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The Role of the Sympathetic Nervous System in Radiation-induced Apoptosis in Jejunal Crypt Cells of Spontaneously Hypertensive Rats

MUTSUMI MATSUU[1]*, KAZUKO SHICHIJO[1], YASUKO NAKAMURA[1], YUJI IKEDA[1], SHINJI NAITO[1], MASAHIRO ITO[1], KUMIO OKAICHI[2] and ICHIRO SEKINE[1]

[1]Department of Molecular Pathology and
[2]Department of Radiation Biophysics, Atomic Bomb Disease Institute, Nagasaki University School of Medicine, 1–12–4 Sakamoto, Nagasaki, 852–8523, Japan

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To evaluate the effect of the sympathetic nervous system on radiation-induced apoptosis in jejunal crypt cells, apoptosis levels were compared in spontaneously hypertensive rats (SHR), animals which are a genetic hyperfunction model of the sympathetic nervous system, and normotensive Wistar-Kyoto rats (WKY). SHR and WKY were exposed to whole body X-ray irradiation at doses from 0.5 to 2 Gy. The apoptotic index in jejunal crypt cells was significantly greater in SHR than in WKY at each time point after irradiation and at each dose. WKY and SHR were treated with reserpine to induce sympathetic dysfunction, and were subsequently exposed to irradiation. Reserpine administration to SHR or WKY resulted in a significant suppression of apoptosis. p53 accumulation was detected in the jejunum in both WKY and SHR after irradiation by Western blotting analysis. There were no significant differences in the levels of p53 accumulation in irradiated intestine between WKY and SHR. These findings suggested that hyperfunction of the sympathetic nervous system is involved in the mechanism of high susceptibility to radiation-induced apoptosis of the jejunal crypt cells.

INTRODUCTION

Spontaneously hypertensive rats (SHR), originally derived from Wistar-Kyoto rats (WKY)[1)], are a well known model for human essential hypertension. It has been demonstrated that the norepinephrine and dopamine content of gastric tissues is higher in SHR than WKY[2)]. The advantage of using the SHR model is that these rats show sympathetic facilitation without any treatment. Therefore, SHR can be considered a useful sympathetic hyperactive model[3)].

*Corresponding author: Phone; +81–95–849–7107, Fax; +81–95–849–7108, E-mail; f1173@cc.nagasaki-u.ac.jp

We previously reported that the decreases of food intake and body weight after whole body X-ray irradiation were greater in SHR than in WKY and that the LD_{50} in SHR was lower than that in WKY. SHR histologically manifested more severe radiation-induced colonic mucosal damage such as degeneration and cystic dilation of crypts with severe inflammatory cell infiltration than WKY[4,5]. These results suggest that the manifestations induced by whole body irradiation are more severe in SHR possessing sympathetic hyperfunction than in WKY.

Exposure of the jejunum to ionizing radiation results in the rapid, apoptotic death of cells in the crypt[6] compared to other tissue[7,8] . Shortening of the villi results from cell loss by apoptosis and the destruction of the stem cells in the crypts that are responsible for repopulating the lining. In radiotherapy patients, damage to the crypts is accompanied by functional changes, including malabsorption, which are expressed clinically as acute small bowel reactions. In extreme cases, the complete loss of the gut epithelial cells results in symptoms of severe gastrointestinal syndrome[9,10].

The purpose of this study is to evaluate the effect of the sympathetic nervous system on radiation-induced apoptosis. We histologically examined the difference in radiation-induced apoptosis of jejunal crypt cells between WKY and SHR to confirm whether the high radiosensitivity of SHR is due to the increased activity of the sympathetic nervous system.

MATERIALS AND METHODS

Animals

Six-week-old male SHR/Izm and WKY/Izm rats were purchased from Funabashi Japan, Inc. (Chiba, Japan). A total of 130 rats were used for examination. The rats were housed to mate in colony cages under conditions of a constant 21–23°C and 12 h daily light/dark cycles at the Laboratory Animal Center for Biomedical Research, Nagasaki University. They were fed chow and tap water *ad libitum*. All animals are kept in a specific pathogen-free facility at the Animal Center in accordance with the rules and regulations of the Institutional Animal Care and Use Committee.

