INTRODUCTION

Human telomerase is a ribonucleoprotein (RNP) complex which is responsible for the elongation of telomeres, and human telomerase reverse transcriptase (hTERT) has been identified as the catalytic subunit, as well as the rate-limiting component, of telomerase. In this study, we attempted to identify the modulators of telomerase, and to determine the molecular mechanisms underlying cisplatin-induced apoptosis.

Methods: To determine the role of telomerase in cisplatin-induced apoptosis, we measured telomerase activity and analyzed apoptosis using PI and trypan blue staining. Also, we inhibited the caspase activations using Z-VAD-fmk to analyze the effects on expression of hTERT protein. Finally, we induced the transient co-expression of the Bcl-2 and Bak genes in HEK293 cells, and then, the telomerase activity and expression of hTERT were evaluated.

Results: In the Bcl-2-overexpressing HeLa cells, telomerase activity was more enhanced, and cell death was reduced to 40-50% that of the mock controls. This finding suggests that Bcl-2-induced telomerase activity exerts an antiapoptotic effect in cisplatin-induced death. As caspase activation was inhibited via Z-VAD-fmk, the hTERT protein was recovered in the mock controls, but not in the Bcl-2-overexpressing cells. This suggests that the expression of hTERT can be regulated by caspases, but Bcl-2 was located within the upstream pathway. Moreover, when the Bcl-2 and Bak genes were co-transfected into the HEK293, both telomerase activity and hTERT protein were prominently reduced.

Conclusions: Bcl-2-induced telomerase activity inhibits cisplatin-induced apoptosis in HeLa cells, and can be regulated via both caspases and the interaction of Bcl-2 and Bak.

Key Words: Apoptosis, Cisplatin, hTERT, Bcl-2, Bak

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thereby allowing for the maintenance of genomic integrity. Human telomerase is comprised of telomerase reverse transcriptase (hTERT), and telomerase RNA component (hTR), as well as some associated factors which regulate the catalytic activity of telomerase[1, 2]. Recent findings have revealed that telomerase confers an additional function required for tumorigenesis, which is independent of its ability to maintain the telomere[3]. Additionally, telomerase contributes to the installation of an immortal cell phenotype via the prevention of apoptosis and may also perform a significant function in cellular resistance against anticancer drugs[4-8].

In previous reports, the suppression of telomerase enzyme activity promotes apoptosis in neuronal cells[9], germ cells[10], and thymocytes[11], whereas the overexpression of hTERT prevents apoptosis via interference with a pre-mitochondrial step in the cell death cascade[12-14]. Enhancements of telomerase activity via Bcl-2 gene overexpression have also been detected in cervical carcinoma, HeLa, and colorectal carcinoma DFi cells[15]. However, this phenomenon, with the exception of the known possibility of a correlation between Bcl-2 and telomerase in cancer progression. For example, in high-grade non-Hodgkin lymphoma, high expression levels of hTERT mRNA may be related to shorter survival, and telomerase activity has been shown to be affected by the expressions of Bax and Bak[16]. However, the molecular mechanisms underlying the interactions between telomerase and these regulators remain unclear, despite the extensive efforts of a number of research groups that have attempted to dissect such mechanisms.

In this study, we focused on the functions of telomerase beyond reverse transcriptase in cancer progression. In an effort to identify the modulators of telomerase, we utilized an in vitro cisplatin (cis-diammine-dichloro-platinum II)-induced apoptosis system.

2. Antibodies and reagents

Anti-Bcl-2 monoclonal antibody (C-2) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the anti-Bak monoclonal antibody was purchased from BD Biosciences (BD PharMingen, San Diego, CA, USA). Rabbit anti-hTERT polyclonal was acquired from Merck Biosciences (Calbiochem, La Jolla, CA, USA), and the broad caspase inhibitor (Z-VAD-fmk) was acquired from Promega (Promega, Madison, WI, USA). All other reagents used in this study were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

3. Cytotoxicity Assay

To determine the role of telomerase in the programming of apoptotic death, we analyzed the cytotoxic effects of cisplatin in cervical cancer, HeLa cells. Exponentially-growing HeLa cells were treated with cisplatin, and the percentage of viable cells was determined using MTS cell proliferation reagent (Promega). The cells were plated in 96-well plates at a concentration of 1 x 10⁴ cells/well in 100 µL of medium. After overnight incubation at 37℃, the media were exchanged with various concentrations of cisplatin (1–20 µg/mL). After an additional 24 hr of incubation at 37℃, 50 µL of MTS solution was added to each well and incubated for further 30 min at 37℃. Optical absorbance was then measured at 490 nm, using a microculture plate reader.

4. Construction of expression vectors and transfection

Wild-type Bcl-2 cDNA was generously provided by the Laboratories of Anticancer Research (KRIIBB, Daejeon, Korea), and the Bcl-2 cDNA fragment was cloned into pcDNA3-neomycin (Invitrogen, Carlsbad, CA, USA). The pME18s-human Bak plasmid was kindly provided by the Genome Research Center (KRIIBB). The fragment of human bak cDNA was transferred to the pCMV Tag plasmid (Invitrogen), and these constructs were verified via DNA sequence analysis. The transient transfection of HeLa or HEK293 with Bcl-2 and/or Bak plasmids was conducted using Lipofectamine/Plus reagent (Invitrogen), in accordance with the manufacturer’s instructions. After 24 hr of incubation, cisplatin was administered, after which the samples were incubated for an additional 24 hr.

