Inhibition of the Epidermal Growth Factor Receptor Suppresses Telomerase Activity in HSC-1 Human Cutaneous Squamous Cell Carcinoma Cells

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Activation of telomerase, which stabilizes the telomere length of chromosomes, is crucial for the continued growth or progression of cancer cells. In a previous study, we showed that telomerase is frequently activated in skin tumors. Because epidermal growth factor plays an important role during the tumorigenesis of epithelial tissue, we have now examined the role of epidermal growth factor signaling in regulating telomerase activity using HSC-1 human cutaneous squamous cell carcinoma cells. Treatment of HSC-1 cells with AG 1478, an inhibitor of the epidermal growth factor receptor, or with a neutralizing antibody to the epidermal growth factor receptor, significantly suppressed their telomerase activity, in association with inhibiting their growth. The suppression of telomerase activity was obvious at day 3 and was maximal at day 5 after treatment with AG 1478. The suppression of telomerase activity correlated with the decreased expression of human telomerase catalytic subunit (hTERT) mRNA, the rate-limiting determinant of its enzyme activity. The expression of c-Myc and of Sp1 proteins, transcription factors for hTERT, were also suppressed by AG 1478 in HSC-1 cells, but the expression of Ets-2 protein, another transcription factor, was not affected. The expression of Mad-1, a competitor of c-Myc, was increased. Inhibition of ERK, Src, or Akt suppressed telomerase activity in HSC-1 cells, but to a lesser extent than did treatment with AG 1478. Serum starvation suppressed telomerase activity, but addition of epidermal growth factor or transforming growth factor α did not increase it, indicating the involvement of other epidermal growth factor receptor ligands in the activation of telomerase in HSC-1 cells. These data indicate that blockade of the epidermal growth factor receptor might be effective in inhibiting telomerase activity of squamous cell carcinomas, which would lead to the suppression of tumor growth. ***Key words: Akt/ERK/human telomerase catalytic subunit (hTERT)/Src. J Invest Dermatol 121:1088–1094, 2003***

The immortalization of human cells is a critical step during tumorigenesis. To immortalize human keratinocytes, the loss of p16^{INK4A} expression and the activation of telomerase are required (Kiyono *et al*, 1998). Telomerase is a ribonucleoprotein enzyme (an RNA-dependent DNA polymerase) that catalyzes the addition of telomeric repeats $(TTAGGG)_n$ to telomeres, the distal ends of eukaryotic chromosomes. Telomeres are essential elements that protect chromosome ends from degradation and ligation. Telomerase is frequently activated in cancer cells, whereas its activity is usually repressed in normal somatic cells except in some self-renewing tissues with high regenerative potential, such as hematopoietic cells and epidermal keratinocytes (Harle-Bachor and Boukamp, 1996). Telomerase activation is thus thought to be crucial for the continued growth of cells by stabilizing telomere length.

We previously reported that telomerase is frequently activated in skin tumors. In addition, telomerase activation could be detected in normal skin and showed a strong association with the level of sun exposure, which suggests that telomerase activation in the skin might be involved at an early stage of human photocarcinogenesis (Ueda *et al*, 1997). We also showed that telomerase activity in skin tumors was strongly correlated with the expression of the human telomerase catalytic subunit (hTERT), as has been shown in tumors of other organs (Wu A *et al*, 1999).

The promoter region of hTERT has been cloned (Horikawa *et al*, 1999; Takakura *et al*, 1999) and the transcription factors c-Myc and Sp1 have been shown to activate hTERT transcription (Horikawa *et al*, 1999; Takakura *et al*, 1999; Wu K-J *et al*, 1999; Kyo *et al*, 2000). Furthermore, Mad-1 has been shown to be a repressor of hTERT gene expression by antagonizing c-Myc (Günes *et al*, 2000; Oh *et al*, 2000). A recent study revealed that epidermal growth factor (EGF) activates the hTERT promoter and that the ETS motif located in the core promoter of hTERT is required for the EGF-induced transactivation of hTERT (Maida *et al*, 2002).

