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J. RADIAT. RES., 39, 165–174 (1998)

# Establishment and Characterization of a Hypocatalasemic Mouse Cell Strain

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(*Received, May 29, 1998*)
(*Revision received, June 23, 1998*)
(*Accepted, June 25, 1998*)

**Hypocatalasemic mouse cell strain/Hydrogen peroxide/ X-rays/Near-UV/Topoisomerase II inhibitor**

Contact-inhibited catalase-deficient fibroblast cell strain has been established from the homozygous hypocatalasemic C3H/Cs$^b$ mutant mouse. This cell strain has low level of catalase enzyme activity and has normal level of enzyme activities of both glutathione peroxidase and superoxide dismutase. Catalase-deficient C3H/Cs$^b$ mutant cell strain is markedly more sensitive to the toxicity of hydrogen peroxide compared to wild-type C3H/Cs$^a$ cell strain. In addition, mutant cell strain is sensitive to X-rays and near-UV compared to wild-type cell strain, but shows the same sensitivities to topoisomerase II inhibitors, adriamycin and 4'-(9-acridinylamino) methanesulfon-m-anisidide (m-AMSA), and the DNA cross-linking agents, cis-diamminedichloroplatinum (II) (cis-Pt) and trans-diamminedichloroplatinum (II) (trans-Pt). These cell strains will be of use in the study of the roles which catalase plays in the intracellular prevention of DNA damage induced by oxidative stress.

## INTRODUCTION

In recent years there has been an increasing awareness of the role which oxidative damage to DNA may play in mutagenesis and carcinogenesis[1)]. Highly reactive oxygen radicals produced

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Abbreviations: SOD; superoxide dismutase, GSHPX; glutathione peroxidase, m-AMSA; 4'-(9-acridinylamino) methanesulfon-m-anisidide, 8-OH-Gua; 8-hydroxyguanine, cis-Pt; diamminedichloroplatinum(II) , trans-Pt; trans-diamminedichloroplatinum(II)

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by ionizing radiation, chemical carcinogens, tumor promoters, and natural cellular processes may lead to the DNA lesions which contribute to cell killing, mutagenesis and tumorigenesis[2]. Among the enzymes thought to protect mammalian cells from oxidative damage are superoxide dismutase (SOD), glutathione peroxidase (GSHPX) and catalase[3]. In 1966, Feinstein et al.[4] reported a case of one catalase-deficient mouse after screening 12,300 progeny of irradiated sires[5]. The back-crossing of this mouse resulted in homozygous C3H/$Cs^b$ mice exhibiting less than 1% of normal blood catalase activity. The mutation present in the $Cs^b$ mouse has been thoroughly studied and is now thought to be a structural mutation in the catalase gene. The mutation does not affect the association of the catalase monomers into the enzymatically active tetramer extremely labile to conditions of alkaline pH and increased temperatures[5,6]. Ito et al. reported that this mutant mouse showed a high risk of developing a duodenal tumor following the oral administration of hydrogen peroxide[7]. The establishment of catalase-deficient cell lines from C3H/$Cs^b$ mice has been reported previously[8]. It is of interest as to whether these cell lines are sensitive to radiation or antitumor drugs. However, no relevant information on these respects has been reported to our knowledge. Therefore, we have established cell strain from C3H/$Cs^b$ mice to elucidate the phenotype of catalase deficiency in cells *in vitro*.

We report here that this cell strain has a low cellular level of a temperature-sensitive catalase activity and is more sensitive to damage by exogenous hydrogen peroxide, X-ray and near-UV compared to wild C3H/$Cs^a$ cell strain.

