Mechanisms of resistance to DNA damage in insect cell Sf9

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Abstract

Insect cells are known to be much more resistant to ionizing radiation (IR) and other DNA-damaged chemicals than mammalian cells in culture. This phenomenon seems to imply that insect cells might possess certain unique mechanisms responsible for resistance to DNA-damaged treatments. For further investigations, a lepidopteran cell line, Sf9, and several cell lines of human origin were included in this study. Insect cells were found to be more resistant to X-irradiation and hydrogen peroxide than human cell lines, and had less DNA damage induced due to the more efficient in DNA repair, which was confirmed by the single cell gel electrophoresis technique (Comet assay). Sf9 also exhibited the higher antioxidant ability to remove reactive oxygen species (ROS) induced by IR and hydrogen peroxide. Insect cells, on the other hand, were found to be equally sensitive to methylating agents as human cells. Methylating agents induce cell death by forming DNA adducts, which later on were cleaved by mismatch repair enzymes thereby generate fatal DNA strand breaks. Methylating agent-induced cytotoxicity consequently is not associated with the intracellular level of ROS. Therefore, the higher efficiency in removing ROS in Sf9 were unable to increase the resistance to methylating agents, as it did to IR, hydrogen peroxide, bleomycin, and streptonigrin in Sf9 cells.

1. Introduction

Ionizing radiation is generally considerer as a harmful agent to organisms. Compared to mammals, insect adults had been proved extremely more resistant to ionizing radiation (Ducoff 1972). However, it seems to be an issue difficult for comparison since there are huge gaps and odds between individuals from mammals and insects. Remarkably, an resemble radioresistance found in cultured insect cells using lepidopteran TN-368 from *Trichoplusia ni* broke through the argument to radioresistance in whole insects, and suggested the intrinsic resistance in cell level to ionizing radiation (Koval et al. 1975). This result confirmed the cellular radioresistance that was previous observed in insects *in vivo*

and opened up a new direction to investigate the mechanism for extreme radioresistant ability in cultured insect cells. Subsequently, many investigations had been carried out to satisfy the puzzle of phenomenon for that cellular radioresistance in cells. Among these studies, DNA damage detecting protocols showed less DNA stand breaks in moth cells induced by X-irradiation or gamma rays (Koval et al. 1979, Chandna et al. 2004). The observations suggested lower DNA damage might be a key contributing factor for higher survival in irradiated insect cells. In further, the superior unscheduled DNA synthesis and DNA single strand break repair in response to X-ray were found in TN-368 insect cells compared to V-79 mammalian cells derived from Chinese hamster (Koval et al. 1978, Koval et al. 1979). The results gave a hint that efficient DNA repair ability involved in the expression of radioresistance. However, the unscheduled DNA synthesis in response to UV irradiation and rejoining rate of DNA double strand breaks caused by gamma rays showed similarity between both cell lines. Although those finds of equal repair rate towards DNA damage appeared to be unsupported for higher survival in insect cells or severer death in mammalian cells, a less-error DNA repair mechanism was somehow proposed for the radioresistant condition (Koval et al. 1977, Koval and Kazmar 1988). These investigations suggested that lower DNA damage induced by ionizing radiation and higher efficiency of DNA repair in insect cells were responsible for its radioresistance. However, study in ionizing radiation-induced cellular stress other than DNA lesion has been infrequent. No doubt, the DNA lesion of single and double stand breaks induced by ionizing radiation is one of the key factors to cell death. In addition to DNA damage, ionizing radiation also leads to generating of reactive oxygen species (ROS). Members of ROS including superoxide anions, hydrogen peroxide, and hydroxyl radicals possess quite high reactivity and destructive ability to various cellular components such as DNA, mitochondrion, protein, and cell membrane (Riley 1994, Wallace 1998). In order to investigate the mechanism for higher radioresistance in cultured insect cells, Sf9, a radioresistant lepidopteran cell line (Chandna et al. 2004) and several cell types from human origins were employed. For this study, radiation induced cellular stress including DNA breaks and ROS were investigated. Factors and mechanisms for radioresistance in insect cells were further analyzed and discussed in this communication.

2. Materials and methods

2.1 Cell cultures

Insect cell line, Sf9, originally established from fall armyworm (*Spodoptera frugiperda*) of Lepidoptera, was kindly gifted by Professor C. H. Wang at National Taiwan University in Taipei. The Sf9 cells were maintained in Sf-900 II serum-free medium (GIBCO BRL) at 28℃ in a humidified incubator. Four human leukemia cell lines including promyelotic HL-60 and NB4 cells, monocytic THP-1 and U937 cells; human gastric carcinoma cell, SC-M1 and

bladder carcinoma cell, NTUB1 were cultured in RPMI 1640 (GIBCO BRL) containing 10% fetal bovine serum (Hyclone), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2mM of L-glutamine, and 2 mg/ml of sodium bicarbonate, and kept at 37℃ in humidified incubator with 5% CO_2 in dark. Cells for each experiment were limited to those being thawed within 1 month.

2.2 Irradiation

Cells were seeded in 6-well dishes and incubated overnight before X-irradiation. Different single doses in log were given as 5, 10, and 20 Gy.

The irradiation was carried out at room temperature by using a Torrex 150D X-ray machine (EG&G, Astrophysics Research, Long beach, CA, USA) operated at 134 kV and 4 mA with a 1.2 mm beryllium filter at a dose rate of 6 Gy/min.

2.3 Growth assay

Growth condition of cells was measured by estimating the cell numbers using UV absorption at 260 nm of DNA contents as proposed previously by Chang (Chang 1991). In brief, cells in each treatment were harvested in a 15-ml tube. The media were removed by centrifugation and washed twice in phosphate buffered saline (PBS). DNA of Cell pellets were fixed in icy methanol at 4℃ for 20 min. Cells were air-dried by inverting the tube open. DNA was re-suspended out in 1 ml of 0.2 N sodium hydroxide for 2 h. The absorption at 260 nm for each cell solution was measured by the spectrophotometer (Hitachi, U2000). The percentage of growth fraction in each sample was calculated by dividing the absorbance value of treated cells by that of control group.

2.4 Apoptosis assay

In DNA ladder assay, apoptotic DNA from insect and human cells treated with X-ray for 3 days was selectively extracted by using DNA ladder assay kit (Suicide Track, CALBIOCHEM, Darmstadt, Germany). DNA was separated on 2% agarose gel in TBE buffer (89 mM Tris-borate, 2 mM EDTA) at 100 V. Ladders of DNA were visioned by staining with 10 μg/ml ethidium bromide.

In annexin-V assay, cells were double stained with annexin V-FITC and propidium iodide (PI) provided by apoptosis detection kit (CALBIOCHEM, Darmstadt, Germany). With filtering through a 40-μm nylon mesh, annexin V positivity was obtained by Epics XL-MCL flow cytometry with EXPO ADC XL4 Color software and data were analyzed using EXPO32 ADC Analysis (Beckman Coulter, Florida, USA).

2.5 DNA damage assay

Single cell electrophoresis, also known as Alkaline COMET assay, was used to measure DNA strand breaks. In brief, fully frosted slides were warmed on a 65℃ hot plate. Eighty µl of 1% normal melting gel (GIBCO BRL) in PBS was dropped onto slides and immediately covered with a 24 x 40 mm cover glass. Slides were transferred on ice-cold 4℃ plate for gel to solidify. Cover glasses were carefully removed in chilled water to set the under coated gel. Treated cells were washed twice in PBS. Fifty µl of cell suspension (containing 5 x 10^5 cells/ml) plus 250 µl 1.2% low melting agarose (GIBCO BRL) in PBS were equally mixed by pippeting. Eighty µl cells-gel mixtures were also dropped on slides which were pre-warmed over a 37℃ plate. The second layers were set as the under ones. In this point, procedures backward were undertaken in dimmed light to prevent non-irradiated DNA damage. Slides were carefully immersed in chilled COMET lysis buffer (2.5 M sodium chloride, 100 mM sodium ethylenediaminetetraacetic acid, and 10 mM Tris base with pH adjusted to 10 with solid sodium hydroxide; 10% dimethyl sulfoxide and 1% Triton X-100 were fresh-added before use) at 4℃ overnight. Next day, slides were denatured in electrophoresis (denatured) buffer containing 0.3 M sodium chloride (pH 13.4) and 1 mM sodium ethylenediaminetetraacetic acid for 20 minutes. Electrophoresis was run at 25 V and 300 mA (voltage was fixed and current was adjusted by buffer volume) for 20 minutes. Slides were briefly rinsed in double distill water and then transferred into 0.4 M Tris-HCl (pH 7.5) for renaturing. Samples could be kept in buffer and analyzed within 48 h.

While observing comets, slides were stained with 10 µl of 20 µM YOYO-1 (Molecular Probes, OR, USA; in glycerol/0.4 M sodium ethylenediaminetetraacetic acid, pH 7.4 (50/50) and 0.1% 8-hydroxyquinoline), then covered with a cover glass and meanwhile immersion oil (Immersol 518F; Zeiss, Oberkochen, Germany) was applied. Comets were observed under a fluorescence microscope (Zeiss, West Germany; shortwave pass filter 450 - 490 nm, chromatic beam splitter 510 nm, long pass filter 520 nm) with 250x amplification. Images of 100 individual cells per treatment randomly selceted from three slides were recorded by a digital camera (DCS-420; Kodak, Rochester, NY, USA). Migration of DNA from the nucleus in each cell was expressed by parameter of tail moment (product of tail length and intensity) (Olive et al. 1991) and measured with COMET Assay III program (Perspective Instruments, Suffolk, UK). The handling protocols were mainly followed upon those described before (Liu and Jan 2000).

2.6 Analysis of intracellular oxidative stress

Intracellular oxidative stress assay took the advantage of highly cellular penetrated character of 2',7'-Dichlorodihydrofluourescin diacetate (DCFH-DA; Molecular Probes, OR, USA). DCFH-DA is rapidly taken in by cells and diacetate (DA) part is removed, leaving

the surplus Dichlorodihydrofluourescin (DCFH) as a fluorescent reporter. DCFH is then oxidized by intracellular peroxides into Dichlorofluourescin (DCF), which fluoresces when excited by 488-nm wavelength. Upon assay, cells were incubated in medium containing 10 μM DCFH-DA for 30 min. DCF fluorescence of each cell population was obtained by flow cytometry. DCF intensity induced by either X-irradiation or hydrogen peroxide was normalized by untreated control in each cell type.

