High Sensitivity of Thymocytes of LEC Strain Rats to Induction of Apoptosis by X-Irradiation

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ABSTRACT. It is known that physical disruption of cell contacts induces apoptosis of thymocytes. When thymocytes from LEC and WKAH rats were incubated *in vitro* at 37°C for 0–6 hr and then the proportion of apoptotic cells was determined using a flow cytometer, it was found that the percentages of apoptotic thymocytes from both LEC and WKAH rats increased with incubation time and that the proportion of apoptotic cells from LEC rats was significantly higher than that from WKAH rats at each incubation time. The fact that cycloheximide, an inhibitor of protein synthesis, did not show significant inhibitory effects on induction of apoptosis of thymocytes indicates that induction of apoptosis during *in vitro* cultivation did not require *de novo* protein synthesis. When thymocytes from LEC and WKAH rats were X-irradiated *in vitro* at 4 and 8 Gy, the percentages of radiation-induced apoptotic cells increased with post-incubation time after X-irradiation in both LEC and WKAH rat thymocytes and the proportions of apoptotic cells from LEC rats were significantly higher than those from WKAH rat cells at 2 and 4 hr post-incubation after X-irradiation. When thymocytes from LEC and WKAH rats were X-irradiated in the presence of cycloheximide, the induction of apoptosis was substantially inhibited, indicating that radiation-induced apoptosis of thymocytes from LEC and WKAH rats required *de novo* protein synthesis. The present results showed high sensitivities of thymocytes of LEC rats to induction of apoptosis during *in vitro* cultivation and by X-irradiation.

KEY WORDS: apoptosis, LEC strain rat, protein synthesis, thymocyte, X-irradiation.

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Apoptosis, or programmed cell death, plays a major role in the development, tissue homeostasis, and elimination of damaged cells. This mode of cell death is characterized morphologically by cell shrinkage, chromatin condensation, membrane blebbing and formation of apoptotic bodies [29] and biochemically by internucleosomal fragmentation of DNA [28]. Apoptosis occurs in response to a diversity of physiological or pathological stimuli and to treatment of cells with various chemical and physical agents such as ionizing radiation. Thymocytes are highly radiosensitive and undergo apoptosis within a few hours after X-irradiation at low doses [3, 16, 22, 30]. Apoptosis induced by X-irradiation requires *de novo* protein synthesis [22], and the tumor suppressor p53 has been shown to regulate this type of apoptosis in thymocytes [5, 12]. However, ionizing radiation can induce apoptosis in thymocytes *via* p53-independent mechanisms [5, 23]. Furthermore, apoptosis is induced more readily in thymocytes by other types of stimuli such as heat shock. It has been reported that heat-induced apoptosis is correlated with activation of mitogen-activated protein kinases such as p38mapk and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) [14] and that induction of apoptosis by heat treatment of thymocytes does not require *de novo* protein synthesis [22].

Radiation has been shown to cause cell death by inducing apoptosis in sensitive target cells, and radiation-induced apoptosis may play a role in killing of tumor cells in radiotherapy. However, normal tissue damage also occurs by radiation in radiotherapy. Individual variation in the level of normal tissue damage resulting from radiotherapy has been

observed in clinics [25, 26]. Part of this variation is genetically predetermined, since there are several syndromes in humans such as ataxia telangiectasia with enhanced sensitivity to ionizing radiation [19, 24]. However, in the absence of a clinical, heritable disorder, no naturally occurring genetic polymorphisms that influence susceptibility to radiation-induced damage to normal tissue in humans have been identified. Therefore, animal models would be useful for understanding the correlation between susceptibility to radiation-induced apoptosis and radiation sensitivity of normal tissue damage in radiotherapy.

The LEC rat strain was established at the Center for Experimental Plants and Animals, Hokkaido University [21, 33]. Rats of this strain suffer from spontaneous fulminant hepatitis associated with severe jaundice at about 4 months of age. Other characteristics of LEC rats are a high incidence of spontaneous liver cancer in long-surviving individuals [33] and an increased sensitivity *in vivo* and *in vitro* to ionizing radiation [9]. In our previous studies using colony-forming assay for determination of cell survival, we found that primary fibroblasts and fibroblast cell lines from LEC rats were more sensitive to X-irradiation, but not to ultraviolet radiation in the short wavelength (ultraviolet C, UVC), than were cells from a control strain of WKAH rats [9–11, 17]. On the other hand, LEC rat cells showed a higher sensitivity to induction of apoptosis by UVC radiation, but not X-irradiation [9–11]. Therefore, the effects of X-irradiation on induction of apoptosis in LEC rat cells are still not clear. In the present study, we found high sensitivities of thymocytes of LEC rats to induction of apoptosis during *in*

vitro cultivation and by X-irradiation.