Irradiation

Irradiation was performed between 9 : 00 a.m. and 12 : 00 p.m. Rats received whole-body X-ray irradiation using a Toshiba EXS-300 X-ray, 200 KV, 15 mA apparatus with 0.5 mmCu + 0.5 mmAl filter at a dose-rate of 0.458 Gy/min. Two rats were simultaneously treated while held in a paper box. A single dose of either 0.5, 1 or 2 Gy was given. Control rats (0 Gy or 0 h) were not irradiated but were otherwise handled identically.

Tissue preparation

Three or four animals in each group of rats were sacrificed by deep anesthesia after irradiation, and the jejunum was immediately resected and immersed in neutral-buffered formalin. After fixation, the jejunum was cut longitudinally and processed for embedding in

paraffin blocks. Thereafter, 3-μm sections were cut and stained with hematoxylin and eosin (H&E). Identification of apoptosis was confirmed using a nonisotopic *in situ* DNA end-labeling (ISEL) technique (APOPTAG™; Oncor, Gaithersburg, MD) which stains the oligofragmented DNA characteristically found in apoptotic nuclei[11]. The jejunum of WKY and SHR were excised at 1, 2, 4, 8, 12 or 24 h after a dose of 2 Gy and at 2 h after a dose of 0.5, 1 or 2 Gy.

Assessing apoptosis

Fifty jejunal crypts per group were selected for analysis. The incidence of cell death (apoptotic index) was quantified by counting the number of dead cells in each crypt by microscopic examination of H&E-stained sections at 400x magnification. The apoptotic index is the mean number of apoptotic cells per jejunal crypt. The distinctive morphological features of apoptosis, as described by Kerr[12] and Walker et al[13], were used to recognize apoptotic cells. Small clusters of dead cell fragments were assessed as originating from one cell and given a single count and any doubtful cells were disregarded[14].

Blood pressure

The blood pressure of WKY and SHR was measured by the indirect tail-cuff method (automatic blood pressure recorder UR-1000 type, Ueda Manufactory, Tokyo, Japan). The blood pressure of reserpine-treated WKY and SHR was measured at 1 and 4 h after reserpine injection. The blood pressure of saline-treated WKY and SHR was measured at 4 h after saline injection.

Reserpine treatment

WKY and SHR were divided into two groups: one was treated with reserpine 5 mg/kg (Daiichi Pharmaceutical Co., Tokyo, Japan) by intraperitoneal injection and the other was treated with saline as a control. At 2.5 h following treatment with reserpine, reserpine-treated rats and vehicle control rats were X-ray irradiated serially at doses of 0.5, 1 or 2 Gy, respectively. Tissue was removed 2 h after irradiation and the apoptotic index of jejunum crypt cells was calculated.

Western blotting

The jejunal tissues of WKY and SHR were removed per time point after 2 Gy irradiation and frozen immediately. The tissues were then suspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.05% SDS, pH 7.4), broken into pieces on ice and subjected to three freeze-thaw cycles[15]. The insoluble cell debris was removed by centrifugation. Supernatants were collected and the protein concentration was quantified using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Protein samples (30 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis, then transferred electrophoretically to Hybond ECL Nitrocellulose Membranes (Amersham Life Science, Buckinghamshire, U.K.) as described previously[16]. Membranes were incubated with an anti-p53 monoclonal antibody, PAb 421 (Oncogene Science Inc., Uniondale, NY),

then treated with a horseradish peroxidase-conjugated anti-mouse IgG antibody (Zymed Labs. Inc., San Francisco, CA). Chemiluminescence (ECL Plus Amersham) was used for analyzing levels of protein according to the manufacturer's protocol. Blots were exposed to Hyperfilm ECL (Amersham).

Statistical evaluation of data

All values were expressed as the mean ± S.E.M. of results obtained from at least three animals per data point. The significance was determined by Mann-Whitney *U*-test. A $P < 0.05$ value was considered to be of statistical significance.

