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# HETEROGENEOUS LOCALIZATION OF TWO CYTOCHROME P-450-DEPENDENT MONOOXYGENASE ACTIVITIES IN DISPERSED CELLS OF MOUSE LUNG*

Yoshihiro Urade[†], Ryotaro Yoshida[†], Hajime Kitamura[§], and Osamu Hayaishi[†]

[†]Department of Medical Chemistry,
Kyoto University Faculty of Medicine,
Kyoto 606, Japan and
[§]Department of Pathology,
Osaka University Medical School,
Osaka 530, Japan

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Cellular localization of two cytochrome P-450-dependent monooxygenase activities (7-ethoxycoumarin deethylase and coumarin hydroxylase) in the mouse lung was examined with the dispersed cells, which were fractionated on a Percoll isopycnic gradient and identified by electron microscopy. The 7-ethoxycoumarin deethylase activity was widely distributed in various types of lung cells. The coumarin hydroxylase activity, on the other hand, was almost exclusively localized in Clara cell-rich fractions (80% purity) in terms of both total and specific activities. Because coumarin hydroxylase is associated with cytochrome P-450, reportedly involved in the activation of various procarcinogens and necrotoxic substances, a high frequency of chemically induced cancer and necrosis in Clara cells may, at least in part, be explained by the exclusive localization of this enzyme activity in this type of cells.

## INTRODUCTION

Cytochrome P-450s catalyze the monooxygenation reactions of various xenobiotics and steroids. Several forms of cytochrome P-450s are heterogeneously distributed in various organs of mammals (1). Some chemical carcinogens or toxins, which are activated to highly reactive products by cytochrome P-450s, induce cancer or acute necrosis in Clara cells (2-4). To investigate the cellular distribution of cytochrome P-450s in the mouse lung,

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two typical cytochrome P-450-dependent monooxygenase activities, 7-ethoxycoumarin deethylase and coumarin hydroxylase activities, were determined in the dispersed lung cells, which were fractionated in an isopycnic gradient and identified by electron microscopy. We report herein that, in the mouse lung, the 7-ethoxycoumarin deethylase activity is widely distributed in various types of cells but the coumarin hydroxylase activity is almost exclusively localized in Clara cells.

## MATERIALS AND METHODS

Chemicals——Collagenase (type 3) was purchased from Worthington. Trypsin (1:250) was obtained from Difco. Soybean trypsin inhibitor and deoxyribonuclease (type 2) were products of Sigma. Percoll was obtained from Pharmacia. 7-Ethoxycoumarin, coumarin and 7-hydroxycoumarin were purchased from Aldrich. All other chemicals were of reagent grade.

Animals——All animals used in this study were male specific pathogen free Slc:ICR mice, weighing 31 ± 2 g, and were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan. All mice were raised under specific pathogen free conditions at 25 ± 2°C and about 50% humidity in the Institute of Laboratory Animals, Kyoto University.

Preparation of Totally Dispersed Cells from Mouse Lung——Mice were anesthetized by an intraperitoneal administration of 50 µl of a pentobarbital solution (50 mg/ml). The chest cavity was opened and the lung was perfused with phosphate-buffered saline (PBS)[1/] (10 mM phosphate buffer, pH 7.4). Following the perfusion, a 0.1% collagenase, 0.1% trypsin and 0.005% deoxyribonuclease solution in PBS was instilled into both vascular and alveolar space via pulmonary artery and trachea, respectively. Five lungs were excised intact, immersed in 10 ml of the protease solution, and incubated at 37°C for 1 h in a spinner flask (Bellco Glass Inc.) with gentle mixing. The incubation mixture was chilled to 4°C, an excess amount of soybean trypsin inhibitor was added, and the cell suspension was filtered through nylon cloth (40 µm mesh). The totally dispersed lung cells were recovered by centrifugation at 500 X g for 10 min at 4°C and washed twice with ice-cold PBS by the same method. About 6 X $10^7$ cells were obtained from the mouse lung by this dissociation procedure, and almost all of these cells were viable by the trypan blue exclusion test (5). The detailed method for the preparation of the dispersed lung cells will be described elsewhere.

