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Abstract

We speculated whether or not the expression level of telomerase reverse transcriptase (hTERT) would be modulated by agents targeting epigenetics in oral cancer cell lines. Although hTERT is known to be targeted by epigenetic changes, it remains unclear how chemoagents targeting epigenetics work on hTERT transcription. In the present study, the epigenetic effects of histone deacetylase (HDAC) inhibitor FR901228 on hTERT transcription were analysed by RT-PCR in oral cancer cell lines. The mRNA expression of hTERT was upregulated after exposure to FR901228 in hTERT-negative Hep2 cells, even in the hTERT highly expressed SAS and KB cells. Moreover, co-treatment of protein synthesis inhibitor cycloheximide (CHX) resulted in the induction of hTERT transcription by FR901228. This suggests that the induction of hTERT by FR901228 requires *de novo* protein synthesis to some extent and is more likely a direct than an indirect effect on epigenetic changes such as histone acetylation / deacetylation. We further examined the effect of FR901228 on c-myc protein, which is one of the main hTERT transcription activators. FR901228 repressed c-myc protein only in the absence of CHX, dependent of the enhancement of de novo protein synthesis. Our results indicate that c-myc protein is repressed indirectly by FR901228 but may not contribute FR901228-induced hTERT transcription. The present study showed that the HDAC inhibitor FR901228 induced the hTERT gene by a complex mechanism that involved other transcription factors except for c-myc, in addition to the inhibition of histone deacetylation.

KEYWORDS: hTERT; FR901228; Oral cancer; HDAC inhibitor

Introduction

Telomerase is an enzyme implicated in the *de novo* synthesis of GGTTAG telomeric DNA onto chromosomal ends to stabilize telomeres, concomitant with immortality in cancer cells [3, 26, 16]. Telomerase is composed of telomerase reverse transcriptase (hTERT) [27], telomerase-associated protein (TEP1) [9], and hnRNP A1 [21]. The *de novo* transcription of the hTERT gene is considered the dominant, rate-limiting step in telomerase activation whereas the expression of hTEP1 and hTR is constitutive [1, 9]. The regulation of hTERT expression is complicated, and it has been reported that hTERT transcription can be activated by overexpression or activation of several transcription activators [8, 17, 20, 24, 33, 36]. Several recent reports have identified c-myc binding sites (E-boxes) in the hTERT promoter and found that c-myc positively regulates hTERT expression [11, 30]. The hTERT gene is likely to be targeted by epigenetic regulation such as histone acetylation or promoter methylation. Some reports showed that the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) could activate hTERT expression in telomerase-negative cells [6, 7, 31, 37]. Hou *et al.* reported that TSA and the protein synthesis inhibitor cycloheximide (CHX) induced hTERT mRNA [13], suggesting that this induction does not require *de novo* protein synthesis and is likely a direct result of HDAC inhibition at the hTERT gene. Although it is reasonable to speculate that epigenetic changes such as histone acetylation or promoter methylation are common underlying features to hTERT transcriptional regulation, it remains unclear how chemoagents targeting epigenetics such as HDAC inhibitors work on hTERT mRNA expression. FR901288, a novel cyclic peptide inhibitor of HDAC, is isolated by the Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan) from a fermantation broth of Chromobacterium

violaceum, and is currently in phase I of clinical trials. As FR901228 has a stronger cytotoxic activity than TSA, several numbers of genes involved are known to increase or decrease their transcriptional levels followed by hyperacetylation of histone [25].

In the present study, we focus on the HDAC inhibitor FR901228 and evaluate the effects of FR901228 on hTERT expression and its transcription activator c-myc in oral cancer cells.

Materials and Methods

Cell lines and culture

Human oral cancer cell lines (HSC4, HSC3, HSC2, KB, SAS, Hep2and HO-1-u-1) obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, were maintained in Dulbecco's modified Eagles medium (MDEM) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., UT, USA), 100 units/ml penicillin (Meiji Seika Ltd., Tokyo, Japan) and 100 μg/ml streptomycin (Meiji Seika Ltd., Tokyo, Japan) in a CO_2 incubator (Sanyo Electric Co., Ltd., Osaka, Japan) with 95% air plus 5% CO_2 at 37°C.

