Effects of Ultraviolet Irradiation on the Migratory Ability of Primordial Germ Cells (PGCs) in the Domestic Chicken

Takeo Minematsu, Yukio Kanai and Atsushi Tajima¹

Institute of Agriculture and Forestry, University of Tsukuba,
1-1-1 Ten-noh Dai, Tsukuba, Ibaraki 305-8572, Japan

The effect of ultraviolet (UV) irradiation on the ability of primordial germ cells (PGCs) to migrate toward the gonadal ridge was investigated in the domestic chicken.

When PGCs were irradiated with UV (254 nm, $0.9 \pm 0.1 \mu\text{W}/\text{cm}^2$) the extent of nuclear damage, as determined by the comet assay, was positively correlated with the duration of UV irradiation in the range from 0 to 60 seconds. However, no further increase in nuclear damage was detected when the duration of UV irradiation was extended from 60 to 120 seconds.

To evaluate the migratory ability of PGCs after UV irradiation, 15 fluorescent-labeled PGCs were transferred into the vascular system of 2-day-old embryos and the fluorescent-labeled PGCs present in the gonads of recipient embryos were counted 5 days later. When the duration of UV irradiation was less than 60 seconds, the number of fluorescent-labeled PGCs found in the gonads of the recipient embryos exceeded the number of transferred PGCs.

In conclusion, the ability of PGCs to migrate toward the developing gonad is retained after exposure to UV irradiation that causes apparent damage to the nucleus.

Key words : UV irradiation, PGCs, Migratory ability, embryo, chicken

Introduction

Since the discovery of the migratory nature of primordial germ cells (PGCs) (Meyer, 1964), the mechanism of PGC migration has been an important and stimulating research topic in developmental biology.

In all known vertebrates, including avian species, gametes are derived from PGCs, which originate in the extra-embryonic region (Fujimoto *et al.* 1989 ; Buehr, 1997 ; Ginsburg, 1997). Kuwana (1993) reviewed the migration of avian PGCs toward the gonadal ridge. In avian species, extra-embryonic PGCs first circulate within the vascular system before migrating to the gonadal ridge (Fujimoto *et al.* 1976). It has been suggested that PGCs are attracted by unidentified chemical substance(s) released from the gonadal ridge (Kuwana and Rogulska, 1999). In addition, fibronectin and tenascin-C are present in the extracellular matrix through which the PGCs migrate to reach the genital ridges (Anstrom and Tucker, 1996). However, the exact mechanism

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¹Correspondence to : Atsushi Tajima, Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan.

Tel : +81-298-53-6688 Fax : +81-298-53-6205 E-mail : tajima@sakura.cc.tsukuba.ac.jp

of PGC migration remains to be resolved.

To elucidate the mechanism of PGC migration, it was thought that a partial impairment of cellular function, such as that induced by exposure to PGCs to ultraviolet (UV) irradiation, would offer an alternative means of studying the mode of PGC migration.

Short-wave UV light induces intrastrand DNA cross-links between adjacent pyrimidines, forming cyclobutane pyrimidine dimers (CPD) and pyrimidine-(6-4)-pyrimidone photoproducts (6-4PP) (Setlow and Carrier, 1966 ; Mori *et al.* 1991 ; Moné *et al.* 2001). The presence of CPDs and 6-4PPs inhibits DNA replication (Rupp and Howard-Flanders, 1968 ; Radman, 1971) and transcription (Herrlich *et al.* 1994 ; Van Hoffen *et al.* 1999 ; Moné *et al.* 2001). Ijiri (1976) revealed that UV irradiation of fertilized eggs resulted in the elimination of germ cell formation in *Xenopus laevis*. Reynaud (1977) showed that UV irradiation of the chick blastoderm significantly decreased the number of germ cells in the gonads of 5-day-old embryos.

Recent progress in the development of techniques for producing germline chimeras by PGC transfer in avian species (Nakamura *et al.* 1991 ; Yasuda *et al.* 1992 ; Tajima *et al.* 1993 ; Naito *et al.* 1994a, 1994b ; Ono *et al.* 1996 ; Tajima *et al.* 1998) has provided a way to assess the effects of manipulating PGCs on their migratory ability.

This study investigated the effect of direct UV irradiation of PGCs on their ability to migrate toward the gonadal ridge in the domestic chicken.