Isopycnic Gradient Centrifugation——The dispersed cells (7 X $10^7$ cells) were resuspended in 4 ml of ice-cold 90% v/v Percoll in PBS (density 1.112 g/ml) and applied to a linear density gradient (total 36 ml), which ranged from 5% v/v Percoll in PBS (density 1.010 g/ml) to 80% v/v Percoll in PBS (density 1.100

1/ Abbreviation used: PBS, phosphate buffered saline.

g/ml). After centrifugation at 400 X g for 25 min at 4°C, the gradient fractions (4 ml/fraction) were recovered sequentially from the bottom by aspirations. An aliquot (0.1 ml) of each fraction was retained for the density determination with a refractometer (Atsube Ltd., Tokyo, Japan). Each fraction was diluted with 5 volumes of ice-cold PBS, centrifuged at 500 X g for 10 min at 4°C, and the pellet was washed twice in the same way. The recoveries of cells, protein and the cytochrome P-450 activities by this fractionation procedure were more than 90%.

Enzyme Assays and Protein Determination——Lungs and the dispersed cells were homogenized with 5 volumes of ice-cold PBS by a Kinematica Polytron homogenizer (Lucerne, Switzerland) and a Bronson Sonifier disrupter (Heat Systems Ultrasonic Inc.), respectively. The 7-ethoxycoumarin deethylase and coumarin hydroxylase activities were determined by fluorometric measurement of 7-hydroxycoumarin production by the method of Aitio (6). The homogenates were incubated with 0.1 mM 7-ethoxycoumarin or 1 mM coumarin in the presence or absence of 1 mM NADPH for 30 min at 37°C in duplicate. The fluorescence of 7-hydroxycoumarin produced was quickly measured 1 min after addition of 10 volumes of 1 M glycine-NaOH (pH 10.0) at an excitation wavelength of 400 nm and an emission wavelength of 460 nm. Protein concentration was determined by the method of Lowry et al. (7) using bovine serum albumin as standard.

Morphological Examination——For electron microscopy, cells were fixed for 3 h in 2% glutaraldehyde in PBS, post-fixed in 2% osmium tetroxide, dehydrated in ethanol, and embedded in Epon by the standard technique. Thin sections were cut with a Porter-Blum ultramicrotome, stained with uranyl acetate-lead citrate, and examined in a JEM 100CX electron microscope. The identification of each cell population was carried out using a group of 400 cells.

## RESULTS

After the enzymatic digestion of the mouse lung, about 40% of protein, and the 7-ethoxycoumarin deethylase and coumarin hydroxylase activities in the lung was recovered in the dispersed cells. The ratio of 7-ethoxycoumarin deethylase activity to coumarin hydroxylase activity in the dispersed cells (20:1) was almost identical with that observed in the lung tissue before the dissociation, suggesting that the dispersed cells contained almost comparable population of cells bearing these two enzyme activities as observed originally in the tissue. Electron microscopy also showed that the destruction of non-cellular structural components occurred time-dependently during the tissue digestion and resulted in the release of heterogeneous population of lung cells.