MATERIALS AND METHODS

Rats: Inbred strains of LEC/Hkm (LEC) and WKAH/Hkm (WKAH) male rats were cared for according to the principles of the 'Guide for the Care and Use of Laboratory Animals' prepared by Rakuno Gakuen University. WKAH rats were used as controls in the present study. All rats were maintained under conditions described previously [9].

Isolation of thymocytes and culture: Thymocytes were prepared from 4-week-old rats essentially as described by Ohyama *et al.* [16]. Briefly, single-cell suspensions were prepared by pressing the thymus through wire mesh screens followed by two passages through a 25-gauge needle. The cells were resuspended in RPMI 1640 medium containing 10% fetal calf serum. The cell cultures were kept at ambient humidity and 37°C in an atmosphere containing 5% CO₂.

X-irradiation: X-irradiation was carried out utilizing a Hitachi MBR-1520R X-ray generator operating at 150 kV and 15 mA with a 0.5 mm-Cu + 1.0 mm-Al filter at a dose rate of 0.95 Gy/min.

Reagent: Cycloheximide (Sigma Aldrich Chemical Co.) was dissolved in dimethyl sulfoxide (DMSO). DMSO efficiently scavenges reactive oxygen species such as hydroxyl radicals produced by X-irradiation. To prevent DMSO from affecting radiation-induced apoptosis, final concentrations of DMSO in the medium were equal in control and cycloheximide-treated cell populations, and were < 0.5%. The cells were exposed to cycloheximide at 37°C for 1 hr, X-irradiated, and then incubated at 37°C in the presence of cycloheximide.

Flow cytometry: The apoptotic cells were labeled with fluorescein-dUTP by using a Mebstain apoptosis kit direct (Medical & Biological Laboratories Co.) according to the manufacturer's instructions. Briefly, X-irradiated cells (1×10^6) were incubated in the medium at 37°C for 0–6 hr and collected. The cells were washed several times with PBS containing 0.2% BSA. The cells were fixed with 0.1 M NaH₂PO₄ containing 4% paraformaldehyde at 4°C for 30 min, washed 2 times with PBS containing 0.2% BSA, and then pelleted by centrifugation at $500 \times g$. The cells were fixed in 5 ml of cold 70% ethanol for 30 min at room temperature and stored at –20°C. Just prior to flow cytometric analysis, individual samples were labeled with fluorescein-dUTP. Fluorescence was measured with a Coulter EPICS EL flow cytometer using a 530-nm filter. The percentage of apoptotic cells was determined using multicycle software.

Statistical analysis: All data are expressed as means $\pm$ standard deviations. Differences between means were analyzed statistically by Student's *t*-test. Values of $p < 0.05$ and $p < 0.01$ were considered significant.

RESULTS

Since it is known that physical disruption of cell contacts induces apoptosis of thymocytes, we examined induction of

Fig. 1. Induction of apoptosis in thymocytes from LEC and WKAH rats during *in vitro* cultivation. Thymocytes were prepared from LEC (▨, ▩) and WKAH (□, ▤) rats and incubated at 37°C for 0–6 hr in the absence (□, ▨) and presence (▤, ▩) of 12.5 µg/ml cycloheximide, and then the number of apoptotic cells was counted using a flow cytometer at each incubation time. Each value represents the average from four separate experiments. Error bars represent the standard deviations of mean values. * $p < 0.05$, ** $p < 0.01$, compared with the values obtained in WKAH rat cells by means of Student's *t*-test.

