Biological Evaluation of the Pollution of the Tsurumi River with 7-Ethoxycoumarin *O*-Deethylase Activity Induced by River Sediment Extracts in HepG2 Cells

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The pollution level of the Tsurumi River flowing into Tokyo Bay was studied longitudinally using river sediment extracts in an assay system based on the arylhydrocarbon receptor (AhR)-dependent induction of 7-ethoxycoumarin *O*-deethylase (ECOD) activity in HepG2 cells. The sampling points of river sediment were as follows: Namamugi, Kami-Sueyoshi, Tsunashima, Shin-Yokohama A and B (downstream and upstream, respectively, from the former open-air industrial waste incineration site), Kozukue and Nakayama (Fig. 1). ECOD activity was induced to different extents in all samples tested. Namamugi, located in the middle of Keihin Industrial District, was considered to be the most polluted, followed by Shin-Yokohama A and Tsunashima. Results from these samples showed reverse U-shaped dose–response curves in terms of ECOD activity as well as 3-methylcholantrene (3-MC) although the expression of CYP1A1 mRNA in HepG2 cells remained roughly constant at higher concentrations as in the case of 3-MC, thus implying post-transcriptional suppression.

Key words —— environmental pollution, river sediment, HepG2 cells, 7-ethoxycoumarin *O*-deethylase, CYP1A1 mRNA, aryl hydrocarbon receptor

INTRODUCTION

The Tsurumi River streams from west to east through the Yokohama City, Kanagawa Prefecture, Japan and flows into Tokyo Bay at Namamugi in the middle of the Keihin Industrial District. Moreover, the inland manufacturing district is located in the river basin. Up to the present, there have been some chemical analysis data publicized concerning the pollution of this river.[1] However, there have been no detailed report on the pollution of the river based on any kind of bioassay.

For the most part, environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated dibenzo-*p*-dioxins and -frans (PCDDs/Fs) are known to induce cytochromes P450 (CYPs), especially CYP1A1 both *in vivo* and *in vitro*.[2–4] Although CYP in cultured cells cannot generally be induced, human hepatoma cell line HepG2 retains this feature and has been applied to a bioassay for environmental pollutants in combination with 7-ethoxycoumarin as a substrate with satisfactory sensitivity.[5]

In a previous study,[6] surface sediment collected at Namamugi on the Tsurumi River estuary was reported to be the most polluted among those collected from several rivers scattered in the Tokyo metropolitan area. This conclution was based on result from a bioassay measuring 7-ethoxycoumarin *O*-deethylase (ECOD) activity in HepG2 cells. Since then, the pollution of the Tsurumi River has been further studied longitudinally at distances up to 20 km upstream from the river estuary. In addition, open-air industrial waste incineration has been performed on the Tsurumi River in Shin-Yokohama halfway upstream from Namamugi. Therefore, the influence of former incineration on river pollution was also studied, although soil stripping and replacement had occurred prior to sampling.

Previously, the authors observed the suppression of ECOD activity at higher concentrations of the sediment extract of Namamugi, showing a reverse