2.7 Measurement and depletion of glutathione (GSH)

Intracellular GSH level was measured using a commercially available Glutathione Assay Kit (CALBIOCHEM, La Jolla, CA, USA). In brief, cells in each treatment were collected and homogenized in 400 μl 5% (v/v) metaphosphoric acid (Merck, Darmstadt, Germany) before two rounds of wash in PBS. The supernatant was obtained by centrifuge with 15000xg at 4℃ for 10 min. Two hundred μl supernatant was added in working solution offered from kit with vortex. The reaction was incubated in dark at room temperature for 10 minutes before the measurement of the absorbance at 400 nm using a spectrometer (Hitachi, U2000). GSH level in each treatment, expressed as nmol/mg protein, was gained by comparison to a standard curve of known GSH concentrations that ranged from 20 to 100 μM, finally, was normalized by protein concentrations in cell extracts determined according to the method derived from Bradford (Bradford 1976) using bovine serum albumin ranging from 1 to 8 μg/ml as standards.

Buthionine sulfoximine (BSO), an agent blocking GSH synthesis by inactivating γ-glutamylcysteine synthetase, was employed. Insect or human cells were cultured in medium containing BSO for 24 h for GSH depletion. In treatment of X-irradiated insect cells with GSH depletion, BSO was removed and fresh Sf-900 II serum-free medium was replaced.

2.8 Preparation of cellular extraction

Cells were harvested and washed twice in PBS and washed another once in HBSS, and then were lysed in 150 μl lysis buffer (containing 20 mM Hepes, pH 7.4, 0.5% (v/v) Nonidet P-40, 100 mM NaCl, 1mM EDTA, 2 mM Na_3VO_4, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml benzamidine; 10 μg/ml leupeptin, aprotinin, pepstatin A were fresh-added before use) for 2 h at 4℃. After centrifuge (16000xg, 5 min), supernatants were collected. Protein concentrations in cell extracts were determined based on the procedure originated by Bradford (Bradford 1976)

2.9 Immunoassay

Protein extracts (50 μg) mixed with 2X protein sample buffer (0.125 M Tris pH 6.8, 4%

SDS, 20% glycerol, 0.02% bromophenol blue, 0.5% 2-mercaptoethanol) were heated at 95℃ for 5 min. Afterward they were separated on 10% SDS-polyacrylamide gel at 100 V and transferred to PVSF membrane at 20 V for 15 min. After blocking with 5% milk buffer for 1 h, the blots were incubated with monoclonal antibody at 4℃ overnight. With 3 repeats of washing in TBST (125 mM NaCl, 25 mM Tris pH 8.0, 0.1% Tween 20), blots were probed with HRP-conjugated secondary antibody for 1 h and then washed in TBST for 3 repeats. Protein signal of interest was visualized by chemiluminescence (Pierce, Rockford, IL, USA). Besides antiserums against actin (Chemicon, Temecula, CA, USA) and MGMT (Lab Vision, Suffolk, UK), other commercial antibodies were purchased from BD Biosciences (San Diego, CA, USA).

3. Results

3.1 Unbiased estimate of cell number using UV 260 nm absorbance

Estimate of cell number through the approximation of DNA amount has been proposed previously (Chang 1991). DNA amount of each sample was determined by their absorbance at UV260 nm. By dividing the absorbance value of treatment by that of control groups, the relative frequency of cell growth after treatment was estimated. Assay on growth inhibition using this protocol in mammalian cell culture has been established in our previous works. In order to verify if this protocol also would work for Sf9 cells in culture, a series of control treatments with cell number varied from 1 to 10 x 10^5 were sampled, and their absorbance at UV 260 nm determined using a Hitachi 2000 spectrophotometer (Fig. 1). A significant linear correlation between cell number and absorbance at UV 260 nm was found with an r^2 value of 0.999. This indicated that the protocol proposed by Chang (1991) provided an unbiased estimation of the cell number for Sf9. Application of this protocol is especially expedient for the growth inhibition assay in Sf9 cells, since Sf9 cells grow in culture by loosely attachment to the bottom of the plate and are unable to form firm colonies.

3.2 Insect cell line Sf9 is much more resistant to X-irradiation than human cells

Sf9 and 6 human cell lines were irradiated with 0, 5, 10 and 20 Gy X-ray. After the removal of X-irradiation, cells were incubated for 72 h before being assayed for their growth inhibition using protocol described in previous section. Dose that induced 50% cell growth inhibition (LD_{50}) was estimated using CompuSyn quantitation program (ComboSyn, Inc., NJ, USA). The promyelocytic NB4 cells was the most sensitive cell line to X-irradiation in this study, with an LD_{50} value of 3.7 Gy. The comparative resistance ratio

of each cells line to X-irradiation was therefore calculated by dividing its LD_{50} by that of NB4. They showed a descending order as follow: Sf9> SC-M1> THP-1> NTUB1>HL-60>U937 >NB4 (Fig. 2). The insect cell line, Sf9, was much more resistant to X-irradiation than all other cell lines of human origin. The growth inhibition induced by X-irradiation in Sf9 cells was almost non-detectable up to doses as high as 20 Gy. In those cells with human origin, on the other hand, cell growth was inhibited by X-irradiation in a dose-dependent manner. Among them, the gastric SC-M1 was the most and the NB4 was the least resistant cells. These 2 human cell lines were picked up as references for further investigations in aid of elucidating the resistance mechanism to X-irradiation in Sf9 cells. Sf9 cells, with LD_{50} of 49.7 Gy, were 13.4 times as resistant as NB4 cells and 3 times as gastric SC-M1. The differential growth inhibition effect of X-irradiation to Sf9, SC-M1 and NB4 was confirmed through microscopic observations (Fig. 3). There was no detectable change in cell number in Sf9 cells after the X-irradiation up to 20Gy. Cell density in SC-M1 and NB4 cells, in contrast, decreased observably as dose of X-irradiation increased.

3.3 X-irradiation did not induce apoptosis in Sf9 as in human cells

Induction of apoptosis has been demonstrated to associate with ionization-induced cell death (Ghosh et al. 1996, Sheard 2001, Zhang et al. 2001, Murakami et al. 2004, Szymczyk et al. 2004, Kanzawa et al. 2006, Yang et al. 2006, Tateishi et al. 2008). Two widely-used approaches including DNA ladder assay and annexin-V staining technique were included to investigate the mechanism of cell death in Sf9, SC-M1 and NB4 cells. Formation of fragmented DNA, one of a typical apoptotic characters detected by DNA ladder assay, was found in both SC-M1 and NB4 cells after X-irradiation (Fig. 4). Sf9 cells, on the other hand, did not show DNA fragment. The results were further confirmed by annexin-V staining, a protocol to detect early apoptosis. A dose-dependent increase in the frequency of annexin-V-stained cells were observed in both SC-M1 and NB4 cells after X-irradiation (Fig. 5). In these 2 human cells, the treatment of X-irradiation at does as low as 5 Gy was sufficient to trigger apoptosis significantly. X-irradiated Sf9 cells, on the contrary, were unable to show apoptosis induction significantly, even at a dose up to as high as 20 Gy.

3.4 X-irradiation induced fewer DNA strand breaks in Sf9 cells

DNA damage induced by X-irradiation was investigated using single cell gel electrophoresis technique, the COMET assay. Immediate after the exposure to X-irradiation, significant increase in tail moment was found in NB4 cells, indicating a substantial induction of DNA damage (Fig. 6, lower panel). The induction of DNA damage,

as shown by the median of the tail moment in COMET assay, increased 50, 70 and 80 times as many in NB4 cells as in the control treatment when exposed to 5, 10 and 20 Gy X-irradiation, respectively. In Sf9 cells, the induction of DNA damage was much lower than NB4 cells, showing only 3, 5 and 15 folds increase in the median of tail moment, respectively, in the same treatment series. The DNA damages induced in SC-M1 cells were in between these 2 cells, which were relevant to its intermediate cytotoxicity response shown earlier in this report. Differences also were found in the morphology of damaged nucleus formed in each cells. Most of the damaged nuclei induced by X-ray in NB4 cells showed a longer tail and smaller head, indicating the loss of DNA migrating to the tail, than those in Sf9 and SC-M1 cells (Fig. 6, upper panel). In Sf9 cells, level of DNA damage correlated positively with growth inhibition (r^2=0.95, Fig. 7, left panel) and apoptosis induction (r^2 = 0.87, Fig. 7, right panel). In SC-M1 and Sf9 cells, induction of DNA damage by exposure to 10 Gy X-irradiation was similar, as indicated by the close median values of tail moment as 25.8 vs. 21.2, and 24.7 vs. 29.6, respectively in 2 independent experiments shown in Figures 6 and 8. Previous results shown in Figures 2, 4 and 5, however, revealed that, in the treatment with same dose, Sf9 showed a much lower levels of growth inhibition and apoptosis induction than SC-M1 cells (Figs. 2, 4 and 5). Therefore the repair of DNA damage in Sf9 and SC-M1 cells was investigated in the treatment of 10 Gy X-ray (Fig. 8). DNA damages were reduced considerably in 2 h after the removal of X-irradiation in both Sf9 and SC-M1 cells. However the Sf9 cells were much more efficient in DNA repair, with median tail moment decreased from 24.7 to 5.6, compared to 19.6 to 10.3 in SC-M1 cells. In addition, the induction of tail moment in SC-M1 cells was found to divide into 2 distinct groups, low and high. Group with high tail moment were about 7% of the total SC-M1 cell population, showing tail moment ranged from 51 to 99, while the rest 93% were under 45. This indicated the heterogeneous response of SC-M1 cells to X-irradiation. As the post-irradiation period was extended to 24 h, frequency of high group, which showed their tail moment between 82 and 163, increased to about 28%. These cells probably were bound to apoptosis thereafter. The SC-M1 cells with low tail moment were capable to repair their DNA damage and showed normal morphology as observed in control group. The co-existence of nuclei with high and low tail moment was shown in Fig. 9B. The heterogeneous response to X-irradiation found in SC-M1 was not observed in Sf9 cells. Sf9 cells remained low in DNA damage induction despite of the increase in post-irradiation incubation period. This result implied that, in insect cells, damaged DNA was repaired more efficiently than in human cells.

