acting aminoarenes (5,6,12). In conclusion, the results in Table 2 demonstrate that the Yodo River system has been continually and heavily polluted with frameshift-type polycyclic planar mutagens and these mutagens have been released mainly from sewage plants located along the tributaries of the Yodo River system for many years.

Levels of PBTA-1 and PBTA-2 in blue rayon extracts from sewage effluents and river waters collected between 1996 and 2005 (Exp. 1): Table 3 summarizes the levels of PBTA-1 and PBTA-2 in all samples collected at six sites between 1996 and 2005. Determination of PBTA-1 and PBTA-2 was performed with 23 sewage effluent samples, 27 river water samples at sites located below sewage plants, and 26 downstream river water samples. The contributions of PBTA-1 and PBTA-2 to the total mutagenicity of water samples are also shown in parenthesis in Table 3. PBTA-1 was detected in 43% of the samples (33/76) and PBTA-2 was detected in 87% of the samples (66/76), and their levels among water samples fluctuated widely (PBTA-1; nd −268 ng/g BRE, PBTA-2; nd −883.1 ng/g BRE). Average levels of PBTA-1 in effluent samples (U-1 and K-1) were 1.76 and 13.8 ng per g BRE; those in river water samples at sites below sewage plants (N-1 and N-2) were 40.6 and 35.8 ng per g BRE; and those in their downstream river water samples (KA-2 and Y-2) were 1.77 and 0.83 ng per g BRE. On the other hand, average levels of PBTA-2 in sewage effluent samples (U-1 and KA-1) were 88.7 and 155.1 ng per g BRE; those in water samples at sites located below sewage plants (N-1 and N-2) were 26.7 and 88.3 ng per g BRE; and those in their downstream river water (KA-2 and Y-2) were 26.7 and 11.1 ng per g BRE, respectively.

The results showed that levels of PBTA-1 and PBA-2 in sewage effluent samples and river water samples at sites located below sewage plants were much higher than those for their downstream river water samples. PBTA-1 and PBTA-2 accounted for 6% and 26% on average, respectively, of the total mutagenicity in all samples analyzed. High levels of PBTA-1, with more than 100 ng/g BRE were detected in four samples from Site N-1 and N-2 (Nishitakase River) before 2001, but they were not detected at all after 2002 except for Site KA-1. On the other hand, such high levels of PBTA-2 with more than 100 ng/g BRE were detected in ten samples before 2001, and in eight samples after 2002. Furthermore, the average contribution (%) of PBTA-2 in samples

Table 4. Levels of PBTA-type mutagens and their contribution ratio of each PBTA-type mutagen to total mutagenicity of water samples

Sampling point	Sampling date	Levels of PBTA-type mutagens (ng /L)								Mutagenicity* (revertants/L)
		PBTA-1	PBTA-2	PBTA-3	PBTA-4	PBTA-5	PBTA-6	PBTA-7	PBTA-8	
Nishitakase River (N-2)	12 November 1999	nd	7.45	1.46	2.55	nd	nd	0.13	0.04	32,300
	17 December 1999	nd	2.11	5.81	5.43	nd	nd	0.44	0.31	35,500
	28 September 2001	5.28	0.54	1.58	0.62	nd	nd	0.19	0.01	19,000
	Average	**1.76**	**3.37**	**2.95**	**2.87**	**0**	**0**	**0.25**	**0.12**	**28,900**
Katsura River (KA-1)	12 November 1999	nd	1.84	3.04	6.55	nd	1.14	0.24	0.2	41,600
	17 December 1999	nd	2.79	1.36	5.29	nd	2.54	0.32	0.18	49,400
	28 September 2001	nd	2.84	6.56	1.66	nd	nd	0.06	0.03	21,000
	Average	**0**	**2.49**	**3.65**	**4.5**	**0**	**1.23**	**0.21**	**0.14**	**37,300**
Uji River (U-1)	12 November 1999	nd	0.8	1	3.4	nd	0.24	0.26	0.26	50,800
	17 December 1999	nd	1.52	4.08	1.47	nd	nd	0.04	0.04	33,300
	28 September 2001	nd	0.57	1.76	1.78	nd	0.52	0.31	0.03	24,300
	Average	**0**	**0.97**	**2.28**	**2.22**	**0**	**0.25**	**0.2**	**0.11**	**36,100**
		Contribution ratio to total mutagenicity (%)*								
		PBTA-1	PBTA-2	PBTA-3	PBTA-4	PBTA-5	PBTA-6	PBTA-7	PBTA-8	Total
Nishitakase River (N-2)	12 November 1999	0	18.1	5.8	18.9	0	0	1.2	0.2	44.2
	17 December 1999	0	4.7	20.9	36.6	0	0	3.7	1.7	67.6
	28 September 2001	24.5	2.2	10.6	7.8	0	0	3	0.1	48.2
	Average	**8.2**	**8.3**	**12.4**	**21.1**	**0**	**0**	**2.6**	**0.7**	**53.3**
Katsura River (KA-1)	12 November 1999	0	3.5	9.4	37.7	0	1.3	1.7	0.9	54.5
	17 December 1999	0	4.4	3.5	25.7	0	2.5	1.9	0.7	38.7
	28 September 2001	0	10.6	40	34.6	0	0	0.2	0.1	85.5
	Average	**0**	**6.2**	**17.6**	**32.7**	**0**	**1.3**	**1.3**	**0.6**	**71.4**
Uji River (U-1)	12 November 1999	0	1.2	2.5	10.6	0	0.2	2.8	1	18.3
	17 December 1999	0	3.6	15.7	16	0	0	0.8	0.2	36.3
	28 September 2001	0	2.1	10.7	20.3	0	0.5	4.4	0.3	38.3
	Average	**0**	**2.3**	**9.6**	**15.6**	**0**	**0.2**	**2.7**	**0.5**	**31.0**

*Mutagenicity towards *S. typhimurium* YG1024 with S9 mix.

Table 5. Estimated amounts of PBTA-type mutagens discharged from sewage plants into river waters

Sampling point*	Discharged effluents volume† (×10 m^3/day)	Estimated amounts of PBTA-type mutagens discharged from sewage plants (g/year)‡								
		PBTA-1	PBTA-2	PBTA-3	PBTA-4	PBTA-5	PBTA-6	PBTA-7	PBTA-8	Total
Nishitakase River (N-2)	637	409	784	686	667	0	0	58	28	2,632
Katsura River (KA-1)	523	0	475	697	859	0	235	40	27	2,333
Uji River (U-1)	155	0	55	129	126	0	14	11	17	352
Total dischargd volume	1,315§	409	1,314	1,512	1,652	0	249	109	72	5,317

*N-2: The site located below sewage plants SP 1 and SP 2 as shown in Fig. 2.
KA-1: The outlet of SP 2 as shown in Fig. 2.
U-1: The outlet of SP 3 as shown in Fig 2.
†The discharged effluents volume was estimated from the treatment capacity of sewage plants (SP 1: 114, SP 2: 1,047 and SP 3: 155×10^3 m^3/day). Discharged volume from SP 2 into KA-1 and N-2 was estimated to be fifty-fifty.
‡Figure is calculated from average levels of PBTA-type mutagens (ng/L) as shown in Table 4 and discharged effluents volume (m^3/day) as shown in this Table.
§This volume occupy more than 70% of sewage treatment capacity of six sewage plants, which their discharges flow into Nishitakase, Katsura and Uji rivers.

Table 6. Mutagenicity of river sediments in the Yodo River system

Sampling site	Sampling date	Extraction solvent	YG1024, +S9 mix (revertants/g dry basis)
U-1	31 May 2002	Methanol	943*
		Methanol:benzene (3:1)	1217
	6 Sep 2002	Methanol	1505
		Methanol:benzene (3:1)	1323
Y-2	31 May 2002	Methanol	233*
		Methanol:benzene (3:1)	111
	6 Sep 2002	Methanol	110
		Methanol:benzene (3:1)	110

*The sample was used for the determination of PBTA-type mutagens.

collected between 2002 and 2005 to the total mutagenicity was ~36%, which was higher than that the 16% between 1996 and 2001. This result demonstrates that PBTA-2 accounts for a large amount of the total mutagenicity of the Yodo River system during recent years.

Estimation of PBTA-type mutagens amounts discharged from sewage plants into river waters (Exp. 2): We estimated amounts of eight kinds of PBTA-type mutagens discharged from sewage plants into the Yodo River system. Water samples were collected at two outlets of sewage plants (U-1 and KA-1) and the site located below two sewage plants (N-2) on 1999 and 2001. Table 4 summarizes the levels of eight kinds of PBTA-type mutagens obtained by the blue rayon column method. All values were corrected for compound recoveries (56, 56, 42, 40, 50, 50, 40 and 46%, respectively for PBTA-1, PBTA-2, PBTA-3, PBTA-4, PBTA-5, PBTA-6, PBTA-7, and PBTA-8). As shown in Table 4, PBTA-2, PBTA-3, PBTA-4, PBTA-6, PBTA-7, and PBTA-8 were detected in most of the tested samples. Their average concentrations were 2.20, 2.96, 3.53, 0.49, 0.22 and 0.12 ng/L. PBTA-1 was detected only in one sample. PBTA-5 was below the detection limit in all samples analyzed. Salmonella mutagenicity assay results for all samples are also shown in Table 4, with the relative contributions of PBTA-type mutagens to total mutagenicity. All samples showed significant potency in strain YG1024 with S9 mix. Seven kinds of PBTA-type mutagens except for PBTA-5 accounted for 31–71% of the total mutagenicity, with an average contribution of 52%. The compounds that made large contributions to the total sample mutagenicity were PBTA-4, PBTA-3 and PBTA-2, accounting for 23, 13 and 6%, respectively. In addition, we estimated the amount of PBTA-type mutagens discharged into the river waters from three sewage plants according to data on average levels of PBTA-type mutagens and discharged effluents volume from three sewage plants (SP 1, SP 2 and SP 3). Table 5 shows estimated amounts of PBTA-type mutagens discharged from sewage plants. In conclusion, ~5.0 kg of seven kinds of PBTA-type mutagens were discharged from sewage plants into the rivers studied here. In addition, the major part of the mutagenicity of river water in the Yodo River system was due to PBTA-type mutagens formed by chemical

modification of industrial discharges in sewage plants, although the compounds in these discharges were diluted or decomposed while moving down the Yodo River system.