RESULTS

Apoptotic index of WKY and SHR after irradiation

Histologic sections of jejunal crypt cells were observed in WKY and SHR (Fig. 1). Two hours after 2 Gy irradiation, a higher frequency of apoptosis was observed in jejunal crypt cells in SHR (Fig. 1c, d) than in WKY (Fig. 1a, b). Although the differences in the frequency of apoptosis between these strains were evident in the sections of the crypts stained with H&E (Fig. 1a, c), to precisely identify these differences, we used the sensitive ISEL technique. In ISEL-positive apoptotic cells or bodies in the crypts, the stained products correlated exactly with the typical morphologic characteristics observed at the light microscopic level (Fig. 1b, d). Figure 2a gives a comparison of the time course of the apoptotic index in SHR and WKY up to 24 h after irradiation with 2 Gy. The apoptotic index in WKY and SHR peaked 2 h after irradiation, and by 24 h a decline in the index was evident. The apoptotic index was consistently higher in SHR than in WKY, significantly so at 2, 4, 8 and 12 h following irradiation. Background levels of jejunal crypt cell apoptosis in both strains did not differ. Apoptosis levels in the two strains at 2 h after 0.5, 1 and 2 Gy irradiation are shown in Fig. 2b. The apoptotic index increased in a dose-dependent manner from 0.5 to 2 Gy in both rat strains. The apoptosis level was significantly higher in SHR than in WKY at every dose of X-ray irradiation ($p < 0.01$).

Effect of reserpine treatment

To confirm the effect of reserpine treatment on SHR and WKY, blood pressure was measured. Blood pressure levels after treatment with reserpine in WKY and SHR, and after treatment with saline in WKY and SHR are shown in Fig. 3. At 4 h, the blood pressure was measured before sacrifice in both strains. At 2.5 h following treatment with reserpine, saline-treated WKY and SHR, and reserpine-treated WKY and SHR were exposed to irradiation of 1 Gy. In WKY treated with reserpine, blood pressure levels at 1 h were decreased significantly (98 ± 2 mmHg) compared with untreated WKY (118 ± 5 mmHg) ($p < 0.01$). The decrease in blood pressure of reserpine-treated WKY was observed at 4 h (95 ± 3 mmHg) ($p < 0.01$). In SHR treated with reserpine, blood pressure levels were decreased significantly (116 ± 11 mmHg) compared with untreated SHR (143 ± 14 mmHg) ($p < 0.01$). The decrease in

