A New Bioassay of Environmental Chemicals Based on Their Effects on Tumor Cell Invasion

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The tumorigenic action of environmental chemical substances has been variously examined, although the effect on malignancies after tumor occurrence has not been studied well. In this study, we established a bioassay system based on tumor cell invasion as a possible index of tumor malignancy, and evaluated the effect of various environmental chemicals on tumor cell invasiveness. We previously established a simple invasion assay system using fluorescent cells and a culture insert with fluorescence blocking micropore membrane (FBM). In this study, the effect of various environmental chemicals on the migration of green fluorescent protein (GFP) gene-transfected HT1080 fibrosarcoma cells through a Matrigel-coated filter was examined. The invasive ability of GFP-HT1080 cells was enhanced in the presence of phenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol, and lindane, and was inhibited with bisphenol A, benthiocarb and others among 31 environmental chemicals tested. Enhancement of the migration of GFP-HT1080 cells with phenol and halogenated phenol derivatives depended on the number of linked chloride moieties. The morphological change of HT1080 cells in the presence of phenol and polychlorophenols was determined by confocal laser fluorescence microscopy, and we observed that it was partly correlated with the invasive characteristics. Finally, we assessed the invasion assay with combinations of chemical substances. As a result, none of the combinations increased the invasion ability of HT1080 cells. Although the effect of environmental chemicals on tumor cell invasiveness may not directly relate to the malignancy of tumor cell itself, it is possible that it relates the tumor progression and metastases.

Key words —— bioassay, environmental chemical, polychlorophenol, invasion, tumor

INTRODUCTION

Environmental chemicals, especially endocrine disrupting chemicals and carcinogenic substances, have been a big concern. Endocrine disrupting chemicals such as bisphenol A and *p*-nonylphenol disrupt homeostasis and affect embryogenesis, growth and behavior.[1–3] Other chemicals such as benzo[*a*]pyrene have tumorigenic activity,[4,5] and heavy metals cause various diseases.[6–8] Since environmental chemicals have multi-functions, the evaluation of unfavorable effects is difficult.[9,10] The tumorigenic action of environmental chemicals, in general, depends on P450 metabolites, which has been studied.[11,12] However, the effect of these substances on the malignancy of generated tumor cells has not been studied well. Tumor cells that acquire malignant characteristics start to invade surrounding tissues and blood vessels, and form secondary colonies. Therefore, the invasion ability of tumor cells is critical for metastases.

Cancer metastasis occurs through a complex cascade of events, including dissociation from the primary site, intravasation, adhesion to the vascular endothelium of the target organ followed by extravasation, and growth at the colonization site.[13,14] The invasion ability of metastatic tumor cells is a key factor in the processes of intravasation and extravasation. At first we established a simple invasion assay system by using florescence blocking membrane and fluorescent tumor cells.[15] The system is quite simple and easy compared with other traditional systems such as the Boyden chamber method.[16] The invasion assay is performed essentially as follows: Filter having size-defined pores is covered with Matrigel, reconstituted basement membrane, or appropriate substrates, and cells which invade from one side of a micropore filter to the other side are counted after fixation and staining. In these methods, cells that do not invade into the filter must be

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removed to reduce the background or false positive counting of invaded cell numbers. The use of fluorescence-labeled cells and fluorescence blocking micropore membrane (FBM) in the cell invasion assay can eliminate the fixation and staining steps. Invaded cells are simply determined under a fluorescence microscope. Furthermore, no false count of non-invaded cells occurs since the cells that do not invade are not detected with fluorescence microscopy.

In the present study, we tried to establish a bioassay system using this invasion assay system, and also to evaluate the environmental chemicals based on their effects on invasion ability of tumor cells, one criteria of tumor malignancy. As a result, some substances actually enhanced tumor cell invasion, although the linkage of the activity and malignancy of the cells is not yet elucidated.

MATERIALS AND METHODS

Chemicals —— Thirty-one environmental chemicals were selected as part of a project of the National Institute for Environmental Studies, Japan Environment Agency. HTS FluoroBlok™ Inserts were kindly provided by Becton Dickinson, Franklin Lakes, NJ, U.S.A. Matrigel was purchased from Becton Dickinson. AlamarBlue™ was from Wako Pure Chemical Industries (Osaka, Japan). Saponin and 4',6-diamidino-2-phenylindole (DAPI) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rhodamine/phalloidin and Anti-fade were from Molecular Probe. All other chemicals used were of analytical grade.