Various reagents, including sex steroid hormones, growth factors, and retinoic acid, have been shown to upregulate or downregulate telomerase activity through various signaling pathways (Kyo *et al*, 1999; Wang *et al*, 2000; Ding *et al*, 2002; Inui *et al*, 2002; Kyo and Inoue, 2002). Rea and Rice (2001) have shown that EGF stimulates telomerase activity in a spontaneously immortalized human epidermal cell line. EGF plays an important role in

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Abbreviations: hTERT, human telomerase catalytic subunit; TRAP, telomeric repeat amplification protocol.

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the development and progression of many solid tumors including squamous cell carcinoma (SCC). Studies using EGF receptor (EGFR) null mice have shown that the EGFR is required as an essential survival signal for skin tumors (Sibilia *et al*, 2000). EGF and EGFR are expressed in head and neck SCC and the level of expression is associated with patient survival (Grandis *et al*, 1998), and inhibition of SCC growth can be achieved by EGFR antisense RNA or by monoclonal antibodies against the EGFR (He *et al*, 1998; Herbst *et al*, 2001). We hypothesized that the EGFR may play a role in regulating telomerase activity and we examined the effect of inhibiting EGFR function on the telomerase activity of HSC-1 human cutaneous SCC cells. A specific inhibitor of the EGFR inhibited telomerase activity in a dose-dependent manner, and this was correlated with the decreased expression of hTERT, c-Myc, and Sp1. ERK, Src, and Akt are suggested to be involved in this signaling.

MATERIALS AND METHODS

Cell, cell culture, and reagents HSC-1 is an SCC cell line derived from human skin (Kondo and Aso, 1981), and was a gift from Dr Katagaka of the Yamagata University School of Medicine. The immortalized human keratinocyte cell line HaCaT was kindly provided by Dr Fusenig (Boukamp *et al*, 1988). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS). An EGFR inhibitor (AG 1478), an MAPK ERK kinase inhibitor (PD 98059, termed an ERK inhibitor here), an Src inhibitor (PP2), a PI3K/Akt inhibitor (LY 294002, termed an Akt inhibitor here), EGF, transforming growth factor α (TGF-α), and a neutralizing antibody to EGFR were purchased from Calbiochem (San Diego, CA). Each inhibitor and neutralizing antibody was added after the cultured cells reached approximately 50%–60% confluency. Dimethylsulfoxide as a solvent at the concentration used in this study (less than 0.2%) had no effect on telomerase activity or other analyses.

For studying the effects of EGF and TGF-α on telomerase activity, HSC-1 cells or HaCaT cells were incubated in low serum medium (DMEM supplemented with 0.5% FBS) for 3 d. The cells were then treated with 10 ng per ml EGF or TGF-α and extracted at day 2 along with control cells incubated with normal serum medium (DMEM supplemented with 10% FBS).

Proliferation assay Cell growth was determined by the MTS cell proliferation assay (Promega, Madison, WI). Briefly, HSC-1 cells were cultured in 96-well plates and, 24 h after seeding, 0.25–2 μM AG 1478 was added to the medium. Cells were incubated for the indicated times, and then MTS reagent was added and allowed to react for 2 h. Absorbance at 490 nm was measured using a microplate reader (Emax; Molecular Devices, Sunnyvale, CA).

Telomerase activity and hTERT expression Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) using the TRAPeze XL Telomerase Detection Kit from Intergen (Purchase, NY) according to the manufacturer's instructions. Briefly, harvested cells were resuspended in CHAPS XL lysis buffer and the lysates were centrifuged at 12,000*g* for 20 min at 4°C. The protein concentration of each supernatant was measured by the Coomassie protein assay (Pierce, Rockford, IL). Two microliters of each extract (0.35 μg per μL protein) were added to the solution containing the TRAPeze XL reaction mix and Taq polymerase (Takara, Shiga, Japan). The solution was subjected to 29 cycles of PCR (94°C for 30 s, 59°C for 30 s, and 72°C for 1 min) followed by a 55°C extension step for 25 min. The PCR products were electrophoresed on 10% nondenaturing polyacrylamide gels and were stained with SYBR Green I nucleic acid gel stain (Molecular Probes, Eugene, OR).

