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Fish Cell Line As an *In Vitro* Test System for Analyzing Chromosome Aberrations

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To develop and evaluate the aquatic environment, several genotoxic monitoring systems (e.g. ^{32}P-postlabelling, micronucleus assay, chromosome aberration, sister chromatid exchange, comet assay, transgenic fish, reporter gene assay) have been utilized in both the field and laboratory (Kligerman et al., 1975, 1984; Chipman and Marsh, 1991; Hayashi et al., 1998; Mitchelmore and Chipman, 1998; Amanuma et al., 2000; Williams et al., 2000). Fish species are used routinely as one of the important test animals for genotoxicity studies. However, most fishes typically have high numbers ($\geq$ 48) of small (1-3 μm) acrocentrics chromosomes. Thus, cytogenetic testing using fish chromosomes is usually difficult.

Kligerman et al. (1975, 1984) demonstrated that the central mudminnow, *Umbra limi*, (Esociformes, Umbridae) has excellent cytogenetic characteristics (e.g. low number [2n=22] of large chromosomes [5-10 μm]). Also, they demonstrated the usefulness of *Umbra limi* as an experimental animal for aquatic genotoxicity testing *in vivo* (Kligerman, 1979). Prein et al. (1978) also suggested that the European mudminnow, *Umbra pygmaea*, could be used to assess mutagenic pollutants, such as aromatic compounds, in the Rhine river. However, *in vivo* testing of clastogens with fishes can be expensive, involving breeding and care of fishes for long periods of time.

For *in vitro* testing, Walton et al. (1984, 1988) reported the establishment and use of *Umbra limi* cell lines (named ULF, ULO, and ULH). Sequentially, Kurihara et al. (1992) independently established and tested their cell line (ULF-23), taking advantages of the cytogenetic characteristics of *Umbra limi*. However, these cell lines were not appropriate for clastogenicity testing as they had high background levels of both sister chromatid exchanges and chromosome aberrations. Here we present the use of mutant cell line (ULF-23HU) from the ULF-23 (Park et al., 1989; Kurihara et al., 1992) for clastogenicity testing using reference direct-acting mutagenic clastogens.

MATERIALS AND METHODS

The mutant cell line, ULF-23HU, is fibroblast-like and has low baseline frequencies of both sister chromatid exchange and chromosome aberrations compared to the original cell line, ULF-23 (Park et al., 1989; Kurihara et al., 1992). Cells were cultured with 5 ml of TC-199 medium in 25 cm^2 flasks supplemented with 10 mM

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Hepes, 10% fetal bovine serum (FBS) (Gibco), penicillin (Sigma, 100U/ml), streptomycin sulfate (Sigma, 100 μg/ml), and fungizone (Sigma, 1.25 μg/ml) at 25°C.

For analysis of chromosome aberrations induced by several standard clastogens, 3 days after subculture (1 x 10^5 cells/ml), cells were treated with different concentrations of 4-nitroquinoline-N-oxide (4-NQO), *cis*-diamminedichloroplatinum II (*cis*-DDP), mitomycin C (MMC), *N*-methyl-*N'*-nitro-*N*-nitrosoguanindine (MNNG) or butylated hydroxyanisole (BHA) for 1 or 24 hr. This experimental scheme is shown briefly in Fig. 1. The chemicals were dissolved in either distilled water [*cis*-DDP, MMC], 20% DMSO [4-NQO, MNNG] or 100% ethanol [BHA]. To check for toxicity of the solvents, we tested the toxicity of DMSO and ethanol with various doses, and determined to keep these solvents below 2% or 1.6% of a final concentration, respectively. The top solvent concentrations did not show any effects on cell viability, on increase chromosome aberrations or sister chromatid exchange frequencies. Also, we used these concentrations as negative controls for testing. One or 24 hr after chemical treatment of the cells, they were washed with phosphate-buffered saline (PBS) twice, were re-fed with 5 ml of freshly-prepared TC-199 medium supplemented with 10% FBS and antibiotics. The cells were then allowed to recover for 24 hr. Based on the cell cycle time (32 hr) of ULF-23HU cells, the recovery time after chemical treatment was established for observing chromatid-type breaks and to avoid the occurrence of more complicated patterns of chromosome aberration due to the occurrence of derived chromosome-type aberrations, that could appear after subsequent cell divisions.

For accumulation of metaphase chromosomes after treatment, colcemid (final concentration 0.15 μg/ml) was added to cultured cells 6 hrs before cell harvest. Slides were prepared using the air-dried method of Park et al. (1989). At least 100 metaphases from each concentration were randomly chosen and analyzed for chromosome aberrations. The tests were replicated once. Briefly, metaphases with 17 to 26 chromosomes were randomly chosen for scoring of chromatid-type breaks under the criteria of Ishidate, Jr. (1988). The data on the frequency of chromosome aberrations was statistically tested using Student's t-test.