3.5 Sf9 cells are lower in both background and induced oxidative stress

In addition to inducing DNA strand break, X-irradiation is also known to increase the intracellular reactive oxygen species (ROS). We therefore aimed to elucidate if the

X-irradiation resistance in Sf9 cells was attributed to their low intracellular ROS level after the treatment. Background and alteration of intracellular ROS levels after X-irradiation were measured by estimating the relative DCF intensity with a Coulter Epics XL-MCL flow cytometer. The treatment of 20 Gy X-irradiation increased DCF intensity in SC-M1 and NB4 cells equally about 2.4 folds as many as the control group (Fig. 9, left panel). The same treatment increased 1.7 folds DCF intensity in Sf9 cells. The oxidative stress produced by X-irradiation in Sf9 was less than in either SC-M1 or NB4 cells. Similar results were obtained in the experiment directly using H_2O_2 (Fig. 9, right panel). Treatment of 0.8 mM H_2O_2 increased DCF intensity in Sf9 cells about 1.7 folds, which was similar to that by X-irradiation. The increase in DCF intensity by the same treatment was as many as 42.4 folds in NB4 cells and 5.8 folds in SC-M1 cells. The ROS level increased in SC-M1 cells was intermediate between that of NB4 and Sf9 cells, which was similar to the pattern of growth inhibition induced by H_2O_2 (Fig. 10). In treatment with 0.4 mM H_2O_2, growth of over 70% NB4 cells were inhibited, while only 25% were in Sf9 cells. Same treatment induced 35% growth inhibition in SC-M1 cells, which was intermediate between NB4 and Sf9 cells. As the concentration of H_2O_2 in treatments approached to 1.5 mM , inhibition of cell growth in either SC-M1 or NB4 cells increased to 85-90%, while the same treatment still allowed 60% Sf9 cells alive. In addition to the differential response to ROS induction, these 3 cell lines also were different in background ROS level (Fig. 11). Background DCF intensity in SC-M1 and NB4 cells were 2.7 and 4.7 times as high as in Sf9 cells. Therefore, Sf9 cells were not only lower in X-irradiation- and H_2O_2-induced but also in background ROS level when compared to human cell lines. These might account for the higher resistance level of Sf9 cells to X-irradiation and H_2O_2.

3.6 GSH depletion enhanced the growth arrest in irradiated Sf9 cells

Reduced glutathione (GSH) is one of the most important mechanisms in the protection of cellular macromolecules against intracellular ROS. Insect cells with a higher background GSH level has been attributed to their resistance to mutagen insults (Wei et al. 1993). Similar phenomenon was observed in our study. Intracellular GSH level in Sf9 cells was found to be about 82.4 nmol/mg protein, which is twice as high as in SC-M1 (36.4 nmol/mg protein) and 5 times as high as in NB4 (17.2 nmol/mg protein) cells (Fig. 12). Depletion of GSH synthesis by buthionine sulfoximine (BSO) revealed that in treatment with 10 μM BSO, GSH synthesis in SC-M1 and NB4 cells was efficiently inhibited. While in Sf9 cells, more than 70% GSH were resistant to the treatment of BSO at this dose level. When the BSO concentration increased to 50 μM, GSH level decreased from 71% to 26% in Sf9 cells. Further increasing in BSO dosage up to 400 μM, however, did not deprive intracellular GSH completely. In such a treatment with extreme high BSO dosage, there were still 20% GSH activity detected in Sf9 cells. Treatments of 10 and 50 μM BSO in

Sf9 cells, though inhibited 29% and 74% GSH activity, respectively, were not cytotoxic to this cell line (Fig. 13). Therefore they were chosen to inhibit the GSH synthesis during the experiment in Sf9 cells. The BSO treatment did enhance the cell growth inhibition induced by X-irradiation in Sf9 cells (Fig. 13). Treatment of 10 μM BSO significantly enhanced the cell growth inhibition of Sf9 cells induced by 10 and 20 Gy X-irradiation ($p < 0.05$, *t*-test). In treatment with 50 μM BSO, Sf9 cells became more vulnerable to X-irradiation. The effective dose of X-irradiation to inhibit cell growth significantly decreased to the level as low as 5 Gy ($p < 0.01$, *t*-test) (Fig. 13). The synergistic effect of GSH depletion to the cytotoxicity of X-irradiation indicated the involvement of ROS. This also confirmed the hypothesis proposed in previous section that the lower ROS level, either induced or background, in Sf9 cells might explain part of reasons why insect cell is more resistant to human cells.

3.7 Sf9 cells were as susceptible to alkylating agents as human cells

Although Sf9 cells were more resistant to ROS-generating agents such as X-irradiation or H_2O_2, they were as susceptible to alkylating agents as human cells. *N*-nitroso propoxur is the *N*-nitroso derivative of insecticide propoxur, insulting human cells by forming O6-methylguanine DNA adduct (Wang et al. 1998). The dose-response curves of Sf9 and SC-M1 cells to *N*-nitroso propoxur shown in Fig. 14 (left panel) indicated that Sf9 was not more resistant than SC-M1 cell as it was to X-irradiation and H_2O_2. The dose-response curves of these 2 cells to MNNG, another alkylating agent inducing O6-methylguanine DNA adduct, were similar to that of *N*-nitroso propoxur (Fig. 14, right panel). The results obtained from the COMET assay were consistent to those observations on growth inhibition, showing that there was no significant difference between Sf9 and SC-M1 cells in the degree of DNA strand break induced by *N*-nitroso propoxur (Fig. 15, left panel). In treatments with *N*-nitroso propoxur, increment in median tail moment induced in Sf9 and SC-M1 cells was similar, with 15.7 vs. 14.5 folds in treatment with 2μg/ml, and 18.8 vs. 22.7 folds in that with 4 μg/ml, respectively (Fig. 15, right panel).

3.8 Sf9 cells are deficient in several DNA repair enzymes

Although Sf9 and SC-M1 cells were similar in *N*-nitroso propoxur- and MNNG-induced growth inhibition and DNA damage, they were quite different in the expression in several enzymes involved in DNA repair (Fig. 16). *N*-nitroso propoxur and MNNG induced cytotoxicity are mainly through the formation of O6-methylguanine adduct on DNA (Wang et al. 1998, Kaina et al. 2001) . The induction of such adduct can be prevented by a specific enzyme, O6-methylguanine DNA methyltransferase (MGMT), which transfers the methyl group from O6-methylguanine to a cysteine residue within the protein (Kaina

et al. 2001, Kaina et al. 2007) . The removing of the O6-methylguanine adduct from DNA depends on the total number of MGMT in cell. Western analysis in our studies indicated that the MGMT protein consistently detected in SC-M1 cells. Expression of MGMT protein in insect has been reported in Drosophila (Kooistra et al. 1999) and documented as a protein with MW about 19 KDa, which is close to the marker antigen we used in this study. Expression of MGMT in Sf9 cells, however, was not detectable in our study. Lack of MGMT probably lead the O6-methylguanine adduct in Sf9 cell to mispair with thymine instead of cytosine during the DNA replication. The mispairing is recognized by mismatch repair enzyme, MutSa, which is a heterodimer composed of MSH2 and MSH6 (Kaina et al. 2007). In our results, expression of MSH2 and MSH6 enzymes was observed in SC-M1 but was absent in Sf9 cells. There was a clear band of protein shown in Sf9 cells at the position approximate twice the molecular weight as that of MSH2 (102 KD) antibody we used in the Western analysis. However, that protein band did not seem to be the dimmer of MSH2 since the SDS PAGE protocol in our experiment would not allow the dimmer to keep intact on the gel. Phenomenon similar to the MSH2 also was found in the Western analysis of MSH3. The dimmer of MSH2/MSH3, which recognized as MutSβ, was a less abundant factor for MMR system and has not been implicated in DNA damage signaling (Stojic et al. 2004) . MutL is another essential MMR protein mediating between MutS and MutH (Jiricny 2006) . MLH1, the major component protein of 3 distinc MutL heterodimers, which expressed significantly in SC-M1 cells, was not detected in Sf9 either. Overall, DNA repair enzymes that involved in O6-methylguanine adduct removing and repairing, were proficient in SC-M1 but deficient in SF9 cells.

4. Discussion

Adult insects are known to be more resistant to ionizing radiation than vertebrate (Ducoff 1972). It was at first attributed to the low or no cell division in most of the cells composing adult insect species. Investigations using cell lines, however, revealed that, although about the same in mitotic indices (Bianchi and Lopez-Larraza 1991), cells isolated from insect are more resistant to the lethal effect of radio-irradiation than those from mammals (Koval et al. 1977, Koval 1983, Koval 1988). Therefore the proliferative status did not seem to interpret the differential radio-resistance between insect and vertebrate appropriately. Factors other than proliferation difference have been proposed to elucidate the mechanism of high radio-resistance in insect and insect cell lines (Koval 1983). Among them, the chromosome size or DNA content per chromosome was suggested to be important in determining insect cell radio-sensitivity by certain authors (Koval 1983). However, results of an investigation on resistance to mutagens in insect cell revealed that mosquito *Ades albopictus* cells, although comparable to mammalian cells in their DNA content (Adams et al. 1979), are more resistant to mutagen

insult (Wei et al. 1993). Cells isolated from lepidopteran insect were found more radio-resistant than those from other insect orders (Koval 1983, Chandna et al. 2004). The holokinetic nature of chromosome in lepidoperan insect, which allows the survival of cells with fragmented chromosome to get through the cell division process, has been proposed as a possible radio-resistance mechanism. This hypothesis, nevertheless, became less convincible when cells from hemipteran insect, which also have holokinetic chromosome, showed to be much less radio-resistant than lepidopteran cells. Results from a series of thorough studies using insect cell line, TN-368, led TM Koval and his colleagues to conclude that insect cells are more resistant to irradiation because of their less sensitive to the DNA damage and more efficient in DNA repair (Koval et al. 1977, Koval 1984, 1986, 1988, 1991). Development of insect cell culture practice provided a useful tool for the elucidation of radio-resistance mechanism. In spite of this, research in this field was not as active as had been expected thereafter. TN-368 was a lepidopteran cell line isolated from cabbage looper *Trichoplusia ni*. In this report, another lepidopteran cell line, Sf9, isolated from fall army worm, *Spodoptera frugiperda*, was used. Compared to TN-368, investigation on radio-resistance using Sf9 has been rare (Chandna et al. 2004). We found it is also a insect cell line with much higher resistance to X-ray irradiation than several human cell lines.

The dose-responsive curve of each cell lines to X-ray irradiation showed that in the treatments that over 50% of human cells were inhibited to grow, Sf9 cells still got more than 90% growth rate. It seemed that, at the dose range that is tolerable for human cells, an increased irradiation dose is still unable to overcome the radio-resistance in Sf9 cells. This phenomenon is comparable to the type of oncogenic resistance categorized by M.V. Blagosklonny (Blagosklonny 2004). Development of oncogenic resistance is due to the alteration in regulation of apoptosis so that the downstream pathways of apoptosis are blocked. Our results from DNA ladder assays and annexin-V staining technique did show that apoptosis induced by X-irradiation occurred in human cells but not in Sf9 cells. This confirmed the view point that radio-resistance in insect cells is an intrinsic nature of insect (Koval 1991).