## MATERIALS AND METHODS

*Establishment of cell culture*

Breeding pairs of C3H/$Cs^b$ and wild-type C3H/$Cs^a$ mice were generously provided by Professor A. Ito of the Research Institute for Radiation Biology and Medicine, Hiroshima University who obtained the strains originally from the Division of Biological and Medical Research, Argonne National Laboratory, ILL[4]. Fibroblast cell cultures were initiated by explantating skin fragments of about 18-day-old embryos from each mouse strain. Skin fragments were removed from the embryo, minced with scissors, and incubated with culture medium in 100-mm plastic Petri dishes (Falcon, Lincoln Park, NJ). When dishes were confluent with the outgrowing fibroblasts from skin fragments, the cells were detached with 0.25% trypsin, suspended in culture medium containing 10% dimethyl sulfoxide (DMSO) and frozen in liquid nitrogen for stock of primary fibroblasts, which were designated passage 0. A part of primary fibroblasts was then serially subcultured every 3 days by seeding at $8 \times 10^5$ cells into a fresh 100-mm plastic Petri dish. The cells were cultured in alpha-modified Eagle's minimum essential medium (alpha-MEM) supplemented with 10% fetal calf serum (FCS)(Hyclone, UT), 20 mM HEPES, penicillin (100 U/ml) and streptomycin (100$\mu$g/ml) and maintained at 37°C in a humidified atmosphere with 5% $CO^2$. At every transfer, the growth rate (GR), or increase in cell number per passage, was calculated as the number of cells in a dish divided by the inoculation size ($8 \times 10^5$). Population doubling number (PDN)[9,10] was obtained by the following equation: PDN = log(GR)/log 2 where GR is the growth rate at every passage. After passage 25, both cell cultures were well established.

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The cells during passages 26–29 were used in the present study.

*Enzyme activity assay*

Pelleted cells were suspended in phosphate buffered saline (PBS) containing 5 mM EDTA, 0.01% digitonin and 0.25% sodium cholate and disrupted by sonication. The homogenate was centrifuged by 12,000 × g for 30 min. The supernatant was used for the determination of enzymatic activities. Enzyme activities for glutathione peroxidase (GSHPX) and catalase were measured by the methods of Flohe and Gunzer[11] and Aebi[12], respectively. Superoxide dismutase (SOD) was measured by using SOD test Wako (Wako, Osaka, Japan) based on the method of McCord and Fridovich[13]. One unit of catalase was defined as the amount decomposing 1$\mu$ mol of $H_2O_2$ per min at 37°C. One unit of GSHPX was defined as the amount of enzyme decomposing 1 nmol of peroxide per min at 25°C. The enzymatic activity of SOD is given as McCord and Fridovich unit[13]. Protein contents were measured by a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard.

*Radiosensitivity*

To measure cell survival, suitable numbers of cells were harvested from actively growing populations, and were inoculated into 60-mm plastic dishes. After overnight growth (about 18 h) the medium was removed, and the cells were irradiated at room temperature with a soft X-ray unit (Softex. Co., Tokyo) operated at 50 kVp and 10 mA, with 0.2 mm Al filtration. The dose rate was 11 Gy/min. After irradiation, the cells were refed with fresh culture medium, and cultured for approximately two weeks to obtain colonies[14–16]. In the case of near-UV, cells were rinsed with phosphate-buffered saline (PBS) and exposed to near-UV through 5 ml of PBS at room temperature and then refed with culture medium for colony formation[17]. The colonies were fixed and stained with methylene blue in methanol, and colonies containing more than 50 cells were counted.

*Drug treatment*

After overnight growth, the plates were exposed to varying concentrations of freshly prepared hydrogen peroxide or other drugs in PBS at 37°C for 30 min. Treatments were terminated by aspirating the PBS containing the compounds, rinsing the dishes with PBS plus 3% FCS, and then adding culture medium for colony formation. The topoisomerase II inhibitor, m-AMSA, a kind gift from Prof. M. M. Elkind (Colorado State Univ.), was dissolved in DMSO as a 10 mM stock solution. Hydrogen peroxide and adriamycin were obtained from Wako (Tokyo) and Sigma Chemicals (St. louis, MO), respectively.

## RESULTS

*Establishment of cell strains*

The fibroblast cultures were initiated by explanting skin fragments of mouse embryos as described in Materials and Methods. There was no significant difference in growth rate between

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the mutant and wild-type embryo cells (Fig. 1). The growth rates of both cell strains decreased after passage 2 and were below 2 during each passage (3 days) until passage 17. After passage 18, however, both cell strains started to proliferate rapidly and maintained stable growth rates at 6–13 thereafter. At this stage, we concluded that both cell strains were permanently established. The established cell strains, C3H/Cs$^a$ for wild-type and C3H/Cs$^b$ for mutant-type, had the ability of contact inhibition. The maximum saturation cell densities achieved were approximately $5.5 \times 10^4$ cells/cm$^2$. The appearance of these cell cultures and their saturation densities are similar to the classical non-tumorigenic 3T3 mouse cell strains[18,19]. The plating efficiencies of these mouse cell strains were 21–45%.