Chemicals

FR901228 was a gift from Fujisawa Pharmaceutical Company (Osaka, Japan). FR901288, 5-Aza-2'-deoxycytidine (5-Aza) (Sigma Chemical Co., St Louis, MO) and CHX (Wako Pure Chemical Industries, Ltd., Osaka, Japan) diluted in water were added to MDEM to the final

concentration indicated in each treatment.

FR901228 treatment

First, 1×10^6 cells were seeded in 5 ml MDEM in a flask (Nalge Nunc International, cat No. 152094, Roslilde, Denmark). Then, 24 h after seeding, the medium was changed for one containing FR901228 at final concentration of 0.5 or 1.0 μM and the flask was immersed in a 37℃ water bath (Taitec, Co., Ltd., Saitama, Japan). Cells were also incubated with FR901228 in the presence of 200 μg/ml of CHX, a potent protein synthesis inhibitor. CHX was added to cells 30 min before the addition of 0.5μM FR901228. Cells were harvested at the indicated times (4 or 16hrs) following FR901228 treatment.

5-Aza-2'-deoxycytidine treatment

Stock solutions of 5-Aza were prepared by dissolving the drug at a concentration of 10 mM in distilled water no more than 2 h prior to use in an experiment; final treatment concentrations were obtained by diluting the stock solution directly into the tissue culture medium. Cells were incubated with several concentrations of 5-Aza of 4.4 and 8.8 μM for 7 days.

RNA isolation and RT-PCR

Following incubation of the cells with agents under each experimental condition, extraction of total cellular RNA was carried out using Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. 1.5μg RNA was reverse-transcribed with Superscript II Reverse

Transcriptase and oligo dT primers (Invitrogen Corp., Carlsbad, CA). Amplification of cDNAs was performed under the following PCR conditions: 7 min at 94℃ for 1 cycle; then 26 cycles at 94℃ for 30 s, 59℃ for 30 s, 72℃ for 30 s; and a final elongation step at 72℃ for 10 min. The primers used for the amplification were as follows:

hTERT primer1: sense: 5'- cgg aag agt gtc tgg agc aa-3', antisense: 5'- gga tga agc gga gtc tgg a -3'; *hTERT* primers1 amplified a 145bp product [18, 27]. hTERT primer2: sense: 5'- act ttg tca agg tgg atg tga cgg -3' (exon6), antisense: 5'- aag aaa tca tcc acc aaa cgc agg -3' (exon10); *hTERT* primers2 amplified a 493bp product spanning exon6 to exon10 [5, 15, 28]. c-myc: sense: 5'-aagtcctgcgcctcgcaa-3', antisense: 5'-gctgtggcctccagcaga-3'. GAPDH;:sense, 5'-gaaggtgaaggtcggagtc-3', antisense: 5'-caaagttgtcatggatgacc-3'. The amplified GAPDH fragment was used as a positive control. The RT-PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and viewed by UV.

Western blotting

Following incubation of the cells with FR901228 treatment, extraction of total cellular protein was carried out using 500μl lysis buffer (1.315mM sucrose, 0.3475mM sodium dodecyl sulfate, 31.25μl 1M Tris (pH6.8), 0.125mg Bromo Phenol Blue powder, 25μl 2-Mercaptoethanol, diluted to 500μl with distilled water). 5μl protein in cell-free extract was loaded per lane and was separated by electrophoresis by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), and the individual proteins transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) using a semi-dry electrophoretic transfer apparatus

