Radiotherapy and Oncology 96 (2010) 259–266

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Radiobiology

Gimeracil sensitizes cells to radiation via inhibition of homologous recombination

Masaru Takagi [a], Koh-ichi Sakata [a,*], Masanori Someya [a], Hiroshi Tauchi [b], Kenta Iijima [b], Yoshihisa Matsumoto [c], Toshihiko Torigoe [d], Akari Takahashi [d], Masato Hareyama [d], Masakazu Fukushima [e]

[a] Department of Radiology, Sapporo Medical University, Hokkaido, Japan; [b] Department of Environmental Sciences, Ibaraki University, Japan; [c] Tokyo Institute of Technology, Research Laboratory for Nuclear Reactors, Tokyo, Japan; [d] First Department of Pathology, Sapporo Medical University, Hokkaido, Japan; [e] Pharmacokinetics Research Laboratory, Taiho Pharmaceutical Co. Ltd., Tokushima, Japan

ARTICLE INFO

Article history:
Received 31 January 2010
Received in revised form 14 May 2010
Accepted 27 May 2010
Available online 26 June 2010

Keywords:
Gimeracil
Radiosensitizer
DNA double strand breaks
Homologous recombination

ABSTRACT

Background and purpose: 5-Chloro-2,4-dihydroxypyridine (Gimeracil) is a component of an oral fluoropyrimidine derivative S-1. Gimeracil is originally added to S-1 to yield prolonged 5-FU concentrations in tumor tissues by inhibiting dihydropyrimidine dehydrogenase, which degrades 5-FU. We found that Gimeracil by itself had the radiosensitizing effect.

Methods and materials: We used various cell lines deficient in non-homologous end-joining (NHEJ) or homologous recombination (HR) as well as DLD-1 and HeLa in clonogenic assay. γ-H2AX focus formation and SCneo assay was performed to examine the effects of Gimeracil on DNA double strand break (DSB) repair mechanisms.

Results: Results of γ-H2AX focus assay indicated that Gimeracil inhibited DNA DSB repair. It did not sensitize cells deficient in HR but sensitized those deficient in NHEJ. In SCneo assay, Gimeracil reduced the frequency of neo-positive clones. Additionally, it sensitized the cells in S-phase more than in G0/G1.

Conclusions: Gimeracil inhibits HR. Because HR plays key roles in the repair of DSBH caused by radiotherapy, Gimeracil may enhance the efficacy of radiotherapy through the suppression of HR-mediated DNA repair pathways.

Radiosensitizers are chemical or pharmacological agents that increase the lethal effects of radiotherapy if administered in conjunction with it. Many compounds that modify the radiation response of mammalian cells have been discovered over the years, but most offer no practical gain in radiotherapy because they do not show a differential effect between tumors and normal tissues. A drug that increases the sensitivity of tumor and normal cells to the same level is considered inefficient [1]. Thus, radiosensitizers are not used routinely in clinical radiotherapy.

5-Chloro-2,4-dihydroxypyridine (Gimeracil) was synthesized as a component of S-1 that is a new oral fluoropyrimidine derivative. S-1 contains Gimeracil and tegafur (prodrug of 5-FU) in a molar ratio of 0.4:1. Gimeracil competitively inhibits dihydropyrimidine dehydrogenase, which degrades 5-FU in the blood [2]. It was originally added to S-1 to yield prolonged 5-FU concentrations in serum and tumor tissues [3]. Clinically, S-1 had shown considerable antitumor efficacy against various cancers [4–8]. Recently, concurrent chemoradiotherapy using S-1 has been performed [9,10]. These results suggest that S-1 might possess the radiosensitizing effect. Altogether, our results indicate that Gimeracil inhibits homologous recombination (HR) and also demonstrates its potential as a radiosensitizer.