Cells —— HT1080 human fibrosarcoma cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, U.S.A.) at 37°C in a 5% CO_2 atmosphere. A green fluorescent protein (GFP)-gene stable transformant of HT1080 cells was prepared as described previously.[15] In brief, plasmid DNA encoding optimized GFP gene (pEGFP, Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.) was linearized by a cleavage with ApaLI, then purified by phenol extraction and ethanol precipitation. Linearized pEGFP was transfected into HT1080 cells by electroporation. After a 48-hr subculture, fluorescently detected clones were isolated with cloning cylinders and amplified. After several cycles of this procedure, stable transformants were isolated by a limiting dilution method.

Cytotoxicity Assay —— The cytotoxic effect of environmental chemical substances on HT1080 cells was examined by AlamarBlue™ assay. HT1080 cells (5×10^4 cells/well) were seeded on a 24-well culture plate and incubated in RPMI1640 medium supplemented with 10% FBS (10% FBS/RPMI1640) for 24 hr at 37°C. After removal of the medium, cells were incubated in the presence of various environmental chemicals (1 ml) for 4 hr at 37°C. After removal of the substances, 250 μl of AlamarBlue™ solution (25-fold diluted with RPMI1640 medium) was added to the culture, and a 1-hr incubation was performed. Viable cells were measured spectrofluorometrically (F-4010, Hitachi) at 535 nm excitation wave length and 583 nm emission wavelength after dilution of the samples with 750 μl of phosphate-buffered saline (PBS, pH 7.4).

Cytotoxicity of combinations of chemical substances was determined by crystal violet dye assay. The cells were soaked in 0.5% crystal violet solution (dissolved in MeOH/H_2O = 1/4 (v/v)) for 10 min, followed by washing in water. The dye was eluted by 1 ml of 33% acetic acid aqueous solution, and viable cells were determined by the absorbance at 630 nm.

Invasion Assay —— An invasion assay was performed according the method previously described.[15] GFP-HT1080 cells (2×10^5 cells/297 μl RPMI1640) were added into a culture insert with an 8 μm-pore FBM (HTS FluoroBlok™ Inserts) precoated with Matrigel with a density of 25 μg/insert. The culture insert was set on a 24-well plate containing 700 μl of 10%FBS/RPMI1640. The serum was used without heat inactivation. Then, 3 μl of an appropriate environmental chemical substance was added to the upper well and incubated for 4 hr in a CO_2 incubator. After incubation, FBM was examined under a fluorescence microscope equipped with a CCD camera. The fluorescent microscopic images were monitored with a computer for counting the invading cell number.

Confocal Microscopy —— HT1080 cells were cultured for 24 hr on a cover glass setting in a culture dish (5×10^4 cells/dish), and after removal of 10%FBS/RPMI1640, chemicals were added. After a 4-hr incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min. Then, the cells were permeabilized with 3% bovine serum albumin (BSA) and 0.1% saponine in PBS, and stained with 5 U/ml rhodamine/phalloidin and 10 μg/ml DAPI in 3% BSA/0.1% saponin/PBS for 20 min. The cover glass was mounted on a

slide glass with anti-fade, and the morphology of the cells was observed under the LSM510 confocal system (Carl Zeiss Co., Ltd., Germany).

RESULTS AND DISCUSSION

Cytotoxic Action of Environmental Chemicals Against HT1080 Human Fibrosarcoma Cells at Doses for Invasion Assay

At first, we determined the cytotoxic action of 31 kinds of environmental chemical substances against HT1080 human fibrosarcoma cells. All substances that showed cytotoxic action were cytotoxic in a dose-dependent manner (data not shown). To confirm that the effect of the substances on tumor cell migration should not be due to the cytotoxic action, 80% viable concentration LC_{20} was determined (Table 1). The concentrations for the invasion assay are indicated in the parentheses of Table 1, and up to 5-fold concentrations of those for the invasion assay were examined. The concentration for the invasion assay of most of the substances was far less, at least 5-fold less, than LC_{20}, indicating that the effect of the substances on the migration of tumor cells is not due to the cytotoxic action. However, potassium dichromate, potassium cyanide, cupric sulfate and nickel chloride may partially damage cells during the invasion assay.