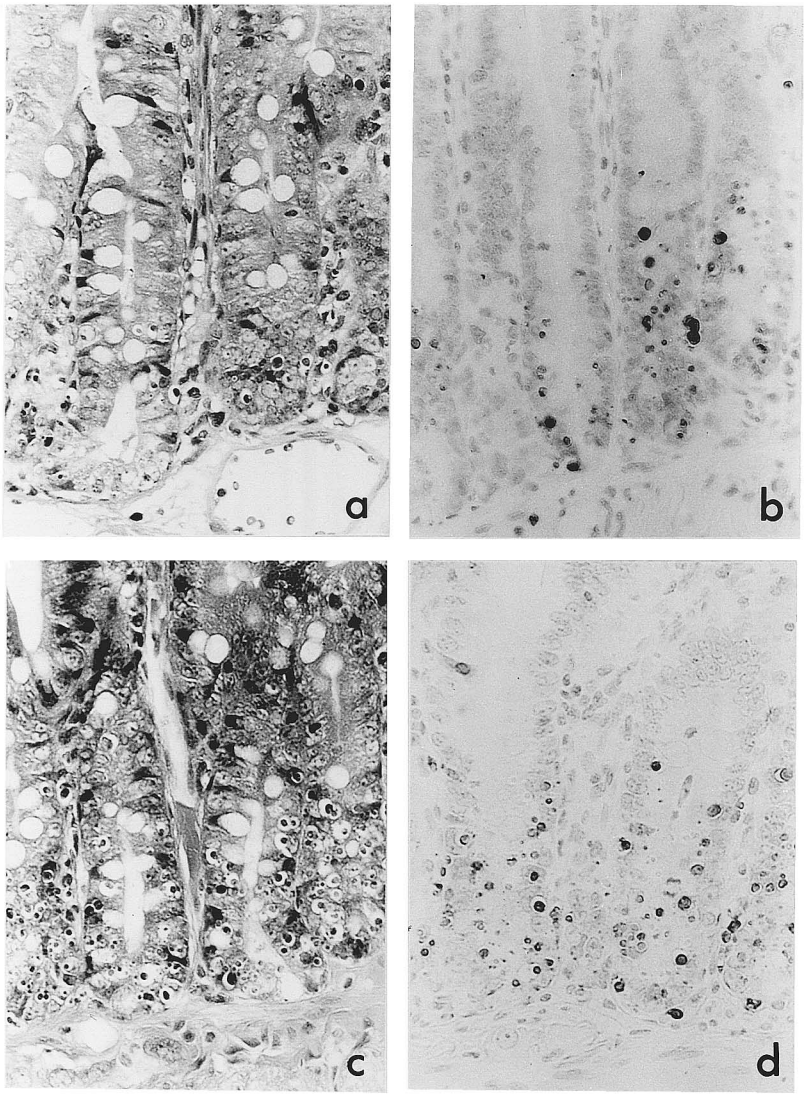

Fig. 1. Histologic sections of jejunal crypt of rats killed 2 h after 2 Gy irradiation. (a) and (b) are WKY, (c) and (d) are SHR, (a) and (c) are H & E × 400, (b) and (d) are apoptotic cells detected by ISEL × 400.

blood pressure of reserpine-treated SHR was observed at 4 h (94 ± 4 mmHg) ($p < 0.01$). Blood pressure levels in saline-treated SHR were increased after irradiation.

Apoptotic index of reserpine-treated WKY and SHR after irradiation

Histologic sections of jejunal crypt cells were examined in WKY and SHR after treatment with reserpine. Apoptosis appeared 2 h after 2 Gy irradiation in saline-treated WKY and SHR, as well as reserpine-treated WKY and SHR jejunal crypts as shown in Fig. 4. The