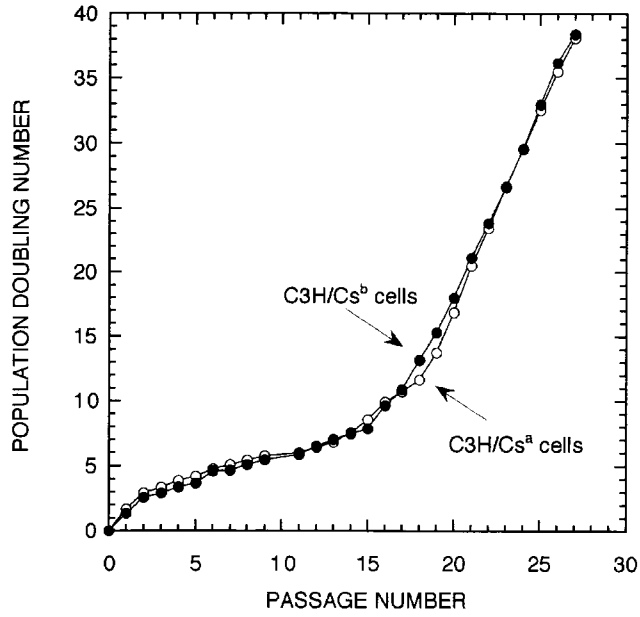


**Fig. 1.** Growth curves of wild-type C3H/Cs$^a$ cells and mutant C3H/Cs$^b$ cells when subcultured as described in Materials and Methods.

*Catalase activity of cell strains*

Oxidative stress was neutralized or removed by not only catalase but also by GSHPX and SOD. Table 1 shows the enzyme activities of catalase, GSHPX and SOD in the cell-free extracts of passage 29. The cell extract from mutant Cs$^b$ cells had only one-fourth of the catalase activity shown by the wild-type cells. However, there were no significant differences in the activities of GSHPX and SOD between the mutant cells and wild-type cells. Lewis reported that the mutant Cs$^b$ catalase is temperature-sensitive[8]. To test this possibility, we preincubated cell extracts from both mutant and wild-type cell strains for 15 min at various temperatures before performing a catalase assay. No catalase activity was detected in the mutant Cs$^b$ extracts, but the activity of the wild-type extracts was unaffected by preheating at 40°C for 15 min (Fig. 2). The mutant Cs$^b$ catalase was thus confirmed to be extremely temperature-sensitive.

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**Table 1.** Enzyme Activities of Catalase, GSHPX, and SOD in Wild-type C3H/Cs[a] Cell and Mutant C3H/Cs[b] Cells at Passage 29

| Enzyme | Wild-type C3H/Cs[a] Cells* | Mutant C3H/Cs[b] Cells* |
|---|---|---|
| Catalase | 10.37 ± 0.27 | 2.27 ± 0.127 |
| GSHPX | 60.0 | 54.0 |
| SOD | 34.5 | 34.0 |

*U/mg protein

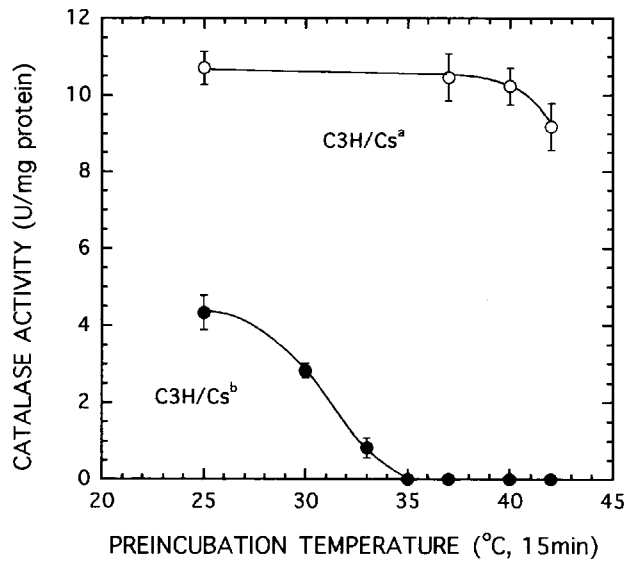


**Fig. 2.** Effect of temperature on catalase activity of cell-free extracts from wild-type C3H/Cs[a] cells and mutant C3H/Cs[b] cells. Cell extracts from both mutant and wild-type cell strains were preincubated for 15 min at various temperatures before the catalase assay was performed.

