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# Development of Altered Hepatocyte Foci by Separate and Combined Treatments with Radiation and Diethylnitrosamine in Neonatal Rats

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*To establish an in vivo radiation carcinogenesis model using glutathione S-transferase placental form positive (GST-P$^+$) hepatic foci, newborn rats were irradiated once by 0.5 Gy and 2 Gy of gamma ray or 0.15 Gy and 0.6 Gy of neutron with or without 0.05% phenobarbital (PB). When the rats were sacrificed at the 12th or 21st week, the incidence of GST-P$^+$ foci induction by radiation alone was very low. The neutron was more sensitive than the gamma ray at week 12 and the reverse phenomenon was observed in the groups at week 21. PB combination showed an increased incidence of GST-P$^+$ foci in gamma ray irradiated groups. The neutron irradiation combined with PB did not show any significant difference compared with the corresponding PB untreated groups. We also investigated the combined effect of diethylnitrosamine (DEN) and 0.75 Gy of gamma ray irradiation. Intraperitoneal injection of 0.15 μmol/g body weight of DEN at 1 hour after gamma ray irradiation showed significantly increased the number and area of GST-P$^+$ foci compared with those of DEN alone or DEN at 1 hour before gamma radiation ($P<0.001$). From these data, after more defined experiments, an in vivo radiation carcinogenesis model will be established by radiation alone or a combination of radiation and carcinogens.*

**Key Words** : *Radiation, Diethylnitrosamine, Hepatic carcinogenesis, Rat.*

## INTRODUCTION

Different factors of a physical, chemical or biological nature in man's environment may act in separation or combination with ionizing radiation giving rise to synergistic effects and this may pose difficult problems in protection (UNSCEAR., 1982). During the past decade, new and important information has become available concerning the carcinogenic effects of radiation. Because such carcinogenic effects occur too infrequently to be demonstrated at low doses, the risks of low-dose radiation can be estimated only by interpolation from the observations at high doses on the basis of theoretical concepts, mathematical models and available empirical evidence, primarily the epidemiological surveys of large populations exposed to ionizing radiation. In spite of a considerable amount of research, only recently have efforts been made to apply the extensive laboratory data in animals to define the dose-response relationship in low dose

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radiation (Ullrich et al., 1987 ; Liniecki et al., 1983 ; Mutsubara et al., 1974 ; Savage and Breckon, 1985).

The liver is a particularly useful model for chemical carcinogenesis and hepatocellular carcinomas can be readily induced by chemicals and radiation, and preceded by a variety of histochemically definable hepatocellular foci and adenomas (Bannasch, 1968 ; Scherer et al., 1972 ; Bannasch et al., 1980). The neonatal exposure of rats to a single treatment of gamma radiation, at moderate to nearly lethal doses, induced small but significant numbers of histochemically detectable foci of altered hepatocytes (Grdina et al., 1985) similar to those induced by chemical carcinogens in this experimental system (Peraino et al., 1984). Therefore, in order to examine the role of ionizing radiation in tumor initiation and promotion, we tried to establish an animal model for radiation carcinogenesis and also examined the tumorigenicity of radiation with or without chemical carcinogen.

## MATERIALS AND METHODS

### Animals

The pregnant Sprague-Dawley rats were obtained 5 days before parturition and were caged individually under constant temperature (23±1℃) and 12 hour light-12 hour dark illumination cycle. The NIH-7 diet (Committe on Standards for Nutrition Studies, 1977) and water were available *ad libitum*. Female offspring were used.

### Chemicals

Diethylnitrosamine (DEN) was obtained from Tokyo Chemical Co., Japan and 0.15 μ mol/g body weight was administered in normal saline by single intraperitoneal injection was administered. Phenobarbital (PB, Daewon Pharmaceutical Co., Korea) was given in the drinking water at a concentration of 0.05%.