Expression of hTERT mRNA was analyzed by RT-PCR amplification as described previously, with slight modifications (Wu A *et al*, 1999). Briefly, total RNA was isolated from the cells using ISOGEN (Nippon Gene, Tokyo, Japan). cDNA was synthesized from 1 μg RNA using an RNA PCR Kit Version 2.1 (Takara, Shiga, Japan) with random primers. PCR primers used were as follows: (1) hTERT, LT5, 5′-CGGAAGAGT GTCTGGAGCAA-3′ (sense) and LT6, 5′-GGATGAAGCGGAGTCT GGA-3′ (antisense); (2) actin, actin1, 5′-GGGAATTCAAAACTGGAACG GTGAAGG-3′ (sense) and actin2, 5′-GGAAGCTTATCAAAGTCCTCGG CCACA-3′ (antisense). To amplify cDNA, 2.5 μL aliquots of reverse-transcribed cDNA were subjected to PCR. The PCR conditions were as follows: (1) hTERT, 36 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s; (2) actin, 25 cycles of denaturation at 90°C for 90 s, annealing at 60°C for 90 s, and extension at 72°C for 90 s. PCR products were electrophoresed in 2.5% NuSieve 3:1 agarose gels (Bioproducts, Rockland, ME). Quantification of TRAP assay and hTERT mRNA was performed using NIH image analysis software (version 1.62f). Briefly, the image files were opened in gray-scale mode by NIH image. For density determination, a density slice of 100–250 was selected and the "measure" command was used. Relative telomerase activity was quantified by measuring the total density of telomerase ladder bands and calculated as the ratio to the internal control. hTERT expression was quantified by measuring the density of the RT-PCR band and calculated as the ratio to actin.

Western blot analysis Western blotting was performed by the method described previously (Kyo *et al*, 2000). Briefly, 30 μg of protein were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gels and were transferred to polyvinylidene difluoride membranes. Membranes were incubated with specific antibodies against c-Myc (N-262; Santa Cruz, Santa Cruz, CA), Sp1 (1C6; Santa Cruz), Ets-2 (C-20; Santa Cruz), Mad-1 (C-19; Santa Cruz), phosphorylated ERK1/2 (Tyr202/Tyr204; Cell Signaling Technology, Beverly, CA), phosphorylated Src (Tyr527; Cell Signaling Technology), phosphorylated Akt (Ser473; Cell Signaling Technology), $p16^{INK4a}$ (H-156; Santa Cruz), or $p21^{CIP1/WAF1}$ (187; Santa Cruz), followed by reaction with horseradish peroxidase linked IgG. Immunoreactive bands were visualized using the ImmunoStar reagents (Wako, Osaka, Japan). After stripping, the membrane was reprobed with polyclonal antiactin (Santa Cruz), anti-ERK1/2, anti-Src, and anti-Akt (Cell Signaling Technology) antibodies that served as gel loading and protein controls. Quantification of protein expression was done using NIH image analysis software (version 1.62f).

Immunoprecipitation of EGFR phosphorylation EGFR antibody (SC03, Santa Cruz) was incubated with protein G-Sepharose (Pharmacia, Uppsala, Sweden) for 2 h at room temperature. Cell lysates (200 μg protein) were then added and immunoprecipitated for 24 h at 4°C. The immuno-complexes were washed, electrophoresed, and analyzed by immunoblotting using a phosphotyrosine antibody labeled with horseradish peroxidase (PY20, Santa Cruz). Re-blotting of the membrane with the EGFR antibody was performed to confirm the equal loading of EGFR.

RESULTS

An EGFR inhibitor, AG 1478, or an EGFR neutralizing antibody suppressed telomerase activity in HSC-1 cells Telomerase activity was high in HSC-1 cells, as shown by the TRAP assay (**Fig 1*a***). In a time-course experiment, an EGFR inhibitor (AG 1478) slightly suppressed telomerase activity in HSC-1 cells at days 1 and 2 after treatment and this suppressive effect became obvious at days 3 and 4, and then reached a maximum level at day 5 (**Fig 1*a***). The suppressive effect on telomerase activity by AG 1478 was concentration dependent. At a dose of 0.25 μM, telomerase activity was not altered, whereas 1 μM AG 1478 induced an obvious suppression and maximum inhibition was seen at a dose of 2 μM (**Fig 1*b***). Blocking of EGFR by a neutralizing antibody also suppressed the telomerase activity similar to the level elicited by AG 1478 treatment (**Fig 1*c***).