(LKB-Produkter AB, Bromma, Sweden) at room temperature. The blotted membranes were blocked for 1 h in TBS-T (containing 0.1% Tween 20) plus 5% powdered skim milk and then probed for 2h with mouse anti-c-myc monoclonal antibody *c-myc* Ab-5 (Clone 67P05) (Neomarkers, Fremont, CA) diluted 1:1000, 2 μg/ml in TBS-T. The membranes were then washed three times in TBS buffer and incubated for 1 h with the appropriate secondary antibody horseradish peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (ImmunoResearch Laboratories Inc., West Grove, PA) in TBS-T. Bound antibody was detected using ECL + plus kit (Amersham Pharmacia Biotech Inc., Little Chalfont, UK) according to the manufacturer's instructions. To quantify the amount of protein that was loaded per lane, we performed western blotting for beta actin as control. The mouse monoclonal antibody for beta actin, beta actin AC-15-ab6276, purchased from Abcam Limited (Cambridge, UK), was diluted 1:5000 in TBS-T and utilized in the same manner.

Results

Comparison of hTERT expression among oral cancer cell lines

The relative levels of hTERT mRNA among seven oral cancer cell lines were determined by RT-PCR (Fig. 1). The great majority of oral cancer cell lines contained detectable amounts of hTERT transcript; HSC4 and SAS cells showed especially high levels of mRNA. Hep2 shows no evidence of hTERT message under the specific experimental conditions employed here.

Comparison of hTERT expressions before and after FR901228 or 5-Aza treatment

The hTERT downregulated cell line Hep2 was incubated with FR901228 at a concentration of 0.5 or 1.0 μM. The hTERT-expressed cell lines SAS and KB were also analyzed as a control. Substantial amounts of hTERT mRNA were present, as shown in Fig. 2a. Following incubation of the cells with FR901228, very dense bands were detected, suggesting the induction of hTERT transcripts in Hep2 cells. The SAS and KB cells also were upregulated the hTERT expression by FR901228 treatment.

To test whether hTERT gene expression was suppressed by hypermethylation of its promoter or not, we exposed 5-Aza, an inhibitor that prevents methylation of newly synthesized DNA, to the hTERT downregulated Hep2 cells. Our results clearly show that the Hep2 cells regained their ability to produce high levels of hTERT mRNA following exposure to graded doses of 5-Aza (Figure 2a).

These data suggest that, in both cases, epigenetic-targeted agent-mediated transactivation of the hTERT gene might result from localized hyperacetylation of histones or CpG methylation at the hTERT promoter.

Effects on hTERT expression of FR901228 treatment combined with CHX

To determine whether the FR901228-mediated induction of hTERT expression was a direct effect or indirect effect, the hTERT downregulated cell line Hep2 and the hTERT-expressed line SAS were incubated with FR901228 in the presence of 200 μg/ml of CHX, a potent protein synthesis inhibitor, and were then analyzed for hTERT mRNA (Figure 2b). In FR901228 treatment, all of

three cells showed inductions of hTERT expression; however, co-treatment of FR901228 with CHX resulted even in the enhancement of the hTERT expression in SAS and KB cells. This clearly reflects a direct role for the HDAC inhibitor in the transcriptional activation of the hTERT gene to some extent, and implies that *de novo* protein synthesis is also involved in the hTERT regulation.

Comparison of c-myc expressions before and after FR901228 treatment

Alternatively, we examined whether or not FR901228 has an effect on the hTERT transcription activator, c-myc. Fig. 3 a and b show that FR901228 reduced endogenous expression of c-myc in SAS, KB and Hep2 cells. We further analyzed for c-myc mRNA in those cells incubated with FR901228 in the presence of CHX (Figure 3c). In contrast when cells were exposed to FR901228 alone, SAS, KB and Hep2 cells showed less decreasing levels of c-myc mRNA when incubated with both FR901228 and CHX. Interestingly, in Hep2 cells, CHX treatment even seems to upregulate baseline expression of c-myc, this seems to depend upon the absence of new protein synthesis in the presence of CHX. The rapid reduction of c-myc mRNA in FR901228 treatment clearly reflects an indirect role for the HDAC inhibitor in the reduction of this gene, but both direct and indirect effects are at play.