apoptosis of thymocytes from LEC and WKAH rats during *in vitro* cultivation (Fig. 1). When thymocytes from LEC and WKAH rats were incubated *in vitro* at 37°C for 0–6 hr and then the proportion of apoptotic cells was determined using a flow cytometer, it was found that the percentages of apoptotic thymocytes from both LEC and WKAH rats increased with incubation time and that the proportion of apoptotic cells from LEC rats was significantly higher than that from WKAH rats at each incubation time. To examine whether induction of apoptosis during *in vitro* cultivation requires *de novo* protein synthesis, thymocytes were incubated in the presence of cycloheximide, an inhibitor of protein synthesis, at concentrations of 5 to 50 µg/ml. No significant difference was observed between the percentages of apoptotic cells from either LEC or WKAH rats cultivated in the presence of 12.5 µg/ml of cycloheximide and those cultivated in the absence of cycloheximide (Fig. 1). Cycloheximide did not show significant inhibitory effects on induction of apoptosis of thymocytes during *in vitro* cultivation at a concentration of 50 µg/ml (data not shown).

When thymocytes from LEC and WKAH rats were X-irradiated *in vitro* at 4 and 8 Gy, the percentages of apoptotic

Fig. 2. Induction of apoptosis in LEC and WKAH rat cells after X-irradiation. After LEC (▥, ▦) and WKAH (□, ▤) rat cells had been exposed to 4 (□, ▤) and 8 (▥, ▦) Gy of X-rays, the cells were incubated at 37°C for 0–6 hr, and the number of apoptotic cells was counted using a flow cytometer at each incubation time. Percentage of radiation-induced apoptotic cells = [(number of apoptotic cells in the X-irradiated cell population — number of apoptotic cells in the unirradiated cell population)/(number of total cells — number of apoptotic cells in the unirradiated cell population)] × 100 (%). Error bars represent the standard deviations of mean values (n=4). * $p < 0.05$, ** $p < 0.01$, compared with the values obtained in WKAH rat cells by means of Student's *t*-test.

cells increased with post-incubation time after X-irradiation in both LEC and WKAH rat thymocytes, and the proportions of apoptotic cells from LEC rats were significantly higher than those from WKAH rats at 2 and 4 hr post-incubation after X-irradiation (Fig. 2). At 2 hr post-incubation after irradiation, the proportion of apoptotic cells from LEC rats was 7- to 8-fold higher than that from WKAH rats. At 6 hr post-incubation after irradiation, no significant difference was observed between the proportions of apoptotic cells in LEC and WKAH rat cell populations.

When thymocytes from LEC and WKAH rats were X-irradiated at 4 Gy in the presence of cycloheximide, the induction of apoptosis was substantially inhibited (Fig. 3). In the case of WKAH rat cells, cycloheximide completely inhibited radiation-induced apoptosis at a concentration of 5 $\mu\text{g/ml}$. In thymocytes of LEC rats, approximately 70% of induction of apoptosis was inhibited by treatment with cycloheximide at a concentration of 5 $\mu\text{g/ml}$, and radiation-induced apoptosis was inhibited by cycloheximide in a

Fig. 3. Effects of cycloheximide on radiation-induced apoptosis in LEC and WKAH rat cells. After WKAH (□) and LEC (▥, ▦, ▧, ▨) rat cells had been exposed to 4 Gy of X-rays in the absence and presence of cycloheximide at 12.5 (▤), 25 (▥) and 50 (▦) $\mu\text{g/ml}$, the cells were incubated at 37°C for 0–6 hr, and the number of apoptotic cells was counted using a flow cytometer at each incubation time. Percentage of inhibition = [1 — (number of radiation-induced apoptotic cells that had been treated with cycloheximide/number of radiation-induced apoptotic cells that had not been treated with cycloheximide)] × 100 (%). Error bars represent the standard deviations of mean values (n=4).

dose-dependent manner. Similar inhibitory effects by cycloheximide on induction of apoptosis were observed in 8-Gy-irradiated thymocytes from LEC and WKAH rats (data not shown).