AG 1478 inhibited proliferation of HSC-1 cells To determine whether the inhibition of telomerase activity by AG 1478 is associated with the inhibition of cell growth, we measured cell proliferation using the MTS assay. The growth of HSC-1 cells was not altered within 2 d of treatment with 0.25–2 μM AG 1478, but a significant inhibition of cell growth of HSC-1 cells was observed after 3 d of treatment with 1 or 2 μM AG 1478 (**Fig 2**).

AG 1478 suppressed expression of hTERT mRNA in HSC-1 cells To examine whether the suppression of telomerase activity by AG 1478 is due to the reduced expression of hTERT, we performed RT-PCR analysis. The expression of hTERT mRNA started to decrease at day 1 after AG 1478 treatment (2 μM), and by

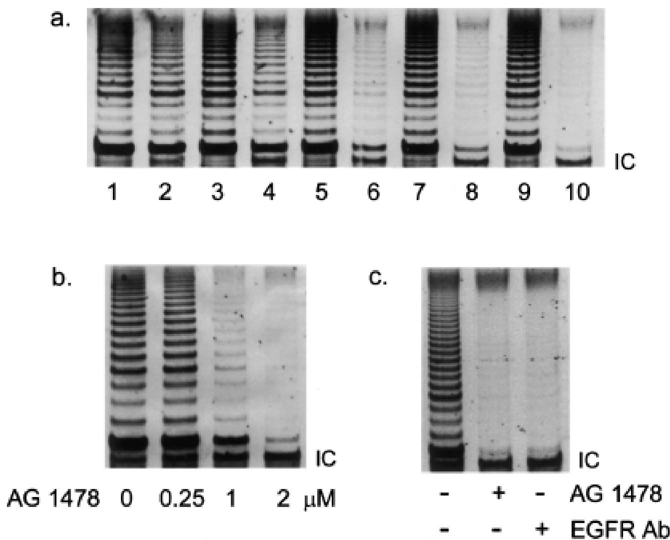


Figure 1. The EGFR specific inhibitor (AG 1478) or an EGFR neutralizing antibody suppressed telomerase activity in HSC-1 cells. (*a*) HSC-1 cells were treated with 2 μM AG 1478 and were extracted at different times along with control solvent-treated cells. *Lanes 1, 3, 5, 7,* and *9*, controls at days 1–5, respectively; *lanes 2, 4, 6, 8*, and *10*, AG 1478 treatment at days 1–5, respectively. (*b*) HSC-1 cells were treated with 0.25, 1, or 2 μM AG 1478 and were extracted at day 5 along with control solvent-treated cells. (*c*) HSC-1 cells were treated with 2 μM AG 1478 or with 10 μg per ml EGFR neutralizing antibody and were extracted at day 5 along with control solvent-treated cells. The TRAP assay was performed to measure telomerase activity. IC, internal control.

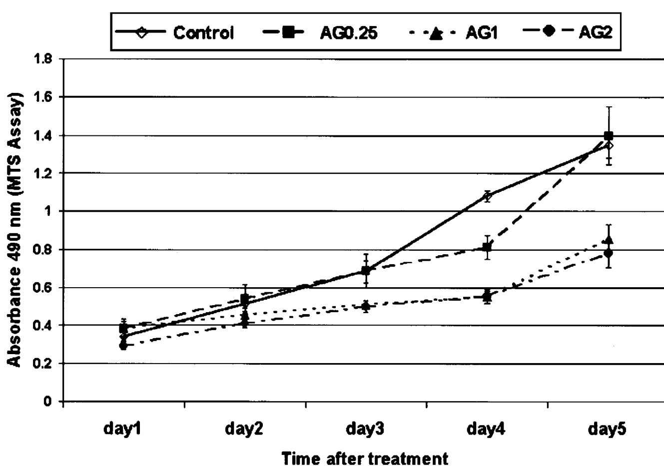


Figure 2. AG 1478 inhibited cell proliferation of HCS-1 cells. HSC-1 cells were treated with 0.25, 1, or 2 μM AG 1478 and were incubated for the indicated times. The MTS assay was performed to measure cell proliferation. Control cells were treated with solvent.

day 3 the hTERT mRNA expression was almost completely suppressed (**Fig 3**).

