

Higher Sensitivity in LEC Rat Cells to a Topoisomerase II Inhibitor, Ellipticine

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(Received 12 February 1998/Accepted 10 April 1998)

ABSTRACT. A concentration of ellipticine, an inhibitor of topoisomerase II, required to reduce cell survival to 37% (D_{37}) is used as an index to compare the cellular sensitivity. D_{37} values of LEC and WKAH rat cells were 1.2 and 2.2 μM , respectively. Thus, LEC rat cells were approximately 1.8-fold more sensitive than WKAH rat cells to ellipticine. There was no significant difference between the topoisomerase II activities in nuclear extracts of LEC and WKAH rat cells. These results suggested that the high sensitivity of LEC rat cells to ellipticine is not associated with the level of topoisomerase II activity. — **KEY WORDS:** ellipticine, LEC strain rat, topoisomerase II.

J. Vet. Med. Sci. 60(8): 969–971, 1998

Active regulation of the superhelical density of eukaryotic cellular DNA is thought to be under the control of DNA topoisomerases [1, 24, 25]. Topoisomerase II can relax supercoiled DNA by virtue of the double-strand-passing activity, and unknot and decatenate DNA molecules. Topoisomerase II has been implicated in the functions (*e.g.*, transcription, replication, recombination and repair) of genomic DNA [19, 25] and in the structural organization of chromatin (*e.g.*, chromatin condensation and separation) [23].

The human genetic disorder ataxia-telangiectasia (AT) is associated with many pathological features, including progressive neuronal degeneration, immunodeficiency, chromosomal instability, and cancer proneness [4, 9, 21]. AT also shows an extremely high sensitivity to ionizing radiation [13, 14, 22] and a high sensitivity to topoisomerase II inhibitors [18, 20]. Smith and Makinson [20] reported that AT cells overproduce topoisomerase II and suggested that the abnormal expression of topoisomerase II is implicated in the hypersensitivity to topoisomerase II inhibitors and ionizing radiation. However, another study has shown unusually low levels of DNA unknotting activity in AT cells [18]. Thus, the relationship among high sensitivity to topoisomerase II inhibitors, topoisomerase II activity, and high sensitivity to ionizing radiation in AT cells remains unclear.

The LEC rat strain was established at the Center for Experimental Plants and Animals, Hokkaido University [17]. Rats of this strain suffered 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 [26] and an increased sensitivity to whole-body irradiation by X-rays [5, 6]. We have reported that the hypersensitivity of LEC rats to whole-body irradiation is controlled by a single autosomal recessive gene, *xhs* [7]. In the present study, we examined the sensitivity to the topoisomerase II inhibitor ellipticine and the activity of topoisomerase II in LEC rat cells.

The rat fibroblast cell lines were established from lungs of LEC and WKAH rats by SV 40 immortalization as

described previously [8]. The cells were grown in a monolayer culture in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS). The cell cultures were kept at ambient humidity and at 37°C in an atmosphere containing 5% CO_2 .

Cells were exposed to ellipticine (TopoGene, Inc.), an inhibitor of topoisomerase II, for 24 hr. Cell-culture dishes were washed twice with phosphate-buffered saline (PBS), pH 7.2, and growth medium was added. After the cells were incubated for 2 weeks, the dishes were methanol-fixed and stained with May-Grunwald and Giemsa. Colonies containing more than 50 cells were counted as survivors under a dissecting microscope.

Nuclear extracts were prepared for the assay of topoisomerase II activity according to the method recommended by Topo Gene, Inc. Briefly, cells (1×10^7 to 5×10^7) were harvested, washed with PBS, and then pelleted by centrifugation at $500 \times g$. The cells were washed with ice-cold TEMP buffer (Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM MgCl_2 , 0.5 mM PMSF) and resuspended in TEMP buffer. After homogenization with Dounce homogenizer, nuclei were pelleted by centrifugation at $1,500 \times g$ for 10 min. The nuclei were washed with TEMP buffer and resuspended in a small volume of TEP buffer (same as TEMP buffer but lacking MgCl_2). After the addition of an equal volume of 1 M NaCl to the nuclear suspension, the suspension was vortex-mixed and left on ice for 45 min. After centrifugation at $12,000 \times g$ for 15 min, activities of the supernatants to catalyze the decatenation of kinetoplast DNA (kDNA) were assayed using a topoisomerase II assay kit (TopoGen, Inc.). The decatenated DNA was separated by electrophoresis through 1.0% agarose gel, stained with ethidium bromide, and then photographed under UV-transillumination. Densitometric analysis was carried out using imaging software (NIH image). The protein contents of nuclear extracts were determined by using a protein assay kit (BioRad).