### Experimental Protocol

*1. The effect of radiation alone on the induction of GST-P$^+$ foci*

Groups 1 and 3 were given a single 0.5 Gy or 2 Gy of whole body gamma-ray irradiation ($^{60}$Co teletherapy unit, Theratron-780) at a dose rate of 210 cGy/min. Groups 5 and 7 were given 0.15 Gy or 0.6 Gy of whole body neutron irradiation (Korea Cancer Center Hospital, Cyclotron, 21 MeV). Groups 2, 4, 6 and 8 were PB treated groups after the irradiation of each dose of gamma ray or neutron. Groups 9 and 10 were untreated and PB alone control groups, respectively. When the rats were 12 or 21 weeks of age, they were sacrificed. The liver and thymus were removed.

*2. The combination effect of radiation and DEN on the induction of GST-P$^+$ foci*

Groups 1, 3 and 4 were given a single 0.75 Gy of whole body gamma ray irradiation. Groups 2, 3 and 4 were DEN treated groups at 1 hour after irradiation for group 2 and 1 hour before irradiation for group 4. Group 5 was an untreated control group. All rats were killed at 150 days after birth.

### Histochemical Analysis

The livers were extracted for the examination of histochemically detectable foci of altered hepatocytes. Four sections of liver from each lobe were fixed in ice-cold acetone and embedded in paraffin and 5 μ m-thick sections were stained with ABC method (Vectastain ABC kit, Vector Labs., Burlingame, CA) for immunohistochemical examination of GST-P$^+$ foci by a slight modification of Jang & Kim's (Jang and Kim., 1988). The number of all GST-P$^+$ hepatocytes was counted microscopically in experiment 1, and the numbers and areas of GST-P$^+$ foci of more than 0.2 mm diameter were measured with a video image processor (VIDEOPLAN, Carl Zeiss, Germany) in experiment 2. They were expressed as foci number/cm$^2$, foci area (mm)$^2$/cm$^2$ and maximum diameter (Dmax, mm).

### Statistical Analysis

The significance of differences between the experimental groups and the corresponding controls were assessed by the Chi-Square test or Student's t-test.

## RESULTS

*1. The effect of irradiation alone on the induction of GST-P$^+$ foci*

Fig. 1 shows the growth curves of gamma ray or neutron treated groups during the experiments. Mean body and relative liver weights are shown in Table 1. The mean body weight did not show any

**Table 1.** Mean body and relative liver weight in rats treated with radiation with or without PB.

| Age (Weeks) | Groups and treatment | Number of rats | Body weight(g) | Relative liver weight (% body weight) |
|---|---|---|---|---|
| 12 | Normal control | 5 | 207±17.5 | 5.43±0.62 |
| | PB control | 10 | 181±0.63 | 5.71±0.63 |
| | Υ-ray 0.5 Gy | 5 | 178±25.9 | 5.92±1.46 |
| | Υ-ray 0.5 Gy+PB | 10 | 193±10.2 | 6.38±0.64[b] |
| | Υ-ray 2 Gy | 5 | 186±20.2 | 5.73±0.33 |
| | Υ-ray 2 Gy+PB | 10 | 185±15.0 | 6.18±0.87 |
| | Neutron 0.15 Gy | 4 | 189± 4.0 | 5.36±0.34 |
| | Neutron 0.15 Gy+PB | 10 | 197±16.9 | 5.48±0.40 |
| | Neutron 0.6 Gy | 5 | 174±11.4 | 5.20±0.56 |
| | Neutron 0.6 Gy+PB | 10 | 181±16.4 | 6.12±0.55[b] |
| 21 | Normal control | 5 | 234±22.1 | 3.90±0.11 |
| | PB control | 10 | 210±14.3 | 4.32±0.92 |
| | Υ-ray 0.5 Gy | 4 | 248±14.2 | 4.00±0.21 |
| | Υ-ray 0.5 Gy+PB | 11 | 231±16.1 | 5.23±0.71[a] |
| | Υ-ray 2 Gy | 4 | 232±19.7 | 3.94±0.19 |
| | Υ-ray 2 Gy+PB | 9 | 201±26.2 | 5.19±0.69[b] |
| | Neutron 0.15 Gy | 5 | 235± 9.8 | 3.86±0.22 |
| | Neutron 0.15 Gy+PB | 9 | 244±18.2 | 5.01±0.26[c] |
| | Neutron 0.6 Gy | 5 | 217±26.5 | 4.45±0.36 |
| | Neutron 0.6 Gy+PB | 10 | 228±17.2 | 4.94±0.34[b] |