Table 1. Cytotoxicity of Environmental Chemicals

No.	Substance	LC_{20} (μM)[a]	
1	2-aminoanthracene	40.8	(10)
2	benzo[a]pyrene	$>$ 50.0	(10)
3	bisphenol-A	34.0	(10)
4	di-2-ethylhexyl phthalate	$>$ 125	(25)
5	2,5-dichlorophenol	51.0	(10)
6	2,4-dichlorophenoxy acetic acid	$>$ 100	(20)
7	formaldehyde	$>$ 25.0	(5)
8	methylmercury chloride	$>$ 1.0	(0.2)
9	4-nitroquinoline-*N*-oxide	$>$ 0.20	(0.05)
10	*p*-nonylphenol	$>$ 10.0	(2)
11	pentachlorophenol	$>$ 10.0	(2)
12	sodium arsenite	3.50	(1)
13	thiuram	$>$ 5.0	(1)
14	tributyltin chloride	$>$ 0.10	(0.02)
15	2,4,5-trichlorophenol	$>$ 50	(10)
16	Trp-P-2 (Acetate)	$>$ 5.0	(1)
17	paraquat	$>$ 50.0	(10)
18	cadmium chloride	$>$ 5.0	(1)
19	lindane (γ-HCH)	$>$ 100	(20)
20	malathion	$>$ 100	(20)
21	maneb	$>$ 50.0	(10)
22	nickel chloride	28.0	(10)
23	potassium dichromate	0.15	(0.1)
24	triphenyltin chloride	0.05	(0.01)
25	phenol	$>$ 250	(50)
26	benthiocarb (Thiobencarb)	86.2	(20)
27	hexachlorophene	5.4	(2)
28	triclosan	5.0	(1)
29	mercuric chloride	8.88	(2)
30	cupric sulphate	24.7	(10)
31	potassium cyanide	104.7	(50)

[a] Twenty percent lethal concentration. Parentheses indicate the concentrations used for invasion assay.

Effect of Environmental Chemicals on the Invasion of HT1080 Cells

Next, we determined the effect of environmental chemical substances on the invasion of HT1080 cells using GFP-HT1080 transformant cells. As shown in Fig. 1, bisphenol A and benthiocarb inhibited the migration 40.9% and 41.9%, respectively, followed by mercuric chloride, formaldehyde, sodium arsenite and cupric sulfate. It is possible that these substrates directly inhibited the motility of the cells. Alternatively, the inhibition of matrix metalloproteinases is also possible, since Matrigel degradation by metalloproteinases is prerequisite for the invasion of HT1080 cells. Interestingly, some substances enhanced tumor cell migration. Phenol enhanced migration to 149.2% of the control, 2,5-dichlorophenol to 138.3%, lindane to 131.0%, and 2,4,5-trichlorophenol to 127.1%.

In a series of studies, halogenated phenol derivatives extremely enhanced the invasion ability of GFP-HT1080 cells. Moreover, the result showed that invasion ability was decreased with an increasing number of chlorides. In fact, pentachlorophenol did not enhance the invasion of the tumor cells. Although the direct relationship between these substances and tumor cell malignancy has not yet been proved, it is possible that these substances affect invasion characteristics of tumor cells *in vivo*.

Effect of Phenol and Polychlorophenol on the Morphology of HT1080 Cells

Since a series of phenol derivatives enhanced the invasion of HT1080 cells, we next examined the morphology of the cells in the presence of these compounds. Cytoskeletal systems are essential for the motility of the cells; therefore, we focused on their effects on actin filaments of the cells. The upper panel of Fig. 2 shows the morphology of HT1080

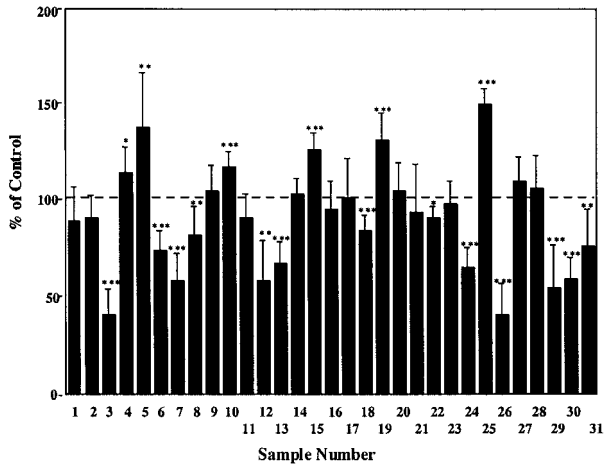

Fig. 1. Effects of Environmental Chemicals on the Invasion of GFP-HT1080 Cells into Matrigel