Discussion

Among those chemoagents targeting epigenetics, HDAC inhibitors are known to exhibit antiproliferative effects on cancer cells through modulating transcription [23, 35], and are

considered as potential therapeutic agents against malignancies. Our results showed a clear increase in hTERT expression following FR901228 treatment of oral cancer cell lines. Although our findings are the results of in vitro studies and the concentrations of FR901228 in our study are higher than those are used in most studies [14, 29], however, we believe that these data may have important clinical implications in the potential utility of FR901228 in oral cancer therapy. Some reports showed that the HDAC inhibitor TSA could activate hTERT expression in telomerase-negative cells [6, 7, 31, 37]. However, it remains unclear how epigenetics work on hTERT mRNA expression. Horikawa *et al.* and others [7, 12] found no general correlation between the hTERT expression and the hTERT promoter methylation status, either overall or at a specific site in cancer cells. Hou *et al.* reported that TSA and CHX induced hTERT mRNA [13]. Wang *et al.* [34] also reported that TSA-induced hTERT transcription and chromatin alterations were not blocked by CHX, suggesting that this induction does not require *de novo* protein synthesis and is likely a direct result of HDAC inhibition at the hTERT promoter. To characterize whether or not the hTERT induction pathway by FR901228 has a direct effect on chromatin, we treated cells with FR901228 and the protein synthesis inhibitor CHX. In SAS or KB cells, co-treatment with CHX and FR901228 resulted even in the enhancement of induction of hTERT transcription compared with the result obtained by FR901228 treatment alone. This suggests that the induction of hTERT by FR901228 is more likely a direct effect than an indirect effect on epigenetic changes such as histone acetylation / deacetylation and involves *de novo* protein synthesis in part.

Overexpression of some transcription factors such as c-myc was recently shown to play some part in activation of endogeneous hTERT transcription [8, 17, 20, 24, 33, 36]. Next, we

sought to discover whether or not the effect of FR901228 on hTERT expression is related to a mechanism through one of the main hTERT transcription activators, c-myc, whose repression has been implicated in several apoptosis pathways [32]. Our results showed that FR901228 treatment clearly decreased in c-myc expression in oral cancer cell lines. HDAC inhibitors, such as TSA and sodium butyrate, have been reported to decrease c-myc mRNA expression and to increase p21 mRNA [35]. Previous studies have shown that butyrate, known as an HDAC inhibitor, reduces c-myc mRNA levels [2, 4, 10, 19]. Our results showed that c-myc expression was less reduced in the presence of the protein synthesis inhibitor CHX. This indicates that the c-myc expression is transcriptionally regulated by FR901228 through histone acetylation/deacetylation indirectly, although the precise mechanism by which HDAC inhibitors abrogate c-myc expression remains to be elucidated. Having demonstrated that FR901228 reduces c-myc expression, the inverse expression patterns of hTERT and c-myc led us to hypothesize that c-myc reduction might play some part in activating hTERT. Li *et al* [22] found a complex consisting of BRCA1, c-myc and Nmi that can inhibit hTERT promoter activity in breast cancer cells. Inconsistent with our hypothesis, the combined treatment with FR901228 and CHX resulted in the enhancement of hTERT expression independent of c-myc expression. It is reasonable to speculate that the decreased expression of c-Myc protein that was observed in our study is not required for activation of the hTERT expression by FR901228. However, precise mechanism between hTERT and c-myc expressions remains to be elucidated.

Our study suggested that hTERT expression may be regulated epigenetically through histone acetylation/DNA methylation to some extent; and other regulatory factors also take part in

increasing hTERT expression through an HDAC-independent mechanism. Our study provides some new insights into an endogenous mechanism for hTERT induction by HDAC inhibitors.

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Figure Legends

Figure 1: Comparison of hTERT expression among oral cancer cell lines.

Extracted RNA was reverse-transcribed to cDNA and the relative levels of hTERT mRNA among seven oral cancer cell lines (HSC4, HSC3, HSC2, KB, SAS, Hep2and HO-1-u-1) were determined by RT-PCR.   The hTERT primers1 amplified a 145bp product and the hTERT primers2 amplified a 493bp product spanning exon6 to exon10.   The two middle bands amplified by primer2 might result from partially spliced hTERT transcripts.   The great majority of oral cancer cell lines contained detectable amounts of hTERT transcript; HSC4 and SAS cells showed especially high levels of mRNA, as indicated by the very dense bands that appear in the relevant lanes.   The gels clearly show that Hep2 cells contained no hTERT mRNA under the specific experimental conditions employed here.