AG 1478 reduced expression of c-Myc and Sp1 but not of Ets-2 in HSC-1 cells; AG 1478 slightly increased expression of Mad-1 in these cells Previous studies have shown that Sp1 cooperates with c-Myc to activate hTERT transcription and telomerase activity (Horikawa *et al*, 1999; Takakura *et al*, 1999; Wu K-J *et al*, 1999; Kyo *et al*, 2000). In contrast, Mad-1 acts as a suppressor for hTERT by antagonizing c-Myc (Günes *et al*, 2000; Oh *et al*, 2000). To investigate whether the suppressive

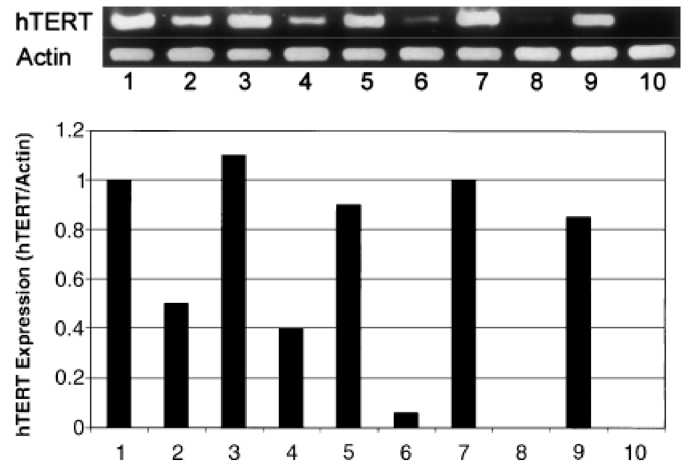


Figure 3. AG 1478 suppressed expression of hTERT mRNA in HSC-1 cells. HSC-1 cells were incubated with 2 μM AG 1478. Treated cells were then extracted for RNA at different times along with control solvent-treated cells and RT-PCR assays were performed using LT5/LT6 primers for the expression of hTERT mRNA. An actin mRNA was used as an internal control. *Lanes 1, 3, 5, 7,* and *9*, solvent treatment at days 1–5, respectively; *lanes 2, 4, 6, 8*, and *10*, AG 1478 treatment at days 1–5, respectively. *Bottom panel*: Relative levels of hTERT mRNA normalized to actin as determined by densitometry using NIH image analysis software.

effect of AG 1478 on hTERT expression was mediated by a modification of expression of c-Myc, Sp1, and/or Mad-1, we performed western blot analysis. AG 1478 significantly reduced the expression of c-Myc and Sp1 in HSC-1 cells (**Fig 4**). This inhibition was observed at day 1 after AG 1478 treatment (2 μM) and reached a maximum at days 4–5 for Sp1 and at days 3–5 for c-Myc. On the other hand, AG 1478 treatment slightly increased the level of Mad-1 protein expression. As the ETS motif is required for EGF induction of hTERT expression (Maida *et al*, 2002), we also examined the expression of Ets-2 in HSC-1 cells. The expression of Ets-2 was not altered by treatment with AG 1478, however (**Fig 4**).

AG 1478 did not alter the expression of $p16^{INK4a}$ or $p21^{CIP1/WAF1}$ in HSC-1 cells To test whether the cyclin-dependent kinase inhibitors $p16^{INK4a}$ or $p21^{CIP1/WAF1}$ are involved in the suppressive effect on telomerase or in the inhibitory effect on cell growth of HSC-1 cells elicited by AG 1478, their expression was examined. AG 1478 (2 μM) did not alter the expression of $p16^{INK4a}$ or $p21^{CIP1/WAF1}$ (**Fig 5**).