DISCUSSION

Lymphoid tissues such as the thymus, spleen and lymph nodes are known to be extremely sensitive to ionizing radiation [reviewed in 13]. In our previous studies using colony-forming assay to determine cell survival, we found that fibroblasts from LEC rats were more sensitive to X-irradiation than were cells from WKAH rats [9, 17]. On the other hand, no significant difference was found in induction of apoptosis of LEC and WKAH rat fibroblasts by X-irradiation [10]. The present results showed that LEC rat thymocytes were more sensitive to induction of apoptosis by X-irradiation than were WKAH rat cells and that apoptosis was induced at an earlier incubation time after X-irradiation in LEC rat thymocytes than in WKAH rat thymocytes. The fact that cycloheximide substantially inhibited radiation-

induced apoptosis indicates that the induction of apoptosis required *de novo* protein synthesis in both LEC and WKAH rat thymocytes. Requirement of *de novo* protein synthesis in radiation-induced apoptosis in rat thymocytes is in good agreement with results reported by Sellins and Cohen [22]. It is thought that ionizing radiation produces reactive oxygen species (ROS) and that DNA damage produced by ROS activates the pathway of apoptosis in thymocytes. Although ionizing radiation produces a variety of lesions in DNA, double-strand breaks (DSBs) seem to be most responsible for radiation-induced cell death [15, 20]. We have shown that the increased radiosensitivity of LEC rat cells is due to a reduced level of repair of DSBs. However, Beitez-Bribiesca and Sanchez-Suarez [4] suggest that the presence of DSBs is not directly associated with induction of apoptotic cell death of rat thymocytes. Therefore, it is not clear whether a deficiency in repair of DSBs is correlated with the high sensitivity of radiation-induced apoptosis in LEC rat thymocytes. Recently, the locus of the main gene responsible for high radiosensitivity in LEC rat cells has been assigned to the rat chromosome 4 [1]. A linkage study of deficiency in repair of DSBs to the high sensitivity of radiation-induced apoptosis in LEC rat thymocytes is now in progress. We have also reported that LEC rat cells show an abnormality of transient cell cycle arrests that occur in normal rat cells following ionizing radiation [7, 8, 10]. Since it is well known that a regulatory pathway of cell cycle progression is associated with induction of apoptosis [12, 23, 32], abnormality of transient cell cycle arrests may be associated with the high sensitivity of radiation-induced apoptosis in LEC rat cells.

Apoptosis of thymocytes is induced by various stimuli other than ionizing radiation, such as glucocorticoids, heat shock, antibodies, Ca^{2+} ionophores, and toxins [6]. It has been shown that after physical disruption of cell contacts, apoptosis of thymocytes is induced during *in vitro* cultivation [27]. The present study showed that apoptosis of thymocytes from both LEC and WKAH rats was induced during *in vitro* cultivation and that the proportion of apoptotic cells from LEC rats was significantly higher than that from WKAH rats at each incubation time. The fact that cycloheximide did not show significant inhibitory effects on induction of apoptosis during *in vitro* cultivation indicates that induction of apoptosis during *in vitro* cultivation did not require *de novo* protein synthesis. The mechanism of induction of apoptosis of rat thymocytes without *de novo* protein synthesis during *in vitro* cultivation remains unclear at this time. It has been suggested that ROS is a mediator of thymocyte apoptosis induced by *in vitro* cultivation [27]. Although ionizing radiation also generates ROS, radiation-induced apoptosis required *de novo* protein synthesis in rat thymocytes, but apoptosis by *in vitro* cultivation did not. In the case of *in vitro* cultivation, the production of ROS in thymocytes is likely to originate mainly from mitochondria [27]. On the other hand, ionizing radiation induces apoptosis via nuclear DNA damage. Therefore, pathways of apoptosis activated by ionizing radiation may be different from

those by *in vitro* cultivation, although induction of apoptosis of thymocytes by both treatments is mediated by ROS. Furthermore, LEC rat thymocytes showed a high sensitivity in induction of apoptosis by both pathways. Since it has been shown that LEC rats have a defect in T cell maturation [2, 31], the difference in the thymocyte subpopulations of LEC and WKAH rats may be associated with the different sensitivity of induction of apoptosis during *in vitro* cultivation.

It has been reported that LEC rat fibroblasts show a high sensitivity in reproductive cell death but not in induction of apoptosis to X-irradiation [9, 10]. On the other hand, in the present study, LEC rat thymocytes showed a high sensitivity to induction of apoptosis by X-irradiation. Thus, the LEC rat is a useful animal model for understanding the correlation between susceptibility to radiation-induced apoptosis and radiation sensitivity of normal tissue damage in radiotherapy.

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