Materials and Methods

Experiment 1 : Cellular damage with UV irradiation of PGCs Sample preparation

Fertilized White Leghorn eggs (Agricultural and Forestry Research Center, University of Tsukuba, Japan) were incubated at 37.8°C for 50 to 65 hours. Embryonic blood was collected from the dorsal aorta of the embryos at stages 13–15 of development (Hamburger and Hamilton, 1951) using fine glass needles prepared by pulling 50 μ l glass pipettes (Drummond Sci. CN) with a micro-pipette puller (PA-81-8811, Narishige, Japan). The collected blood was dispersed in 100 μ l of Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS).

The PGCs were isolated from embryonic blood using a modification of the Ficoll density gradient centrifuge method reported by Chang *et al.* (1992), and washed using the filtration method of Tajima *et al.* (2003). Ficoll (Sigma Chem. MO) was dissolved at 16% (w/v) in MEM containing 10% FBS. Blood samples were overlaid on 1 ml of 16% Ficoll solution in 1.5-ml centrifuge tubes. After centrifugation at 800 $\times$ g for 30 minutes, 200 μ l of the PGC-rich supernatant was recovered and placed on 5- μ m mesh nylon filters (Small Parts, FL). The nylon filters were dampened with MEM containing 10% FBS just before use. The PGCs on the nylon filters were washed three times with 50 μ l of MEM containing 10% FBS. The nylon filters were then placed in 100 μ l of MEM containing 10% FBS in 1.5-ml centrifuge tubes and agitated using a Vortex mixer. To recover the cells from the nylon filters, the filters were fixed at the top of the centrifuge tubes, adjacent to the cap, and centrifuged at 800 $\times$ g for 30 minutes.

After centrifugation, the samples were divided into two fractions of equal volume

: one fraction was subjected to UV irradiation, whereas the remaining fraction was used as an untreated control. A total of 24 samples were prepared and each sample was randomly assigned to one of the four periods of UV irradiation (10, 30, 60, or 120 seconds).

A dual monochromator microfluorometer (JASCO, Japan) was used for UV irradiation. The samples were placed on Heavy Teflon-coated Slides (HTCS ; Erie Sci. NH) and incubated at room temperature for 5 min to allow the cells to settle. The samples were exposed to 254 nm UV irradiation at $0.9 \pm 0.1 \mu\text{W}/\text{cm}^2$, for 0 to 120 seconds.

After incubation for 4 hours at 37°C, the DNA damage and PGC viability were evaluated using single-cell gel electrophoresis (comet assay) and a fluorescent double-staining technique, respectively.

DNA damage assay

Approximately 100 PGCs were recovered from each sample using fine glass needles and dispersed in 10 μl of 0.5% low melting-point (LMP) agarose gel. The PGC-agarose suspensions were placed on frosted glass slides, which were pre-coated with a thin layer of 0.65% normal melting point agarose. The samples were immediately covered with 18 $\times$ 18 mm cover glasses and placed at 4°C for 15 minutes. The cover glasses were then gently removed, a second layer of LMP agarose was added, the coverslip replaced, and the slide returned to 4°C for an additional 15 minutes.

After removing the cover glasses, the slides were immersed in a lysing solution (2.5 M NaCl, 100 mM Na₂ EDTA, 10 mM Tris, 3 M NaOH, 1% sodium lauroyl sarcosine, 1% Triton X-100, 10% DMSO) at 4°C for 1 h to lyse the PGCs and permit DNA unfolding. The slides were then washed gently with distilled water, and placed in a horizontal gel electrophoresis unit filled with fresh electrophoretic buffer (300 mM NaOH, and 1 mM Na₂ EDTA) for 20 min to allow the DNA to unwind. Electrophoresis was conducted for 5 min at 300 mA, 25–27 V using a Power Pac 1000 (Bio-Rad, CA).

After electrophoresis, the agarose on the glass slides was gently washed with distilled water and soaked at room temperature in three changes of 0.4 M Tris buffer (pH 7.5) for 15 minutes each, to remove alkali and detergents. A few drops of SYBR Green I (Takara, Japan), diluted 1 : 10,000 in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na₂ EDTA), were placed on each agarose gel, and the gels were covered with cover glasses. The slides were placed at room temperature in the dark for 30 minutes. Stained nuclei and DNA were visualized using an inverted fluorescent microscope (IMT-2, Olympus, Japan) with a 490-nm excitation filter. The length of each "comet" (Fig. 1) of 50 randomly selected PGCs was measured using a micrometer.