Inhibitors for ERK, Src, or Akt suppressed telomerase activity in HSC-1 cells To reveal the signal transduction pathway that is involved in the inhibition of hTERT expression by AG 1478, we treated HSC-1 cells with inhibitors of signaling molecules that are known to function downstream of the EGFR. First, we examined the effects of the inhibitors on the phosphorylation status of their target molecules. Immunoprecipitation analysis confirmed that 2 μM AG 1478 suppressed phosphorylation of the EGFR in HSC-1 cells (**Fig 6*a***). Furthermore, AG 1478 suppressed the phosphorylation of ERK1/2, Src, and Akt in HSC-1 cells (**Fig 6*a***). An ERK inhibitor, PD 98059, at a dose of 50 μM suppressed the phosphorylation of ERK1/2 but not of Src or of Akt. An Src inhibitor, PP2, at a dose of 20 μM, suppressed the phosphorylation of Src but not of ERK1/2 or of Akt. Finally, an Akt inhibitor, LY 294002, at a dose of 20 μM, suppressed the phosphorylation of Akt but not of ERK1/2 or of Src (**Fig 6*b***).

Next, we investigated the effects of these inhibitors on hTERT mRNA expression by RT-PCR analysis. Fifty micromoles of PD 98059 or 20 μM PP2 suppressed hTERT mRNA expression but 20 μM LY 294002 did not alter it (**Fig 6*c***). Telomerase