GFP-HT1080 cells (2×10^5 cells) in 300 μl were added into a culture insert with an 8 μm-pore FBM precoated with Matrigel. The culture insert was set on a 24-well plate 700 μl of 10% FBS/RPMI1640, and each environmental chemical substance was added to the upper well and incubated for 4 hr. After incubation, the invaded cell number was counted under a fluorescence microscope. Asterisks represent significant difference from control: *, < 0.05; **, < 0.01; ***, < 0.001. The chemicals and concentrations: 1, 10 μM 2-aminoanthracene; 2, 10 μM benzo[*a*]pyrene; 3, 10 μM bisphenol-A; 4, 25 μM di-2-ethylhexyl phthalate; 5, 10 μM 2,5-dichlorophenol; 6, 20 μM 2,4-dichlorophenoxy acetic acid; 7, 5 μM formaldehyde; 8, 0.2 μM methylmercury chloride; 9, 0.05 μM 4-nitroquinoline-*N*-oxide; 10, 2 μM *p*-nonylphenol; 11, 2 μM pentachlorophenol; 12, 1 μM sodium arsenite; 13, 1 μM thiuram; 14, 0.02 μM tributyltin chloride; 15, 10 μM 2,4,5-trichlorophenol; 16, 1 μM trp-P-2 (Acetate); 17, 10 μM paraquat; 18, 1 μM cadmium chloride; 19, 20 μM lindane (gamma-HCH); 20, 20 μM malathion; 21, 10 μM maneb; 22, 10 μM nickel chloride; 23, 0.1 μM potassium dichromate; 24, 0.01 μM triphenyltin chloride; 25, 50 μM phenol; 26, 20 μM benthiocarb (tiobencarb); 27, 2 μM hexachlorophene; 28, 1 μM triclosan; 29, 2 μM mercuric chloride; 30, 10 μM cupric sulphate; 31, 50 μM potassium cyanide.

cells at concentrations used for the invasion assay, and the lower shows that at 10-fold concentrations. Phenol induced rounding of the cells at both concentrations, suggesting that the interaction of the cells with substrate, cover glass in this, was weakened by treatment with phenol. This phenotype is similar to malignant tumor cells in some sense, and it is possible that this phenotype caused increased migration of the cells by treatment with phenol. In contrast, polychlorophenols altered the cell morphology to a spindle-like shape. This alteration was enhanced with increased numbers of chlorides on the phenol moiety of the compounds and with their concentration. Interestingly, 2,5-dichlorophenol, which is known to have mutagenicity,[17] also altered the cell shape to round at the concentration used for the invasion assay, although it transformed the cells to a spindle-like shape at a higher concentration. Therefore, we speculate that spindle-like changes in cell morphology might result from direct interaction of the chemicals with the cell membrane. In fact, the increased chloride moiety may increase the hydrophobic interactions with the cell membrane, although further studies would be needed to clarify this. The mechanism by which phenol and a low concentration of polychlorophenols causes rounding of the cells is not clear at present, but it is possible that they enhance motility rather than migration of the cells.