RESULTS AND DISCUSSION

Based on the cell cycle time (32 hr) of ULF-23HU cells, we examined the chromosome aberration frequency by simply quantifying the numbers of chromatid-type breaks induced after chemical exposure. This was accomplished by harvesting the metaphase cells after a short recovery (20 hr) time. Thus, we did not observe any chromosome-type breaks.

For 4-NQO, at a concentration of 5 x 10^{-8} M, the numbers of chromatid breaks increased 2 times compared to the control group. At a higher concentration, 4-NQO was toxic (Table 1). The 4-NQO forms bulky adducts on DNA strands, resulting in distortion of the DNA helix (Darroudi et al., 1989). In our study, ULF-23HU cells did not show a strong concentration-related increase in chromosome damage after 4-NQO

exposure. This is similar to human fibroblast cells and Chinese hamster ovary (CHO) cells treated at a concentration of 7.5 x 10^{-7} M and 5 x 10^{-7} M, respectively (Weinstein et al., 1977; Darroudi et al., 1989).

The clastogen *cis*-DDP, a cancer chemotherapeutic agent and bifunctional alkylating agent, induces inter- and intra-strand crosslinks (Zwelling et al., 1979). This chemical caused a sharp increase in aberrations in ULF-23HU cells. At a concentration of 5 x 10^{-6} M *cis*-DDP, chromatid breaks were induced and increased in a concentration-dependent fashion (at a concentration of 5 x 10^{-5} M about 60 times compared to basal level [Table 1]). Compared to ULF-23HU cells, human lymphocyte and mouse bone marrow cells have been shown to be less sensitive to *cis*-DDP (Tandon and Sohdi, 1985 ; Ohe et al., 1990).

MMC, another chemotherapeutic agent and bifunctional alkylating agent, induces inter- and intra-strand crosslinks and forms free hydroxy radicals (Tomasz et al., 1987; Durse et al., 1990). MMC produced a concentration-related increase in chromatid breaks of about 18 times over the basal level at a concentration of 5.0 x 10^{-8} M (Table 1). This indicates that ULF-23HU cells are more sensitive to MMC than are CHO cells (Natarajan et al., 1983). Also, ULF-23HU cells showed a clear concentration-response even at a low concentration of MMC. Another fish, *Boleophthalmus dussumieri*, found off the Bombay coast, has also shown a remarkable sensitivity to MMC (Krishnaja and Rege, 1982).

MNNG, a monofunctional alkylating agent containing a sensitive nucleophilic center, also induced chromatid breaks in a concentration-dependent manner [Table 1]. In Chinese hamster lung (CHL) cells, chromatid breaks were induced at a concentration of 1.7 x 10^{-2} M and 6.8 x 10^{-2} M upon MNNG exposure for 24 and 48 hrs, respectively (Ishidate, Jr., 1988). However, in ULF-23HU cells, there was a significant increase in chromosome damage at lower concentration [1 x 10^{-5} M]. The increase was concentration dependent. Thus, ULF-23HU cells are likely more sensitive to MNNG than are mammalian cells.

As a negative control, the antioxidative BHA was tested. BHA has the effect of inhibiting mutagenicity and carcinogenicity (Ito et al., 1983; Ohkawa et al., 1986). We exposed cells over a wide range of concentration, but no significant increase in chromatid breaks was observed (Table 1). However, BHA was toxic at a concentration of 5 x 10^{-4} M and 1.0 x 10^{-4} M exposed for 1 hr and 24 hr, respectively. In CHL cells, chromatid breaks were observed with BHA and its analogues BHA-OH and BHA-o-Q after exposure with S9 metabolic activation (Matsuoka et al., 1990). Thus, it would be interesting to check the induction of chromatid breaks in ULF-23HU with and without the S9 fraction.

Overall, the fish cell line ULF-23HU cells was either equal or more sensitive to 4-NQO, *cis*-DDP, MMC, and MNNG than some mammalian cell lines. Regan et al. (1983) reported that fish have a tendency to be more sensitive to UV radiation and mutagenic carcinogens than mammalian cells. Thus, our results support this. This also

Table 1. Frequency of chromatid breaks per cell in the fish cell line ULF-23HU exposed to 4-NQO, *cis*-DDP, MMC, MNNG and BHA.