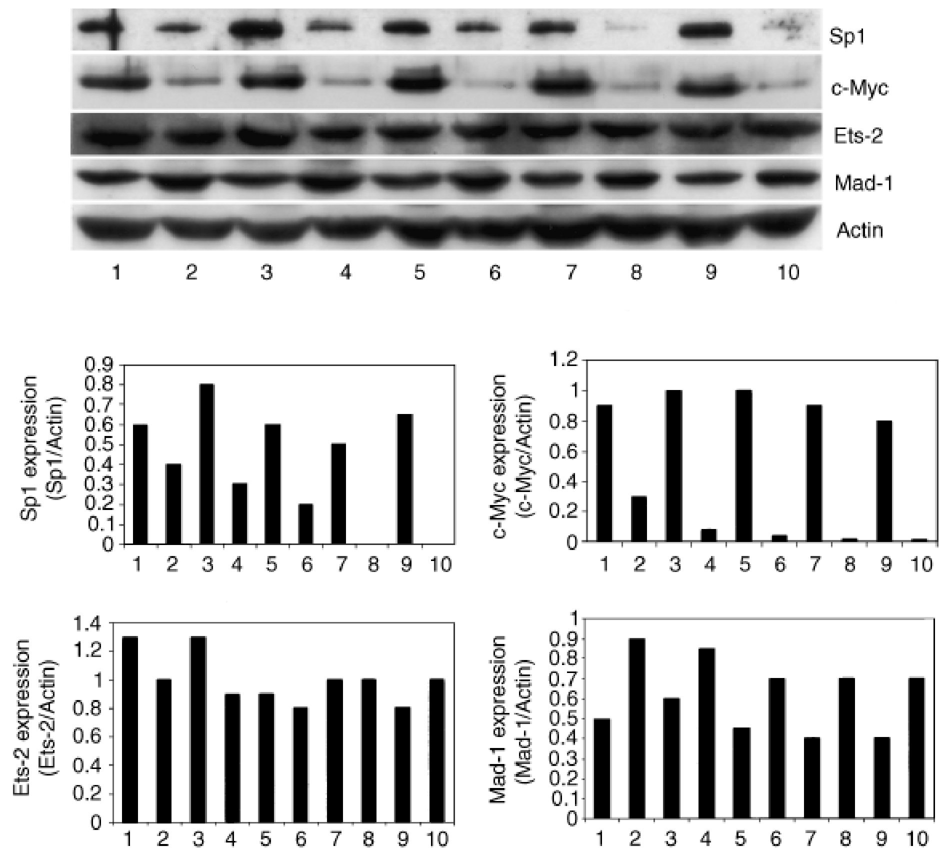


Figure 4. AG1478 reduced expression of c-Myc and of Sp1 but not of Ets-2 and slightly increased the expression of Mad-1 in HSC-1 cells. HSC-1 cells were incubated with 2 μM AG 1478. Treated cells were then extracted at different times along with control solvent-treated cells and western blot analysis of cell lysates was performed to detect the expression of c-Myc, Sp1, Ets-2, and Mad-1 proteins. The actin protein was used as an internal control for normalization of protein loading. *Lanes 1, 3, 5, 7,* and *9,* control solvent-treated cells at days 1–5, respectively; *lanes 2, 4, 6, 8,* and *10,* AG 1478 treatment at days 1–5, respectively. *Bottom panel*: relative levels of Sp1, c-Myc, Ets-2, and Mad-1 normalized to actin as determined by densitometry using NIH image analysis software.

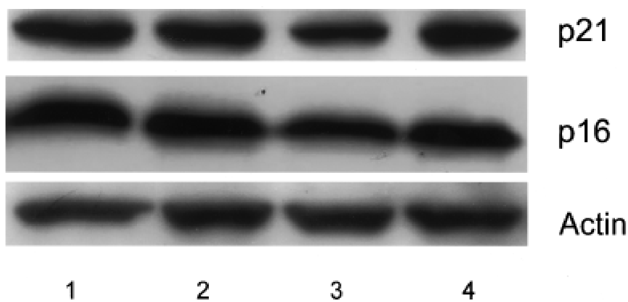


Figure 5. AG 1478 did not alter the expression of $p16^{INK4a}$ or $p21^{CIP1/WAF1}$ in HSC-1 cells. HSC-1 cells were incubated with 2 μM AG 1478 and were extracted at different times along with control solvent-treated cells. Western blot analysis of cell lysates was performed to detect the expression of $p16^{INK4a}$ and $p21^{CIP1/WAF1}$ proteins. The actin protein was used as an internal control for normalization of protein loading. *Lane 1,* solvent treatment; *lanes 2, 3,* and *4,* AG 1478 treatment at days 3, 4, and 5, respectively.

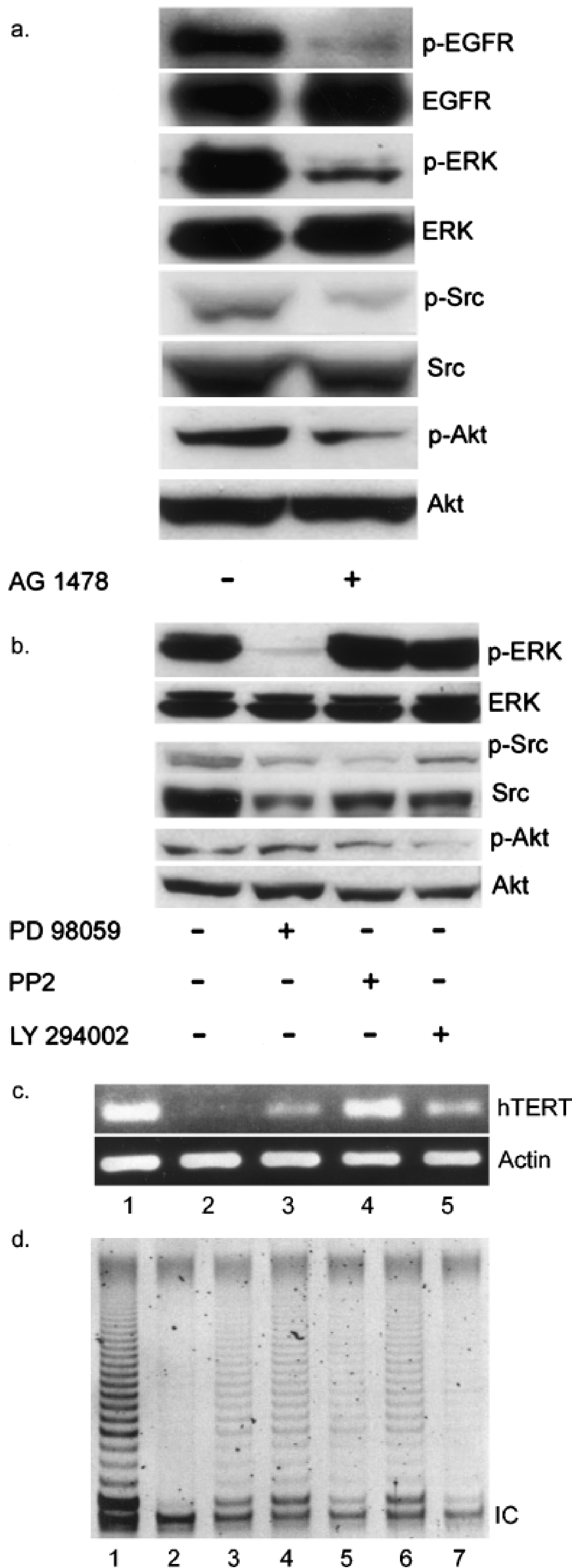
activity of HSC-1 cells after treatment with these inhibitors was also measured. The ERK inhibitor reduced the telomerase activity (**Fig 6*d***). Similar results were also observed when HSC-1 cells were treated with an Src inhibitor or with an Akt inhibitor. The extent of the suppressive effects on telomerase by each of these inhibitors alone was less than that elicited by AG 1478, however. Combinations of the inhibitors (PD 98059 + PP2 or PD 98059 + LY 294002) suppressed telomerase to a similar extent to AG 1478 (**Fig 6*d***).