Salmonella mutagenicity and levels of PBTA-type mutagens in river sediment and drinking water (Exp. 3 and 4): In order to evaluate the genotoxic burden of river sediments, we performed the determination of mutagenicity and levels of PBTA-type mutagens in sediment samples collected at two sites. The extracts of sediments collected at Site U-1 located below the sewage plant showed mutagenicity towards YG1024 with S9 mix, and the extract possessed the same frameshift-type mutagenicity as the sewage effluents and river waters (Table 6). The revertants produced with the extracts at Site U-1 were about ten times greater, on average, than those from its downstream Site Y-2. However, the potency of the sediment sample from Site U-1 was not unexpectedly so high considering the result mentioned above showing water samples from Site U-1 had "extreme mutagenicity" in the highest frequency as shown in Table 2 and PBTA-type mutagens were discharged from sewage plant (SP 3) as shown in Table 5, although they were higher compared with the study of the Po River, Italy (50–660 revertants/g, YG1024, +S9 mix) (36), and they were much lower compared with the Cristais River, Brazil (37,000 revertants/g, YG1041, +S9 mix) (11). Furthermore, only trace levels of PBTA-2 and PBTA-3 were detected in sample at Site U-1 (PBTA-2; 0.08 ng and PBTA-3; 0.02 ng, respectively, per g) and they accounted for less than 10% of the total mutagenicity. It was suggested that PBTA-type mutagens in water might not easily accumulate in the river sediment, though aromatic compounds with three or more fused rings are known to be adsorbed to particle matter or bottom sediments in aquatic environments (28,36-38).

The Yodo River system flows through the prefectures of Kyoto and Osaka and it is a source of drinking water for more than seventeen million people living in the downstream area. There are several sources for drinking water that are located downstream and upstream from Site Y-2 shown in Fig. 2. We tried to analyze the mutagenicity and the level of PBTA-type mutagens in drinking water that comes from the source located below Site Y-2. Result showed that no significant mutagenicity was detected (<400 revertants/L), and the levels of PBTA-type mutagens were also less than the detection limit (<0.02 ng/L) in these drinking water samples (Data are not shown). The present result showed the absence of mutagenic polycyclic aromatic compounds involving PBTA-type mutagens in drinking water.

Mutagenicity monitoring of the Yodo River system using Sep-Pak C18 column and Blue-Chitin column

Table 7. Mutagenicity monitoring of the Yodo River system using Sep-Pak C18 cartridge and Blue-Chitin column methods

Column	Mutagenicity in YG1024 with S9 mix (revertants/L)											
	Uji River		Katsura River		Nishitakase River		Kizu River	Yodo River				
	U-1	U-2	KA-1	KA-2	N-1	N-2	KI-1	Y-1	Y-2	Y-3	Y-4	Y-5
Sep-Pak C18	26,200±9,500	5,200±4,100	40,200±36,800	12,700±6,200	1,500±800	15,600±7,800	800±600	5,600±3,400	3,000±2,100	2,200±2,200	1,600±1,300	1,600±1,100
Blue-Chitin	11,400±7,400	2,600±1,400	21,100±16,000	6,400±4,100	1,900±500	11,600±6,400	1,500±1,500	4,100±2,300	2,100±1,400	1,200±1,300	2,000±1,300	1,900±1,900

Figure means average±SD (n=7, 4 at N-1).

method (Exp. 5): Water samples were collected at 12 sites 7 times along the Yodo River system as shown in Fig. 2 between June 2004 and December 2005 (4 times at N-1). The Yodo River system is composed mainly of the Uji River, Katsura River, Kizu River, Nishitakase River, and the rest. Large volumes of effluents from many sewage plants, treating industrial, agricultural and domestic wastes, located along the Yodo River system are released into these rivers. Seventy-two samples among 81 (89%) were mutagenic using the Sep-Pak C18 cartridge column method, and 75 samples among 81 (93%) were mutagenic using the Blue-Chitin column method. The average mutagenicity value determined by the Sep-Pak C18 cartridge method was 10,000 revertants/L; it was 5,800 revertants/L for the Blue-Chitin column method (Table 7). For the highly contaminated sampling sites mentioned above (U-1, KA-1, N-1 and N-2), the average mutagenicity values were 23,200 and 12,600 revertants/L by the Sep-Pak C18 and Blue-Chitin methods, respectively. In less contaminated sites (U-2, KA-2, KI-1, Y-1, Y-2, Y-3, Y-4 and Y-5), these values were 4,100 and 2,700 revertants/L, respectively. In this mutagenicity monitoring, the levels of PBTA-type mutagens were not analyzed. To clarify the actual contribution ratio against the total mutagenicity should be clarified in future work. But, there was a significant correlation between Sep-Pak C18 cartridge column method and Blue-Chitin column method ($n=81$, $r^2=0.81$, $p<0.001$). This result shows that there exists not only planar polycyclic aromatic mutagens with more than three rings, but also a wide range of chemical mutagens including polar and apolar mutagens in the Yodo River system. Furthermore, these chemicals are released mainly from sewage plants into the Yodo River system. More studies will be needed to elucidate the unknown mutagens beyond the PBTA-type mutagens and to determine of the sources of such mutagens. Rigid regulation also should be made to reduce the levels of mutagenicity.

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Regular article

Detection of 3,3′-Dichlorobenzidine in Water from the Waka River in Wakayama, Japan

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In a previous study, we found that water concentrate from the Waka River, which flows through an industrial area in Wakayama, Japan, showed significant mutagenicity in *Salmonella typhimurium* YG1024 without a mammalian metabolic system (S9 mix), and 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl (ADDB) was identified as a major direct-acting mutagenic constituent in the water concentrate. ADDB induced 428,000 revertants/μg in YG1024 without S9 mix, and this activity was 48 times as high as that with S9 mix. In this study, to clarify whether other mutagenic contaminants were present in the river, water concentrates were collected at three sites along the Waka River and examined for mutagenicity by the Ames test using YG1024 and YG1029 with and without S9 mix. All water concentrates showed potent mutagenicity in YG1024 with and/or without S9 mix. ADDB was detected in water concentrate that was extremely mutagenic without S9 mix. An indirect-acting mutagen, which accounted for more than 30% of the total mutagenicity of the water concentrates in YG1024 with S9 mix, was isolated from two extremely mutagenic water concentrates by HPLC separation. On the basis of spectral data and co-chromatography using authentic chemicals, this mutagen was identified as 3,3′-dichlorobenzidine (DCB). DCB showed strong mutagenicity in YG1024 with S9 mix, inducing 5,186 revertants/μg. DCB was detected in water concentrates collected on the other three sampling dates in the range from 15.8 to 28.5 μg/g of blue rayon. These results suggest that Waka River water might be continually contaminated with mutagens, and DCB was thought to be the major mutagenic constituent of the river water.

Key words: Waka River, river water, mutagen, 3,3′-dichlorobenzidine

Introduction

Surface water including river water is an essential element for human life as a supply of drinking water and for agricultural and industrial purposes. However, there are many reports on the contamination of surface water with chemicals from anthropogenic sources such as pesticides and industrial wastes (1–7).

In a previous study (8), we examined the mutagenicity of 540 water concentrates collected from 130 rivers in Japan by the Ames assay using *Salmonella typhimurium* YG1024 and YG1029 with and without the mammalian metabolic system (S9 mix). Four hundred and sixty-seven (87%) of the water concentrates were mutagenic, and most of the positive water concentrates showed the highest activities in YG1024 with S9 mix. To date, we have detected seven 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA) congeners, which show strong mutagenicities toward YG1024 with S9 mix, as major mutagenic constituents in highly mutagenic river water concentrates collected in Kyoto, Aichi, and Fukui Prefectures, Japan (9–14). Moreover, four non-chlorinated derivatives of PBTA, i.e. non-ClPBTA-2, non-ClPBTA-3, non-ClPBTA-4, and non-ClPBTA-7, were isolated and identified as major mutagens in water concentrate from the Ho River in Shizuoka Prefecture (15). From their synthesis studies, these PBTA congeners were hypothesized to be formed from azo dyes as byproducts within textile dyeing factories and/or municipal sewage plants and to be released into rivers.

Moreover, we found that water concentrate from the Waka River, which was collected in August 1997, showed potent mutagenicity in YG1024 without S9 mix and identified 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl (ADDB) as the major mutagenic constituent (16). ADDB exerted strong mutagenicity without S9 mix, inducing 428,000 revertants/μg in YG1024 and activated human aryl hydrocarbon receptor-mediated transcription in a *lac Z* reporter gene assay. For identification, ADDB was successfully synthesized from 3,3′-

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dichlorobenzidine (DCB) and 3,3′-dichloro-4,4′-dinitrobiphenyl, which are used as dyes and ingredients of dyes and polymers. The Waka River flows through an industrial area, where the chemical industry has developed, in Wakayama City. Many factories producing dyes and ingredients of dyes, polymers, and other chemicals are found in that area. Wastewater from these factories is thought to be released into this river after insufficient treatment. ADDB could be formed unintentionally via postemission modification of drainage water containing parent chemicals such as DCB. The major mutagenic constituents are thought to fluctuate according to the products manufactured in the factories. The purpose of this study was to clarify whether other mutagenic contaminants were present in the Waka River and to identify mutagens in the river water if that was the case.

Materials and Methods

Chemicals: DCB (CAS 91-94-1) was purchased from Sigma Chemical Co. (St. Louis, MO). ADDB (CAS 413567-74-5) was synthesized as described previously (16). Blue rayon was obtained from Funakoshi Co. Ltd. (Tokyo, Japan). HPLC-grade acetonitrile, methanol and trifluoroacetic acid (TFA) were purchased from Nacalai Tesque Inc. (Kyoto Japan). All other chemicals were of analytical grade.

