Cladribine Enhances Apoptotic Cell Death in Lung Carcinoma Cells Over-Expressing DNase γ

Akemi Hayakawa, a,b Haruhiko Suzuki, c Yuzuru Kamei, d Sei-ichi Tanuma, d and Junji Magae* a,e

a Faculty of Science and Engineering, Tokyo University of Science; 1–1–1 Daigaku-dori, Yamaguchi, Yamaguchi 756–0884, Japan; b Department of Plastic and Reconstructive Surgery, Nagoya University Graduate School of Medicine; c Department of Immunology, Nagoya University Graduate School of Medicine; 65 Tsurumai-cho, Showa-ku, Nagoya 466–8550, Japan; d Department of Biochemistry, Faculty of Pharmacy, Tokyo University of Science; 2641 Yamazaki, Noda, Chiba 278–8510, Japan; and e Radiation Safety Research Center, Nuclear Technology Research Laboratory, Central Research Institute of Electric Power Industry; 2–11–1 Iwado-Kita, Komae, Tokyo 201–8511, Japan.

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Worldwide, lung cancer is the most common form of cancer and often has a poor prognosis. Establishment of effective therapies for lung cancer is a major concern in clinical cancer research. We compared the cytotoxic effects of chemotherapeutic agents including cisplatin, 5-fluorouracil, vinorelbine and cladribine, on a human lung cancer cell line, A549, and its derivative transfected with the DNase γ gene. We observed selective cytotoxicity of cladribine on the DNase γ-expressing sub-cell line of A549. Cladribine induces selective apoptosis in DNase γ-expressing A549 cells, which depends on activation of caspases. These results suggest that a combination therapy that includes cladribine along with the introduction of DNase γ has potential as a new therapeutic strategy for lung cancer.

Key word cladribine; DNase γ; apoptosis; lung cancer; gene therapy; A549

Lung cancer recently replaced gastric cancer as the number one cause of cancer-related deaths in Japan. Lung cancers often have poorer prognoses than other cancers and rapidly progress to an advanced stage, which makes treatment difficult. In the late 1990s, several anti-cancer drugs for lung carcinoma, including paclitaxel, docetaxel, irinotecan, vinorelbine and gemcitabine, were developed, and molecule-targeted drugs using gefitinib also became a popular strategy for treating lung carcinoma. Although a combination therapy of cisplatin along with one of these drugs is a common chemotherapy for lung carcinoma, this strategy is not sufficiently effective, and the prognosis for lung cancer remains poor. Accumulated studies using A549 cells derived from human lung cancer have demonstrated that the anti-cancer effect of chemotherapy drugs often is mediated by apoptotic cell death. 1–5 Several chemotherapeutic agents that have been used against ovarian cancer and breast cancer, as well as photodynamic therapy, also involve the induction of apoptosis in cancer cells. 6–8

Apoptosis proceeds along several distinct signalling pathways. Cross-linking of the extracellular domains of death receptors such as Fas results in intracellular recruitment of the adaptor molecule Fas-associated death domain protein (FADD) to the receptor cluster, which subsequently recruits procaspase-8, forming the Fas/FADD/procaspase-8 complex, known as the death-inducing signalling complex (DISC). 9,10 DISC catalyzes the proteolytic conversion of procaspase-8 into active caspase-8, which activates the downstream effector caspases, triggering cell death. Apoptosis also occurs via a mitochondria-mediated process, which begins with a transition of mitochondrial membrane permeability, followed by the release of apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF). 11,12 The initiator caspase-9 then becomes activated through the formation of the cytochrome c/AIF-1/procaspase-9-containing apoptosome complex in the cytosol. 13 Endoplasmic reticulum (ER) stress induces the unfolded protein response, which activates signalling pathways involving both caspases-8 and -9 that lead to apoptosis. 14 These apoptotic signals ultimately activate effector caspases that induce a series of morphological and biochemical alterations.

DNA fragmentation into a nucleoscale unit is one of the most characteristic features of apoptotic cells. Several mammalian DNases including caspase-activated DNase (CAD)/H1001 and endonuclease G 22,23 are suggested to be responsible for the apoptotic DNA fragmentation. DNase γ is a member of DNase I family, identified in apoptotic rat thymocytes. 20,21 It is active at neutral pH, dependent on the presence of Co2+/Mg2+, and inhibited by divalent cations such as Co2+, Ni2+, Cu2+, and Zn2+. DNase γ is activated during a specific stage of cell development. DNase γ is expressed during myogenic and neuronal differentiation, and is responsible for DNA fragmentation in differentiated cells, while CAD is expressed in undifferentiated proliferating neuroblastoma and is responsible for staurosporine-induced apoptosis in these cells. 24–26 In the immature B cell line WEHI-231, DNase γ is responsible for the DNA fragmentation induced by B cell receptor ligation, while CAD plays a major role in DNA fragmentation induced by cytotoxic drugs such as etoposide, staurosporine, and tunicamycin. 26 Moreover, it is suggested that DNase γ is involved in DNA breaks occurring in the gene rearrangement of the immunoglobulin variable (V) region in chicken DT40 B cell line, which also generates somatic hypermutation in immunoglobulin V region. 27 HeLa cells with ectopically expressed DNase γ respond to ceramide to promote apoptotic DNA fragmentation, which never is observed in the parental cell line. 28 These results suggest that DNase γ could be used in gene therapy to induce apoptotic DNA degradation in solid tumors.