Assessment of Chemical Mixtures Through the Effect on the Tumor Cell Migration

Finally, we assessed the effect of chemical mixtures on tumor cell invasion by using various combinations of the substances. Combination 1 consisted of 10 nM bisphenol A, 1 nM Trp-P-2, 1 nM cadmium chloride, 2 nM maneb and 10 pM triphenyltin chloride, which represent chemicals from the following combinations. The concentrations indicated were the final concentrations used for invasion assay. Combination 2 consisted of heavy metals: 200 pM methylmercury chloride, 4 nM sodium arsenite, 1 nM cadmium chloride, 50 nM potassium dichromate, 10 pM tripheyltin chloride. Combination 3 was composed of possible endocrine disruptors: 10 nM bisphenol A, 25 nM di-2-ethylhexylphthalate, 2 nM *p*-nonylphenl, 20 pM tributyltin chloride, and 2 nM maneb. Combination 4 was composed of antimicrobial substances: 2 nM pentachlorophenol, 1 nM thiuram, 10 nM 2,4,5-trichlorophenol, 40 nM malathion and 1 nM triclosan. Combination 5 consisted of miscellaneous substances not included in former combinations except triclosan: 5 nM benzo[*a*]pyrene, 5 nM formaldehyde, 10 nM paraquat, 1 nM triclosan, 2 nM mercuric chloride. Combinations 6 to 9 consisted of substances classified based on their cytotoxicity data: combination 6 was the weakest and combination 9 was the strongest. Combination 6: 5 nM benzo[*a*]pyrene, 10 nM bisphenol A, 25 nM di-2-ethylhexylphthalate, 10 nM paraquat, and 40 nM malathion. Combination 7: 5 nM formaldehyde, 2 nM *p*-nonylphenl, 2 nM pentachlorophenol, 10 nM 2,4,5-trichlorophenol, and 1 nM Trp-P-2. Combination 8: 4 nM sodium arsenite, 1 nM cadmium chloride, 2 nM maneb, 1 nM triclosan, and 2 nM mercuric chloride. Combination 9: 200 pM methylmercury chloride, 1 nM thiuram, 20 pM tributyltin chloride, 50 nM potassium dichromate, and 10 pM tripheyltin chloride. Combination 10 was composed

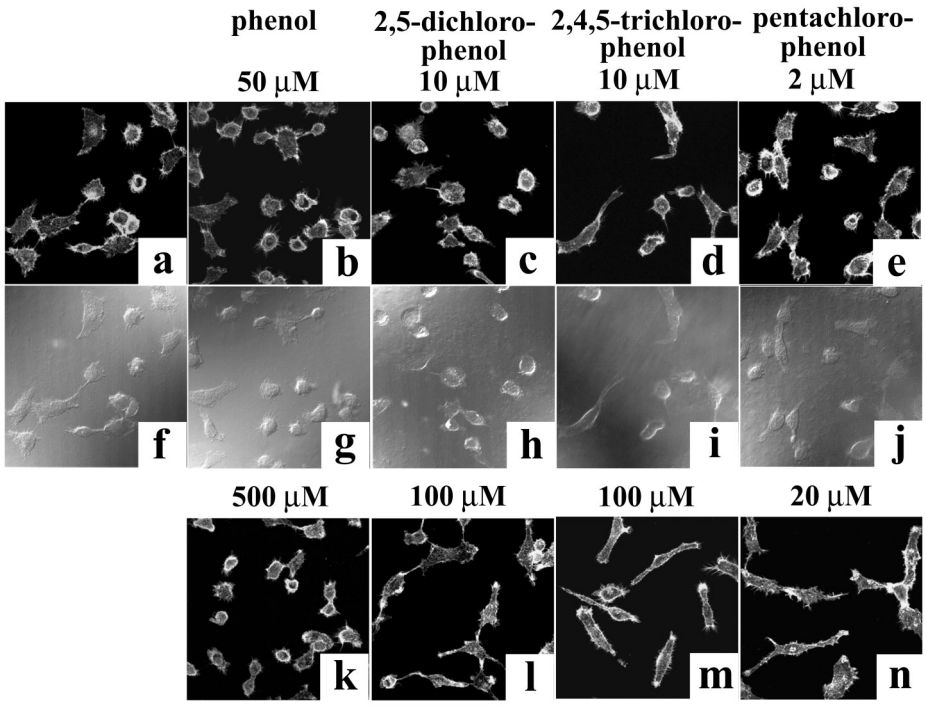

Fig. 2. Morphological Change of HT1080 Cells in the Presence of Phenol or Polychlorophenols

HT1080 cells preincubated in 10% FBS/RPMI1640 for 24 hr were co-incubated without (a, f) or with phenol (b, g, 50 μM; k, 500 μM), 2,5-dichlorophenol (c, h, 10 μM; l, 100 μM), 2,4,5-trichlorophenol (d, i, 10 μM; m, 100 μM), or pentachlorophenol (e, j, 2 μM; n, 20 μM). F-actin was labeled with rhodamine/phalloidin (a–e, and k–n, confocal microscopy), and the nucleus was labeled with DAPI (f–j, confocal microscopy merged with Nomarski differential-interference-contrast microscopy).

of the substances used in an international program for validation of cytotoxicity tests: 2 nM pentachlorophenol, 4 nM sodium arsenite, 10 nM paraquat, 40 nM malathion, and 2 nM mercuric chloride. Combination 11 was formed of the chemicals used to examine the effect on cladoceran and studfish: 5 nM formaldehyde, 200 pM methylmercury chloride, 1 nM thiuram, 20 pM tributyltin chloride, and 10 nM paraquat.