MATERIALS AND METHODS

1. Cell culture

Human cervical cancer cell line HeLa and human embryonic kidney 293 cells were maintained in DMEM medium (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS (HyClone, Road Logan, Utah, USA) in a humidified incubator at 37℃.
5. Telomeric repeat amplification protocol (TRAP) assay

Telomerase activity was measured using a TRAPeze kit (Intergen, Purchase, NY, USA) in accordance with the manufacturer’s recommendations. In brief, the cisplatin-treated cells were lysed in CHAPS buffer and incubated on ice for 30 min, after which the soluble proteins were obtained. The concentrations of proteins were then measured using a BioRad protein assay kit (BioRad Laboratories, Richmond, CA, USA). To allow for the quantitative assessment of the activity, 0.5 μg of protein extracts were used in the presence of an internal TRAP assay standard (36 bp). The telomeres were extended via 30 min of incubation at 30℃, then amplified via two-step PCR (94℃ for 30 sec and 60℃ for 30 sec) conducted for 25-30 cycles. The radioactive TRAP products were separated on 12.5% neutral polyacrylamide gel, then autoradiographed.

6. Flow cytometry analysis of apoptosis

For the cell death analyses, the cells were harvested and washed in PBS. After 30 min of fixation with 75% ethanol at 4℃, the cells were washed with PBS three times and stained with propidium iodide (20 μg/mL PI, 0.1 μg/mL RNase A in PBS) in a dark room. Apoptotic cell death was then visualized via flow cytometry, using the FACS Caliber and CellQuest software packages (Becton Dickinson, Mountain View, CA, USA).

7. Trypan blue staining

The cells were stained with trypan blue (Sigma) and were counted using a hemocytometer. The percentage of cell death was expressed as the ratio of the number of trypan blue-permeable cells to the total cell count (trypan blue-permeable cell number/total cell number).

8. Western blot analysis

For Western blot analyses, the cisplatin-treated or transfected cells were lysed in a lysis buffer (20 mM Tris, pH 7.4, 2 mM EDTA, 150 mM sodium chloride, 1 mM sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 5 μg/mL aprotinin, and 10 μg/mL leupeptin), and the protein concentrations were determined using a BioRad protein assay kit (BioRad): 50-150 μg of protein was subjected to 12-15% SDS-PAGE and transferred onto PVDF membranes (Hybond-P, Amersham Biosciences, Buckinghamshire, England). Subsequently, the membranes were incubated for 2 hr at room temperature in a 5% skim milk solution, then probed overnight at 4℃ with the appropriate primary antibodies. The bound antibodies were then visualized using a suitable secondary antibody conjugated with horseradish peroxidase, using enhanced chemiluminescence reagents (ECL, Amersham Biosciences).

RESULTS

1. Telomerase plays an antiapoptotic role in cisplatin-induced cell death

Cisplatin inhibited the viability of HeLa cells dose-dependently (Fig. 1A), and telomerase activity was transiently increased as the consequence of cisplatin treatment, but its activity with regard to Bcl-2 expression was enhanced to a greater degree than that of controls (Fig. 1B). Therefore, we concluded that the enhanced activity effected by the expression of Bcl-2 also affected cisplatin-induced cell death, and the phenotype of cell death was determined via staining with propidium iodide (Fig. 1C) or trypan blue (Fig. 1D). As shown in Fig. 1C, apoptotic cell death was reduced to 40-50% in the Bcl-2 overexpressed cells as compared to the mock controls, and the trypan blue staining data was similar to that of the PI staining data (Fig. 1D). In conclusion, telomerase activity is closely associated with cisplatin-induced cell death, and the enhanced activity affected by Bcl-2 exerts an anti-apoptotic effect.

2. TERT expression was dependent on caspase activation in cisplatin-induced cell death

As shown in Fig. 2, about a two-fold increase in the quantity of hTERT protein was recovered as the result of Z-VAD-fmk inhibition in the mock control, but no change was detected in the Bcl-2 overexpressed cells. This finding suggests that the expression of hTERT was dependent upon the activation of caspase, but not on Bcl-2, which was located within the upstream pathway.
3. Co-expressions of Bcl-2 and Bak modulate the telomerase activity and TERT expression

In order to elucidate the interactions between wild type Bcl-2 and pro-apoptotic Bak on the function of telomerase, we transiently co-transfected a pcDNA-neo plasmid encoding for wt Bcl-2, or a pCMV Tag-neo plasmid encoding for Bak or control vectors, into the HEK293 cells. As shown in Fig. 3A, telomerase activity was found to be increased slightly by Bak expression, just as can be seen with cisplatin treatment in Fig. 1B. However, in case Bcl-2 and Bak were co-expressed at the same level, telomerase activity was reduced markedly. Evidently, hTERT protein was decreased to a greater degree on the Western blots than was seen in the vector control cells. Also, Bcl-2 protein expression was affected by Bak expression, but not Bak. The targeting of Bcl-2 by Bak may have an influence on the expression of hTERT (Fig. 3B). Therefore, our findings suggest that both telomerase activity and hTERT expression may be regulated via intracellular Bcl-2 expression, and that this can be regulated in turn by the interactions occurring between Bcl-2 and Bak.