Materials and methods

Cells

Human cell lines (DLD-1, HeLa, LC-11, M059J, M059K, GM7166VA7, XRCC3, BRCA2, MRC5SV, and FAD423SVT), rodent cell lines (CHO-K1, xrs-6, V79, XR-V15B, and XRCC3), and chicken cell line (DT40) were used.

DLD-1 is a human colorectal carcinoma cell line whereas HeLa and LC-11 are derived from a uterine cervix carcinoma and lung carcinoma, respectively. M059J and M059K are glioma cell lines derived from the same biopsy specimen but M059J lacks DNA-PKcs expression. GM7166VA7 is derived from an Nijmegen breakage syndrome (NBS) patient [11]. XR-V15B and xrs-6 are defective in the gene encoding Ku 86 and are derived from V79 [12,13] and CHO-K1 (Chinese hamster ovary cells) [14,15], respectively. XRCC3, also derived from Chinese hamster cells, was a kind gift from Dr. John Thacker. MRC5SV, an immortalized normal lung fibroblast cell line, was obtained from RIKEN Cell Bank, Japan. FAD423SVT, an immortalized skin fibroblast from a Fanconi anemia D1 patient (BRCA2 mutation), was a kind gift from Dr. Kenshi Komatsu. DT40 is the hyper-recombinogenic chicken B-cell line [16].

* Corresponding author. Address: Department of Radiology, Sapporo Medical University, School of Medicine, Hokkaido, Japan.
E-mail address: sakatako@sapmed.ac.jp (K.-i. Sakata).

0167-8140/$ - see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.radonc.2010.05.020

Cell culture and clonogenic assay

M059K, M059J, CHO-K1, xrs-6, V79, XR-V15B, and XRCC3 were cultured in Eagle's MEM supplemented with 10% fetal calf serum. DLD-1 and LC-11 were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. HeLa, GM7166VA7, MRC5SV, and FAD423SVT cell lines were cultured in DMEM supplemented with 10% fetal calf serum. All these cell lines were cultured at 37 °C. DT40 cell line was cultured in RPMI-1640 medium supplemented with 15% fetal calf serum and 1.5% chicken serum at 39 °C.

Trypsinized cells were dispensed for colony formation assay in 60 mm plates. A range of concentrations of Gimeracil was added to the cells at 24 h after seeding cells. The cells were then irradiated with 120 kV, photons at a dose rate of 1.17 Gy/min at various time points. Gimeracil was obtained from the Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) and was dissolved in DMSO (dimethyl sulfoxide). Equal amounts of DMSO were added to mock irradiated and irradiated cells. Experiments were repeated three times. Average values and standard deviations were expressed. *t*-Test was used to examine the significance.

γ-H2AX focus assay

In order to measure the ionizing radiation-induced histone H2AX focus (γ-H2AX focus) formation in cells, they were first irradiated with 4 Gy. At 24 h post-irradiation, the cells were centrifuged, diluted to appropriate numbers and grown on a slide glass. Next, they were fixed with cold methanol for 20 min, rinsed with cold acetone for 10 s, and then air-dried. Anti-γ-H2AX (Upstate) was used as primary antibody. Alexa-488-conjugated anti-rabbit IgG (Molecular Probes) was used as secondary antibody for visualization of γ-H2AX foci. Slides were mounted with antifade reagent (Mounting medium, DAKO). The foci were observed with an Olympus fluorescent microscope under oil immersion of 10 × 100 magnification. For quantification, clear and easily distinguishable spots of certain brightness were considered as positive foci. Two hundred cells of each sample at each time point were examined visually and average number of foci per cell was calculated. Since the foci became gradually larger and more distinguishable, here we used an average number obtained at 24 h after irradiation for quantification. The experiments were repeated three times. Average values and standard deviations were expressed. *t*-Test was used to examine the significance.