Similar to the penomena observed in TN-368 cells as described previously in this report, our results indicated that X-irradiation induced much less DNA damage in Sf9 than in human cells. The growth inhibition and apoptosis induction correlated significantly with the degree of DNA damage among treated cells. The COMET assay which allowed us to estimate the efficiency of DNA repair by scoring the disappearance of DNA tail during the post-irradiation period revealed that Sf9 cells decreased their DNA damage in a rate mush faster than human cells. In addition, repair of DNA damage did not seem to implement homogenously on every X-irradiated human cells. Certain integrate portion of damaged human cells kept at a higher level of their tail moment after the treatment

removal indicating the lack of complete repair in damaged DNA. The percentage of those cells with damaged DNA left un-repaired increased as the post-incubation period extended. This unique phenomenon was not observed in Sf9 cells, and does not seem to have been described previously by other authors either.

It is well established that irradiation generates reactive oxygen species (ROS), which in turn acts as a common mediator for apoptosis (Ozben 2007). Among previous documents, radio-resistance in insect cells, however, has never been correlated with the induction of apoptosis and ROS. In our results, Sf9 cells are lower in background intracellular ROS level. The induction level of ROS after the treatments with X-irradiation also was lower than those in human cells. The significant role of intracellular ROS in the radio-resistance in insect cells was corroborated by the results that Sf9 cells were more resistant to and induced less intracellular ROS by the treatment of hydrogen peroxide, a typical ROS-generating agent, than human cell lines. The relationship between ROS and insect radio-resistance became even clearer when intracellular GSH level was investigated. GSH is known to be a powerful antioxidant to reduce intracellular ROS and frequently associated with the decrease in the ROS-induced cytotoxicity (Griffith 1999). In Sf9 cells, background GSH level is more than twice as much as in human cells. The concentration of GSH inhibitor, buthionine sulfoxide (BSO), needed to decrease GSH significantly in Sf9 cells is more than 40 times higher than for human cells. Deprivation of GSH by pretreatment with BSO significantly decreased the radio-resistance in Sf9 cells. Our results therefore suggested that the higher background antioxidant and lower induced ROS level play a significant role in determining the higher radio-resistance in insect cells.

In addition to X-irradiation, insect cells were found more resistant than human cells to other mutagens, including UV (Koval 1986), bleomycin (Bianchi and Lopez-Larraza 1991) and possibly also many others (Wei et al. 1993). However, our investigation showed completely different results when N-nitroso alkylating agents, including MNNG and an insecticide derivative, N-nitroso propoxur, were used to target the insect and human cell lines. These 2 N-nitroso mutagens are equally toxic to Sf9 and human cell lines. Degree of DNA damage induced by these 2 mutagen which known to form O6-methylthioguanine adduct on DNA are also similar between insect and human cells. Despite of the similar response in cytotoxicity and DNA damage induced with human cells, Sf9 cells were lack of the expression in enzymes responsible for the removing of the O6-methylthioguanine adduct from DNA and the damage repair thereafter. Similar phenomenon has been described in human cells that in cells without the activity of MGMT, deficiency in MMR enzyme, such as MutSα or MutSβ, on the contrary, enable to cells with damage survived. It is because that MMR enzyme, which originally play the role in repairing the mismatched DNA pairing due to the O6-methylthioguanine adduct, also induced DNA strand break

during the process of their repair. Lack of MMR enzyme allows the cells to escape the cytotoxicity caused by DNA strand break, and go one way another to develop the resistance to the treating chemicals. Whether this would explain the similar resistance in Sf9 and human cells despite of their significant difference in repair enzyme involved is await for further investigation.

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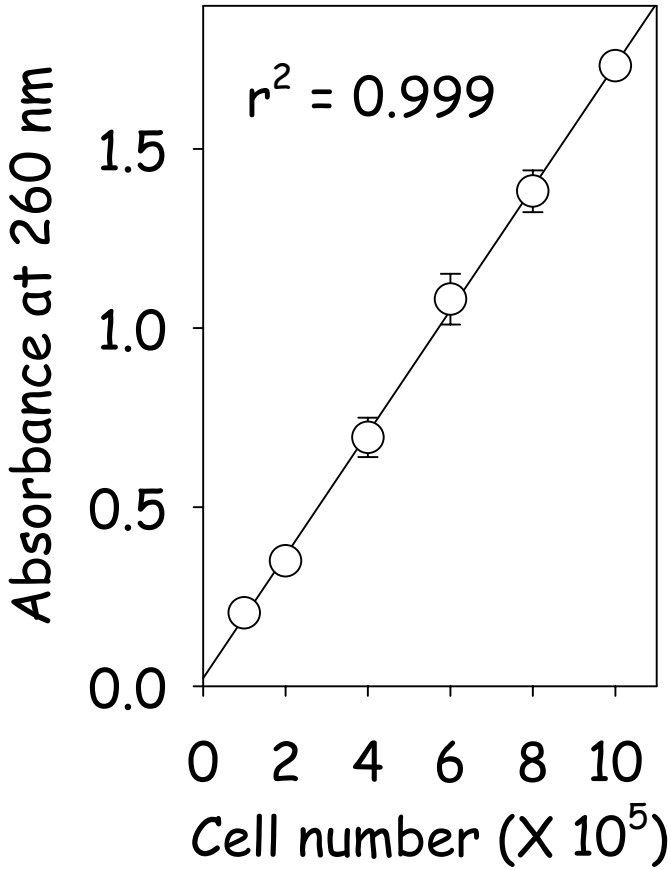
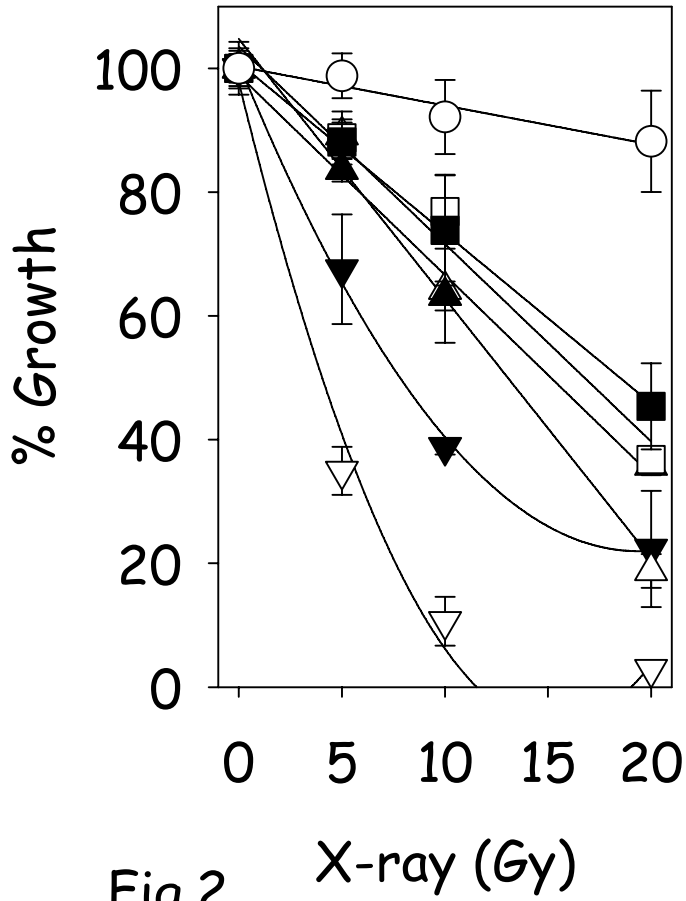


Fig.1

Linear correlation between cell number and absorbance at UV 260 nm in Sf9 insect cells



Cell lines	LD50 (Gy)	Resistance level
○Sf9	49.7	13.4
■SC-M1	18.2	4.9
□THP-1	16.3	4.4
▲NTUB1	13.9	3.8
△HL-60	11.7	3.2
▼U937	8.0	2.2
▽NB4	3.7	1.0

Fig.2

Cytotoxicity of X-irradiation to the insect cell and other human cell lines

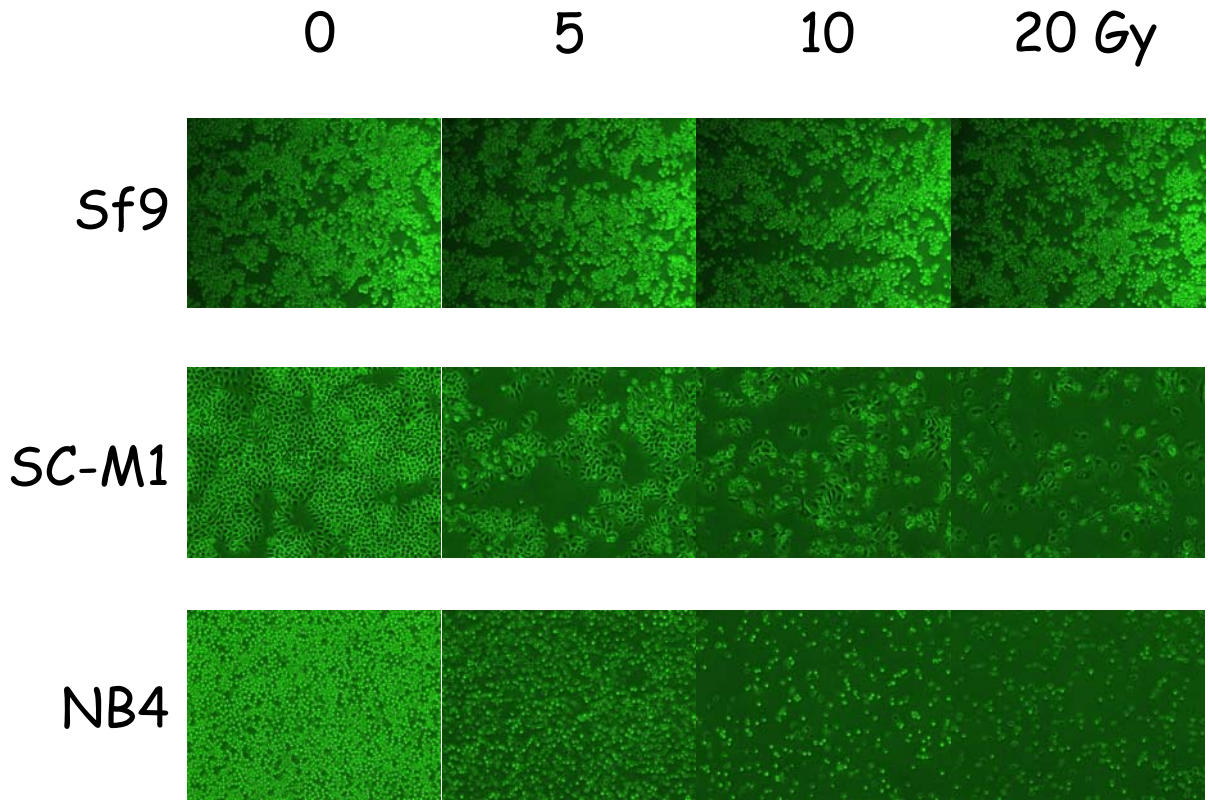


Fig.3

Photographs (50 X) indicating effect of cell population growth of X-ray to insect and human cell lines