The experiment was repeated 6 times and t-test was used to analyze the statistical difference in comet length between control and UV irradiated sample within each period of UV irradiation. A net increase in the comet length was calculated and subjected to a statistical analysis using one-way ANOVA followed by Duncan's multiple range test.

Cell viability assay

Fig. 1. DNA damage assay (Comet assay).

Ultraviolet-irradiated PGCs were embedded in a low melting point agarose gel. After lysis, the sample was electrophoresed, and stained with SYBR Green I. The effect of UV irradiation was estimated by measuring the lengths of the observed "comets".

The viability of PGCs was assessed by staining with propidium iodide (PI) and fluorescein diacetate (FDA). Only the plasma membranes of dead cells permit PI to penetrate and emit red fluorescence on binding to DNA (Johnson, 1995). By contrast, FDA emits green fluorescence only in viable cells that possess the esterase activity necessary to convert the non-fluorescent precursor into fluorescein (Kasten, 1981).

Propidium iodide (Wako, Japan) was diluted with Dulbecco's PBS at a concentration of 1 mg/ml. Fluorescein diacetate (Wako, Japan) was diluted with acetone at a concentration of 10 mg/ml. These stock solutions were stored at 4°C and -20°C, respectively.

Each PGC sample was combined with 10 μ l each of the PI and FDA stock solutions and incubated at 37.8°C for 5 minutes. Stained PGCs were examined by fluorescent microscopy using 490-nm and 546-nm excitation filters for FDA and PI, respectively (Fig. 2), and the dead cells (PI-positive and FDA-negative) were counted. The experiment was repeated 6 times. The viability of randomly selected 50 PGCs was measured for each treatment and t-test was used to analyze the statistical difference between control and UV irradiated samples within each period of UV irradiation.

Experiment 2 : Evaluating the migratory ability of UV-irradiated PGCs

Approximately 5 μ l of blood collected from a Rhode Island Red embryo was dispersed in 50 μ l of MEM supplemented with 10% FBS. The sample was placed on an HTCS and incubated at room temperature for 5 min. Twenty-five samples were prepared and an equal number of samples assigned to each of the five periods of UV irradiation (0, 10, 30, 60, and 120 seconds). Ultraviolet irradiation was performed using the same method as in Experiment 1.

After UV irradiation, 15 PGCs were picked up using a fine glass needle after labeling with PKH-26 (Zynaxis, PA). The PGCs were injected into recipient embryos

Fig. 2. Cell viability assay.

(a-1) and (b-1): Light photomicrographs of PGCs after UV irradiation.

(a-2) and (b-2): Fluorescent photomicrographs of UV-irradiated PGCs after staining with PI, with a 546-nm excitation filter.

(a-3) and (b-3): Fluorescent microscopic picture of UV-irradiated PGCs after staining with FDA, with a 490-nm excitation filter.

The PGCs in (a-1), (a-2), and (a-3) are PI-negative and FDA-positive, whereas the PGCs in (b-1), (b-2), and (b-3) are PI-positive and FDA-negative.

using the method of Tajima *et al.* (1998). Briefly, fluorescent-labeled PGCs were injected into the dorsal aorta of a 2-day-old RIR recipient embryo at stages 15–16 of development, via a small window cut in the shell. Approximately $5\mu\text{l}$ of blood had been removed 2 or 3 hours before the injection of PGCs. After injection, the window was sealed with plastic tape and the egg was incubated at 37.8°C .

Five days after injection, the recipient embryos were dissected under a dissection microscope (SZH, Olympus, Japan) and both the right and left gonadal ridges were collected. The collected gonadal ridges were immersed in $50\mu\text{l}$ of 0.1% trypsin in Dulbecco's PBS(–) on an HTCS, minced under the dissection microscope, and incubated at 37.8°C for 5 minutes. After mixing, $50\mu\text{l}$ of bovine adult serum was added to terminate the enzyme reaction. The total number of PGCs labeled with PKH-26 recovered from each gonadal ridge was counted under an inverted fluorescent microscope, using a 546-nm excitation filter. The experiment was repeated 5 times. A statistical analysis was performed by one-way ANOVA followed by Duncan's multiple range test.