Data represents Mean±SD.
PB : Phenobarbital.
Significantly different from the corresponding PB untreated control groups at [a]$P<0.05$, [b]$P<0.005$ and [c]$P<0.001$.

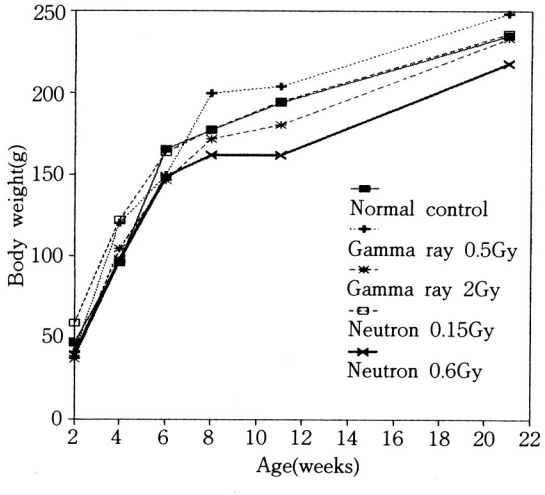


**Fig. 1.** Growth curve of gamma ray or neutron irradiated rats.

significant difference between the groups. The liver weight of the gamma ray (0.5 Gy) or neutron (0.6 Gy) alone groups was significantly different from the corresponding PB combined groups at week 12. The liver weights of all PB treated groups were increased compared with the corresponding PB untreated groups at week 21.

Table 2 shows the data on GST-P$^+$ foci in rats treated with radiation with or without PB. The groups of 0.5 Gy or 2 Gy of gamma ray did not induce GST-P$^+$ foci at week 12 and the groups of 0.15 Gy or 0.6 Gy of neutron induced 25% and 100% incidence of GST-P$^+$ foci, respectively. The number/cm$^2$ was 2.0 and 1.09 in the 0.15 Gy and 0.6 Gy of neutron irradiated groups. The incidence of GST-P$^+$ foci was 50% and 100% and the number was 1.26 and 10.35 in the 0.5 Gy and 2 Gy gamma ray irradiated groups at week 21, respectively. The incidence was 60% and 40%, and the number was 1.21 and 3.70 in the 0.15 Gy and 0.6 Gy neutron irradiated groups. PB combination increased the incidence of GST-P$^+$ foci in the gamma ray irradiated groups but significant difference only showed in the 2 Gy of gamma irradiation at week 12 and in the 0.5 Gy of gamma irradiation group at week 21. The neutron irradiation combined with PB showed increased