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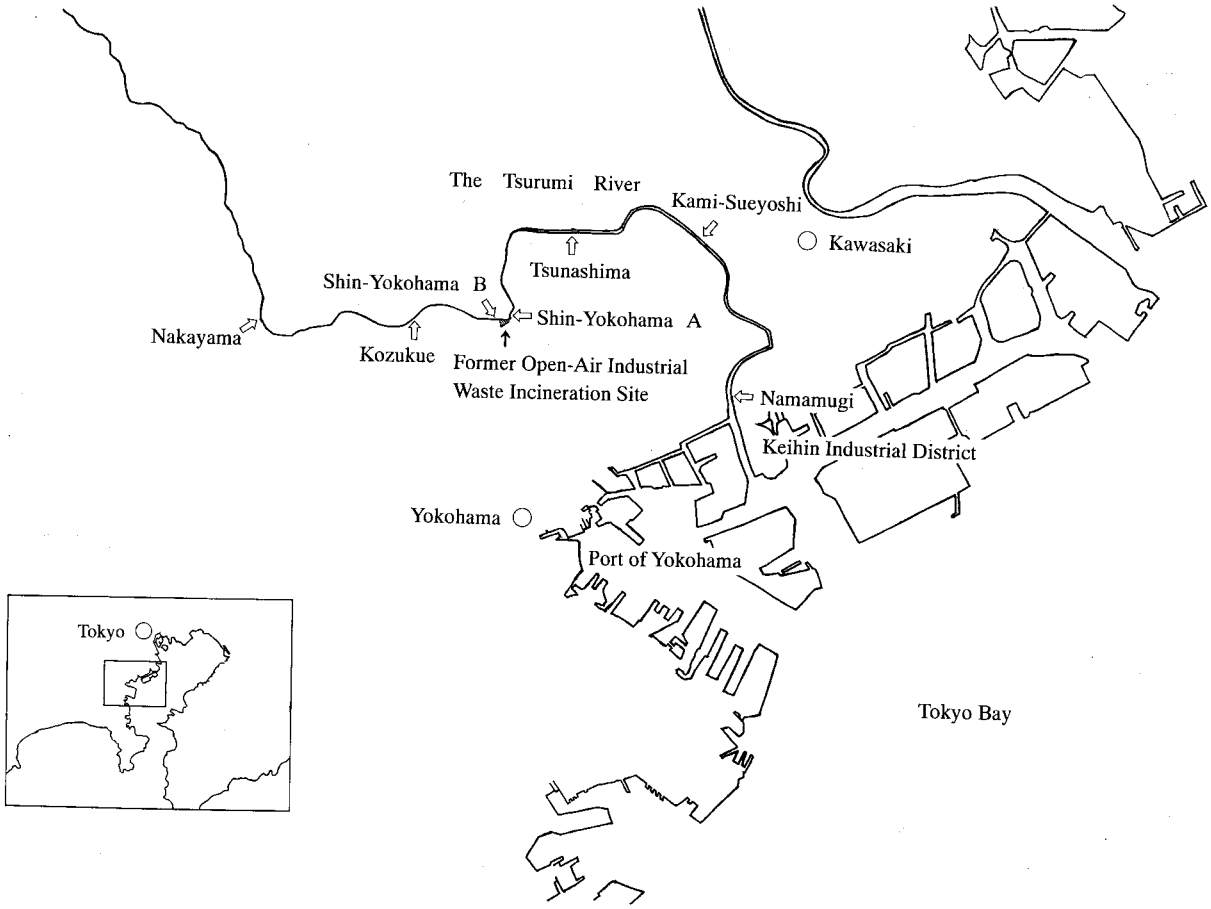

Fig. 1. The Location of River Sediment Sampling Points

U-shape dose–response curve. Based on lactate dehydrogenase activity in the cultured supernatants, these observations could not be attributed to the cytotoxicities.[6] Therefore, the mechanism of suppression in the expression of CYP1A1 induction by river sediment extract was investigated as well as 3-methylcholanthrene (3-MC) as a model compound.

MATERIALS AND METHODS

Sediment Samples —— Surface sediment samples were collected at several points on the Tsurumi River, central Japan, at the sampling points indicated in Fig. 1. Sampling dates for individual points were as follows: Namamugi, Mar. 2000; Shin-Yokohama A and Shin-Yokohama B, May 2001; Tsunashima and Kozukue, Jul. 2001; Kami-Sueyoshi and Nakayama, Jan. 2002.

Reagent —— Dichloromethane and ethylacetate of a grade used for the test of pesticides, acetone, dimethylsulfoxide (DMSO) and 3-MC of guaranteed reagent grade, and β-glucuronidase of the grade suitable for biochemistry were obtained from WAKO Pure Chemical Industries, Ltd. (Japan) Dulbecco's modified Eagle's medium (DMEM) and phenol red-free Eagle's minimum essential medium (MEM) were obtained from Gibco BRL (U.S.A.).

Hybond-N+ was obtained from Amersham Pharmacia Biotech (U.K.). Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA control probe was purchased from Clontech Laboratories. BcaBEST labeling kit (TAKARA, Japan) and [α-^{32}P]dCTP were used for the labeling of probes.

Cell Cultures —— Human hepatoma HepG2 cells were obtained from Cell Resource Center for Biomedical Research, Tohoku University. The cells were grown in DMEM supplemented with 15% fetal bovine serum and subcultured every 7–10 days at a 1 : 3 split ratio.