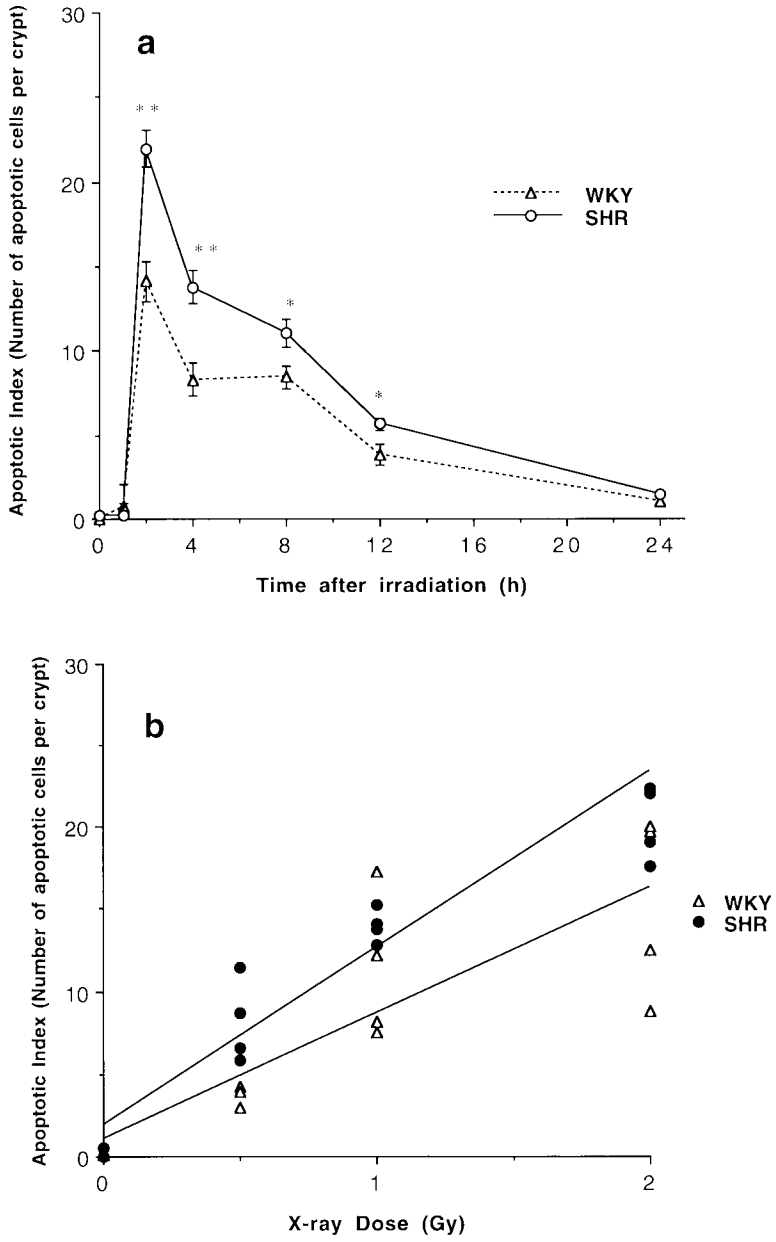

Fig. 2. (a) Jejunal crypt cell apoptotic index in WKY(△) and SHR(O) at various times following irradiation with 2 Gy. (b) Dose dependent effect on jejunal crypt cell apoptotic index in WKY(△) and SHR (●) at 2 h following irradiation with 0.5, 1 and 2 Gy. Data are the mean ± S.E.M. for three or four rats per data point. *$p < 0.05$ and **$p < 0.01$ vs WKY, respectively. The lines were fitted by linear regression analysis ($r = 0.81$ in WKY, $r = 0.79$ in SHR).

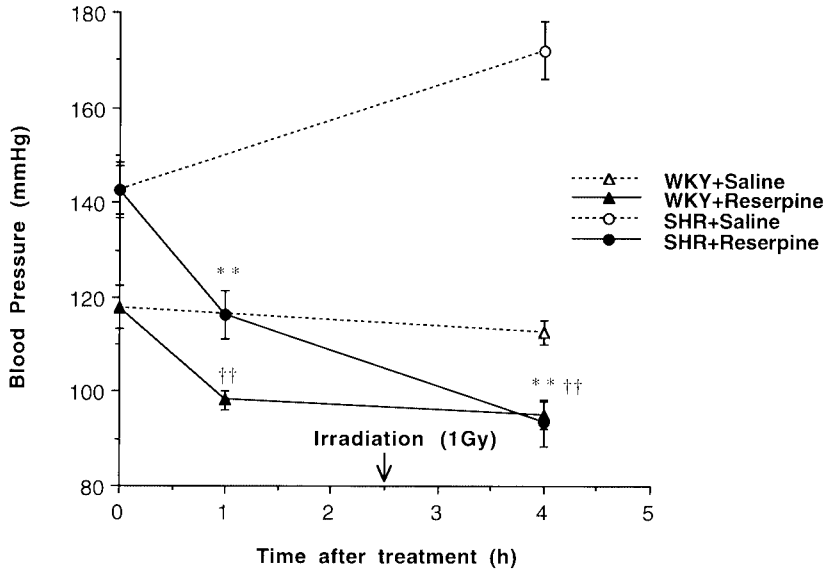

Fig. 3. Effect of treatment with reserpine on blood pressure. Data are the mean ± S.E.M. ††$p < 0.01$ vs untreated (0 h) WKY, **$p < 0.01$ vs untreated (0 h) SHR. At 2.5 h following treatment with reserpine and saline, rats were irradiated.