**Table 2.** The incidence and number of GST-P$^+$ foci in rats treated with radiation with or without PB.

| Age (Weeks) | Groups and treatment | Number of rats | GST-P$^+$ foci | |
|---|---|---|---|---|
| | | | Incidence(%) | No./cm$^2$ |
| 12 | γ-ray 0.5 Gy | 5 | 0 | 0 |
| | γ-ray 0.5 Gy+PB | 10 | 4(40) | 0.66±0.34 |
| | γ-ray 2 Gy | 5 | 0 | 0 |
| | γ-ray 2 Gy+PB | 10 | 7(70)[a] | 1.05±0.75 |
| 21 | γ-ray 0.5 Gy | 4 | 2(50) | 1.26±0.84[c] |
| | γ-ray 0.5 Gy+PB | 11 | 11(100) | 2.68±3.10 |
| | γ-ray 2 Gy | 4 | 4(100)[c] | 10.35±10.31[c] |
| | γ-ray 2 Gy+PB | 9 | 6(66.6)[a] | 1.33±0.82[a] |
| 12 | Neutron 0.15 Gy | 4 | 1(25) | 2.00±0.00[a] |
| | Neutron 0.15 Gy+PB | 10 | 4(40) | 0.52±0.16[a] |
| | Neutron 0.6 Gy | 5 | 5(100)[c] | 1.09±0.53[d] |
| | Neutron 0.6 Gy+PB | 10 | 7(70) | 0.75±0.60 |
| 21 | Neutron 0.15 Gy | 5 | 3(60)[c] | 1.21±0.67[d] |
| | Neutron 0.15 Gy+PB | 9 | 5(55.5) | 1.70±1.05 |
| | Neutron 0.6 Gy | 5 | 2(40) | 3.70±1.92[e] |
| | Neutron 0.6 Gy+PB | 10 | 3(30) | 0.94±0.24[b] |

Data represents Mean±SD.
PB : Phenobarbital.
Significantly different from the corresponding PB untreated groups at [a]P<0.05 and [b]P<0.001.
Significantly different from the untreated control group at [c]P<0.05, [d]P<0.005 and [e]P<0.001.

tendency in the incidence of GST-P$^+$ foci but significant differences were not observed compared with the corresponding PB untreated groups.

*2. The combination effect of radiation and DEN on the induction of GST-P$^+$ foci*

Table 3 shows mean body and relative liver weights and GST-P$^+$ foci data in rats treated with radiation with or without DEN. The mean body and relative liver weights of all experimental groups were not significantly different from those of the corresponding control group. GST-P$^+$ foci data in rats treated with DEN alone were much higher than those in rats given gamma ray irradiation alone. DEN administration at 1 hour after gamma irradiation significantly increased the incidence of GST-P$^+$ foci compared with the DEN or radiation alone groups. However, DEN administration at 1 hour prior to irradiation did not show any synergism for the induction of GST-P$^+$ foci.

**Table 3.** Quantitative data for GST-P$^+$ foci in rats treated with gamma radiation with or without DEN.

| Groups and treatment | Number of rats | Body weight (g) | Relative liver weight (% body weight) | GST-P$^+$ foci | | |
|---|---|---|---|---|---|---|
| | | | | Number/cm$^2$ | Area/cm$^2$ | $D_{max}$(mm) |
| Untreated control | 15 | 228.7±22.6 | 3.33±0.58 | 0 | 0 | 0 |
| γ-ray 0.75 Gy | 15 | 234.2±22.1 | 3.47±1.06 | 4.51±1.73 | 0.18±0.07 | 0.24±0.05 |
| DEN | 15 | 227.7±13.1 | 3.25±0.65 | 14.24±2.27 | 0.56±0.41 | 0.28±0.08 |
| γ-ray 0.75 Gy+DEN | 15 | 230.1±16.2 | 3.37±0.37 | 43.57±10.37[b] | 2.64±0.59[b] | 0.35±0.06[a] |
| DEN+γ-ray 0.75 Gy | 15 | 236.8±30.7 | 3.27±0.27 | 13.50±2.71 | 0.67±0.20 | 0.35±0.10 |

Data represents Mean±SD.
DEN : Diethylnitrosamine.
Significantly different from DEN alone group at [a]P<0.005 and [b]P<0.001.