Collection and preparation of organic extracts from river water: Water concentrates were collected at 3 points, i.e. sites A, B, and C, along the Waka River in Wakayama between January 2003 and December 2005 by the blue rayon hanging method (8,17). Site B is located at 3.3 km down stream of site A, and site C is on the opposite site of the sluice from site A (Fig. 1). In brief, 5 or 8 g of blue rayon was hung in the river for 24 hours at each sampling site. The blue rayon was then washed several times with distilled water. The materials adsorbed by the blue rayon were extracted by three rounds of shaking in 60 mL of methanol-ammonia water (50:1, v/v) per gram of blue rayon for 20 min. The extracts were combined and evaporated to dryness. Aliquots of the residues were dissolved in dimethyl sulfoxide for the mutagenicity assay or 50% methanol for fractionation by HPLC.

Isolation of a mutagen from river water samples: The water concentrate from sites A was injected into a semipreparative YMC-Pack ODS-AM 324 column (10 ×300 mm; YMC Co., Kyoto, Japan) for HPLC. Elution was performed at a flow rate of 3 mL/min with the following gradient system of methanol in distilled water: 0–25 min, 75%; 25–40 min, 75–80%; 40–60 min, 80–100%; 60–90 min, 100%. The mutagenic fractions with retention times of 10–11 min and 25–26 min on the YMC-Pack ODS-AM 324 column were separately evaporated to dryness. The residue from the latter fraction was dissolved in 50% acetonitrile, injected into a Luna 5μ Phenyl-Hexyl column (4.6×250 mm; Phenomenex Co., California, USA), and eluted at a flow rate of 0.7 mL/min with the following gradient system of acetonitrile in distilled water: 0–40 min, 60%; 40–60 min, 60–100%; 60–90 min, 100%. The former fraction, which had retention times of 10–11 min on the YMC-Pack ODS-AM324 column, was dissolved in 50% acetonitrile, applied to a YMC-Pack ODS-A303 column (4.6×250 mm; YMC Co., Kyoto, Japan) and eluted at a flow rate of 0.8 mL/min with the following gradient system of acetonitrile in distilled water: 0–40 min, 35%; 40–60 min, 35–100%; 60–90 min, 100%. The mutagenic fraction with retention times of 55–56 min was evaporated to dryness.

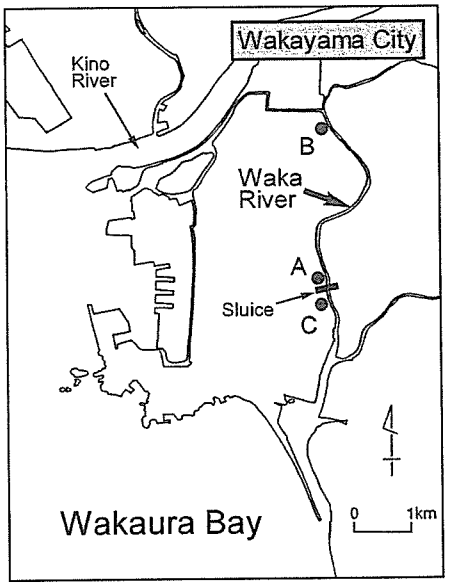

Fig. 1. Geographic locations of the sampling sites of water concentrates and the Waka river in Wakayama City.

The water concentrate from site B was fractionated by HPLC using the YMC-Pack ODS-AM 324 column. The fraction with retention times of 10–11 min was further fractionated by HPLC with the YMC-Pack ODS-A303 column. These HPLC fractionations were performed under the conditions described above.

All HPLC procedures were carried out at 30°C, and the eluates were monitored for UV absorption at 254 nm using an SPD M10Avp diode array detector (Shimadzu Co., Kyoto, Japan). An aliquot of each fraction from the water concentrate was used for mutagenicity assay.

Spectral measurement: UV absorption spectra were measured with the Shimadzu LC-VP HPLC system using a SPD M10Avp diode array detector. Samples were dissolved in 50% methanol or 50% acetonitrile and injected into the YMC-Pack ODS-AM 324, YMC-Pack ODS-A303, or Luna 5μ Phenyl-Hexyl columns.

Table 1. Mutagenicities of water concentrates from the Waka River*

Sampling Site	YG1024 (revertants/g of blue rayon)†		YG1029 (revertants/g of blue rayon)†	
	Without S9 mix	With S9 mix	Without S9 mix	With S9 mix
A	266,000	189,000	2,100	25,300
B	33,400	127,000	769	11,860
C	3,220	24,600	ND‡	2,540

*Water concentrates were collected on January 24, 2003.
†The slope of dose-response curve (revertants/g of blue rayon) was calculated by least-squares linear regression from the first linear portion of the dose-response curve.
‡ND: not detected.

Mass spectra were measured by electron spray ionization-mass spectrometry (ESI-MS) using an LCQ Advantage ion trap mass spectrometer (Thermo Electron Co., Massachusetts, USA) equipped with an Agilent 1100 HPLC system (Agilent Technologies Inc., California, USA). Electronspray ionization was carried out at 5.0 kV spray voltage. Data acquisition was performed in a positive ion mode. Ion source temperature was set at 300°C. Samples, dissolved in 50% methanol, were injected into an HY purity C_{18} column (5 μm particle size, 2.1 × 150 mm) and eluted with 50% methanol including 1% acetic acid at a flow rate of 0.35 mL/min.

Mutagenicity test: Mutagenicity of samples was examined by the preincubation method (18) using *Salmonella typhimurium* YG1024 (19) and YG1029 (19), which were *O*-acetyltransferase-overproducing derivatives of strain TA98 and TA100, respectively (19), and were highly sensitive to the mutagenicity of aromatic nitro, amino, and hydroxylamino compounds. The S9 mix contained 0.05 mL of S9, prepared from livers of male Sprague-Dawley rats treated with phenobarbital and β-naphthoflavone, in a total volume of 0.5 mL. The concentration of protein in S9 was 18 mg/mL. Mutagenic potencies of samples were calculated from linear portions of the dose-response curves, which were obtained with three or four doses and duplicate plates at each dose. The slope of the dose-response curve was adapted as the mutagenic potency. When the samples induced two-fold increases over the average yield of spontaneous revertants and showed well-behaved concentration-response patterns, the samples were judged positive.

Quantification of DCB: The water concentrate dissolved in methanol was fractionated by HPLC using an Inertsil ODS-3 column (10 × 300 mm; GL Sciences Inc., Tokyo, Japan) with a mobile phase of 85% methanol in 0.01% TFA at a flow rate of 1.6 mL/min. The fraction with a retention time of 14.4–16.4 min, corresponding to DCB, was collected and evaporated to dryness. Similarly, the residue was further purified using a Luna 5μ Phenyl-Hexyl column (4.6 × 250 mm) and 45% acetonitrile in 0.01% TFA as a mobile phase and then a STR-ODS-II column (4.6 × 250 mm, Shinwa Chemical Industries Ltd., Kyoto, Japan) and 45% acetonitrile in 0.01% TFA. The fraction corresponding to DCB was collected and evaporated to dryness. The residue, dissolved in acetonitrile, was finally analyzed using a COSMOSIL $5C_{18}$ AR-II column (4.6 × 250 mm; Nacalai Tesque Inc., Kyoto, Japan) and 60% acetonitrile in 25 mM phosphate buffer (pH 6.5) at a flow rate of 0.8 mL/min. All HPLC procedures were carried out at 30°C, and the absorption of the eluate at 260 nm was monitored. The amount of DCB in the specimen was estimated from standard curves obtained from various doses of the authentic compound and was corrected the recovery, i.e. 68.5%, of the compound during purification.

Results

Mutagenicity of water concentrate from the Waka River: Water concentrates were collected at 3 sites along the Waka River by the blue rayon hanging method in January 2003 (Fig. 1). As shown in Table 1, all water concentrates showed mutagenicity in *S. typhimurium* YG1024 and YG1029, and the activities were higher in YG1024 than YG1029. Mutagenic potencies of the water concentrate from site A were extreme in YG1024 with and without S9 mix, inducing 189,400 and 266,000 revertants/g of blue rayon, respectively. The water concentrate from site B, about 3 km downstream from site A, showed comparably high mutagenicity in YG1024 with S9 mix, but not without S9 mix. The sample from site C, which is on the opposite side of the sluice from site A, showed the lowest mutagenicity among the three samples, and its potency was less than 13% of that of site A.

Identification of mutagens from water concentrate of the Waka River: To clarify the chemical structures of mutagens in the samples from sites A and B, the water concentrates were applied to a YMC-Pack ODS-AM324 column for HPLC. In the absence of S9 mix, many fractions from the water concentrate collected at site A exhibited mutagenicity in YG1024 as shown in