DISCUSSION

In an effort to identify the modulators of telomerase in the progression of cancers, we utilized a cisplatin-induced
apoptotic program as an *in vitro* model. Since its introduction into clinical trials, cisplatin has had a significant impact in the field of cancer medicine, changing the course of therapeutic management of several cancers, including those of the ovary, testis, head, and neck[17]. However, the primary obstacles to anticancer chemotherapy include the cytotoxicity of anticancer agents to normal cells, as well as the occurrence of tumor cells that exhibit resistance to chemotherapeutic agents. In the majority of cases, the level of resistance is less than 50-fold: however, even a small increase in the cisplatin-resistance of a tumor can prove clinically important, as large dose escalations result in severe multiorgan toxicity[18]. Therefore, novel chemotherapeutic strategies, which might serve to reduce the cytotoxicity of normal cells and reverse the chemoresistance observed in tumor cells, represent an important target for the development of selective cancer therapies.

Currently, in order to inhibit telomerase, a variety of strategies have been adopted, including the introduction of TERT antisense oligomer[19, 20], G-quadruplex-interactive agents[21, 22], or small interference RNA[23]. In this study, we hypothesized that the targeting of the signaling modulators of telomerase might induce apoptosis in cancer cells. Therefore, we transiently overexpressed Bcl-2 cDNA in HeLa cervical cancer cells. The telomerase activity and apoptotic cell death induced by Bcl-2 were measured and evaluated (Fig. 1B–D). As shown in Fig. 1B, telomerase activity was augmented by cisplatin signals, but Bcl-2 expression enhanced its activity prominently. This suggests that telomerase activity may be more closely associated with cisplatin-induced apoptosis, and also exert an antiapoptotic effect.

Next, to determine in which caspase proteins affect telomerase function, we inhibited caspase activations via the application of Z-VAD-fmk. The expression of hTERT, the catalytic subunit of telomerase, was recovered markedly; however, no change was observed in the Bcl-2-overexpressing cells (Fig. 2). Thus, we conclude that telomerase activation is located downstream of the caspases, even though Bcl-2 existed in the upstream signal pathway, and also that the HeLa cells in our study underwent a cas-
요 약

배경: 인간 끝분절효소(telomerase)는 텔로미어(telomere) 반복서열을 합성하는 리보핵산 합성효소이며, 인간 hTERT (human telomerase reverse transcriptase)는 끝분절효소의 rate-limiting 요소일 뿐 아니라 활성단위체로서 등장되었다. 본 연구에서는 끝분절효소 조절자를 동정하고, 시스플라틴 유도 세포사멸에서의 분자적 메커니즘을 분석하고자 하였다.

방법: 시스플라틴 유도 세포사멸에서의 끝분절효소의 기능을 규명하기 위해, 끝분절효소 활성을 측정하고, 세포사멸을 PI 염색과 trypan blue 염색으로 분석하였다. 또한, hTERT 단백질 발현에 있어 이의 효과를 결정하기 위해 Z-VAD-fmk로 caspase 활성을 억제하였다. 마지막으로 HEK293 세포주에 Bcl-2와 Bak 유전자를 일시적으로 동시에 과발현시킨 다음, 끝분절효소 활성 및 hTERT 발현을 분석하였다.

결과: Bcl-2를 과발현하는 HeLa 세포에서는 끝분절효소 활성은 더욱 증가되었고, 세포사멸이 mock 대조군에 비해 40-50%정도 감소되었다. 이는 Bcl-2에 의해 유도된 끝분절효소활성이 시스플라틴-유도 세포사멸에서 항-세포사멸의 효과를 발휘함을 시사한다. Z-VAD-fmk로 caspase의 활성화를 억제함으로써 Mock 대조군 세포에서는 hTERT 단백질 발현이 회복되나, Bcl-2 발현 세포에서는 변화가 없었다. 이는 hTERT의 발현이 caspase에 의해 조절될 수 있었으나, Bcl-2는 상위 신호전달 체계에 존재하고 있음을 시사한다. 또한, HEK293 세포주에 Bcl-2와 Bak 유전자를 동시에 과발현한 시험을 때, 끝분절효소 활성과 hTERT 단백질 발현이 두법이 감소되었다.

결론: Bcl-2 발현에 의해 유도된 끝분절효소활성은 HeLa 세포에서의 시스플라틴-유도 세포사멸을 억제하며, 이는 caspase와 Bcl-2, Bak의 상호작용에 의해서도 조절될 수 있음을 제시한다.

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REFERENCES


