾ These observations and the results of our study may be in some way related to each other by future studies.

The observed bioactivities of PI on *in vitro* animal cells are at least in accord with the supposition that PI suppresses TPA-induced tumor promotion, even though it is too early to relate them directly to its previously observed effect on Ehrlich carcinoma.

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