Figure 2: Epigenetic-targeted agent-mediated transactivation of the hTERT gene.

a : Comparison of hTERT expressions before and after FR901228 or 5-Aza-2'-deoxycytidine(5-Aza) treatment.

Cells of the hTERT-downregulated cell line Hep2 cells were incubated with FR901228 at a concentration of 0.5 or 1.0 μM.   The hTERT-high-expressed cell lines, SAS and KB were also analyzed as a control.   Substantial amounts of hTERT mRNA were determined by RT-PCR. The gels clearly show that the hTERT expression was induced after 4h-16 h of treatment with FR901228 in all of three cell lines.   Additionally, the hTERT-downregulated Hep2 cell was

incubated for 7 days with several concentrations, ranging from 4.4 to 8.8 μM, of 5-Aza, an inhibitor that prevents methylation of newly synthesized DNA.   The Hep2 cells regained their ability to produce high levels of hTERT mRNA.

b: Effects on hTERT expression of FR901228 treatment combined with cycloheximide (CHX).

To determine whether FR901228-mediated induction of hTERT expression was a direct effect or not, Hep2, KB and SAS cells were incubated with FR901228 in the presence of 200 μg/ml of CHX and were then analyzed for hTERT mRNA by RT-PCR.   SAS, KB and Hep2 cells showed slight inductions of hTERT expression; however, co-treatment with CHX resulted even in the enhancement of the hTERT expression in comparison with FR901228 alone.

Figure 3: Comparison of c-myc expression before and after FR901228 treatment.

a: Comparison by RT-PCR of c-myc mRNA before and after FR901228 treatment

b: Comparison by Western blotting of c-myc protein before and after FR901228 treatment.

To examine whether or not FR901228 has an effect on the hTERT transcription activator c-myc, we analyzed c-myc expression before and after FR901228 treatment in SAS, KB and Hep2 cells. FR901228 reduced endogenous expressions of c-myc in both cell lines.

c: Effects on c-myc expression of FR901228 treatment combined with CHX.