DNA-PK assay

Protein extraction and DNA-PK assay were performed as described in our previous report [17]. Cells were suspended in high salt buffer and lysed by three rounds of freeze–thaw cycles. The lysate was clarified by centrifugation at 15,000 rpm for 7 min at 4 °C. Protein concentration was estimated using a BCA protein assay kit (Pierce) with bovine serum albumin as the standard. Cell lysates were diluted with high salt buffer to 0.25 mg/ml. The lysate was then mixed with kinase assay buffer, synthetic peptide hp53-S15 (sequence: EPPLSQEAFADLWKK; synthesized in Sawady Biotechnology), and with or without sonicated salmon sperm DNA, respectively. This reaction mixture was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 30% acetic acid and absorbed onto a phosphocellulose filter disc (2.3 cm in diameter, Whatman). The filter discs were sequentially washed in 15% acetic acid and 99% ethanol; the remaining radioactivity was counted in a liquid scintillation counter. The net phosphorylation of hp53-S15 was calculated as follows: (phosphate incorporation in the reaction with DNA) − (phosphate incorporation in the reaction without DNA) divided by the specific radioactivity of ATP. We used the same sample from a human origin as an internal control for all DNA-PK assays. The experiments were repeated three times. Average values and standard deviations were expressed. *t*-Test was used to examine the significance.

SCneo assay

Gene conversion analysis was performed as described elsewhere [18]. Briefly, the SCneo construct, provided by M. Jasin, was used for site-specific DNA repair analysis. For this study, the hygromycin-resistant marker for stable transformant selection was substituted with Ecogpt (mycophenolic acid resistance), and a fragment containing the genomic sequence of the chicken ovalbumin gene was added to both the 5′- and 3′-ends of the SCneo [18]. The plasmid was transfected into cells by electroporation, and stable transformants with one copy of the SCneo were isolated. For induction of DNA double strand breaks at the I-Sce I site, pCMV-3× nls I-Sce I (or pBluescriptII for negative control) was introduced into the cells by electroporation. The electroporated cells were incubated with or without Gimeracil for 48 h in order to induce DSB and allow them to repair. Then the cells were grown in medium containing G418 (Calbiochem) at concentration of 1.6 mg/ml for DT40 cells, 400 mg/ml for HeLa and MRC5SV cells. After 10–14 days incubation, numbers of G418-resistant colonies were scored and HR frequencies were calculated. The experiments were repeated three times. We used a *t*-test to examine the statistical significance in HR frequency between 0 mM and various concentrations of Gimeracil.

It is known that there is an inherent variation in the frequency of SCneo assay, which differs among individual clones. Therefore, we used the same clone that shows the average HR frequency. The used clones of HeLa and DT40 and MRC5 cells have been reported previously [18,19].

Flowcytometry

The estimation of the distribution of cells in different phases of the cell cycle was analyzed with the help of FACS. Harvested cells were fixed with 70% ethanol and stored at 4 °C until further use. Fixed cells were washed once in PBS, then incubated in PBS supplemented with RNase A (0.25 mg/ml) for 30 min at 37 °C and finally stained in a propidium iodide-containing solution (50 μg/ml in PBS) for 10 min at 4 °C. The cells were analyzed by a flowcytometer (FACS Calibur, Becton–Dickinson, USA).

Synchronization of cells

In order to measure the cell-cycle phase-specific radiation sensitivity, cells were synchronized with nocodazole (Sigma). The cells were enriched at M-phase by the addition of 0.05 μg/ml nocodazole for 16 h and then washed thrice with pre-warmed medium, followed by irradiation at 0, 6, and 12 h.

Results

Clonogenic assay in human cancer cells

To examine the effects of Gimeracil on the radiosensitivity of various cells, we performed the clonogenic cell survival assay (Fig. 1a). The surviving fraction was significantly reduced after pretreatment with Gimeracil in DLD-1, HeLa, and LC-11 cell lines.

We also examined the relationship between varied time of exposure or concentrations and the radiosensitizing effects of Gimeracil in DLD-1 and HeLa cells. It was observed that the radiosensitizing effects of Gimeracil increased as time of exposure