*Sensitivity of cell strains to exogenous hydrogen peroxide*

If the endogenous catalase has a protective role against both internal and external oxidative stress, then the mutant cells should be more sensitive to hydrogen peroxide toxicity. Figure 3 shows the sensitivities of the mutant and wild-type cells after treatment for 30 min with varying concentrations of hydrogen peroxide. The mutant Cs[b] cells had about five-fold higher sensitivity compared to the wild-type cells.

*Sensitivities to radiation and drugs*

Highly reactive oxygen radicals produced by radiation may lead to cell killing via oxidative damage. Therefore, we irradiated the cell strains by X-rays and by near-UV. Figure 4 shows the X-ray sensitivities of each cell strain at passage 26. The wild-type cells were slightly resistant to X-rays than the mutant cells. In addition, the mutant cells were more sensitive to the near-UV

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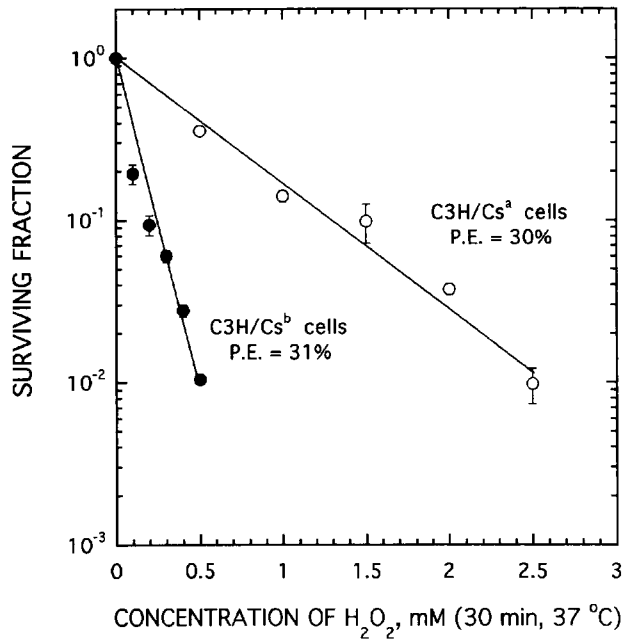


**Fig. 3.** Sensitivity of wild-type C3H/Cs$^a$ cells and mutant C3H/Cs$^b$ cells to treatment with varying concentrations of hydrogen peroxide for 30 min at 37°C. P. E. = plating efficiency. Uncertainties (standard errors) are shown where larger than the data points.

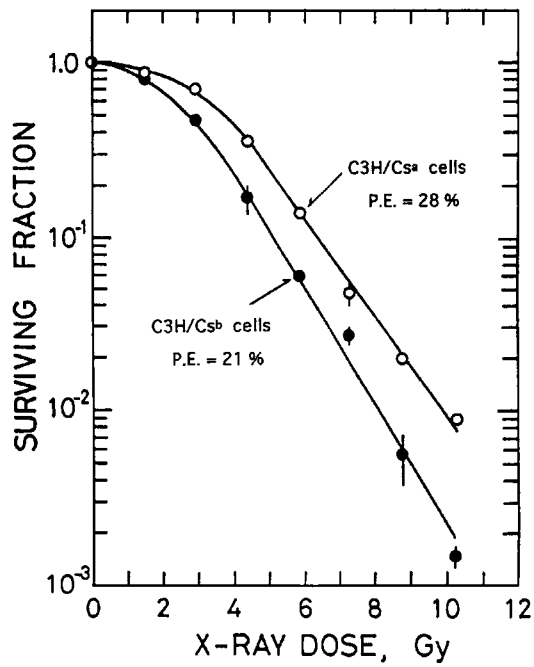


**Fig. 4.** X-ray sensitivities of wild-type C3H/Cs$^a$ cells and mutant C3H/Cs$^b$ cells. Other details are as in Fig. 3.