Cladribine (2-chlorodeoxyadenosine, Fig. 1A) is a synthetic anti-cancer drug that is popularly used to treat
leukemia. Cladribine is a prodrug requiring intracellular phosphorylation to become an active purine nucleoside analogue.\textsuperscript{20} The prodrug is able to enter cells via purine nucleoside transporters, where it is then converted to the active 2-chlorodeoxyadenosine monophosphate. The accumulation of cladribine nucleotides leads to breaks in DNA strands, interfering with DNA synthesis and repair.\textsuperscript{30,31} Although cladribine has been reported to induce apoptosis in B cell leukemia, the effect of cladribine on solid tumors including lung cancers remains to be established, mainly because of cladribine-resistance of solid tumors.\textsuperscript{32} In this study, we investigated the anti-cancer effect of cladribine on human lung carcinoma and found that lower levels of cladribine induce apoptotic death in a human lung cancer cell line transformed with the DNase $\gamma$ gene. Our results suggest that a combination of cladribine and DNase $\gamma$ gene therapy could be one of the strategies to overcome cladribine-resistance in lung cancer treatment.

MATERIALS AND METHODS

Cell Culture A human lung adenocarcinoma epithelial cell line, A549, was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). It was cultured in MEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin at 37 $^\circ$C in 5% CO$_2$ atmosphere. To obtain a stable DNase $\gamma$ transformant, the expression plasmid for human DNase $\gamma$ cDNA\textsuperscript{30} that was inserted into pcDNA3 expression vector, was introduced into A549 through the phosphate-calcium precipitation method with the Cellfect Transfection Kit (GE Healthcare Japan, Tokyo, Japan). Clones were isolated after incubation with 0.7 mg/ml G418 for 72 h, and maintained in the same manner as the parent A549 cell line. A human cervical carcinoma, HeLa S3, and its stable transformant expressing rat DNase $\gamma$ (HeLa S3/$\gamma$) are described previously.\textsuperscript{28}

Chemicals and Antibodies Cladribine was purchased from Sigma (St. Louis, MO, U.S.A.), and vinorelbine was kindly provided by Kyowa Hakko Co., Ltd. (Tokyo, Japan). 5-Fluorouracil was obtained from Kyowa Hakko Co., Ltd. (Tokyo, Japan) and cisplatin from Bristol-Myers Squibb (New York, NY, U.S.A.). Anti-DNase $\gamma$ antibody is used as described previously.\textsuperscript{34} and diluted to a final concentration of 0.001%. Antibodies for poly-(ADP-ribose) polymerase (PARP), caspase 8, caspase 9, and $\beta$-actin were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). Second antibodies for species-specific immunoglobulin G (IgG) conjugated with horseradish peroxidase were purchased from MBL (Nagoya, Japan). An inhibitor of DNase $\gamma$, DR396,\textsuperscript{35} was obtained from Sigma.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay Cells were seeded in 96-well plates at $3 \times 10^4$ cells per well and cultured for 24 h, allowing cells to adhere to the bottom of the culture plates. Cells were then treated with culture medium containing different concentrations of chemotherapeutic agents for 72 h in culture medium. After the cells were incubated with 1 mg/ml (MTT, Sigma) for another 2 h, the medium was removed, and 100 $\mu$l of 0.04 $\mu$m HCl in isopropanol was added to extract the reduced formazan product. The resulting optical density at 590 nm was determined.

Nuclear Staining Cells were detached from a dish by trypsinization, and one million cells were harvested by centrifugation, washed in phosphate buffered saline (PBS), fixed with 1% glutaraldehyde for 30 min, washed once with PBS, and stained with 0.2 $\mu$m Hoechst33258 (Sigma) for 5 min. Cells with intact nuclei were counted under a fluorescent microscope.

Flow Cytometry Cells were detached from a dish by trypsinization, and one million of cells were harvested by centrifugation, washed in PBS, fixed with ice-cold 70% ethanol for 24 h, and treated with 20 $\mu$g/ml RNase for 30 min. Propidium iodide (PI) was added to a final concentration of 20 $\mu$g/ml. Thirty minutes after the addition of PI, DNA contents of the samples were analyzed by flow cytometry (FACS Calibur, Becton and Dickinson, NJ, U.S.A.).

Western Blot Total protein from $5 \times 10^5$ cells was separated by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis, followed by electroblootting onto a nitrocellulose membrane. Membranes were blocked in TBST (20 mM Tris–HCl, 400 mM NaCl, 0.05% Triton-X100, pH 8.0) containing 5% skim milk overnight, and probed with a specific first antibody, followed by corresponding species specific IgG antibodies conjugated with horseradish peroxidase. Signals were visualized by fluorescence emission using commercial detection kits (GE Healthcare Japan) according to the manufacturer’s instructions.