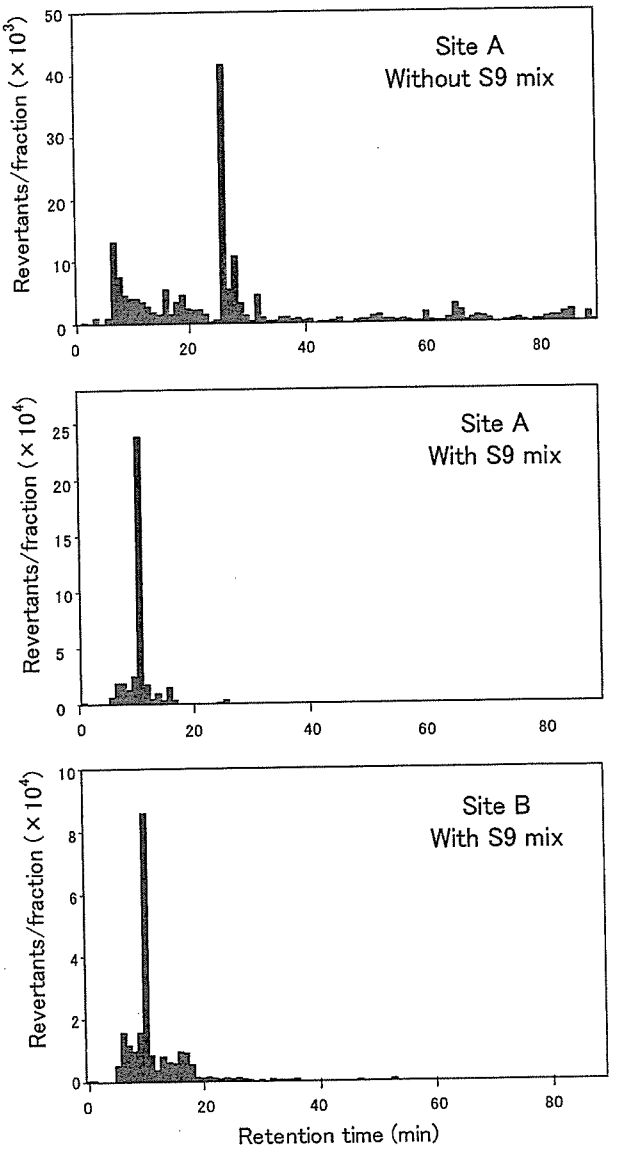

Fig. 2. HPLC profile of mutagens in water concentrates collected at sites A and B of Waka river in January 2003. The mutagenicity of 1-min fractions was examined in *Salmonella typhimurium* YG1024 without or with S9 mix. HPLC was performed on a YMC-Pack ODS-A324 column and eluted with the following gradient system of methanol in distilled water: 0–25 min, 75%; 25–40 min, 75–80%; 40–60 min, 80–100%; 60–90 min, 100%, at a flow rate of 3 mL/min.

Fig. 2. High potency was detected in the fraction with retention times of 25–26 min, which accounted for 24% of total mutagenicity of the water concentrate in YG1024 without S9 mix. This mutagenic fraction was further separated by HPLC using a Luna 5μ Phenyl-Hexyl column. Strong mutagenicity was detected in only the fraction at retention times of 25–27 min, and accounted for 13% of total mutagenicity of the water concentrate. The retention times of these mutagenic fractions on the YMC-Pack ODS-AM324 and Luna 5μ Phenyl-Hexyl columns were consistent with those of synthesized ADDB. Moreover, UV absorption spectrum of the peak fraction with retention times of 25–27 min on the Luna 5μ Phenyl-Hexyl column coincided with that of ADDB. These results indicated that the direct-acting mutagen isolated from the water concentrate from site A was ADDB.

In the presence of S9 mix, many fractions obtained from the water concentrates from sites A and B by HPLC separation using the YMC-Pack ODS-AM324 column showed mutagenicity (Fig. 2). For both concentrates, strong activities were detected in the fractions with retention times of 10–11 min. The mutagenic fractions from the water concentrates from sites A and B accounted for 37% and 47%, respectively, of the total mutagenicity of the water concentrates. These mutagenic fractions were further separated by HPLC using a YMC-Pack ODS-A303 column. Figures 3 and 4 show HPLC profiles of the mutagenic fractions from sites A and B, respectively, on that column, which were monitored by absorption at 254 nm and mutagenic activity of one-minute fractions of the effluent toward YG1024 with S9 mix. An indirect-acting mutagen was isolated as a single UV peak at a retention time of 55.4 min for each concentrate. These mutagens accounted for 32% and 39% of the total mutagenicity of water concentrates from sites A and B, respectively. Figure 5 shows the UV absorption spectrum of the indirect-acting mutagen from site A. Absorption maxima were observed at 213 nm and 286 nm. The UV absorption spectrum of the mutagen isolated from site B was consistent with that from site A. For structural determination, these indirect-acting mutagens were analyzed by LC-MS. Both mutagens had the same retention times, i.e. 20.1 min, on an HY purity C_{18} column and showed the same putative molecular ion peak at m/z 253 $[M+1]^+$ and isotopic ion peaks at m/z 255 $[M+3]^+$ and m/z 257 $[M+5]^+$. From these mass spectra, we assumed that these mutagens isolated from concentrates from sites A and B were DCB, which was a synthetic precursor of ADDB (16). When DCB standard was subjected to LC-MS analysis, DCB was eluted at 20.1 min on an HY purity C_{18} column and its mass spectrum was identical to those of mutagens isolated from sites A and B. On the basis of these results, the indirect-acting mutagens isolated from water concentrates collected at sites A and B of the Waka River were concluded to be DCB. The chemical structure of DCB is shown in Fig. 6.

Mutagenicity of DCB: It has been revealed that DCB is mutagenic in *S. typhimurium* TA98, TA100, and some other strains with and/or without S9 mix (20–24), and that the most potent mutagenicity was detected in TA98 with S9 mix. However, there was no report on the mutagenicity of DCB in *S. typhimurium* YG1024, an *O*-acetyltransferase-overproducing deriva-

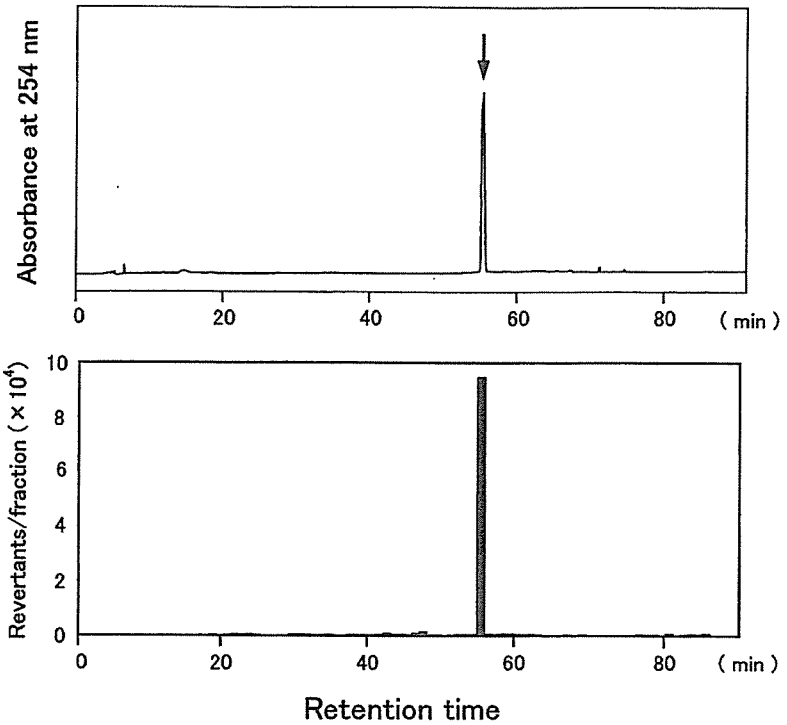

Fig. 3. Purification of an indirect-acting mutagen from water concentrates from site A by HPLC. A mutagenic fraction from a YMC-Pack ODS-AM324 column with retention times of 10–11 min was purified on a YMC-Pack ODS-A303 column. A mutagen was eluted at a retention time of 55.4 min as a single peak, indicated by an arrow. UV absorbance and mutagenicity are shown in the upper and lower panels, respectively.

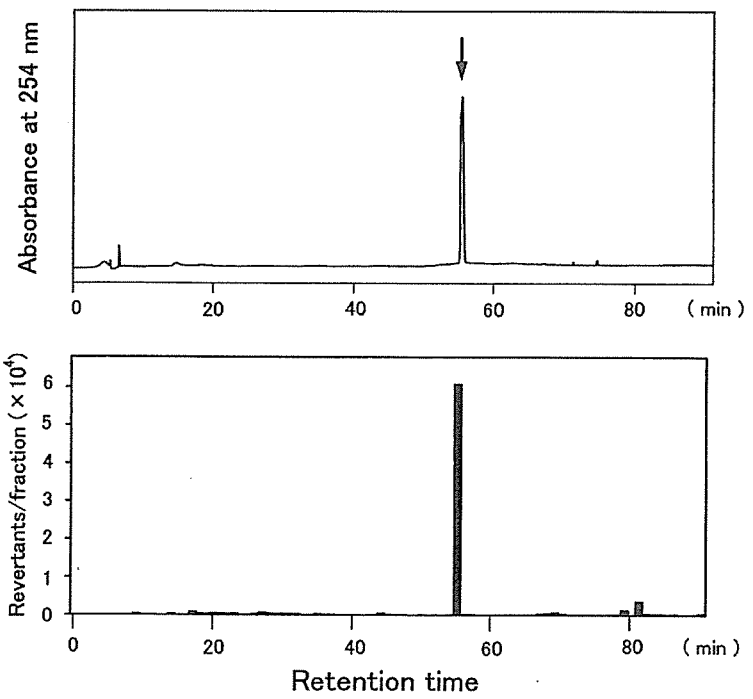

Fig. 4. Purification of an indirect-acting mutagen in water concentrates from site B by HPLC. A mutagenic fraction from a YMC-Pack ODS-AM324 column with retention times of 10–11 min was purified on a YMC-Pack ODS-A303 column. A mutagen was eluted at a retention time of 55.4 min as a single peak, indicated by an arrow. UV absorbance and mutagenicity are shown in the upper and lower panels, respectively.

tive of TA98. As shown in Table 2, DCB showed high mutagenicity in YG1024 with S9 mix, inducing 5,186 revertants/μg.

Quantification of DCB in Waka River water and contribution toward mutagenicity: To clarify whether contamination of the Waka River with DCB was temporary, water concentrates were collected at site A in July, October, and December 2005 by the blue rayon hanging method, and DCB in these concentrates was analyzed. Table 3 summarizes the results of DCB quantification and mutagenicity of the water concentrates toward YG 1024 with S9 mix. DCB was detected in all water concentrates, ranging from 15.8 to 28.5 μg/g of blue rayon. Water concentrates showed mutagenicity in YG1024 with S9 mix, inducing from 306,000 to 531,000 revertants/g of blue rayon. As shown in Table 3, the contribution ratios of DCB toward the mutagenicity of these water concentrates in YG1024 with S9 mix were from 23 to 28%.