Extraction —— Sediment samples were air-dried at room temperature and passed through a 10-mesh sieve to remove gravel. Then, 20 g of the sieved fraction was extracted with dichloromethane using

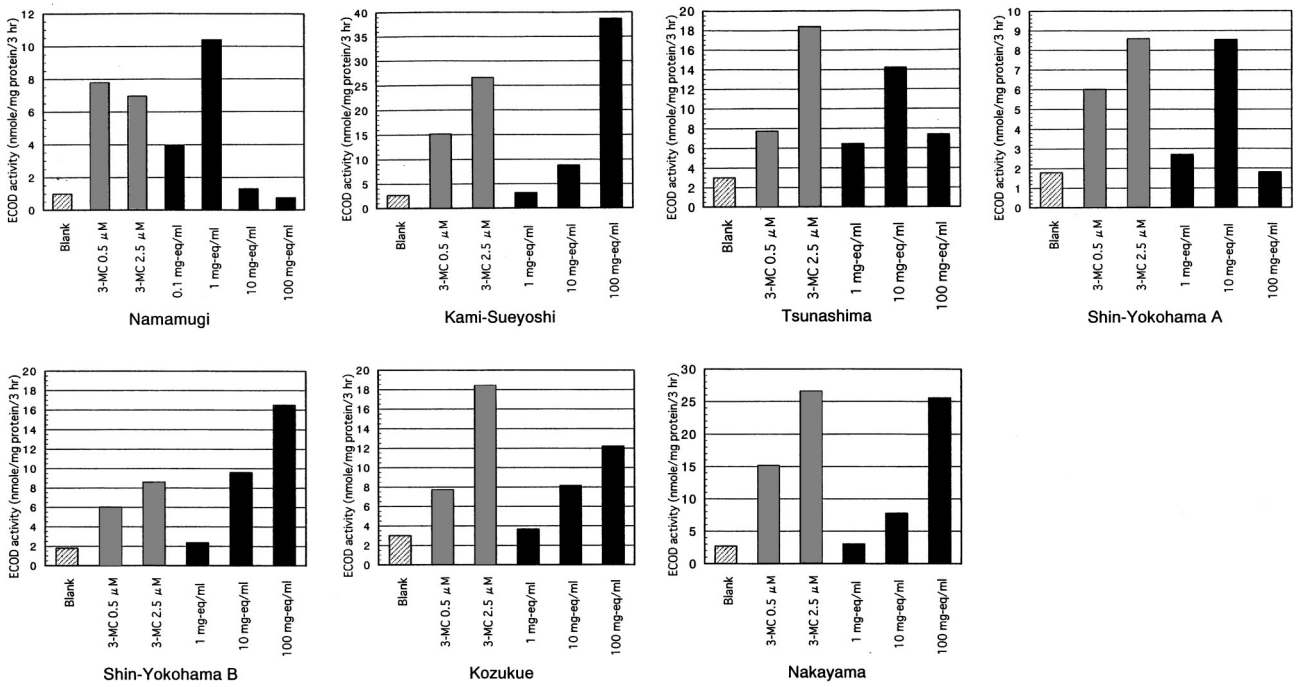

Fig. 2. 7-Ethoxycoumarin *O*-deethylase Activity Induced by River Sediment Extracts

ECOD activity was measured at 37°C for 3 hr using HepG2 cells after being induced in duplicate at 37°C for 16 hr with sediment extracts at final concentrations of 0.1 to 100 mg-sediment equivalent (mg-eq in short)/ml or 3-MC at 0.5 and 2.5 μM.

Soxhlet extractor for 16 hr continuously. The extracts were concentrated to approximately 5 ml with a Kuderna-Danish concentrator, transferred to a 10-ml conical tube, and evaporated to dryness under a N_2 gas stream. The residues were dissolved in 400 μl of DMSO and further diluted 10, 100 and 1000 times; each solution of 12 μl corresponds to 600, 60, 6 or 0.6 mg of sediment.

Determination of ECOD Activity —— The induction and determination of ECOD activity were performed as described previously.[6] A Type F-3000 fluorescence spectrophotometer (Hitachi, Japan) was used for fluorescence spectrophotometry.

Total RNA Preparation —— Total RNA was prepared with an Rneasy kit (Quiagen, Germany) from the HepG2 cells. RNA yield and purity were assessed by A260/A280 ratio.

Northern Blotting —— Total RNA (30 μg) was separated electrophoretically on a denatured 1.2% agarose/2.2 M formaldehyde gel and stained with ethidium bromide for the analysis of mRNA. The gel was equilibrated in 20 × SSPE (3 M NaCl, 200 mM sodium phosphate, 20 mM EDTA, pH 7.4), and the RNA was transferred on to Hybond-N+ using a capillary blotting unit (Scotlab, U.K.). The membrane was prehybridized for 3 hr at 42°C, and hybridized for 20 hr at 42°C separately with either ^{32}P-labeled CYP1A1 cDNA from HepG2 CYP1A1 mRNA or G3PDH cDNA probe. The membrane was exposed to an Imaging Plate and analyzed by Image Analyzer STORM 860 (Amersham Biosciences, U.S.A.).