SAS, KB and Hep2 cells were incubated with FR901228 in the presence of CHX and analyzed for c-myc mRNA by RT-PCR.   As when cells were exposed to FR901228 alone, SAS, KB and Hep2 cells showed less decreasing levels of c-myc mRNA when incubated with both FR901228 and CHX.
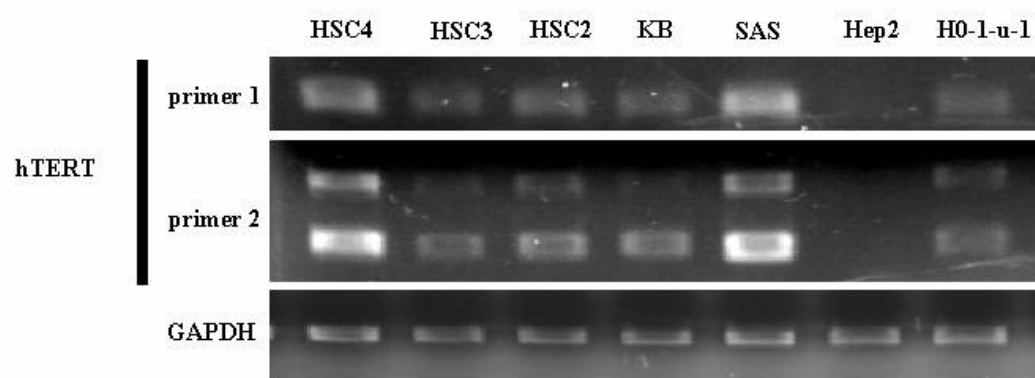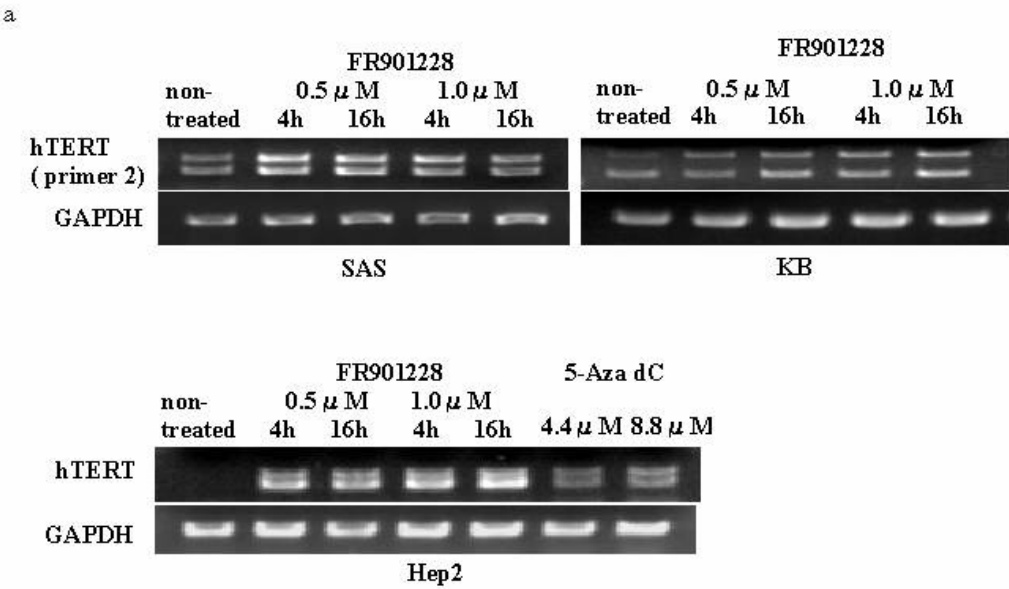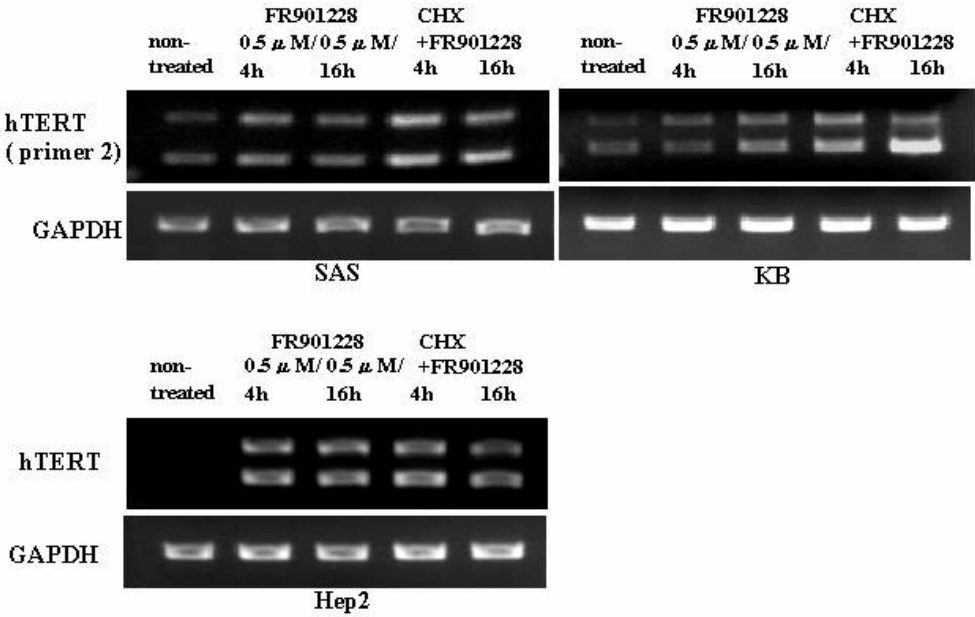
Figure 1

Figure 2a

Figure 2b

Figure 3a

Figure 3b

Figure 3c