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irradiation than the wild-type cells, as shown in Fig. 5.

It is generally agreed that the topoisomerase II inhibitors will not induce cell killing through oxidative damage[20]. Figures 6 and 7 show that for the topoisomerase II inhibitors adriamycin[21]

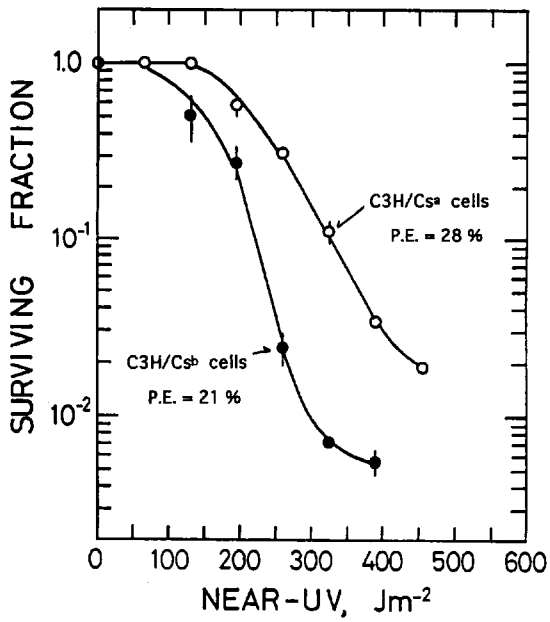


**Fig. 5.** Near-UV sensitivities of wild-type C3H/Cs[a] cells and mutant C3H/Cs[b] cells. Other details are as in Fig. 3.

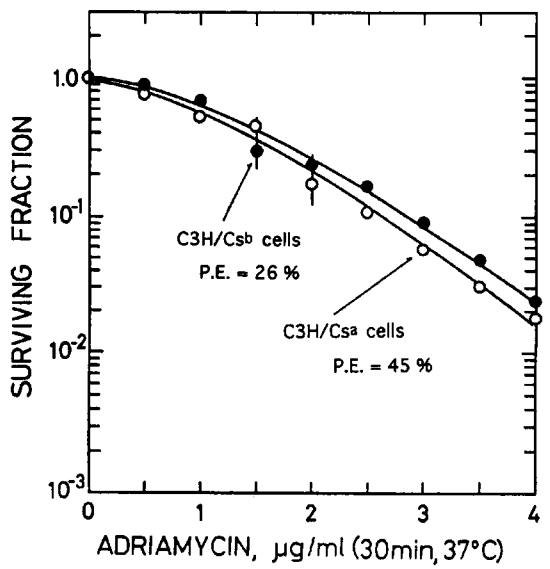


**Fig. 6.** Sensitivity of wild-type C3H/Cs[a] cells and mutant C3H/Cs[b] cells to treatment with varying concentrations of adriamycin for 30 min at 37°C. Other details are as in Fig. 3.

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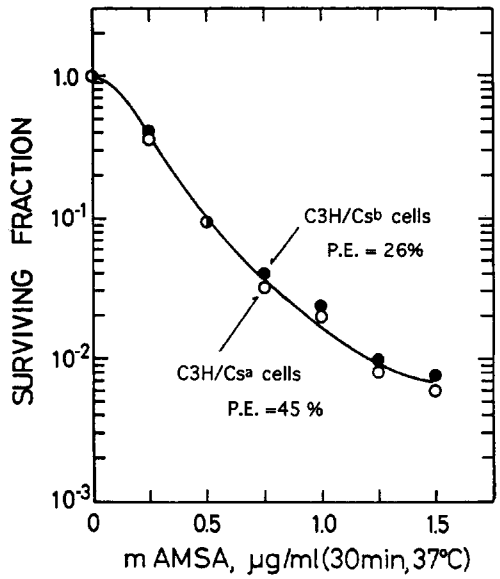


**Fig. 7.** Sensitivity of wild-type C3H/Cs$^a$ cells and mutant C3H/Cs$^b$ cells to treatment with varying concentrations of m-AMSA for 30 min at 37°C. Other details are as in Fig. 3.
Other details are as in Fig. 3.

and m-AMSA[22], there were no significant differences in sensitivity between the mutant cells and wild-type cells.