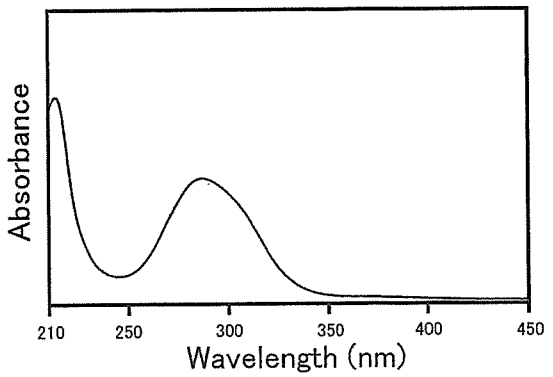

Fig. 5. UV absorption spectrum of the indirect-acting mutagen isolated from the water concentrate collected at site A. UV absorption spectrum was measured in 80% acetonitrile.

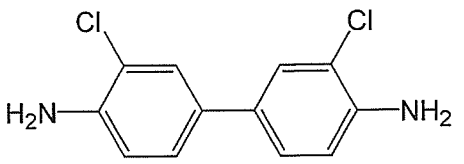

Fig. 6. Chemical structure of 3,3′-dichlorobenzidine (DCB).

Discussion

In this study, we collected water concentrates at 3 sites, A, B, and C, along the Waka River in January 2003 and, subsequently, at site A in July, October, and December 2005. As shown in Tables 1 and 3, potent mutagenicity was detected in YG1024 with S9 mix for all of the water concentrates collected at site A and the sample from site B, 3 km downstream from site A. Their activities ranged from 127,000 to 531,000 revertants/g of blue rayon. In a previous study in which we examined the mutagenicity of 540 water concentrates from 130 rivers in Japan, the water concentrates could be classified into four levels, i.e. low (up to 1,000 revertants/g of blue rayon), moderate (1,000–10,000 revertants/g of blue rayon), high (10,000–100,000 revertants/g of blue rayon), and extreme (more than 100,000 revertants/g of blue rayon), on the basis of the mutagenic potency in YG1024 with S9 mix (8). The percentages of water samples classified as extremely mutagenic ($n=52$) and highly mutagenic ($n=99$) were 10 and 18%, respectively. The mutagenic potencies of the water concentrates obtained at sites A and B along the Waka River in this study were classified as extreme. DCB was detected in all water concentrates from sites A and B, and the contribution ratios of DCB were from 23 to 39% of the total mutagenicity of the water concentrates. These results suggest that Waka River water is continually and heavily contaminated with mutagens and DCB is the major mutagenic constituent in this river

Table 2. Mutagenicities of DCB in *S. typhimurium* test strains

Strain	Mutagenicity (revertants/μg)*	
	Without S9 mix	With S9 mix
YG1024	18	5,186
TA98	7[†]	103[†]
TA100	ND[†,‡]	2[†]
TA98/1,8-DNP$_6$	11[§]	43[§]

*The slope of dose-response curve was calculated by least-squares linear regression from the first linear portion of the dose-response curve.
[†]From ref 20.
[‡]ND: not detected.
[§]From ref 22.

Table 3. Amount and contribution of DCB to the mutagenicity of water concentrates from site A of the Waka River in YG1024 with S9 mix

Sampling Date	Mutagenicity (revertants/g of blue rayon)*	Amount of DCB (μg/g of blue rayon)	Contribution ratio of DCB (%)[†]
July 14, 2005	531,000	28.5	28
October 20, 2005	450,000	20.3	23
December 15, 2005	306,000	15.8	27

*The slope of dose-response curve (revertants/g of blue rayon) was calculated by least-squares linear regression from the first linear portion of the dose-response curve.
[†]The mutagenic potency of DCB used to calculate the contribution ratios was 5,186 revertants/μg.

water.

Many researchers have reported the mutagenicity of DCB in *Salmonella* test strains, i.e. DCB was mutagenic in TA98, TA100, TA98/1,8-DNP$_6$, TA1535, TA1537, and TA1538 with S9 mix, and the highest potency was detected in TA98 (20–24). In this study, DCB was revealed to be highly mutagenic in YG1024, *O*-acetyltransferase-overproducing derivative of TA98, with S9 mix, and its activity was about 50 times as high as that in TA98 with S9 mix (Table 2). These results are consistent with previous results that TA98/1,8-DNP$_6$, an *O*-acetyltransferase-deficient derivative of TA98, was partially resistant to DCB mutagenesis, as compared to TA98, indicating that metabolic activation by *O*-acetyltransferase plays a significant part in DCB mutagenicity (22,24). DCB was positive in some *in vivo* genotoxicity assays, e.g. the chromosomal aberration test (22), the micronucleus assay (25), and the alkaline single cell gel electrophoresis assay (26). Moreover, there is sufficient evidence that DCB is carcinogenic in mice, rats, hamsters, and dogs (27), and DCB has been assigned to a probable human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC) (28).

DCB was photochemically unstable in aqueous solution and degraded to monochlorobenzidine (MCB), benzidine, and some unidentified compounds (29,30). Furthermore, MCB was revealed to be a transient intermediate and benzidine was formed as a stable photoproduct by subsequent photodechlorination of MCB. Benzidine is mutagenic (20–24) and carcinogenic in several animals (28); moreover, it has been recognized as a human carcinogen (Group 1) by IARC (31).

Since DCB was detected in all water concentrates from the Waka River and there were mutagenic HPLC fractions corresponding to chemicals other than DCB, bendizine might exist in Waka River water and be eluted in mutagenic fractions. To estimate the risk of DCB to human health and to organics in the river, more detailed quantification of the river water is necessary. In this study, water concentrates were semi-quantitatively prepared by the blue rayon hanging method. To determine DCB and benzidine quantitatively, we are preparing river water concentrates from the Waka River by extracting organic compounds from certain amounts of river water using a column method, and analyzing DCB and benzidine in the concentrates. Furthermore, we are attempting to clarify the chemical structures of mutagens, other than DCB, in the water concentrates. Extensive studies on the biological effects of contaminants, including DCB, on fish and other organics inhabiting the river will be needed.

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Inhibition of peroxisome proliferator-activated receptor gamma activity in esophageal carcinoma cells results in a drastic decrease of invasive properties

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Esophageal cancer is difficult to treat because of its rapid progression, and more effective therapeutic approaches are needed. The PPARγ is a nuclear receptor superfamily member that is expressed in many cancers. PPARγ expression is a feature of esophageal cancer cell lines, and in the present investigation, the PPARγ antagonists T0070907 and GW9662 could induce loss of invasion but could not induce growth reduction or apoptosis at low concentrations (<10 mM). A high concentration of antagonists (50 μM) inhibited cell growth and induced apoptosis, but these effects did not explain our result at the low concentration. Morphological change, decreased expression of the cell signaling pathway and inhibition of cancer cell invasion were observed in the low concentration. This suggested that PPARγ antagonists inhibited esophageal cancer cell invasion as well as cell adherence, most likely due to alteration in the FAK–MAPK pathway, and this was independent of apoptosis. These results suggested that PPARγ plays an important role in cancer cell invasion and that it might be a novel target for therapy of esophageal cancer. (*Cancer Sci* 2006; 97: 854–860)

Esophageal cancer is associated with a high mortality rate due to its typically late presentation and rapid progression. For tumors that are not amenable to surgical curative resection, chemotherapy and radiotherapy are commonly applied. But most patients continue to have a poor prognosis, along with an increased morbidity due to treatment-related side-effects.[1] Clearly, new therapies for esophageal cancer are needed.

The nuclear transcription factor PPARγ has recently become a putative therapeutic cancer target for a variety of cancers.[2–4] As PPARγ is mainly expressed in adipose tissue and activation plays a central role in adipocyte differentiation and insulin sensitivity,[5] activating synthethic ligands, TZDs, are commonly used as oral antihyperglycemic agents in control of diabetes mellitus type 2. However, PPARγ is also overexpressed in many tumors, including examples in the esophagus, stomach, breast, lung and colon, suggesting that modulation of PPARγ function might impact on tumor survival.[2–4,6,7] Initial efforts have focused on activation with the TZD ligands, as these have been shown to induce G1 cell cycle arrest in a variety of tumor cell lines.[8,9] However, the results of clinical trials with TZDs have shown modest, if any, benefit.[10,11] With esophageal cancers, PPARγ activation by TZDs in cell lines has been reported to inhibit *in vitro* cell growth and/or induce apoptosis.[12–14]

Several observations suggest that inhibition of PPARγ function might be beneficial in treating neoplasms.[15,16] PPARγ is overexpressed in many cancer cell types, but loss-of-function mutations are rare,[17] suggesting that the receptor is a tumor cell survival factor. The hypothesis that PPARγ function might contribute to carcinogenesis or cancer cell survival is also supported by the observation that in one murine model of colon cancer, PPARγ activation led to an increase in tumor formation.[18,19]

Little is known about inhibition of PPARγ function in esophageal cancer cells. In this study, we investigated the effect of PPARγ inhibition on esophageal cancer cell lines using PPARγ-specific antagonists T0070907 and GW9662 in high (50 μM), low (<10 μM) and very low (<10 μM) concentrations. The PPARγ antagonist could prevent cell attachment to the ECM in high concentrations in our previous studies, however, the effect of PPARγ antagonists in low concentrations was not clear.[20,21] A better understanding of the PPARγ function might lead to it being further utilized for cancer treatment.

Materials and methods

Reagents

The PPARγ-specific antagonists T0070907 and GW9662 were purchased from Cayman Chemical (Ann Arbor, MI, USA) and Sigma Chemical (St Louis, MO, USA), respectively.

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Abbreviations: DMEM, Dulbecco's minimum essential medium; ECM, extracellular matrix; Erk, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FITC, fluorescein-isothiocyanate; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; p-Erk, phosphorylated extracellular signal-regulated kinase; p-FAK, phosphorylated focal adhesion kinase; PPARγ, peroxisome proliferator-activated receptor gamma; TZDs, thiazolizinediones.