RESULTS AND DISCUSSION

The results for the induction of ECOD activity in HepG2 cells by river sediment extracts are summarized in Fig. 2. With all the samples from the Tsurumi River, the induction of ECOD activity was observed to different extents. Based on the data obtained, the pollution of Namamugi located at the estuary of the Tsurumi River ranked top in terms of inductivity determined as the reciprocal of the lowest dry-weight equivalent of river sediment giving a significant ECOD activity. The sediment extract of Namamugi induced marked ECOD activity as low as 0.1 mg sediment-equivalent/ml (hereafter referred to as mg-eq/ml). However, at higher concentrations, a decrease in ECOD activity was noted, exhibiting a reverse U-shaped curve with a peak response at 1 mg-eq/ml. Since Namamugi is located in the middle of the Keihin Industrial District, the extensive pollution of the river sediment seems to be the

result of the long-lasting accumulation of pollutants on the river bed. The pollution of river sediment at this sampling point was estimated to be 2 to 20 μg PAHs/g from previous findings.[5)]

With the extract of river sediment sampled at Kami-Sueyoshi located 5 km upstream from Namamugi, the induction of ECOD activity was recognized at concentrations of 10 mg-eq/g or higher in a dose–dependent manner within the highest concentration tested (100 mg-eq/ml). In comparison with Namamugi, Kami-sueyoshi is considered to be less polluted with the putative AhR-dependent inducers of CYP1A. Further, the pollution of Kami-Sueyoshi is less significant than those of Tsunashima and Shin-Yokohama A, both located further upstream on the river.

The extracts of river sediment sampled at Tsunashima and Shin-Yokohama A just downstream from the former open-air industrial waste incineration site induced a maximal response at 10 mg-eq/ml, showing suppression of enzyme activity at 100 mg-eq/g, a one order of magnitude higher concentration than that of Namamugi.

Shin-Yokohama B is located upstream of the above-described former incineration site on the other side of Shin-Yokohama A. The sample of Shin-Yokohama B induced the ECOD activity dose–dependently up to the highest concentration of 100 mg-eq/ml as well as that of Kami-Sueyoshi. The same was true in the cases of Kozukue and Nakayama further upstream of the Tsurumi River where drainage from many manufacturing facilities was probably responsible for the still significant pollution of the river sediments. It is well known that many kinds of causative agents for environmental pollution such as PAHs and dioxins are generated by municipal solid waste incineration[7–9)] and open-air industrial waste incineration. The leaching of the ECOD-inducers in HepG2 cell-assay from the ex-incineration site, though remediation had reportedly been completed, might be responsible for the pollution of the river sediment of Shin-Yokohama A but not Namamugi, because the river sediments of Namamugi and Shin-Yokohama A/Tsunashima were more polluted than that of Kami-Sueyoshi located between Namamugi and Tsunashima along the Tsurumi River.

The reverse U-shaped dose–response curve in the ECOD-induction assay was observed with the river sediments collected at extensively polluted sampling points on the Tsurumi River, *i.e.*, Namamugi, Tsunashima and Shin-Yokohama A. However, the suppression at higher concentrations could not be elucidated by their cytotoxicities against HepG2 cells.[6)] The extract of river sediment collected at Namamugi was evaluated for its effect on the expression of CYP1A1 mRNA in HepG2. The transcriptional effect of 3-MC, by which the down-regulation of 7-ethoxyresorufin *O*-deetylase (EROD) activity had been reported,[10)] was also tested as a representative of PAHs. As shown in Figs. 3 and 4, the expression of CYP1A1 mRNA increased dose–dependently in parallel with the increase in ECOD activity by both 3-MC and the river sediment ex-