RESULTS AND DISCUSSION

First we tried to establish A549-derived cell lines that expressed higher levels of DNase $\gamma$. A549 cells were transfected with an expression plasmid containing a human DNase $\gamma$ cDNA. After selection with G418 for 72 h, we picked up a single clone, named A549$\gamma$C2. Western blot analysis demonstrated over-expression of DNase $\gamma$ in A549$\gamma$C2 cells, compared with that in parental A549 cells (Fig. 1B). We used this cell line to identify anti-cancer drugs that were selectively cytotoxic toward the DNase $\gamma$ transformants, and, through a 72 h-MTT assay, found that cladribine had the most selective cytotoxic effect on the transformants.
One million of A549/C2 cells were incubated with cladribine and DR396 (0.1 mM) for 72 h, and stained with Hoechst 33258. Cells with intact nucleus were counted under a fluorescent microscope. Effect of cladribine was calculated as percent of cell number compared to that without cladribine. Values represent as mean and S.D. of independent experiments. *Statistically significant compared to corresponding control without DR396 (p<0.05, two-sided t-test). Closed circles: cells cultured with DR396. Open circles: cells cultured without DR396. Treatment with DR396 alone did not affect the cell number significantly (103.9±3.2%, compared to control).

Next, we examined whether cladribine caused apoptosis associated with nuclear DNA fragmentation. A549 cells and A549/γC2 cells were treated with cladribine for 72 h, and flow cytometric analysis was used to assess DNA fragmentation. Consistent with MTT assay results, A549 cells treated with 50 μM cladribine contained more DNA fragmentation, shown as sub-G1 population by flow cytometric analysis, whereas those treated with cladribine at concentrations lower than 10 μM did not (Fig. 3A). By contrast, A549/γC2 cells underwent DNA fragmentation at 5 μM cladribine, which supports the observation of elevated sensitivity to cladribine in A549/γC2 cells as compared with parent A549 cells. In Western blot analysis, 116 kDa poly(ADP ribose) polymerase (PARP), a typical effector caspase substrate, was cleaved to 86 kDa protein in cells treated with cladribine at doses above 1 μM in A549/γC2 cells, but at least 50 μM was required before this cleavage was observed in the parental cell line (Fig. 3B), correlating to induction of a sub-G1 DNA population and PARP cleavage. While cladribine hardly increased active form of caspase 8 (42 kDa), it partly increased caspase 9 cleavage at the doses above 5 μM. This result is consistent with its activities on DNA fragmentation and PARP cleavage, and suggests that these activities are at least in part dependent on the mitochondria-mediated apoptotic process.

Cladribine is a highly effective cytotoxic agent against leukemia cells which works by inducing apoptosis. Here we have shown the cytotoxic effects of cladribine against non-leukemic tumor cells, indicating a novel application for cladribine as an apoptosis enhancer in combination with up-regulation of DNase γ in lung carcinomas. While efficacy of cladribine on leukemia is clinically established, it is known that solid tumors including lung cancers are highly resistant to cladribine, which prevents the clinical use of cladribine for this kind of cancers. Although the mechanism of resistance is not fully understood, it is reported that loss of p53 or its downstream target p21 renders human colon cancer cell lines cladribine-resistant. While IC50 values of p53 and p21 deficient cell lines are 520 μM and 141 μM, that of a cell line with wild type p53 is 31.7 μM, which is consistent with that of parent A549 cells observed in this study (38 μM). Ectopic expression of DNase γ further increased sensitivity to caldribine more than 10 times (IC50=3.2 μM). This result suggests that depression of DNase γ is another mechanism for the cladribine-resistance in human lung cancers, and demonstrates clinical application of cladribine for lung cancers that have high DNase γ activity spontaneously or artificially.

DNase γ is synthesized as an inactive precursor protein and converted into an active mature enzyme by removal of the N-terminal precursor peptide. Although DNase γ is activated in response to cellular signals leading to apoptosis, the precise mechanism of activation remains to be elucidated. DNase γ is present in non-apoptotic thymocytes in an active form, and activity is kept constant during apoptosis induced by X-ray irradiation or dexamethasone, indicating that DNase γ is present in a different state in the non-apoptotic cells and exerts its activity through multiple post-translational events. Cladribine, but not other chemotherapeutic agents, might stimulate the signalling pathways leading to the activation of DNase γ. Notably, the increased activity of DNase γ, a direct executor of apoptosis-associated DNA cleavage, conversely enhanced activation of caspases that provide signals for activation of DNA fragmentation machinaries. This observation implies a positive feedback mechanism, in which DNase γ-mediated DNA
fragmentation further enhances activation of caspases and the downstream DNases including CAD, leading to full activation of cellular apoptotic activity. In fact, apoptosis induced by DNA-damage is caused mainly through mitochondria-dependent process, which is consistent with our observation that caspase-9 but not caspase-8 was preferentially activated in cladribine-treated A549 cells expressing ectopic DNase γ. Further study of cladribine’s effects may contribute to elucidate how DNase γ is activated in apoptotic cells, and how DNase γ contribute to apoptosis and cladribine-resistance in lung cancer cells.

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