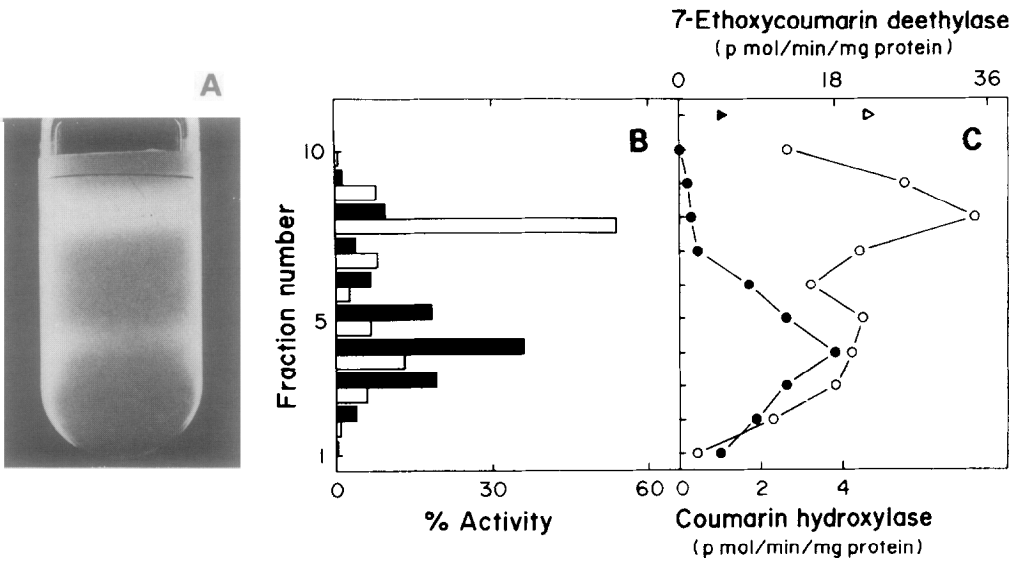


Fig. 1. Distribution of cells, 7-ethoxycoumarin deethylase activity and coumarin hydroxylase activity after isopycnic gradient centrifugation of totally dispersed cells of the mouse lung. Experimental procedures for the dispersion and fractionation of lung cells, and assay methods of these enzyme activities in the fractionated cells were described under Materials and Methods. A. Distribution of the dispersed cells after centrifugation. B. Distribution profile of the total activities of 7-ethoxycoumarin deethylase (□) and coumarin hydroxylase (■). The total enzyme activity recovered in the fractionated cells was expressed as 100% activity. C. Distribution profile of the specific activities of 7-ethoxycoumarin deethylase (o) and coumarin hydroxylase (●). Triangles (△, ▲) represent the specific activities of these enzymes in the cells before fractionation.

When the dispersed cells were fractionated in a Percoll isopycnic gradient (1.010 to 1.100 g/ml), the cells were distributed with two major bands (about 60% and 15% of the total cells) at the densities of 1.040 and 1.080 g/ml, respectively (Fig. 1A). The 7-ethoxycoumarin deethylase activity was mainly (about 65% of the total activity) distributed in the upper band (fractions 7-9), and the distribution pattern was similar to that of the dispersed cells. The coumarin hydroxylase activity was mainly present in the lower band (fractions 3-5) and 80% or more of the total activity was recovered in these fractions (Fig. 1B). When the specific activities of these two enzymes were determined in each fraction (Fig. 1C), the 7-ethoxycoumarin deethylase activity was concentrated only 1.3-fold even in the peak fraction 8 as compared

Table I

Distribution of various types of mouse lung cells in an isopycnic gradient

| Fraction | Density (g/ml) | %Cells | Cell population (%cells in each fraction) |
|---|---|---|---|
| Upper band | 1.040 | 60 | Endothelial cells (42%)<br>Alveolar interstitial cells (36%)<br>Type I epithelial cells (10%) |
| Middle fractions | 1.050-1.070 | 25 | Type II epithelial cells (50%)<br>Alveolar macrophages (20%) |
| Lower band | 1.080 | 15 | Clara cells (65%)<br>Ciliated epithelial cells (30%) |

with that in the unfractionated cells, indicating that this enzyme activity was evenly distributed in various types of lung cells. In contrast, the coumarin hydroxylase activity appeared to be localized in certain types of lung cells because the specific activity was 4- to 5-fold higher in the peak fraction 4 than that in the unfractionated cells.

To determine the types of lung cells, in which two cytochrome P-450-dependent monooxygenase activities were heterogeneously localized, the population of lung cells was examined in these two band fractions and the fractions between two bands by electron microscopy (Table I). The upper band was composed mainly of endothelial cells (42% of cells in these fractions), alveolar interstitial cells (36%) and type I epithelial cells (10%). In the middle fractions, type II epithelial cells (50%) and alveolar macrophages (28%) were enriched. The lower band was mainly composed of Clara cells (65%) and ciliated epithelial cells (30%). The fraction 4, the peak fraction of the coumarin hydroxylase activity, was most enriched with Clara cells (80% or more) (Fig. 2).