Results

Experiment 1 : Cellular damage with UV irradiation of PGCs

The DNA damage induced by UV irradiation of PGCs was investigated using single-cell gel electrophoresis (comet assay) (Fig. 1). In this assay, electrophoresis of individual lysed cells in agarose permits damaged DNA to migrate away from the nucleus, forming the “tail” of a “comet”, consisting of a nuclear body and a migrated DNA tail. In this study, the length of a comet was defined as the total length of the comet from the leading edge of the nucleus to the tip of the tail. The intensity of DNA damage corresponds to the length of a comet (Sastre *et al.* 2001).

As shown in Table 1, UV irradiation significantly increased the length of a comet as compared with non-UV-irradiated controls when the period of UV irradiation exceeded 10 seconds. A significant dose-dependent net increase in comet length was observed in all of the irradiated samples when the period of irradiation was less than 60 seconds ($p < 0.05$). However, no further increase in comet length was observed when the UV irradiation time was increased from 60 to 120 seconds.

The viability of PGCs was investigated by fluorescent double staining with PI and FDA. No significant differences in the percentage of PI-positive/FDA-negative PGCs were observed after UV irradiation for 10 to 120 seconds. Although there was no significant difference, a slight increase in the percentage of PI-positive/FDA-negative PGCs was observed when the period of UV irradiation was 120 seconds (Table 1).

Experiment 2 : Migratory ability of irradiated PGCs

Figure 3 shows the numbers of PKH-labeled PGCs recovered from gonadal ridges

Table 1. The effects of UV irradiation on PGCs (Mean $\pm$ SE)

Period of UV irradiation	Treatment	Comet length ¹ (μ m)		Percentage of dead PGCs	
		Average	Net increase (UV-Control)	PI-positive	FDA-negative
10 sec	Control	40.8 $\pm$ 0.6		0.8 $\pm$ 0.8	4.0 $\pm$ 1.1
	UV	45.3 $\pm$ 0.7*	4.5 $\pm$ 0.5 ^D	1.6 $\pm$ 0.8	3.2 $\pm$ 1.5
30 sec	Control	42.2 $\pm$ 0.2		1.6 $\pm$ 0.8	3.6 $\pm$ 1.2
	UV	50.8 $\pm$ 0.2*	8.6 $\pm$ 0.6 ^C	2.4 $\pm$ 1.2	6.8 $\pm$ 2.1
60 sec	Control	39.1 $\pm$ 0.1		2.4 $\pm$ 0.8	2.8 $\pm$ 1.2
	UV	55.9 $\pm$ 0.2*	16.8 $\pm$ 1.9 ^A	1.6 $\pm$ 0.8	4.0 $\pm$ 0.9
120 sec	Control	41.8 $\pm$ 0.1		1.6 $\pm$ 0.8	4.8 $\pm$ 0.8
	UV	55.0 $\pm$ 0.1*	13.2 $\pm$ 0.7 ^B	6.0 $\pm$ 4.6	12.4 $\pm$ 4.8

¹ Comet length : The total length of the comet, including the diameter of the nuclear body and the length of the tail.

* Comet length of UV treatment was significantly longer than that of the control ($p < 0.05$).

^{A-D} Values with different letters in the same column are significantly different ($p < 0.05$).

Fig. 3. Migratory ability of PGCs after UV irradiation.

Fifteen UV-irradiated PKH-labeled PGCs were injected into the dorsal aorta of embryos at stages 15-16 of development. The injected embryos were incubated for 5 days. The total number of PKH-labeled PGCs in both the right and left gonadal ridges was counted after digestion with 0.1% trypsin.

^{a-c} Values marked with different letters are significantly different ($p < 0.05$).

5 days after transfer. There were 83.6 ± 13.66 , 90.6 ± 8.66 , 41.4 ± 3.59 , 33.0 ± 4.64 , and 9.2 ± 2.60 PKH-labeled PGCs with 0, 10, 30, 60, and 120 seconds of UV irradiation, respectively.