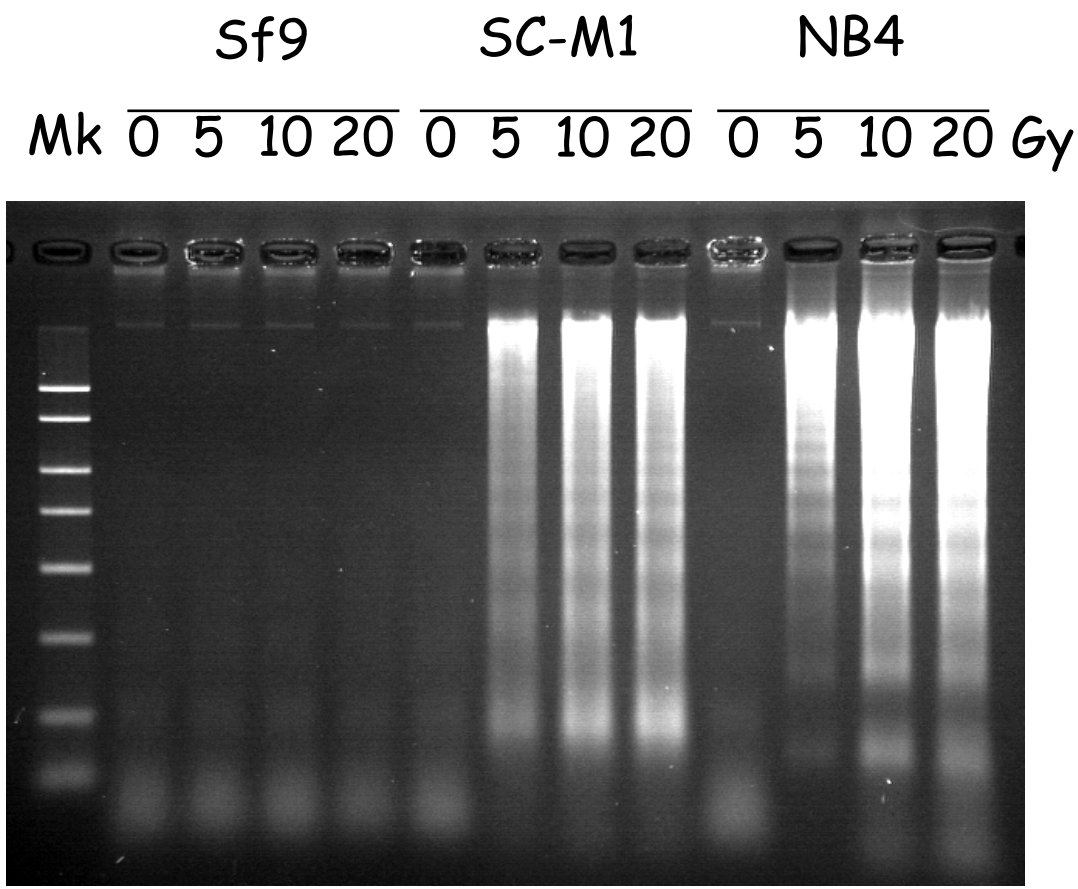


Fig.4

Effect of DNA fragmentation of X-ray to insect and human cell lines

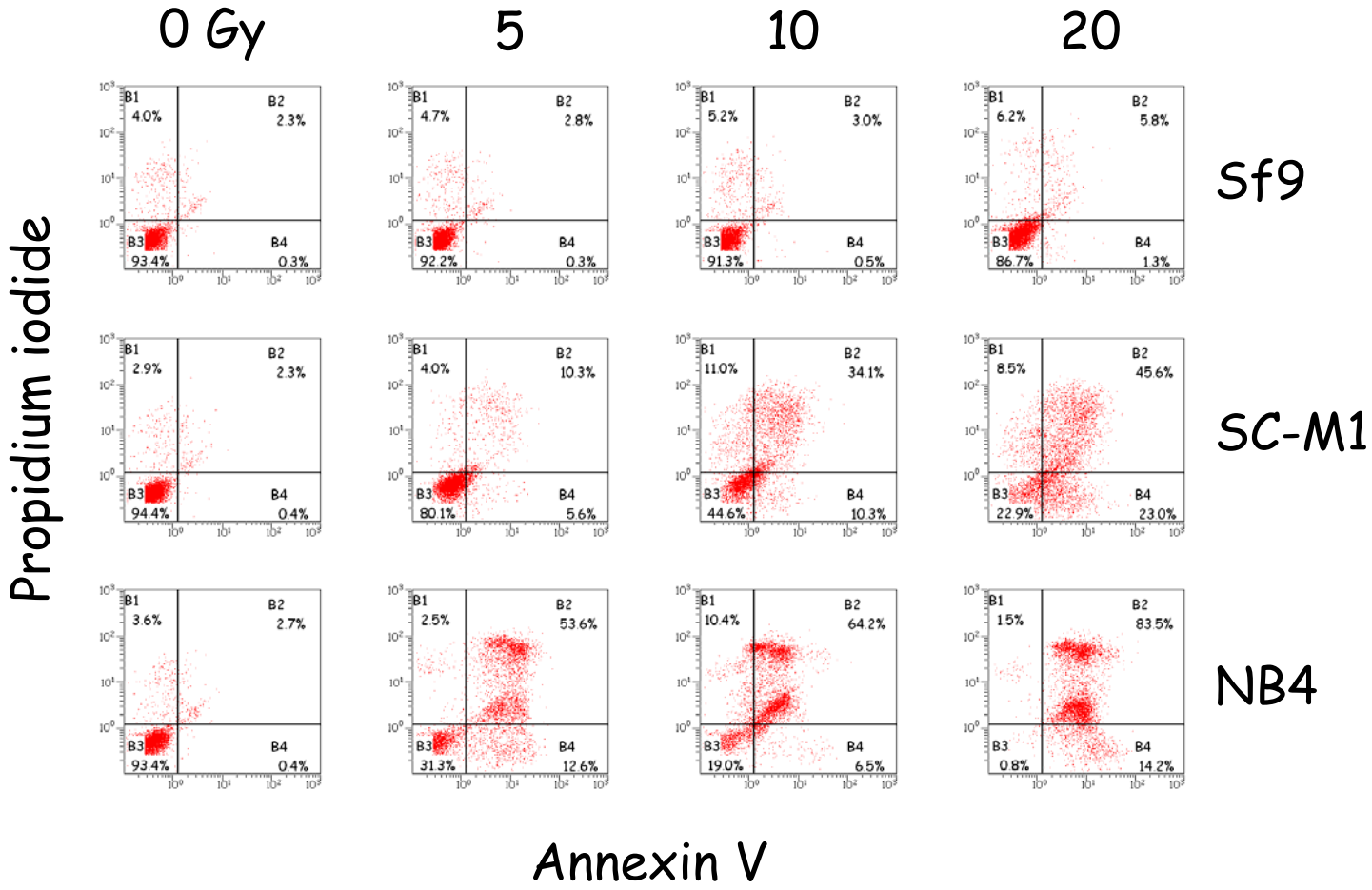


Fig.5

Effect of apoptosis of X-ray to insect and human cell lines

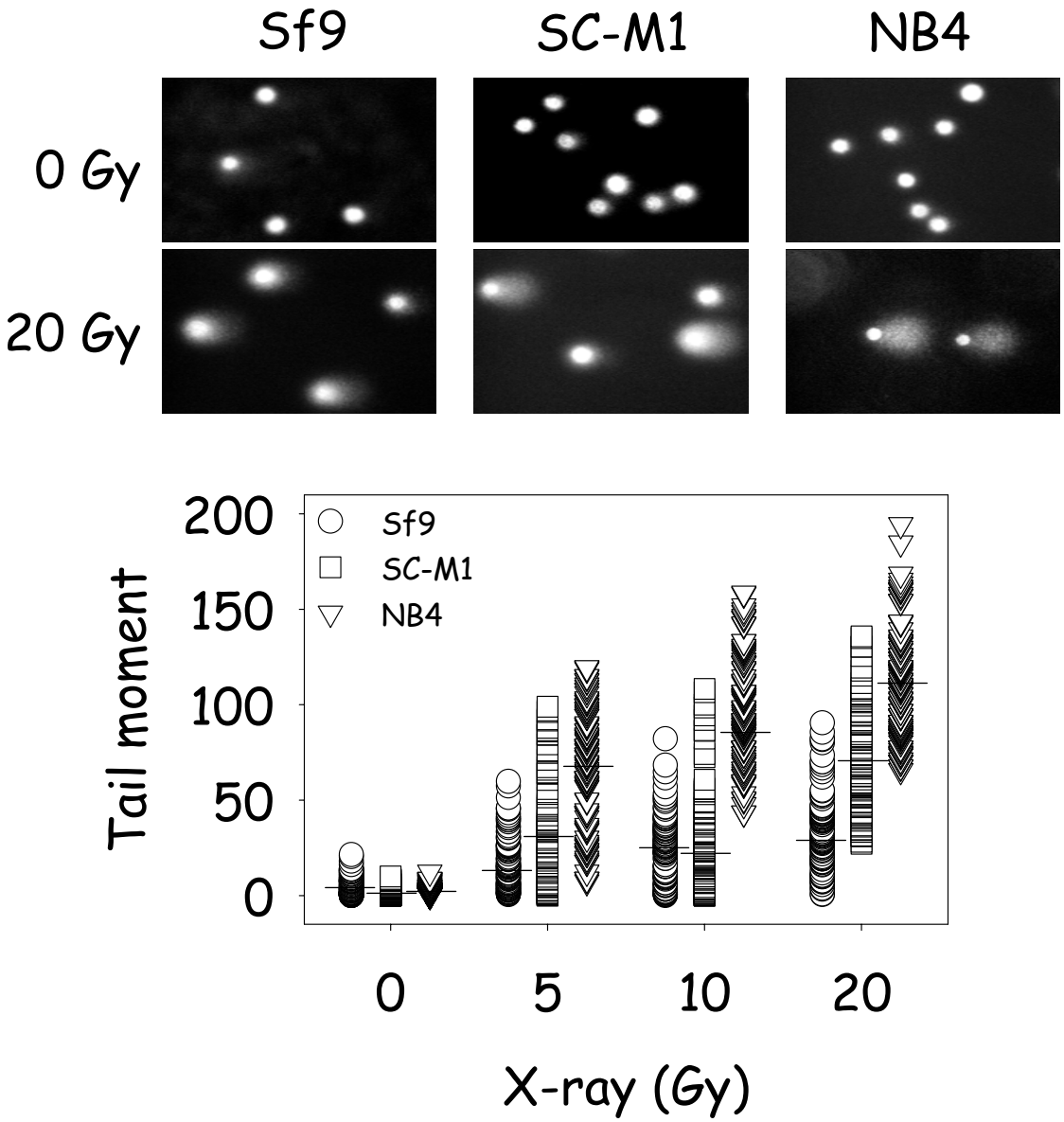


Fig.6

Effect of DNA stand breaks of X-ray to insect and human cell lines and Photographs (250 X) indicating effect of DNA stand breaks of X-ray to insect and human cell lines