## DISCUSSION

Cell strains derived from C3H/He, BALB/c and C57BL/10 mouse embryos[10] have been reported to be spontaneously transformed and grow rapidly after passage 12. The mouse cell strains described in the present report showed stable growth after passage 18 (Fig. 1), and the cell strains were thought to be established by passage 25. We found that the mutant C3H/Cs$^b$ cell strain had normal levels of both GSHPX and SOD activities, but had only low level of catalase activity, approximately 28% of the wild-type cell strain level (Table 1). Our findings on the temperature sensitivity (Fig. 2) agree with the results of Lewis[8] in that the mutant catalase activity was extremely temperature-sensitive, but the wild-type activity was not (Fig. 2). The plating efficiencies of our established cell strains were 21–31% for mutant cells and 28–45% for wild-type cells, but those of Lewis's established cell lines were 5–15%[8]. Because of this low plating efficiency, he reported the data of the hydrogen peroxide sensitivity using SV40-transformed cell lines[8].

In the present study, as expected, the hydrogen peroxide sensitivities depended strongly on catalase activity (Fig. 3). The mutant cells were slightly sensitive to X-rays (Fig. 4) and were more sensitive to near-UV (Fig. 5) than the wild-type cells. Since hydrogen peroxide itself can be

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produced by near-UV radiation on tryptophan in the cells[23], present results support the view that catalase neutralized or removed (directly or indirectly) the reactive oxygen radicals produced by ionizing radiation and especially by near-UV.

Since cell killing by adriamycin has been proposed to involve a free radical process[24], we expected that the mutant cells would be more sensitive to adriamycin than wild-type cells. However, the mutant cells showed the same sensitivity to this drug as the wild-type cells (Fig. 6). This might be explained by the fact that adriamycin functions as a topoisomerase II inhibitor just like m-AMSA (Fig. 7)[21,22]. We also confirmed that there are no significant differences in sensitivity to the DNA cross-linking agents, cis-Pt and trans-Pt between the two cell strains (data not shown).

8-Hydoroxyguanine (8-OH-Gua) is one of the major modified bases in DNA induced by reactive oxygen species; it is widely used as a marker for oxidative damage to DNA. We recently developed a sensitive new method with which altered bases are detected and quantified by gas chromatography/mass spectrometry (GC/MS) selected-ion monitoring, which detected at least seven kinds of altered bases[25]. With this method, we found that the 8-OH-Gua content in the present mutant cell strain is three-times higher than that in the wild-type cell strain at the same passage (28th) (Tano et al., manuscript in preparation). These results suggest that catalase plays an important role in preventing oxidative damage to DNA. SOD and catalase are thought to protect mammalian cells from oxidative damage[3]. The rate of radiation-induced cellular transformation of primary hamster fibroblasts and mouse C3H/10T1/2 cells is significantly reduced if SOD is present in the culture medium[26,27], and the addition of exogenous catalase significantly enhances this protective effects of SOD[26,27]. Presumably, intracellular activities of these enzymes also contribute to the reduction in cellular transformation. We expect the catalase-deficient 3T3-like cell strain C3H/Cs$^{b}$ to be useful in determining the intracellular roles which catalase plays in preventing cell killing, mutation or transformation induced or promoted by oxidative damage.

## ACKNOWLEDGEMENTS

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. The results reported are the responsibility of the authors and do not represent the official views of the Ministry.

## REFERENCES

1. Ames, B. N. (1983) Dietary carcinogens and anticarcinogens. Science **221**: 1256–1264.
2. Pryor, W. A. (1976–1982) Free Radicals in Biology. Vols. 1–5: Academic Press, New York.
3. Fridovich, I. (1978) The biology of oxygen radicals. Science **201**: 875–880.
4. Feinstein, R. N., Howard, J. B., Braun, J. T. and Seaholm, J. E. (1966) Acatalsemic and hypocatalasemic mouse mutants. Genetics **53**: 923–933.
5. Feinstein, R. N., Suter, H. and Jaroslow, B. (1968).Blood catalase polymorphism: Some immunological aspects. Science **159**: 638–640.
6. Srivastava, S. and Ansari, N. (1980). The peroxidatic and catalatic activity of catalase in normal and acatalasemic