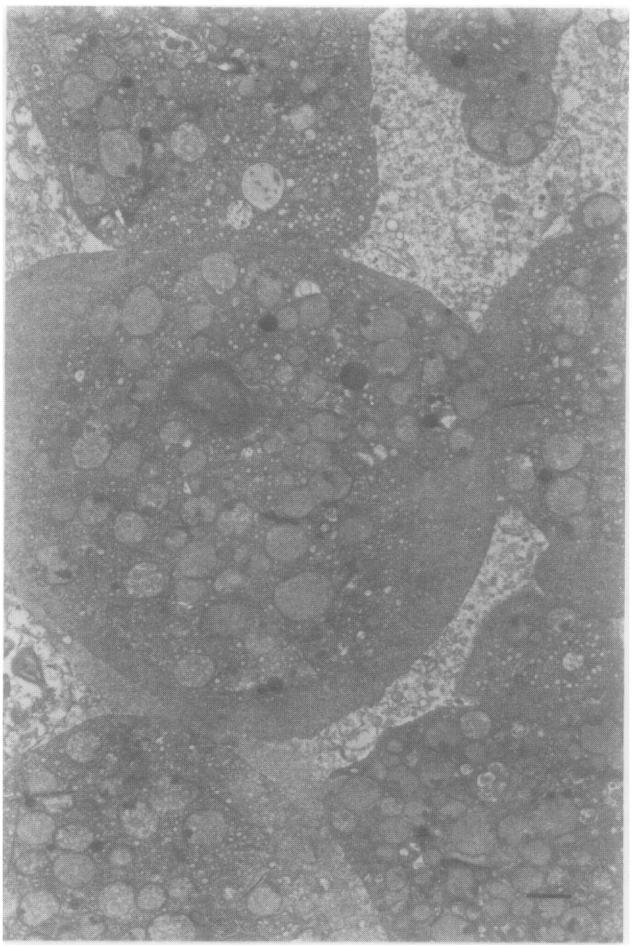

Fig. 2. Electron micrograph of Clara cells concentrated in the peak fraction of the coumarin hydroxylase activity (X 6,000). Bar represents 1 μm. Clara cells were identified by their unique morphological characteristics: 1) abundance of agranular endoplasmic reticulum and 2) numerous membrane-bound ovoid granules (8).

The distribution profile of Clara cells was associated well with that of the coumarin hydroxylase activity.

These findings indicated that, in the mouse lung, the 7-ethoxycoumarin deethylase activity was widely distributed in various types of cells while the coumarin hydroxylase activity was almost exclusively localized in Clara cells.

## DISCUSSION

Devereux and Fouts (9) reported recently the isolation method of Clara cells from the rabbit lung, in which the cells

were fractionated by a centrifugal elutriation and a two-polymer aqueous phase system, but the recovery of Clara cells by their method was less than 10%. In the present study, by only one-step fractionation on a Percoll isopycnic gradient, Clara cells of mice were easily separated from the bulk of other lung cells and concentrated in a single band without significant loss. The purity of Clara cells was more than 80% in the peak fraction 4, and almost all of these cells were viable and morphologically intact (Fig. 2).

The results described in this report indicated that the 7-ethoxycoumarin deethylase activity was widely distributed in various types of mouse lung cells (Fig. 1C). The wide cellular distribution of the enzyme activity was also observed in the rabbit lung by Devereux et al. (10). On the other hand, 80% or more of the coumarin hydroxylase activity was recovered in the Clara cell-rich fractions and the distribution profile was associated well with that of Clara cells (Fig. 1B and C). Chemical procarcinogens such as benzo(a)pyrene were converted to carcinogenic metabolites by the cytochrome P-450 in the mouse lung (11). The benzo(a)pyrene-induced cancer occurs in Clara cells of the mouse lung with a high frequency (2). Thus, the exclusive localization of the coumarin hydroxylase activity in Clara cells may be a critical reason why these cells are the target cells for the chemical carcinogenesis. In addition, Boyd (3) and Boyd et al. (4) reported that some toxins and toxic chemicals such as 4-ipomeanol, various furan derivatives and $CCl_4$ induced acute necrosis specific for Clara cells. They also showed that the toxicity was mediated by a cytochrome P-450-dependent metabolic activation of the parent compounds to highly reactive products. Therefore, the intensive susceptibility of Clara cells to these toxic compounds may also be due to the exclusive localization of the coumarin hydroxylase activity in these cells.

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