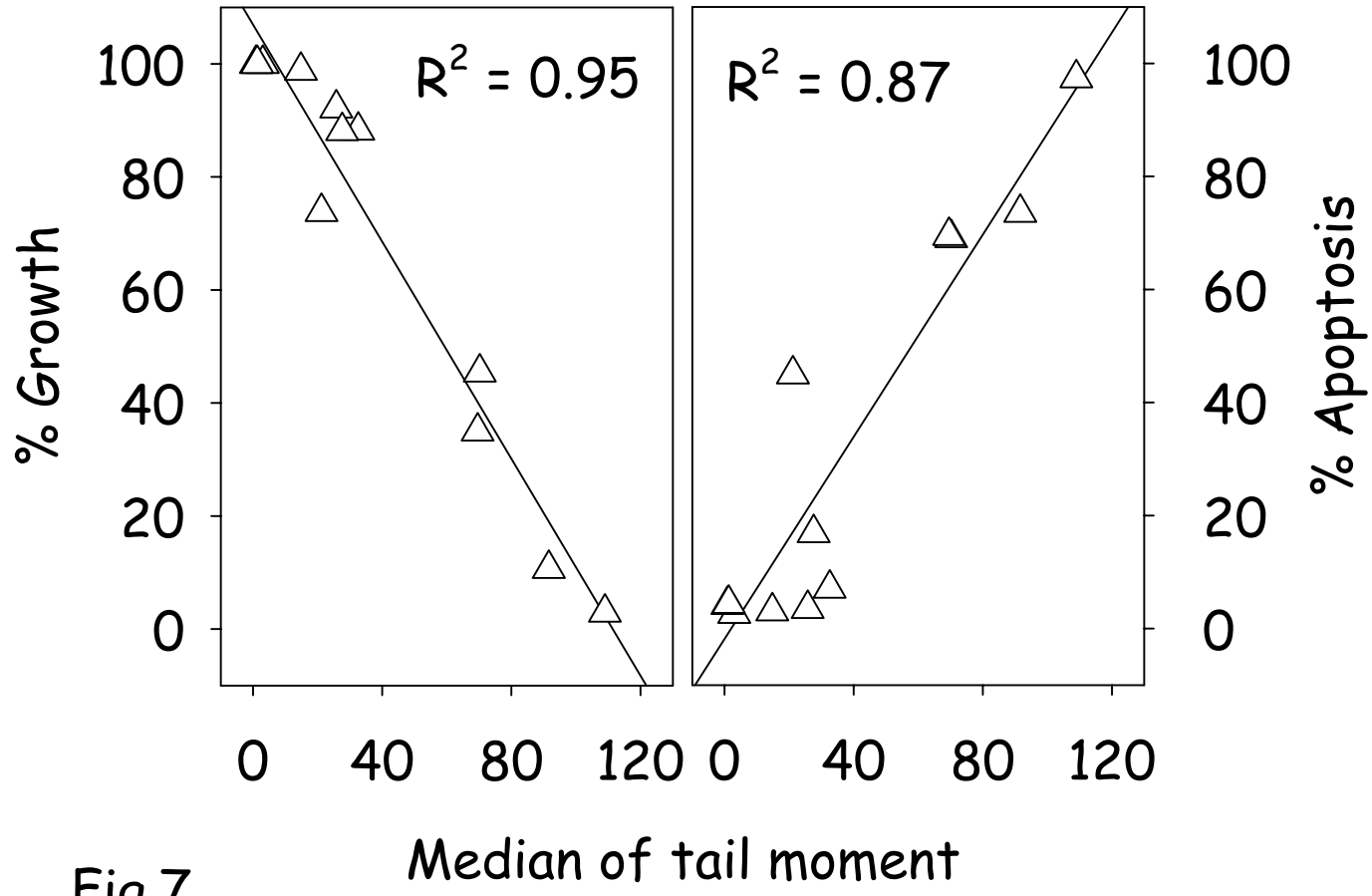
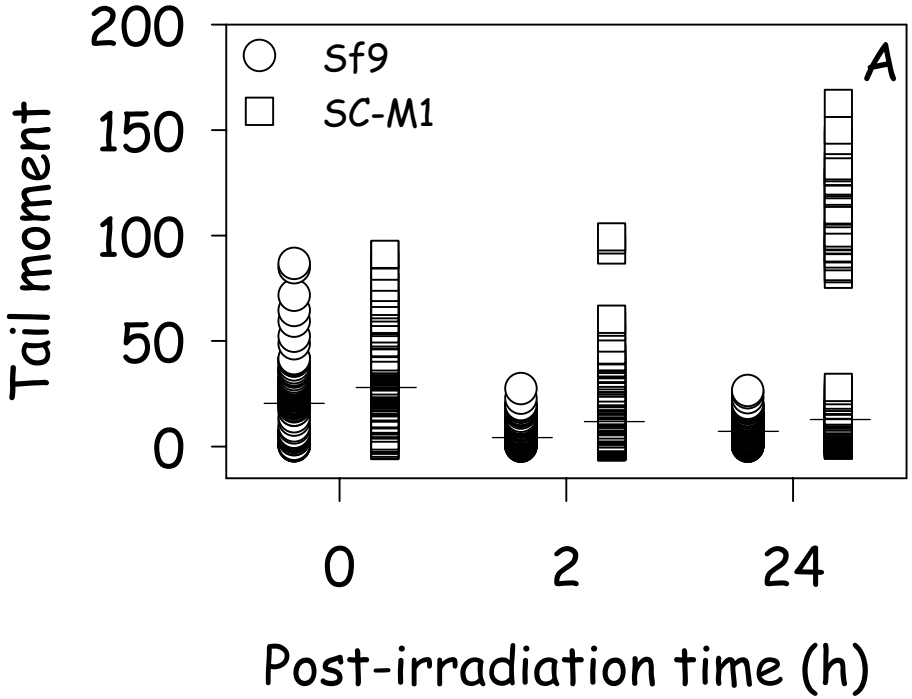


Fig.7

Regression relationship between DNA damage to cell growth and apoptotic level



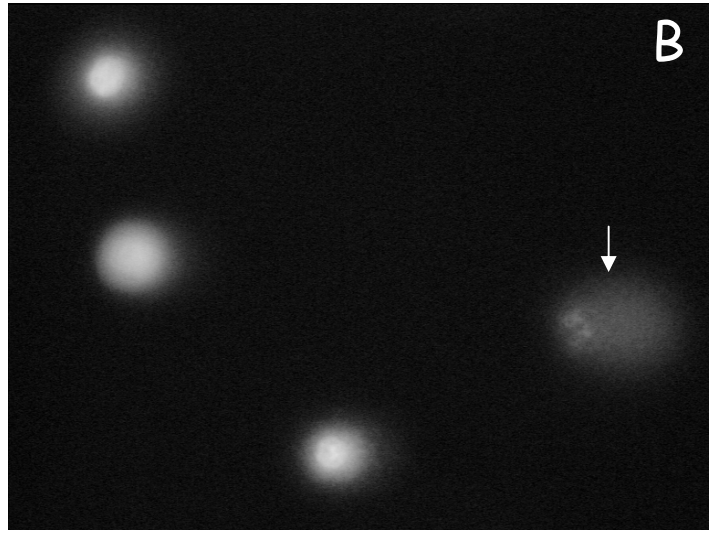


Fig.8

(A) DNA repair conditions in 10-Gy-X-irradiated insect and human cell lines. (B) The photograph (250 X) indicating DNA repair conditions in SC-M1 human cells after X-irradiation treatment for 24 h, and arrows pointing the cell incapable of repairing X-ray induced damaged DNA and carrying distinct DNA breaks