At first, the cytotoxicity of each combination on HT1080 cells was examined. The data in Table 2 are expressed as the concentration amplification of the test solution that caused 20% or 50% cell death. These data suggest that these chemical mixtures did not cause notable cytotoxic action during the invasion assay at the indicated concentrations. Thus, we next examined the effect of these mixtures on the migration of HT1080 cells by the same procedures used for evaluating the effect of individual chemicals. As shown in Fig. 3, the invasion of tumor cells was strongly suppressed by combinations 2, 8 and 10. Heavy metals showed a tendency to suppress the tumor cell invasion. One possible explanation is that heavy metals inhibited matrix metalloproteinases. In fact, the activity of enamel matrix proteinases were inhibited by heavy metals such as lead, cadmium and zinc.[18] Haga and coworkers reported that cadmium-resistant HT-1080 cells showed high invasiveness into Matrigel through the induction of matrix metalloproteinase-9 (MMP-9) activity.[19] This phenomenon is not directly related

Table 2. Cytotoxicity of Environmental Chemicals in Combination

Combination[a)]	LU_{20}[b)]	LU_{50}[c)]
Combination 1	9.5	18.3
Combination 2	6.3	29.7
Combination 3	7.5	18.7
Combination 4	6.4	14.5
Combination 5	6.6	15.4
Combination 6	13.1	24.4
Combination 7	6.0	11.2
Combination 8	5.0	13.7
Combination 9	8.3	17.9
Combination 10	8.8	29.6
Combination 11	45.2	189.2

a) Substances in each combination were described in the text, and concentrations of each shown in the text were arbitrarily decided as one unit. *b*) Units required for 20% cell death. *c*) Units required for 50% cell death.

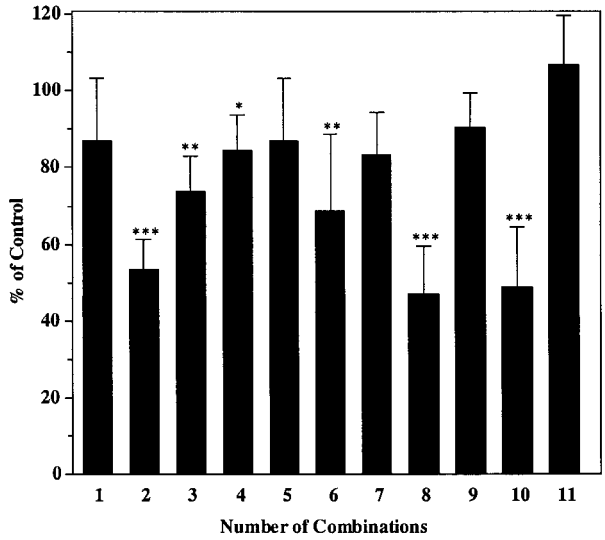

Fig. 3. Effect of Environmental Chemicals in Combination on the Invasive Property of GFP-HT1080 Cells

The effect of combinations of environmental chemical substances on the invasion of GFP-HT1080 cells was evaluated by the same procedure described in the legend of Fig. 1. Chemical substances and their concentrations of each combination are shown in the text. Asterisks represent significant difference from control: *, < 0.05; **, < 0.01; ***, < 0.001.

to the present study. However, if heavy metal inhibits MMPs, the enhanced secretion of MMPs by heavy metal resistant cells is reasonable. Actually, MMPs are deeply involved tumor cell invasion.[20)]

In the present study we have investigated the effects of environmental chemicals on the invasion ability of tumor cells. Although this phenomenon cannot yet be directly correlated to tumor malignancy, this is the first attempt to assess environmental chemicals from the view-point of tumor malignancy. Since the tumorigenicity of environmental chemicals and their enhancement of tumor malignancy are quite important for our health, a step-by-step accumulation of this kind of information should be continued.

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