Chemicals	Dose (M)	Exposure time (hr)	No. of metaphase	Chromatid breaks per cell ± SD	t-test[#]
4-Nitroquinoline -N-oxide	0.2% DMSO	1	200	0.18 ± 0.01	-
	1.0×10^{-9}	1	200	0.13 ± 0.06	-
	5.0×10^{-9}	1	200	0.22 ± 0.14	-
	1.0×10^{-8}	1	200	0.36 ± 0.00	+
	5.0×10^{-8}	1	200	0.36 ± 0.01	+
	1.0×10^{-7}	1	-	toxic[&]	
cis-Diammine-dichloro-platinum II	0 (water)	1	200	0.06 ± 0.02	-
	5.0×10^{-7}	1	200	0.06 ± 0.01	-
	1.0×10^{-6}	1	200	0.13 ± 0.00	-
	5.0×10^{-6}	1	200	0.63 ± 0.02	+
	1.0×10^{-5}	1	200	1.69 ± 0.03	++
	5.0×10^{-5}	1	100	3.50 ± 0.16	++
	1.0×10^{-4}	1	-	toxic	
Mitomycin C	0 (water)	1	200	0.16 ± 0.02	-
	5.0×10^{-8}	1	200	0.17 ± 0.02	-
	1.0×10^{-7}	1	200	0.45 ± 0.01	+
	2.5×10^{-7}	1	200	1.29 ± 0.04	++
	5.0×10^{-7}	1	200	1.72 ± 0.16	++
	1.0×10^{-6}	1	200	2.80 ± 0.12	++
	2.5×10^{-6}	1	-	toxic	
N-Methyl-*N'*-nitro-*N*-nitroso-guanindine	1.6% DMSO	1	200	0.10 ± 0.02	-
	1.0×10^{-6}	1	200	0.14 ± 0.01	-
	2.5×10^{-6}	1	200	0.18 ± 0.00	-
	5.0×10^{-6}	1	200	0.51 ± 0.03	+
	1.0×10^{-5}	1	200	0.90 ± 0.08	+
	2.5×10^{-5}	1	-	toxic	
Butylated hydroxyanisole (negative control)	1.0% ethanol	1	200	0.07 ± 0.02	-
	1.0×10^{-5}	1	200	0.06 ± 0.03	-
	2.5×10^{-5}	1	200	0.12 ± 0.05	-
	5.0×10^{-5}	1	200	0.06 ± 0.02	-
	1.0×10^{-4}	1	200	0.13 ± 0.02	-
	2.5×10^{-4}	1	200	0.17 ± 0.00	-
	5.0×10^{-4}	1	200	toxic	
	1.0% ethanol	24	200	0.14 ± 0.01	-
	5.0×10^{-6}	24	200	0.10 ± 0.02	-
	1.0×10^{-5}	24	200	0.09 ± 0.02	-
	2.5×10^{-5}	24	200	0.10 ± 0.02	-
	5.0×10^{-4}	24	200	0.19 ± 0.01	-
	1.0×10^{-4}	24	200	toxic	

[&]toxic: no mitosis/cell death; [#]+: significant ($P < 0.01$); ++: very significant ($P < 0.001$); -: not significant.

indicates that ULF-23HU cells are useful for assessing the genotoxicity of some chemicals at low concentrations, which is more realistic to exposure in the environment.

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REFERENCES

Amanuma K, Takeda H, Amanuma H, Aoki Y (2000) Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments. Nature Biotechnol 18: 62-65

Chipman JK, Marsh JW (1991) Bio-techniques for the detection of genetic toxicity in the aquatic environment. J Biotechnol 17: 199-208

Darroudi F, Natarajan AT, Lohman PHM (1989) Cytogenetical characterization of UV-sensitive repair deficient CHO cell line 43-3B. II. Induction of cell killing chromosomal aberrations and sister chromatid exchange by 4 NQO, mono- and bi-functional alkylating agents. Mutat Res 212: 103-112

Durse L, Rajagopalan S, Eliot HM, Covey JM, Sinha K (1990) DNA interstrand cross-link and free radical formation in a human multidrug resistant cell line from mitomycin C and its analogues. Cancer Res 50: 648-652

Hayashi M, Ueda T, Ueno K, Wada K, Kinae N, Saotome K, Tanaka N, Takai A, Sasaki YF, Asano N, Sofuni T, Ojima Y (1998) Development of genotoxicity assay systems that use aquatic organisms. Mutat Res 399: 125-133

Ishidate Jr M (1988) Databook of chromosomal aberration test *in vitro*. Elsevier, Amsterdam

Ito N, Fukushiman S, Hagiwara A, Shibata M, Ohiso T (1983) Carcinogenicity of butylated hydroxyanisole in F344 rats. J Natl Cancer Inst 70: 343-352

Kligerman AD, Bloom SE, Howell WM (1975) *Umbra limi*: a model for the study of chromosome aberrations in fishes. Mutat Res 31: 225-233.