LEC and WKAH rat cells were treated with ellipticine at concentrations from 0.1–5 μM for 24 hr, and the cellular survival was assayed by colony formation (Fig. 1). The concentration of ellipticine required to reduce cell survival

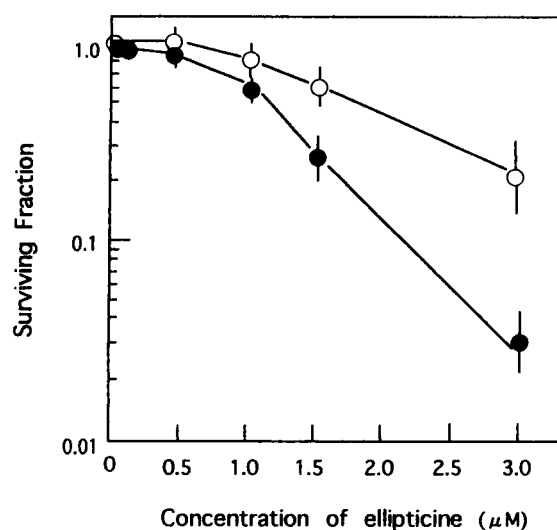


Fig. 1. Survival curves of LEC rat cells (○) and WKAH rat cells (●) after exposure to ellipticine. Points represent the average from four separate experiments. Error bars represent the standard deviation of mean values.

to 37% (D_{37}) is used as an index to compare the cellular sensitivity. D_{37} values of LEC and WKAH rat cells were 1.2 and 2.2 μM , respectively. Thus, LEC rat cells were approximately 1.8-fold more sensitive than WKAH rat cells to ellipticine. LEC rat cell lines other than those used in the present study and primary fibroblast cells from LEC rats

also showed a higher sensitivity to ellipticine than did other WKAH rat cell lines and primary WKAH rat fibroblasts (data not shown).

When the activities of topoisomerase II were assayed in nuclear extracts from LEC and WKAH rat cells, 3–4 DNA bands were observed (Fig. 2a). Band I included a linear form of DNA (lane 6) and a nicked, open circular form of decatenated kDNA (a in lane 7). Bands II and III represent supercoiled and relaxed forms of decatenated kDNA, respectively. Although the activities of topoisomerase II are determined by the sum of the intensities of a nicked, open circular form of decatenated kDNA in band I + band II + band III, each intensity of linear and nicked, open circular forms in band I was indistinguishable in densitometric analysis under the conditions used in this study. Therefore, we compared the sum of the intensities of bands I + II + III and bands II + III produced by LEC rat nuclear extracts with those produced by WKAH rat extracts (Fig. 2b). No significant difference was observed between the intensities of decatenated DNA in LEC and WKAH rat cells. When kDNA was reacted with more than 0.5 μg of nuclear proteins, smears of DNA that were difficult to analyze accurately appeared (Fig. 2a). There was no significant difference in the kinetics of production of decatenated kDNA using nuclear extracts with incubation time between LEC and WKAH rat cells (data not shown).

Since DNA conformation, dependent on topoisomerase function, can influence the efficiency of a repair process, topoisomerases capable of sensing and changing the topology of DNA are highly attractive components of model