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Cell culture

Human esophageal cancer cells (KYSE30, KYSE70, KYSE140) used in this study were obtained from the Human Science Foundation (Osaka, Japan). The histology of KYSE30 was well differentiated, KYSE70 poorly, and KYSE140 moderately. KYSE30 and KYSE70 were maintained in DMEM and KYSE140 in Ham's F12 supplemented with 2% fetal bovine serum. Cultures were maintained at 37°C, with an atmosphere of 5% CO_2 and saturated humidity.

Western blot analysis

Adherent cells were washed in phosphate-buffered saline, and cell extracts were prepared in Laemmli lysis buffer. Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA, USA) following the manufacturer's suggested procedure. After electrophoresis of 20 μg aliquots using 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA), blocked for 1 h in tris-buffered saline with bovine serum albumin at room temperature, and incubated with primary monoclonal antibody for 1 h. The anti-PPARγ antibody (E-8) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-pFAK(pY397) from BD Biosciences (San Jose, CA, USA) and anti-p-Erk from Cellsignaling Technology (Beverly, MA, USA). After three washings, the membranes were incubated for 1 h at room temperature with secondary antibody, and immune complexes were visualized using the enhanced chemiluminescence detection kit (Amersham, London, UK) following the manufacturer's procedure.

Immunofluorescence and cell morphology

Cells (5×10^5 per well) were grown on collagen-1 coated glass cover slips in six-well flat bottom plates for 24 h. T0070907 and GW9662 were added and the cells were pre-incubated for 24 h. The cells were fixed with 4% formaldehyde followed by 100% ethanol at −20°C. Permeabilization was carried out with 0.1% Triton-X, and non-specific binding was blocked with 2% normal swine serum. Cells were incubated with antipaxillin monoclonal antibody (BD Biosciences) followed by FITC-labeled secondary antibody. Alexa fluoro 594-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) was used to visualize F-actin. The samples were then mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined by confocal laser scanning microscopy (Carl Zeiss, Oberkochen, Germany). All experiments were done in triplicate.

Assessment of cell growth

Cell proliferation was measured by MTT assay.[22] KYSE70 cells were plated in 96-well plates at a concentration of 5×10^3 cells in 100 μL of DMEM. After 24 h incubation, the medium was changed with various concentrations of T0070907 and GW9662 added (1–50 μM), and the cells further incubated for 24–72 h. MTT solution (0.5%) was then added to each well. After the plates were incubated for 4 h, 20% sodium dodecylsulfate solution was incubated and absorbance at 595 nm was determined using a microplate reader (Model 550; Bio-Rad). Control wells were treated with dimethylsulfoxide alone. Three independent experiments were carried out.

Apoptosis assay

To evaluate the apoptotic cell death, annexin V staining was carried out using an annexin V–FITC apoptosis detection kit I (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's recommendations. Cells were subsequently analyzed by FACScan flow cytometry (Becton Dickinson).

Transwell invasion assays

In vitro cell invasion was assayed in BD BioCoat Matrigel invasion chambers (24 wells, 8 μm pore size; BD Biosciences). The top chamber was seeded with 5×10^4 KYSE70 cells in DMEM. The bottom chamber was filled with DMEM supplemented with 2% fetal bovine serum as a chemoattractant. Cells were preincubated with T0070907 and GW9662 (1–10 μM) in the top chamber, followed by incubation for 24 h in a humidified tissue culture incubator at 37°C under a 5% CO_2 atmosphere. Noninvasive cells were removed from the upper surface of the membrane with a cotton swab, and cells on the lower surface of the membrane were fixed and stained with toluidine blue and mounted on glass slides. Five random fields/well were counted for quantitation of cell invasion. Triplicate wells were counted for each assay.

Statistical analysis

All results are expressed as means ± standard errors of the mean. Statistical comparisons were made using either Student's *t*-test or Scheffe's method after ANOVA. Differences were considered significant at $P < 0.05$.

Results

Esophageal cancer expresses PPARγ protein

Human esophageal cancer tissues were stained using anti-PPARγ-specific antibody, and the expression was high in area of cancer invasion (Fig. 1a). To test whether inhibiting PPARγ activity affects esophageal cell growth or survival, three esophageal cell lines, KYSE30, KYSE70 and KYSE140, were examined. Western blot analysis revealed differential PPARγ protein expression in each. The expression level of PPARγ was very low in normal mucosa, low in KYSE30 cells (well differentiated), moderate in KYSE140 cells (moderately differentiated) and high in KYSE70 cells (poorly differentiated). As the degree of cell differentiation decreased from well differentiated to poorly differentiated, PPARγ protein expression increased. The expression of PPARγ was increased in esophageal cancer tissues compare with normal esophageal epithelial cells (Fig. 1b). The cell line with the highest expression of PPARγ, KYSE70, was used in subsequent investigations into the inhibitory effect of PPARγ activity.

Treatment with PPARγ antagonists decreases cell adhesion to the ECM

Cells treated with 10 or 50 μM of T0070907 and GW9662 underwent morphological changes by 24 h, but those treated with 1 μM did not undergo any morphological change (Fig. 2). At this time point, most cells were still adherents to the plate. However, rather than becoming the normal elongated shape, they were rounded. This morphological change was not the result of apoptosis in cells treated with

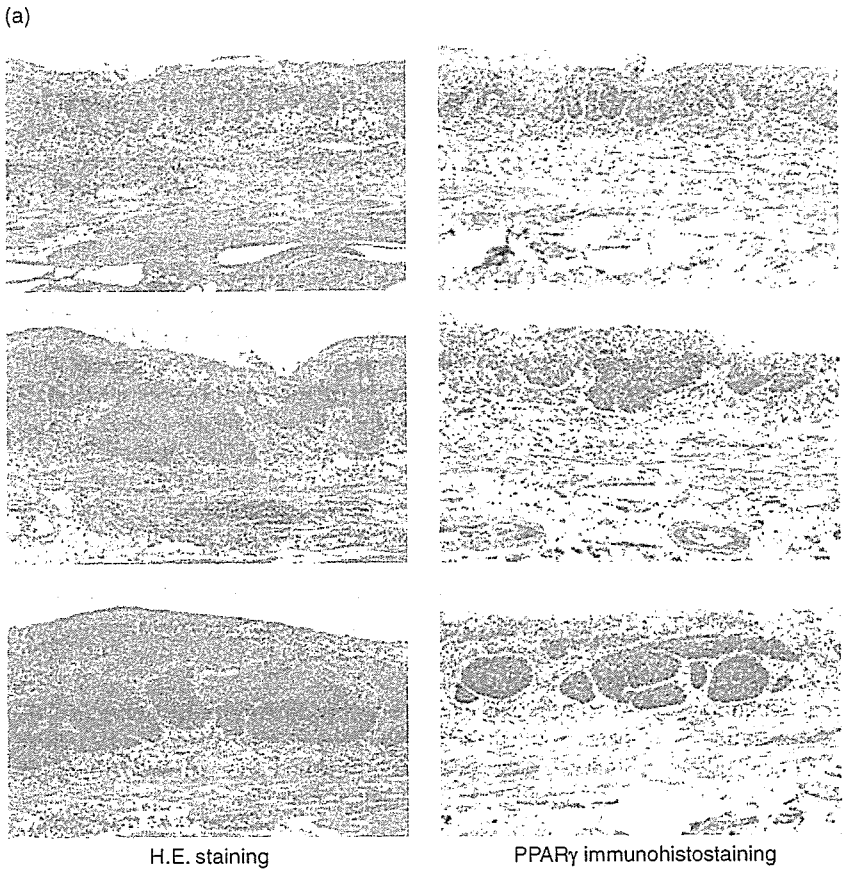

Fig. 1. PPARγ expression in esophageal cancer. (a) Surgical resection of human esophageal cancer tissue stained using HE and antibody specific for PPARγ. The expression was high in area of cancer invasion. (b) The expression level of PPARγ was very low in normal mucosa, low in KYSE30 cells (well differentiated), moderate in KYSE140 cells (moderately differentiated) and high in KYSE70 cells (poorly differentiated). As the degree of cell differentiation decreased from well differentiated to poorly differentiated, PPARγ protein expression increased.

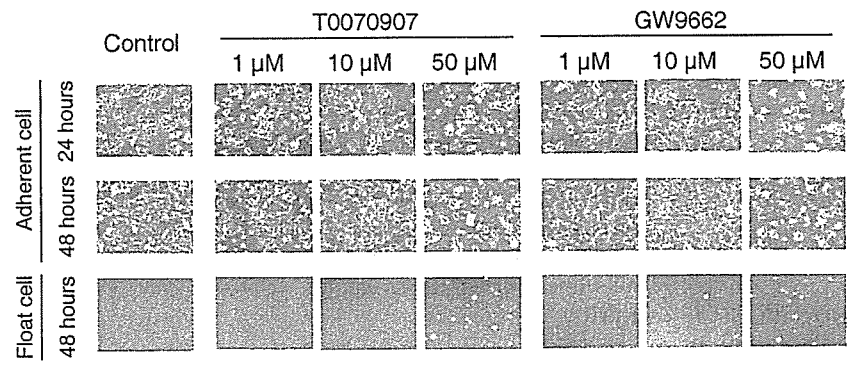

Fig. 2. Morphological changes in esophageal cancer cell lines induced by PPARγ antagonists. KYSE70 cells were incubated with dimethylsulfoxide (control), and 1–50 μM T0070907 and GW9662. Cells treated with 10 and 50 μM of PPARγ antagonists underwent morphological changes by 24 h. By 48 h, 10 μM induced morphological changes but did not inhibit cell adherence; 50 μM induced morphological changes and inhibited cell adherence.

10 μM T0070907 and GW9662, as cells at this time point were not positive for annexin V (Fig. 3). By 48 h, almost half of the cells treated with 50 μM T0070907 and GW9662 were nonadherent. In other words, 1 μM of antagonists induced no change, 10 μM induced morphological changes but did not inhibit cell adherence, and 50 μM induced morphological changes and inhibited cell adherence.