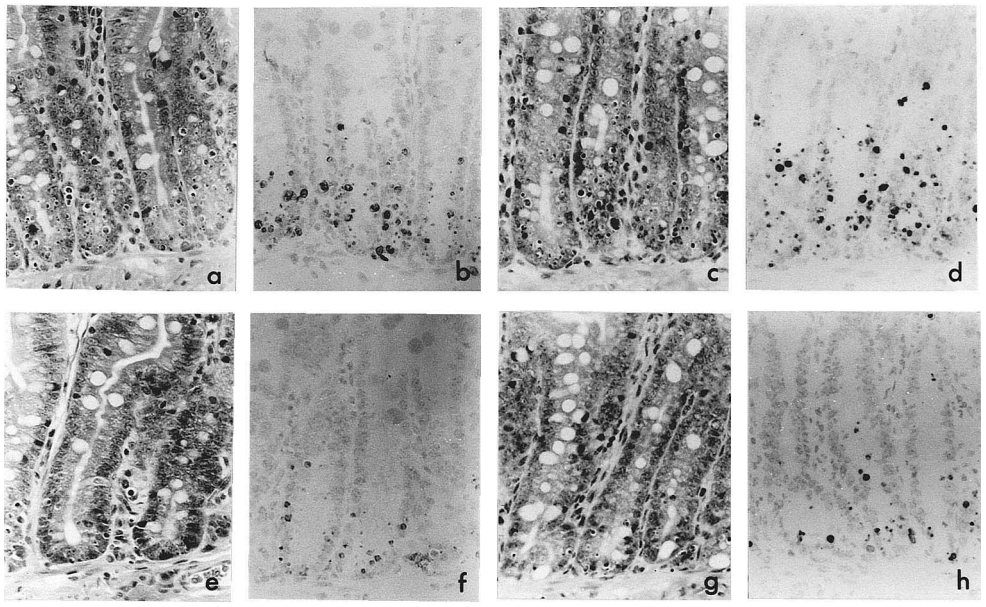

Fig. 4. Jejunal crypt apoptosis in WKY and SHR treated with reserpine 2 h after irradiation at 2 Gy. (a) and (b) are saline-treated WKY, and (c) and (d) are saline-treated SHR. In vehicle control rats, apoptotic cells were observed in jejunal crypts (a, c; H&E, b, d; ISEL × 400). (e) and (f) are reserpine-treated WKY, and (g) and (h) are reserpine-treated SHR. Reserpine pretreatment inhibited the occurrence of radiation-induced apoptosis (e, g; H&E, f, h; ISEL × 400).

frequency of apoptosis of jejunal crypt cells in reserpine-treated WKY (Fig. 4e, f) and SHR (Fig. 4g, h) was decreased compared to in saline-treated WKY (Fig. 4a, b) and SHR (Fig. 4c, d). The appearance of apoptosis in WKY and SHR 2 h after 2 Gy irradiation was suppressed by treatment with reserpine. The apoptotic indices of crypt cells in reserpine-treated WKY and SHR, and in saline-treated control WKY and SHR 2 h following 0.5, 1 or 2 Gy irradiation are shown in Fig. 5. The value at 0 Gy did not differ between reserpine-treated rats and control rats. The apoptotic indices in the jejunal crypt cells in WKY treated with reserpine were 20.5% at 0.5 Gy, 82.5% at 1 Gy and 78.7% at 2 Gy of the values for the vehicle-treated WKY at the same dose. The apoptotic indices in SHR treated with reserpine were 65.4% at 0.5 Gy, 74.9% at 1 Gy and 47.4% at 2 Gy of the levels at the same dose in the vehicle-treated SHR. The apoptotic index in WKY treated with reserpine was significantly suppressed compared to that in control WKY after 0.5 Gy ($p<0.01$) or 2 Gy ($p<0.05$) irradiation. The apoptotic index in SHR treated with reserpine was significantly suppressed compared to that in control SHR after 0.5 Gy ($p<0.01$), 1 Gy ($p<0.01$) or 2 Gy ($p<0.01$) irradiation.

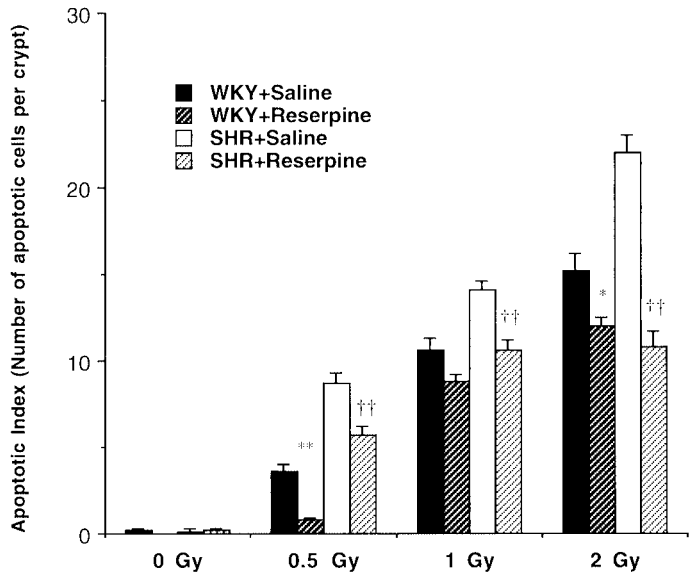

Fig. 5. Jejunal crypt cell apoptotic index at 2 h following irradiation with 0.5, 1 or 2 Gy in saline-treated WKY, reserpine-treated WKY, saline-treated SHR and reserpine-treated SHR. Data are the mean ± S.E.M. for three or four rats per data point. *$p < 0.05$ and **$p < 0.01$ vs saline-treated WKY, ††$p < 0.01$ vs saline-treated SHR.