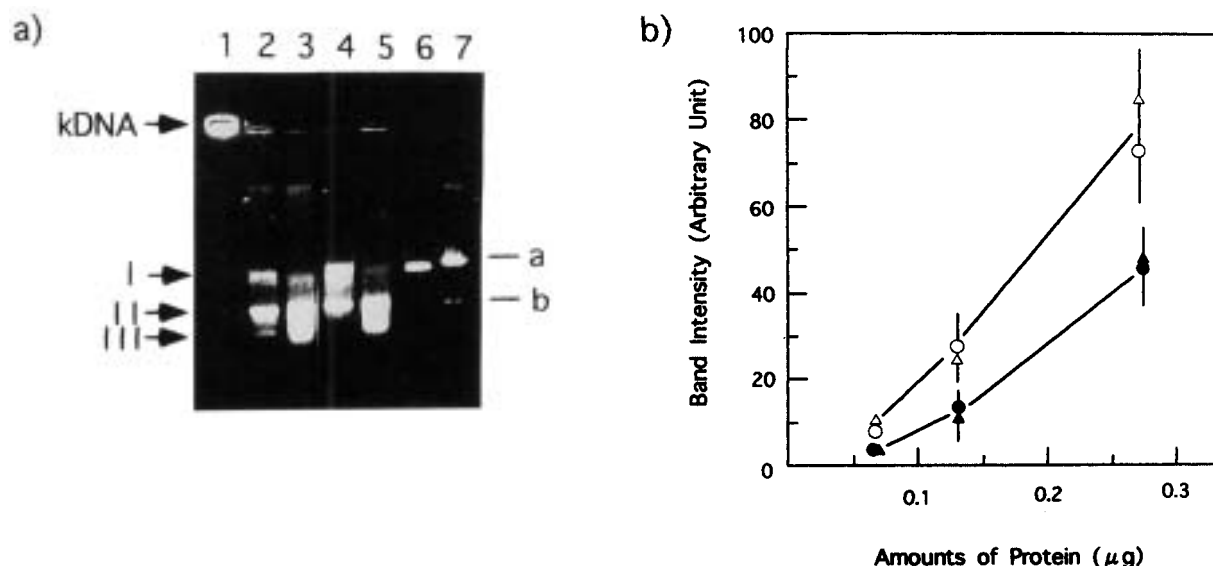


Fig. 2. Assays of topoisomerase II activity. a) After incubation in a reaction mixture containing 0.27 μg (lanes 2 and 4) and 0.5 μg (lanes 3 and 5) of nuclear proteins from LEC (lanes 2 and 3) and WKAH (lanes 4 and 5) rat cells at 37°C for 30 min, DNAs were separated by electrophoresis through 1% agarose gel. Lanes 1, 6 and 7 represent untreated control kDNA, linear kDNA and decatenated kDNA markers, respectively. Bands a and b in lane 7 represent nicked, open circular and supercoiled forms of decatenated kDNA, respectively. Band I included a linear form of kDNA and a nicked, open circular form of decatenated kDNA. Bands II and III represent supercoiled and relaxed forms of decatenated kDNA, respectively. b) Densitometric analysis of decatenated kDNA bands. Intensities (arbitrary unit) of the sum of bands I + II + III (○, ●) and II + III (△, ▴) produced by nuclear extracts from LEC (○, △) and WKAH (●, ▴) rat cells. Error bars represent the standard deviation of mean values ($n=3$).

pathways for repair or misrepair of DNA lesions [19]. X-ray-hypersensitive AT and mouse lymphoma L5178Y cells show a high sensitivity to topoisomerase II inhibitors [3, 18, 20]. The present results also showed a high sensitivity of X-ray-hypersensitive LEC rat cells to the topoisomerase II inhibitor ellipticine. Therefore, the sensitivity of these cells to ionizing radiation may be associated with the sensitivity to topoisomerase II inhibitors. Furthermore, AT cells [15], mutant Chinese hamster ovary cell lines with a high sensitivity to topoisomerase II inhibitors [16] and LEC rat cells [12] show an enhanced sensitivity to a variety of DNA-damaging agents.

The relationship between high sensitivity to topoisomerase II inhibitors and the level of topoisomerase II activity in AT cells remains unclear, because conflicting results have been reported [18, 20]. The present results showed no significant difference between the activities of topoisomerase II in nuclear extracts of LEC and WKAH rat cells. Therefore, in the case of LEC rat cells, the high sensitivity to the topoisomerase II inhibitor might not be associated with the level of topoisomerase II activity. The reason why X-ray-hypersensitive cells, such as LEC and AT cells, show a high sensitivity to topoisomerase II inhibitors remains unknown. We have previously shown that the repair process of DNA double-strand breaks (dsbs) induced by X-irradiation is slower in LEC rat cells than in WKAH rat cells [7]. Therefore, the defects of repair of DNA dsbs might affect the sensitivity to ellipticine in LEC rat cells.

AT cells are frequently associated with an increase of spontaneous and induced chromosome aberrations [4]. Topoisomerase II plays a role in the structural organization of chromatin [23]. Therefore, the high sensitivity to topoisomerase II inhibitors may be associated with the high frequency of induction of chromosome aberrations in AT cells. Indeed, X-ray-sensitive mutants of Chinese hamster ovary cells that are known to be deficient in the repair of DNA dsbs show abnormally elevated levels of chromosome aberrations induced by a topoisomerase II inhibitor [2]. LEC rat cells also show high frequencies of chromosome aberrations induced by X-irradiation *in vivo* and *in vitro* [10, 11]. These findings indicate that there is a resemblance between LEC rat cells and AT cells. Therefore, the LEC rat could provide a useful animal model to assist in an understanding of the repair process of radiation-induced DNA damage, induction of high frequency of chromosome aberrations, and radiosensitivity in AT cells.

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