PPARγ antagonists induce change of actin organization

Actin fibers play an important role in maintaining the cytoskeletal structure, and paxillin is functionally important in transducing intracellular messages that are associated with growth factor signaling and cell–ECM interactions.[23] To determine whether the observed cell rounding was associated with alterations in cytoskeletal function, these proteins were

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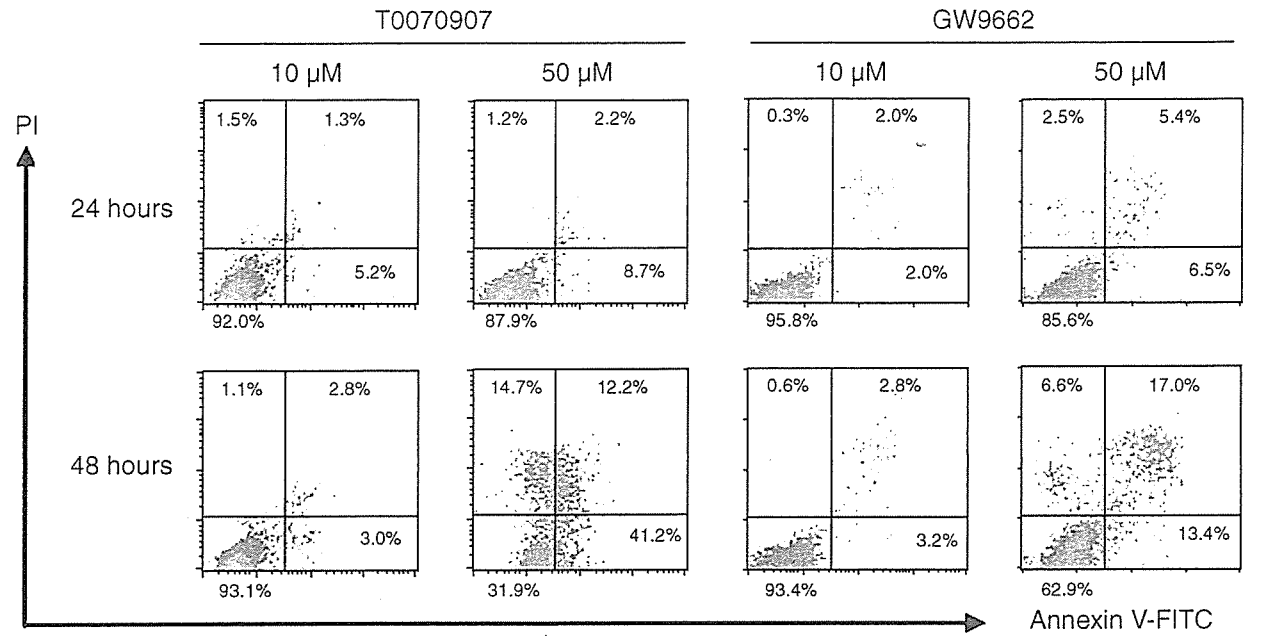

Fig. 3. PPARγ antagonists induced apoptosis in KYSE70 esophageal cancer cells at 48 h. Cells were incubated with 10 and 50 μM T0070907 and GW9662, followed by flow cytometry analysis using annexin V and propidium iodide double staining. Apoptotic cells were observed in 50 μM antagonists at the 48 h time point, but not in 10 μM.

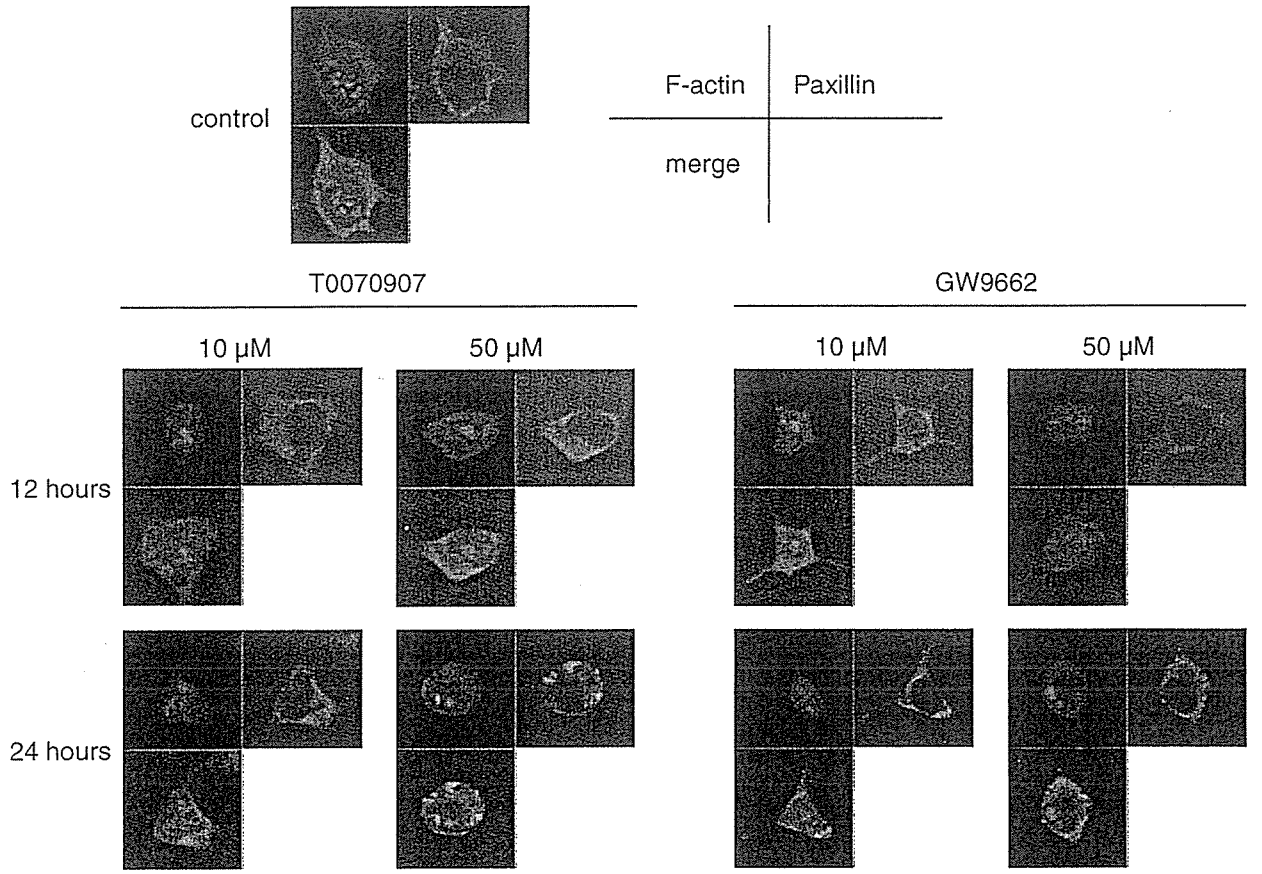

Fig. 4. Before PPARγ antagonist treatment of KYSE70 esophageal cancer cells, actin fibers and paxillin were visible. After 12 h of treatment with 10 and 50 μM T0070907 and GW9662, the cells began to lose their actin fibers, and by 24 h almost all of the cells had changed to a round shape with complete loss of actin fibers.

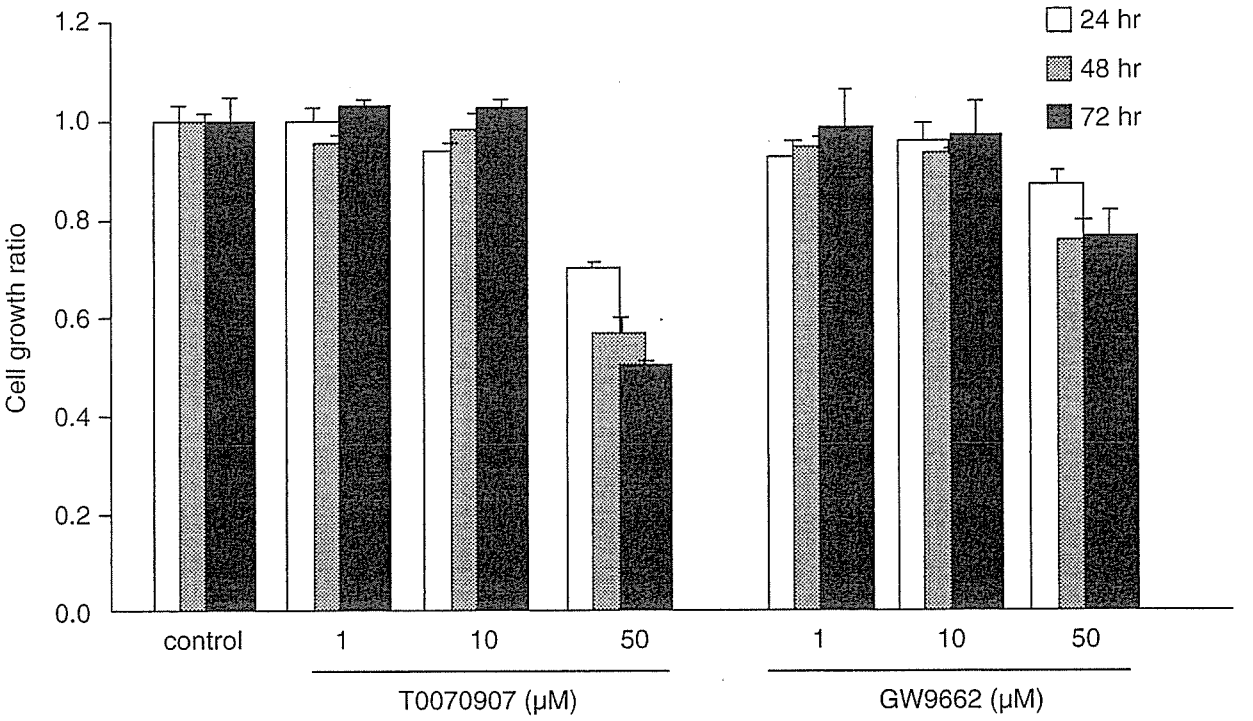

Fig. 5. The PPARγ antagonists T0070907 and GW9662 prevented cell growth in KYSE70 esophageal cancer cells. MTT assay showed the cell growth ratio was significantly reduced in cells treated with 50 μM PPARγ antagonists, and this effect was time-dependent. PPARγ antagonists at concentrations below 10 μM did not reduce the cell growth ratio.

examined by confocal microscopy. Before antagonist treatment, actin fibers and paxillin were visible (Fig. 4). After 12 h of treatment in 10 and 50 μM PPARγ antagonists, the cells began to lose their actin fibers, and by 24 h almost all the KYSE70 cells had changed to a round shape with loss of actin fibers. Similar results were obtained with KYSE30 and KYSE140 cells (data not shown).