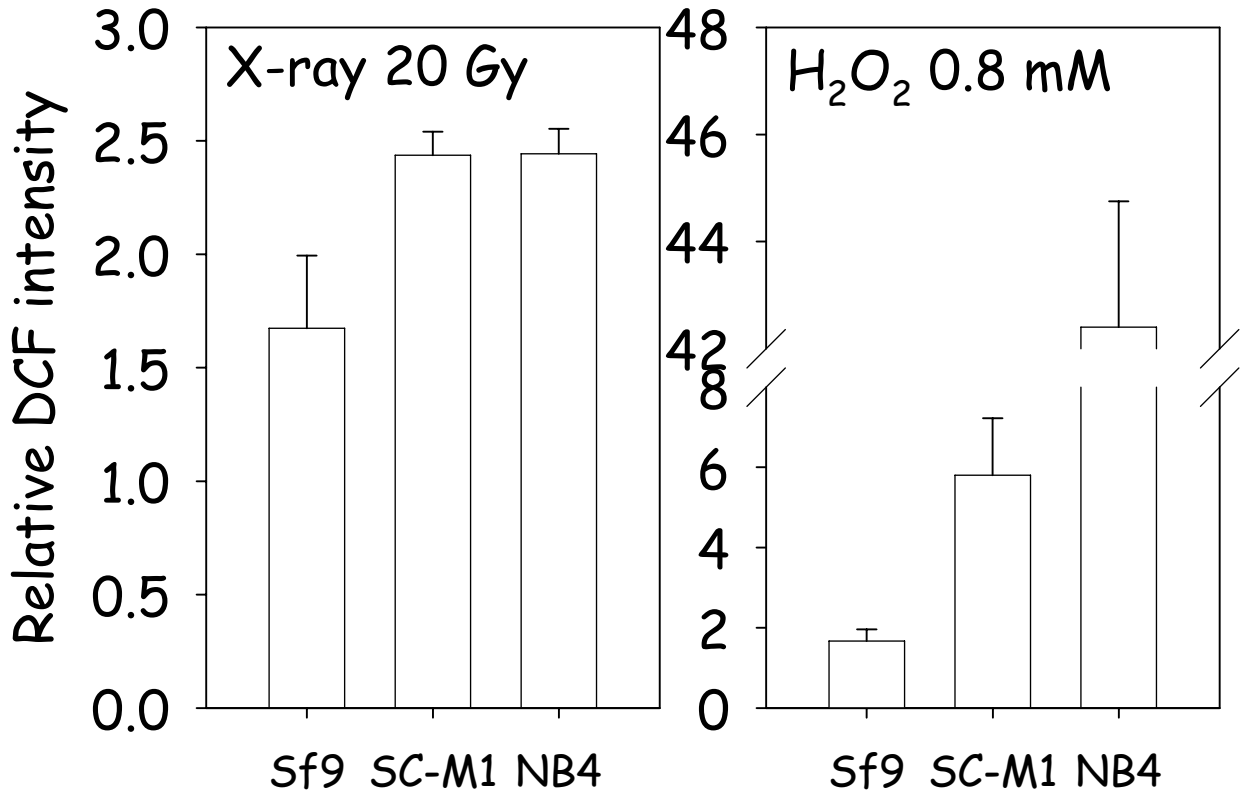


Fig.9

Effect of intracellular ROS alteration of X-ray and hydrogen peroxide to insect and human cell lines

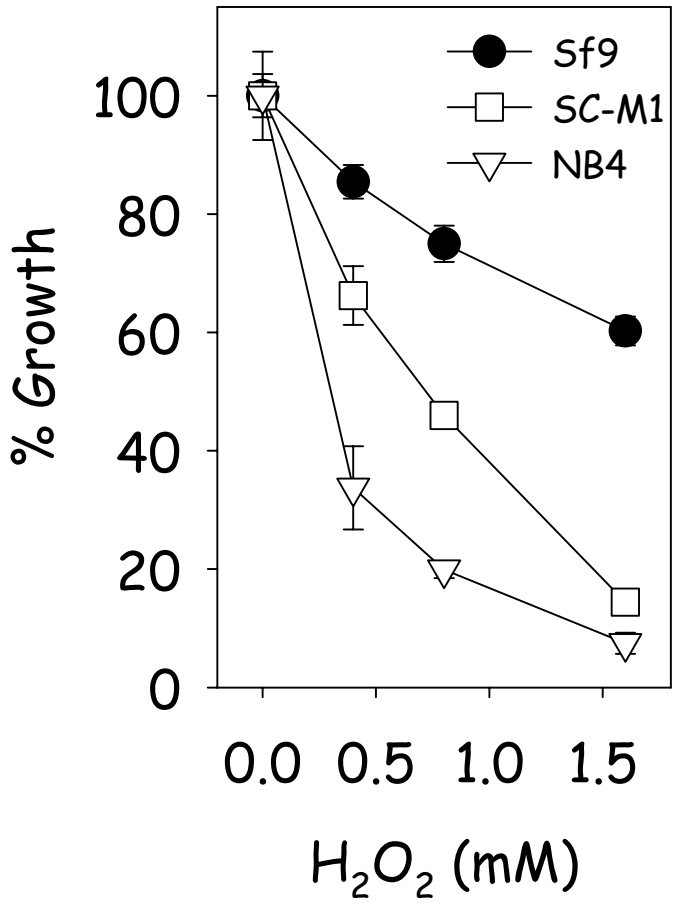


Fig.10

Cytotoxicity of hydrogen peroxide to the insect and human cell lines

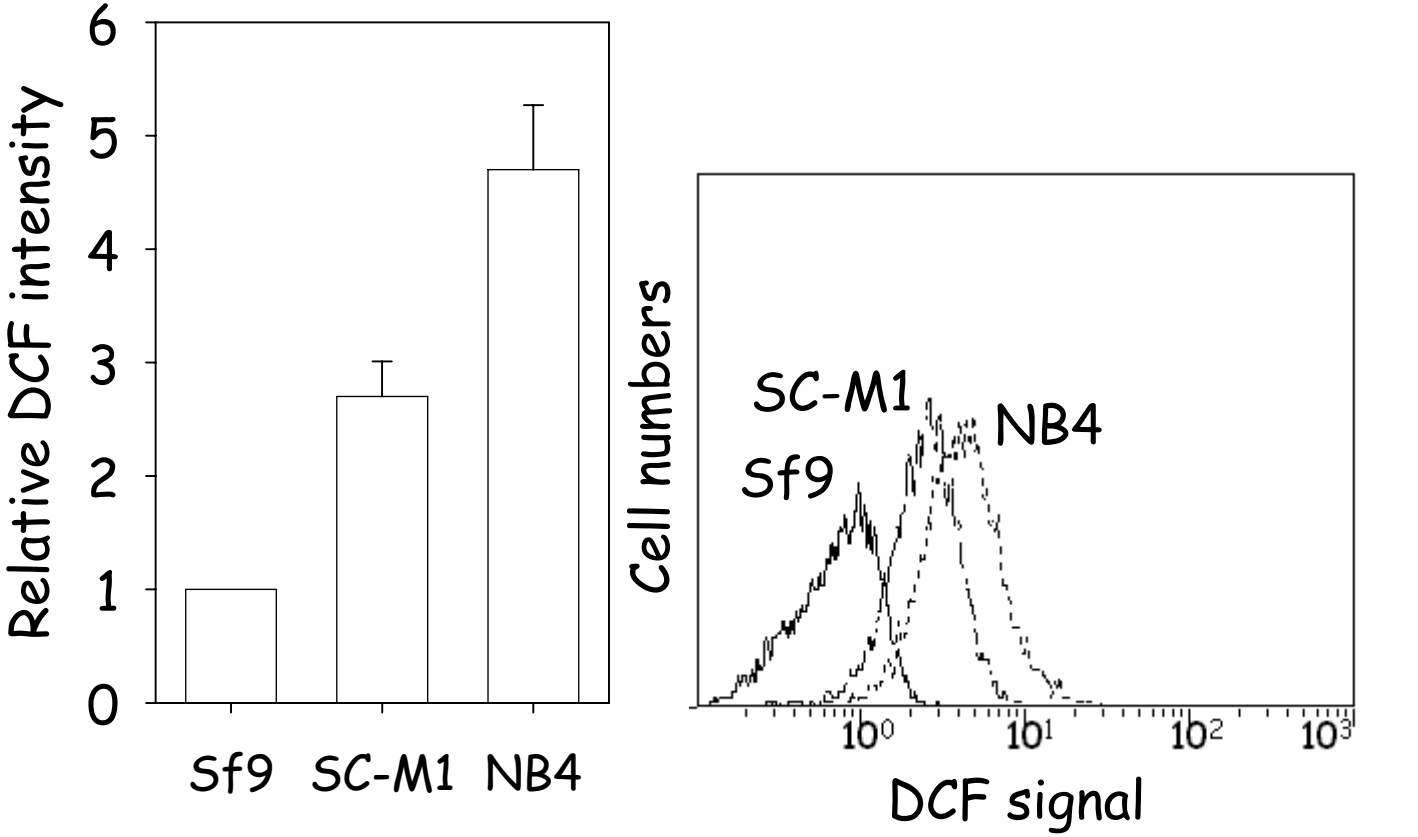


Fig.11

Intracellular ROS basal level in insect and human cell lines

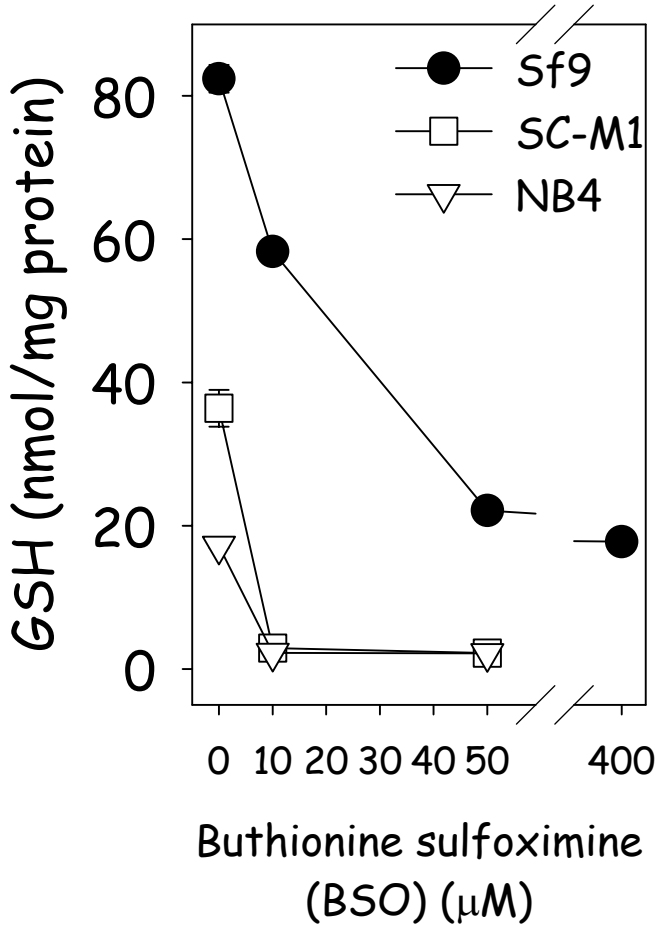


Fig.12

Effect of GSH depletion by BSO in insect and human cell lines

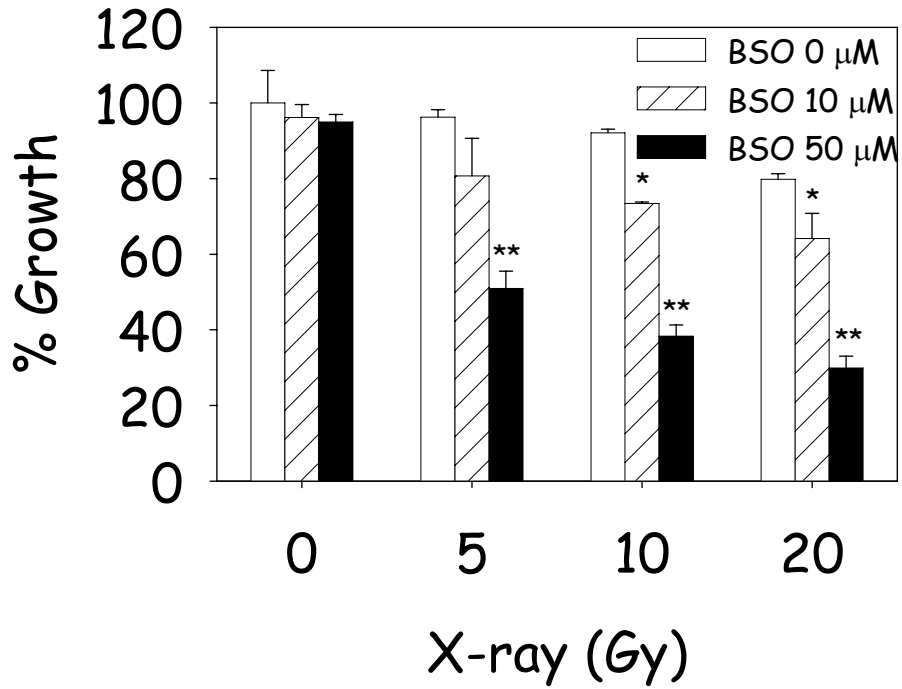


Fig.13

Effect of cytotoxicity of X-irradiation to Sf9 insect cells with GSH depletion by BSO