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mouse liver. Biochim. Biophys. Acta **633**: 317–322.
7. Ito, A., Watanabe, H., Naito, M. and Kawashima, K. (1984) Correlation between induction of duodenal tumor by hydrogen peroxide and catalase activity in mice. Gann **75**: 17–21.
8. Lewis, W. H. (1985) Establishment of mouse cell lines homozygous for temperature-sensitive mutation in catalase gene. Somat. Cell Mol. Genet. **11**: 319–324.
9. Ban, S., Nikaido, O. and Sugahara, T. (1980) Acute and late effects of a single exposure of ionizing radiation on cultured human diploid cell populations. Radiat. Res. **81**: 120–130.
10. Yagi, T. (1982) DNA repair ability of cultured cells derived from mouse embryos in comparison with human cells. Mutation Res. **96**: 89–98.
11. Flohe, L. and Gunzer,W. A. (1984) Assay of glutathione peroxidase. Methods in Enzymol. **105**: 114–121.
12. Aebi, H. (1984) Catalase in vitro. Methods in Enzymol. **105**: 121–126.
13. McCord, M. J. and Fridovichi, I. (1969) Superoxide dismutase. An enzymic action for erythrocuprein (hemocuprein). J. Biol. Chem. **244**: 6049–6055.
14. Utsumi, H. and Elkind, M. M. (1985) Two forms of potentially lethal damage have similar repair kinetics in plateau- and in log-phase cells. Int. J. Radiat. Biol. **47**: 569–580.
15. Utsumi, H. and Elkind, M. M. (1989) Bleomycin-induced potentially lethal damage and its repair. Radiat. Res. **119**: 342–344.
16. Utsumi, H., Tano, K., Mizuma, N., Kobayashi,T. and Ichihashi, M. (1996) Cellular effect of thermal neutron capture treatment using 10B1-para-boronophenylalanine: Lethal effect on melanoma cells with different degrees of X-ray sensitivity. J. Radiat. Res. **37**: 193–198.
17. Utsumi, H. and Elkind, M. M. (1979) Photodynamic cytotoxicity of mammalian cells exposed to sunlight-simulating near ultraviolet light in the presence of the carcinogen 7,12-dimethylbenz(a)anthracene. Photochem. Photobiol. **30**: 271–278.
18. Todaro, G. J. and Green, H. (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into the established lines. J. Cell Biol. **17**: 299–313.
19. Aaronson, S. A. and Todaro, G. J. (1968) Basis for the acquisition of malignant potential by mouse cells cultivated in vitro. Science **162**: 1024–1026.
20. Gellert, M. (1981) DNA Topoisomerases. Ann. Rev. Biochem. **50**: 879–910.
21. Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. M. and Liu, L. F. (1987) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science **226**: 466–468.
22. Utsumi, H., Shibuya, M. L., Kosaka, T., Buddenbaum, W. E. and Elkind, M. M. (1990) Abrogation by novobiocin of cytotoxicity due to the topoisomerase II inhibitor amsacrine in Chinese hamster cells. Cancer Res. **50**: 2577–2581.
23. McCormick, J. P., Fischer, J. R., Pachlatko, J. P. and Eisenstark, A. (1976) Characterization of a cell-lethal product from the photo oxidation of tryptophan: hydrogen peroxide, Science **191**: 468–470.
24. Powis, G. (1987) Anthracycline metabolism and free radical formation. In: Metabolism and Action of Anti-cancer Drugs, Eds. G. Powis and R. A. Prough, pp. 211–260, Taylor & Francis, London.
25. Mori, T., Tano, K., Takimoto, K. and Utsumi, H. (1998) Formation of 8-hydoroxyguanine and 2,6-diamino-4-hydoroxy-5-formamidopyrimidine in DNA by riboflavin mediated photosensitization. Biochem. Biophys. Res. Commun. **242**: 98–101.
26. Zimmerman, R. and Cerutti, P. (1984) Active oxygen acts as a promoter of transformation in mouse embryo C3H/10T1/2/C18 fibroblasts. Proc, Natl. Acad. Sci. USA **81**: 2085–2087.
27. Borek, C. and Troll, W. (1983) Modifiers of free radicals inhibit in vitro the oncogenic actions of x-rays, bleomycin, and the tumor promoter 12-O-tetradecanoylphorbol 13-acetate. Proc, Natl. Acad. Sci. USA **80**: 1304–1307.

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