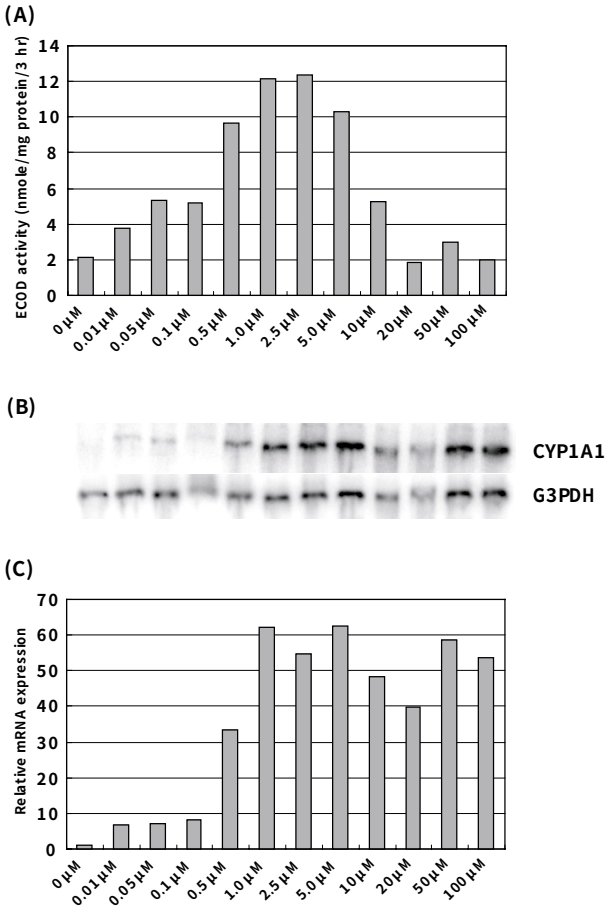

Fig. 3. 7-Ethoxycoumarin *O*-deethylase Activity and Normalized CYP1A1 mRNA Expression in HepG2 Cells in the Presence of 3-MC

(A) ECOD activity. The ECOD activity in HepG2 cells was measured as described in the legend to Fig. 2 except that 3-MC was used in the range of 0.01 to 100 μM instead of 0.5 and 2.5 μM. (B) Northern blots. Total RNA was isolated from HepG2 cells cultured at 37°C for 16 hr in the presence of 0.01 to 100 μM of 3-MC. The 30 μg-portions were used for Northern blots. (C) Readings from Northern blots. Readings are the ratios of the band intensities of CYP1A1 and G3PDH mRNAs normalized by that of the vehicle-treated cells.

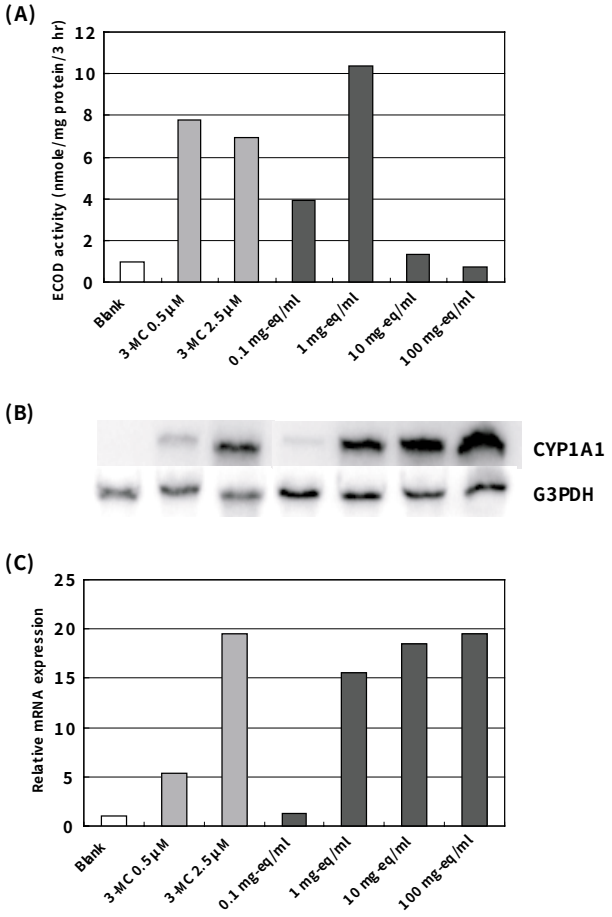

Fig. 4. 7-Ethoxycoumarin *O*-deethylase Activity and Normalized CYP1A1 mRNA Expression in HepG2 Cells in the Presence of River Sediment Extract

(A) ECOD activity. ECOD activity in HepG2 cells was measured as described in the legend to Fig. 2 using the sediment extract of Namamugi at the same final concentrations. (B) Northern blots. Total RNA was isolated from HepG2 cells and processed as described in the legend to Fig. 3 using the river sediment of Namamugi. (C) Readings from Northern blots. The readings are the ratios of the band intensities of CYP1A1 and G3PDH mRNAs normalized by that of the vehicle-treated cells.

tract at lower concentrations free from the decrease in ECOD activity. However, the expression of mRNA was kept roughly constant at higher concentrations in both cases, implying a post-transcriptional nature of the suppression in the enzyme activity. Further studies of their effects on the expression of CYP1A1 apoprotein are now under progress in order to detail the mechanism by which the expression of CYP1A1 is controlled in HepG2 cells.

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