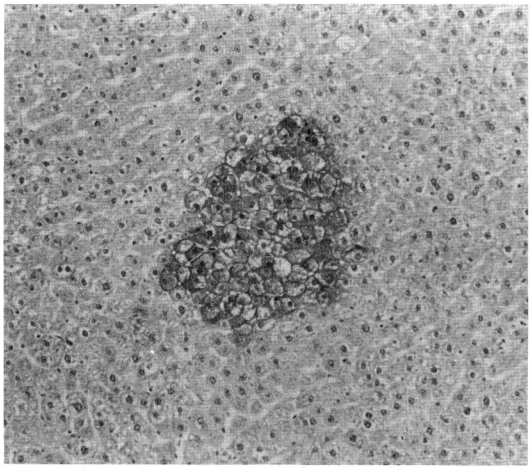

**Fig. 2.** Immunohistochemical staining of GST-P$^+$ foci in rat livers autopsied at the 150th day after diethynitrosamine administration after 0.75 Gy of gamma ray irradiation(× 200).

## DISCUSSION

It has been clearly established that histochemically detectable foci of altered hepatocytes invariably appear in rats treated with chemical hepatocarcinogens (Peraino et al., 1983 ; 1984). The present study provides evidence that such foci can be induced by radiation in the absence of any known chemical initiating carcinogenic stimulus. Other investigators have reported the induction of gamma glutamyl transpeptidase (GGT) activity by X-ray or gamma ray irradiation (Castanera et al., 1968 ; Enomoto et al., 1984 ; Grdina et al., 1985 ; Peraino et al., 1987). The gamma ray and neutron showed different sensitivities in the induction of GST-P$^+$ foci in these experimental doses. This difference may originate from different LET (Linear Energy Transfer) between gamma ray and neutron. Neutron might be more sensitive than gamma ray for the induction of GST-P$^+$ foci at week 12. However, the gamma ray might be more sensitive than neturon at week 21. PB combination showed the increased tendency of the incidence of GST-P$^+$ foci in gamma ray irradiated groups but this was not a consistent result. In the case of neutron, PB did not exert any promoting activity. From these results, it is evident that gamma ray or neutron can induce GST-P$^+$ foci but the incidence was very low and a dose dependent relationship was not observed in neutron treated groups. So it is suggested that gamma ray or neutron alone is not sufficient to establish an *in vivo* model system by radiation using GST-P$^+$ foci.

The next trial was of the possible combined effects of the initiator diethylnitrosamine (DEN) with gamma ray on the induction of GST-P$^+$ foci in the neonatal rat liver. There are some data about combining the gamma ray and DEN treatments. It synergistically enhanced the frequencies of GGT$^+$ foci, but not those of iron exclusion-negative foci or positive foci of both markers (Grdina et al., 1985 ; Peraino et al., 1986 ; 1987). These results could be interpreted in that combinations of qualitatively different types of genetic damage may mutually facilitate the induction of phenotypically altered cells ; specific sets of gene loci are differentially sensitive to the combined actions of gamma ray and DEN (Peraino et al., 1987), and DEN and gamma radiation affect different but complementary elements of the initiation mechanism (Peraino et al., 1986). In our results, 75 rad of single gamma irradiation induced altered hepatocyte foci that were significantly much lower than those from a single DEN treatment. DEN administration at 1 hour after irradiation induced a much higher number and area of GST-P$^+$ foci than those of DEN treatment at 1 hour before irradiation. DEN administration at 1 hour before irradiation induced a similar number and area of GST-P$^+$ foci compared with the DEN alone group. These results suggest that gamma radiation can be regarded as a tumor initiator and the combining actions of gamma ray and DEN facilitate the induction of preneoplastic changes, and that this GST-P$^+$ hepatic foci assay may be a useful tool for identifying preneoplastic changes involved in radiation carcinogenesis.

From the above, with radiation alone it might be impossible to establish an *in vivo* model which examines the radiation carcinogenesis using GST-P$^+$ foci in 21 weeks. However, DEN treatment after radiation may be a sensitive indicator of certain types of genetic damage. In the future, other strains, sexes, ages, radiation doses, and durations must be considered to establish a model of radiation carcinogenesis.

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