A significant decrease in the number of PKH-labeled PGCs in the recipient gonad was observed when the period of UV irradiation increased from 10 to 30 seconds and from 60 to 120 seconds ($p < 0.05$). By contrast, no significant difference in the number of PKH-positive germ cells was observed between 0 and 10 seconds or between 30 and 60 seconds of UV irradiation.

Discussion

Low level DNA damage to a single cell caused by UV irradiation (Sastre *et al.* 2001), X-ray irradiation, or H_2O_2 treatment (Singh *et al.* 1988) can be quantified using the comet assay because DNA lesions, such as CPDs and 6-4 PPs produced in the cell by UV irradiation, are removed by a mechanism generally known as nucleotide excision repair (NER) (Van Hoffen *et al.* 1995).

Preliminary experiments showed that 4 hours of incubation after UV irradiation are required before a measurable "tail" of a "comet" can be observed. It is thought that the DNA fragments seen as the "tail" are produced by the NER reaction during the incubation period.

Comet tails were observed in all of the untreated control cells. Although we did not determine the exact reason for the presence of tails in the control cells, we speculate that the major component was RNA, which is abundant in the cells. In addition, the comet tails in the control cells could be due partly to the naturally occurring NER

activity of PGCs. Therefore, the difference in the comet length between the controls and UV-irradiated cells was thought to be the result of NER activity in the PGCs after UV irradiation.

When PGCs were irradiated with UV (254 nm, $0.9 \pm 0.1 \mu\text{W}/\text{cm}^2$) for 0–60 seconds, the intensity of DNA damage, as determined by the NER activity of PGCs, was positively correlated with the period of UV irradiation. However, no further increase in the comet length was detected when the period of UV irradiation was extended from 60 to 120 seconds. Our results indicate that the NER activity of PGCs plateaued at 60 seconds of UV irradiation with our experimental conditions.

The effect of UV irradiation on the viability of PGCs was estimated by double staining with PI and FDA. No significant increase in the proportion of dead cells was observed when the period of the UV irradiation was less than 60 seconds. Although the difference was not statistically significant, a slight increase in the proportion of dead PGCs was observed when the period of UV irradiation was increased to 120 seconds (Table 1). In addition, in the PGC transfer experiment, the number of PKH-positive cells recovered from the recipient gonads was less than the number of transferred cells only when the period of UV irradiation was 120 seconds. These results indicate that the nucleus of PGCs was irreversibly damaged by 120 seconds of UV irradiation, which is likely to result in cell death.

A significant decrease in the total number of PKH-positive cells recovered from the gonads of recipient embryos was observed when the period of UV irradiation was increased from 10 to 30 seconds. Conversely, the total number of PKH-positive cells in the gonads of the recipient embryos always exceeded the number of injected PGCs, for all periods of irradiation less than 60 seconds.

It has been reported that the UV irradiation inhibits DNA replication (Rupp and Howard-Flanders, 1968 ; Radman, 1971) as well as gene transcription (Herrlich *et al.* 1994 ; Van Hoffen *et al.* 1999 ; Moné *et al.* 2001). Therefore, we hypothesized that UV irradiation of PGCs for less than 60 seconds inhibits the proliferation of PGCs rather than their migration. In order to examine the proliferating ability of PGCs after UV exposure, histological as well as *in vitro* culture studies need to be carried out in the future.

In a recent study of polar plasm in *Drosophila*, factors necessary for germ cell migration, such as Nanos protein (Kobayashi *et al.* 1996) and *Polar granule component* RNA (Nakamura *et al.* 1996), were reported to exist within the polar plasm. Tsunekawa *et al.* (2000) reported the presence of a germ-plasm-like cytoplasmic structure in chick germ cells, and proposed that the chicken germline is determined by maternally inherited factors in the germ plasm. Cellular factors related to PGC migration may also be present in the germ plasm in avian species. If UV irradiation inhibits gene transcription in PGCs, all of the cellular factors required by the PGCs for successful migration toward the gonadal ridge should be present in the circulating PGCs.

In conclusion, the ability of irradiated chicken PGCs to migrate toward the gonadal ridge was retained even after apparent damage to the nuclear DNA. In order

to clarify the mechanism of migration, future research should be directed toward searching for and analyzing cellular factor(s) that play key role(s) in PGC migration.

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