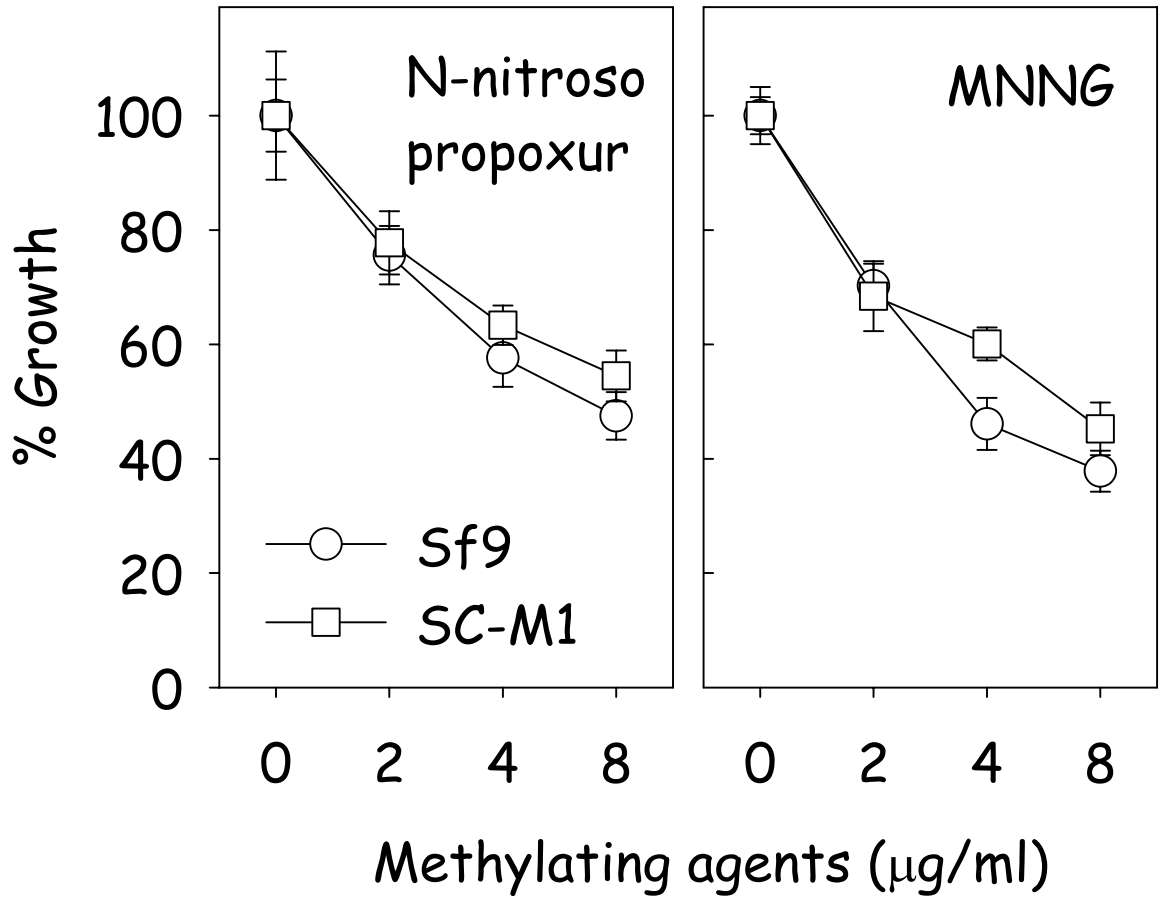


Fig.14

Cytotoxicity of methylating agents including N-nitroso propoxur and MNNG to insect (Sf9) and human (SC-M1) cell lines

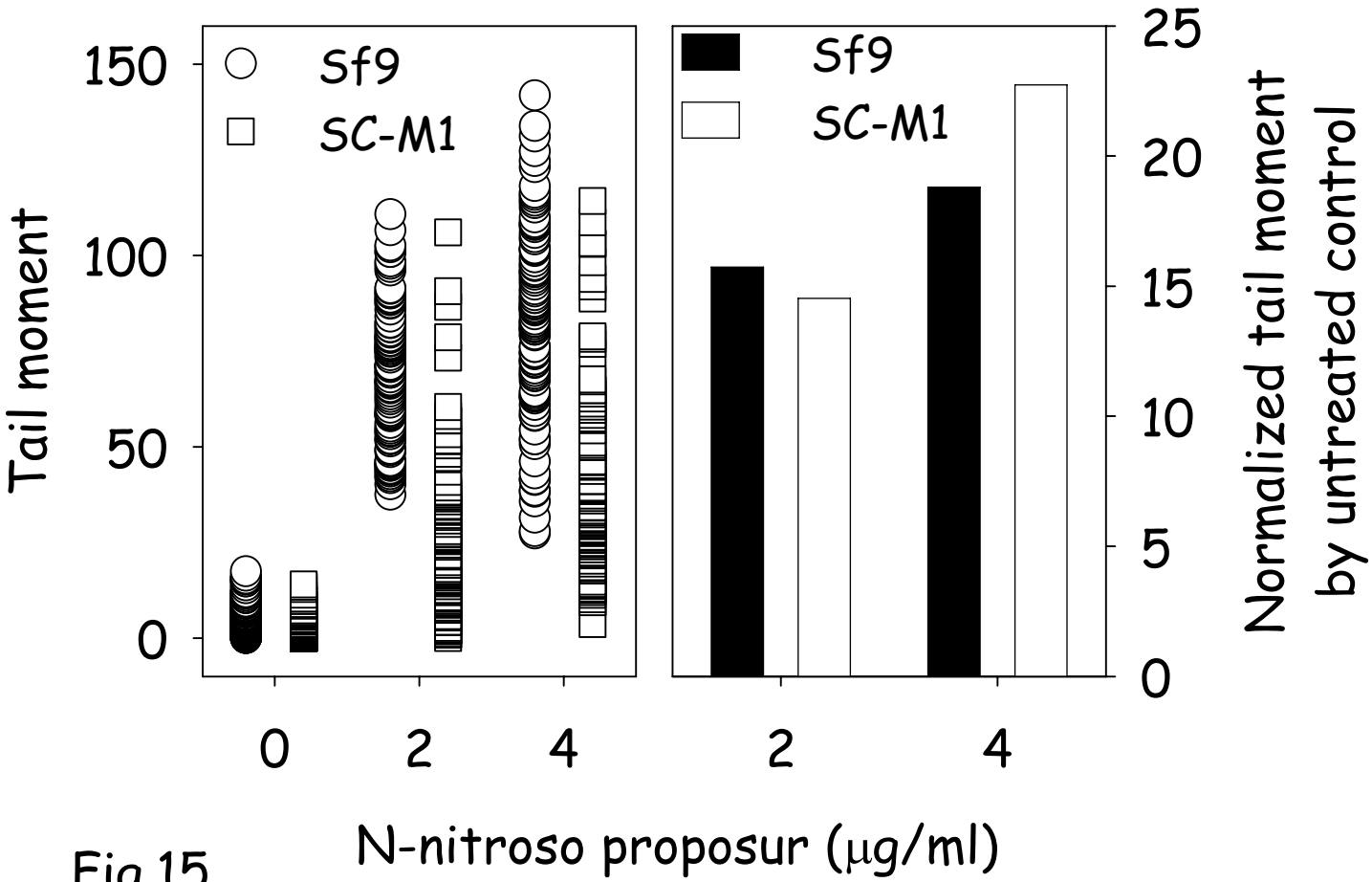


Fig.15

DNA damage induced by N-nitroso propoxur to insect (Sf9) and human (SC-M1) cell lines

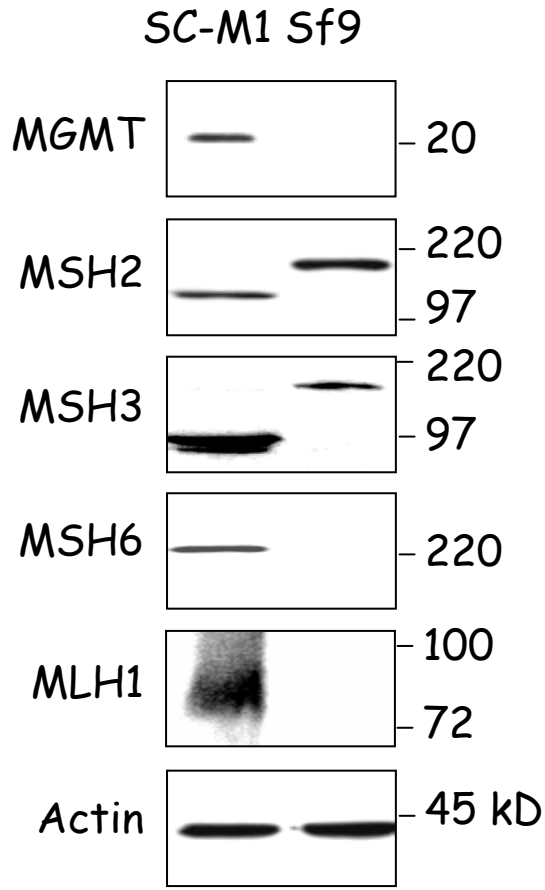


Fig.16

Detection of some DNA repair enzyme in insect (Sf9) and human (SC-M1) cell lines