Kligerman AD (1979) Induction of sister chromatid exchanges in the central mudminnow following *in vivo* exposure to mutagenic agents. Mutat Res 64: 205-217

Kligerman AD, Bishop WE, Valentine LC (1984) Use of the mudminnow, *Umbra* sp., in an *in vivo* sister chromatid exchange test. Natl Cancer Inst Monogr 65: 111-118

Krishnaja AP, Rege MS (1982) Induction of chromosomal aberrations in fish *Boleophthalmus dissumieri* after exposure *in vivo* to mitomycin C and heavy metals mercury, selenium and chromium. Mutat Res 102:71-82

Kurihara Y, Rienkjkarn M, Etoh H (1992) Cytogenetic adaptive response of cultured fish cells to low doses of X-rays. J Radiat Res 33: 267-274

Matsuoka A, Matsui M, Miyata N, Sofuni T, Ishidate Jr M (1990) Mutagenicity of 3-*tert*-butyl-4-hydroxyanisole (BHA) and its metabolites in the short-term tests *in vitro*. Mutat Res 241: 125-132

Mitchelmore CL, Chipman JK (1998) DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat Res. 399: 135-147

Natarajan AT, Tates AD, Meijers M, Neuteboom I, de Vogel N (1983) Induction of sister-chromatid exchanges (SCEs) and chromosomal aberrations by mitomycin C and methyl methanesulfonate in Chinese hamster ovary cells. Mutat Res 121: 211-223

Ohe T, Tsuda S, Sakata Y, Taniwaki M, Misawa S, Abe T (1990) *cis*-diammine-dichloroplatinum (II)-induced sister chromatid exchange and chromosomal aberration formation in cultured human lymphocytes and their inhibition by sodium thiolsulfonate. Mutat Res 244: 279-285

Ohkawa MY, Inui N (1986) Retinoic acid and butylated hydroxyanisole inhibit promoter-enhanced transformation *in vitro*. Cancer Lett 33: 161-165

Park E-H, Lee J-S, Yi A-K, Etoh H (1989) Fish cell line (ULF-23HU) derived from the fin of the central mudminnow (*Umbra limi*): suitable characteristics for clasto-genicity assay. In Vitro Cell Dev Biol 25: 987-994

Regan JD, Snyder RD, Francis AA, Olla BL (1983) Excision repair of ultraviolet- and chemically-induced damage in the DNA of fibroblasts derived from two closely related species of marine fishes. Aquat Toxicol 4: 181-188

Prein AE, Thie GM, Alink GM, Koeman JH, Poels CL (1978) Cytogenetic changes in fish exposed to water of the river Rhine. Sci Total Environ 9: 287-291

Tandon P, Sohdi A (1985) *cis*-dichlorodiammineplatinum (II) induced aberration in mouse bone-marrow chromosome. Mutat Res 156: 187-193

Tomasz M, Lipman R, Chowdary D, Pawlak J, Verdine GL, Nakanish K (1987) isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. Science 235: 1204-1208

Walton DG, Acton AB, Stich HF (1984) Comparison of DNA-repair synthesis, chromosome aberrations and induction of micronuclei in cultured human fibroblasts, Chinese hamster ovary and central mudminnow (*Umbra limi*) cells exposed to chemical mutagens. Mutat Res 129: 129-136

Walton DG, Acton AB, Stich HF (1988) Chromosome aberrations in cultured central mudminnow heart cells and Chinese hamster ovary cells exposed to polycyclic aromatic hydrocarbons and sediment extracts. Comp Biochem Physiol 89C: 395-402

Weinstein D, Katz ML, Kazmer S (1977) Chromosomal effects of carcinogens and non-carcinogens on WI-38 after short term exposures with and without metabolic activation. Mutat Res 46: 297-304

Williams TD, Lee J-S, Sheader DL, Chipman JK (2000) The cytochrome P450 1A gene (CYP1A) from European flounder (*Platichthys flesus*), analysis of regulatory regions and development of a dual luciferase reporter gene system. Mar Environ Res 50: 1-6

Zwelling LA, Bradley MO, Sharkey NA, Anderson T, Kohn KW (1979) Mutagenicity, cytotoxicity and DNA crosslinking in V79 Chinese hamster cells treated with *cis*- and *trans*-Pt(II) diamminedichloride. Mutat Res 67: 271-280