Western blotting for p53

To examine whether p53 accumulation is related to high susceptibility to radiation-induced apoptosis in SHR, p53 accumulation was observed in the jejunum in both WKY and SHR after irradiation by Western blotting analysis. Representative results of p53 accumulation are shown in Fig. 6. Accumulation of p53 in the jejunum of WKY was increased at 1 and 4 h

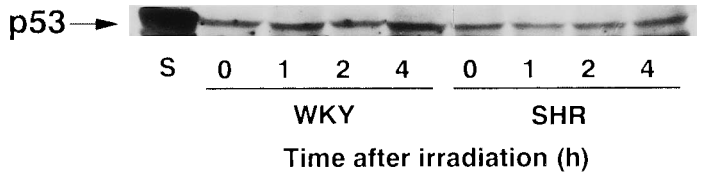

Fig. 6. Western blot analysis of p53 protein accumulation in jejunum of WKY and SHR after irradiation (2 Gy). **S**; SV40 transformed scid cells (SC3VA2).

after irradiation. On the other hand, accumulation of p53 in the jejunum of SHR was not increased at 1 h but was increased at 4 h after irradiation. No significant differences in p53 levels between WKY and SHR could be detected at 4 h after irradiation.

DISCUSSION

The experiments reported here were designed to investigate the effects of the sympathetic nervous system on radiation-induced apoptosis of jejunal crypt cells. To investigate how the sympathetic nervous system is involved in the apoptosis induced by radiation, an animal model SHR which has sympathetic facilitation was used. This study demonstrated that SHR have higher levels of radiation-induced apoptosis in jejunal crypt cells than control WKY with both increases in dose and with the passage of time after irradiation (Fig. 2).

To further evaluate whether the high levels of radiation-induced apoptosis in SHR were influenced by the sympathetic nervous system, chemical sympathectomy was performed by reserpine-treatment of animals. Reserpine is a classic pharmacological agent for amelioration of hypertension and is widely used for its action on storage vesicles for monoamines, which leads to the depletion of dopamine, norepinephrine, epinephrine and 5-hydroxytryptamine. Reserpine was administered in both WKY and SHR to induce sympathetic dysfunction[17,18]. To examine the effect of reserpine, the blood pressure of subjects was measured. Blood pressure levels in WKY and SHR were significantly decreased at 1 h and 4 h after treatment with reserpine compared with untreated rats (Fig. 3). Chemical sympathectomy with reserpine resulted in a decreased radiation-induced apoptotic level in jejunal crypt cells in WKY and SHR, but did not influence the normal steady state level of apoptosis (Fig. 5). These findings indicated that chemical sympathectomy suppressed radiation-induced apoptosis. Although murine strain differences in radiosensitivity have been suggested, indicating that it may be due to genetic variation[19,20], in this study we found chemical sympathectomy with reserpine suppressed radiation-induced apoptosis in both strains.

With regard to the sympathetic nervous system, it has been reported that catecholamines produced by lymphocytes down-regulate proliferation and differentiation of mouse lymphocytes by the induction of apoptosis[21]. Chemical or surgical sympathectomy inhibited cell proliferation of the gastric mucosa[22–24]. Radiation sensitivity also has been known to be cell-cycle dependent[25,26]. The relation between the acceleration of cell proliferation and the autonomic nervous system may play a role in the high degree of radiation susceptibility and

the high apoptotic levels in SHR.

The p53 tumour suppressor gene is one of the primary cellular factors which determines the nature of growth arrest and/or cell death in response to ionizing radiation[27–29]. Although there is some evidence for p53-independent apoptosis[30,31], the roles of p53 in G1 arrest and apoptosis suggest that the p53 status of the cell could be a fundamental component of radiosensitivity, especially in cells which undergo radiation-induced apoptosis[32]. We tried to examine the accumulation of p53, which is the candidate gene to regulate radiation-induced apoptosis, at several time points after 2 Gy irradiation in WKY and SHR jejunum by Western blotting (Fig. 6). Although the amount of p53 in WKY was increased at 1 h after 2 Gy irradiation, it was not increased in SHR at 1 h after irradiation. And there is no obvious difference at 4 h between WKY and SHR. This result indicates that the high level of apoptosis in SHR may be due to p53-independent apoptosis.

This is the first report demonstrating the participation of sympathetic nervous system in radiation-induced apoptosis. Regulation of the sympathetic nervous system may prove clinically useful in suppressing radiation-induced damage of intestinal epithelia. In conclusion, these findings suggested that hyperfunction of the sympathetic nervous system is involved in the mechanism of high susceptibility to radiation-induced apoptosis of the jejunal crypt cells.

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