Effect of PPARγ antagonists on cell growth

In order to compare the effects of PPARγ antagonism on esophageal cancer cell growth, KYSE70 cells were incubated with T0070907 and GW9662. At 24, 48 and 72 h, the number of cells was measured using MTT assay (Fig. 5). The results were similar in all cell lines, therefore we used KYSE70 cells because they had the highest expression of PPARγ. The compounds did not reduce the growth ratio at concentrations below 10 μM, but did at 50 μM.

Effect of PPARγ antagonists on apoptotic cell death

The results of propidium iodide–annexin V–FITC staining showed that the PPARγ-specific antagonists both induced apoptosis in KYSE70 cells by 48 h at a concentration of 50 μM (Fig. 3), but did not induce apoptosis at 10 μM.

PPARγ antagonism affects the ability of esophageal cancer cell invasion

The PPARγ antagonists had the potential to decrease cell invasiveness. In transwell invasion assays, the number of invasive KYSE70 cells significantly decreased with antagonist treatment below 10 μM, and this effect dose-dependent (Fig. 6). At this concentration (10 μM), the PPARγ antagonists did not induce apoptosis, suggesting that the effect of invasion reduction was independent of apoptosis.

PPARγ antagonism inhibits the phosphorylation of FAK and Erk

To determine possible mechanisms of the cell growth inhibition by PPARγ antagonism, important adhesion and survival cell signaling pathways were investigated. PPARγ antagonists altered FAK (Tyr397) and Erk phosphorylation. KYSE70 cells were incubated with 10 μM of each antagonist.

The results of Western blot analysis revealed decreased expression of p-FAK and p-Erk by the treatment with the antagonists (Fig. 7). p-FAK decreased after 9 h and p-Erk decreased after 12 h.

Discussion

Currently, there is very little information about the inhibition of PPARγ in cancer cells, including esophageal cancer cells. Our previous studies using hepatocellular carcinoma and tongue cancer cells have demonstrated that PPARγ antagonists (high concentration, 50 μM) could prevent cell attachment to ECM, leading to loss of adhesion-induced apoptosis.[20,21] Tongue and esophageal cancer are similar in that they are both squamous cell carcinomas, but the treatment approaches are different. However, esophageal cancer is clinically very important because its mortality rate is very high.

In this study, we demonstrated using esophageal cancer cells that a very low concentration (<10 μM) of PPARγ

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antagonists could induce the inhibition of invasive properties, but not induce growth reduction or apoptosis. MTT assay (Fig. 5) showed that a low and very low concentration of PPARγ antagonists did not inhibit the growth ratio, even after 72 h. Similarly, a low concentration (10 μM) of PPARγ antagonists did not induce apoptosis (Fig. 3). However, a low concentration of PPARγ antagonists could inhibit the cancer cell invasion in the transwell migration assays (Fig. 6). These results suggested that the mechanism by which the PPARγ antagonists inhibited the cancer cell invasion at low concentrations was different from the mechanism by which the high concentration induced apoptosis and cell growth reduction. Therefore, our results suggested that PPARγ might play an important role in cancer cell invasion.

Several reports have clearly demonstrated that PPARγ agonist ligands, the TZDs, could inhibit cell growth and apoptosis of adenocarcinomas, as well as esophageal cancer tissues.[14,24–26] The PPARγ antagonists T0070907 and GW9662 could induce a very similar inhibition of cell growth at a high concentration (50 μM) (Fig. 4). Although it appears paradoxical that both over-activation and inhibition of PPARγ activity could lead to reduced cell growth, this might be a result of different mechanisms. In the TZD setting, a well-recognized G0–G1 cell cycle arrest occurs. In contrast, with PPARγ antagonists, apoptosis appeared to follow loss of adhesion, which was not observed using TZDs. It is suggested that both PPARγ antagonists and TZDs should be considered important candidates for further development as anticancer agents.

PPARγ antagonists were found to first affect cell morphology, with almost all cells changing their cytoskeletal structure, involving both actin fibers and paxillin, within the first 12 h. After adopting a rounded shape, the cells then began detaching from the ECM by 24 h. At this time point, the cells were clearly not apoptotic, as determined by flow cytometric analysis in low concentration (10 μM). This suggested there were different mechanisms dependent on PPARγ activity.

FAK, a 125 kDa non-receptor tyrosine kinase, is an important regulator of cell survival, invasion, migration, and cell cycle

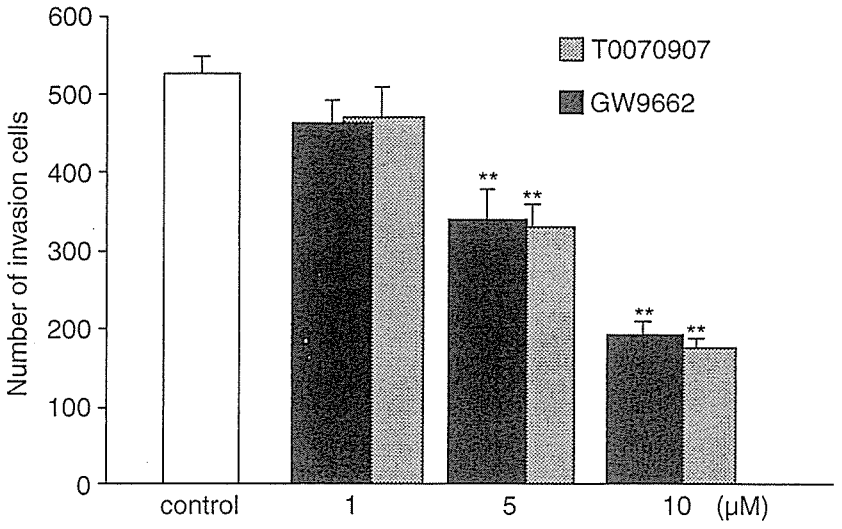

Fig. 6. KYSE70 esophageal cancer cells were incubated with PPARγ antagonists for 24 h during a transwell invasion assay. The number of invasive cells was decreased with antagonist treatment below 10 μM, and this effect was dose-dependent. Error bars represent standard errors of the mean for three replicates. *$P < 0.05$, **$P < 0.01$.

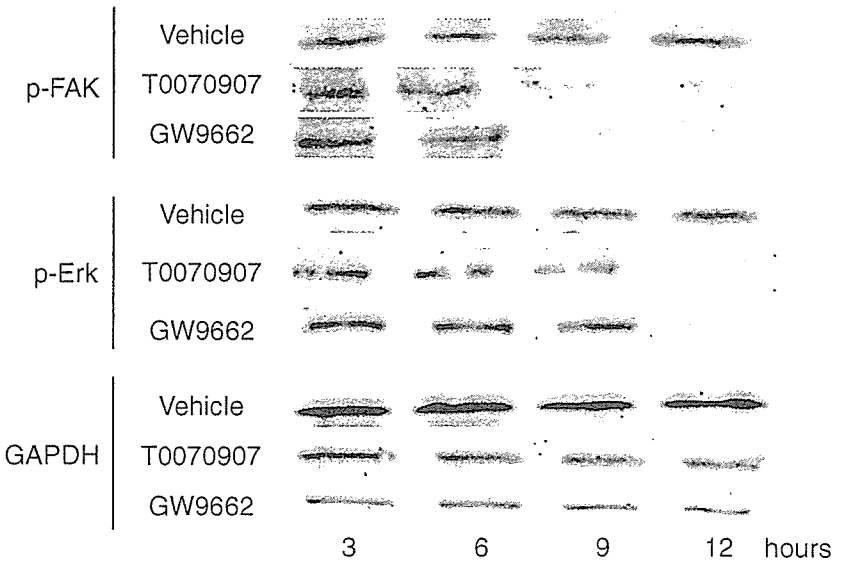

Fig. 7. Expression of FAK (Tyr397) and Erk phosphorylation was altered in KYSE70 esophageal cancer cells incubated with 10 μM PPARγ antagonists. p-FAK was decreased at 9 h and p-Erk was decreased at 12 h.

progression.[27–29] This overexpression of FAK has been observed in a number of human malignant cells, and this might play an important role in determining cellular invasion and metastasis.[30,31] FAK is functionally important in transducing intracellular messages associated with growth factor signaling, cell–ECM interactions, modifying the cytoskeleton and activating MAPK cascades, including Erk. In the present study, the inhibition of phosphorylation of FAK in KYSE70 cells treated with antagonists (10 μM) was observed at 9 h, followed by a reduction in Erk phosphorylation at 12 h. Inhibition of Erk phosphorylation occurred after the inhibition of FAK phosphorylation by PPARγ antagonists, suggesting that PPARγ might play an important role in the MAPK pathway.

High concentration (50 μM) PPARγ antagonists induced apoptosis and cell detachment, and reduced cell growth, but a low concentration (10 μM) could reduce cell invasion and alter the MAPK signaling pathway. These results suggest that the effects of a low concentration of antagonists were independent of the effects of apoptosis, detachment and cell growth inhibition. One study has reported that the difference in effect depends on PPARγ concentration,[32] but further investigation is necessary.

In summary, PPARγ antagonists inhibited esophageal cancer cell invasion as well as cell adherence to ECM, most likely due to alteration in the FAK–MAPK pathway, and this was independent of apoptosis. Our results suggest that PPARγ plays important roles in cancer cell invasion, therefore it might be a novel target for esophageal cancer therapy.

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