EGF and TGF-α, ligands for the EGFR, induced telomerase activity in serum-starved HaCaT cells but not in serum-starved HSC-1 cells HSC-1 cells cultured in low serum medium (0.5% FBS) showed a suppression of telomerase activity to about 50% the level found in cells cultured in normal serum medium (10% FBS). Addition of EGF or TGF-α did not increase telomerase activity in HSC-1 cells (**Fig 7*a***). In contrast, HaCaT cells cultured in low serum medium showed a decrease of telomerase activity to about 30% that of the control and the addition of EGF or TGF-α could restore it (**Fig 7*b***).

DISCUSSION

Our results indicate that signaling from the EGFR is important for the activation of telomerase in HSC-1 cells. The suppression of telomerase correlated with the decrease of hTERT mRNA expression, which was presumably due to the suppressed expression of c-Myc and Sp1. The decrease of hTERT, Sp1, and c-Myc at earlier time points than telomerase is consistent with the fact that telomerase is a very stable complex with a half-life of more than 24 h. The increased expression of the c-Myc antagonist Mad-1 could also have a suppressive effect on hTERT transcription. On the other hand, the expression of Ets-2 was not altered after treatment of HSC-1 cells with AG 1478. This is consistent with a previous study that showed that levels of Ets expression were not altered by treatment with EGF (Maida *et al*, 2002). Modifications of the Ets protein, such as phosphorylation, will be necessary to investigate the contribution of Ets to responses elicited by AG 1478.

AG 1478 inhibited the proliferation of HSC-1 cells. There are several studies on the link between telomerase activity and cell proliferation (Holt *et al*, 1996; Zhu *et al*, 1996; Maida *et al*, 2002). One study showed that telomerase activity was repressed when cells were growth arrested in either a quiescent state or a differentiated state (Holt *et al*, 1996), whereas another study showed that quiescent and dividing cells have similar levels of telomerase activity (Zhu *et al*, 1996). One recent study showed that EGF activated telomerase in NIH3T3 cells that overexpressed the



EGFR and in A-431 cells (Maida *et al*, 2002). EGF stimulated the growth of NIH3T3 cells, however, but inhibited the growth of A-431 cells, suggesting that there was no direct relationship between telomerase activity and cell proliferation (Maida *et al*, 2002). It is thus of interest to know whether growth suppression was caused directly by the inhibited telomerase activity in HSC-1 cells. This will require further study using methods to inhibit telomerase specifically, such as an antisense oligonucleotide to hTERT.

The roles of signaling molecules activated by the EGFR, including ERK, Src, and Akt, on the telomerase activity of HSC-1 cells were examined using specific inhibitors. Inhibitors of ERK, Src, or Akt inhibited telomerase activity, but to a lesser extent than did the EGFR inhibitor. Combinations of inhibitors (ERK inhibitor + Src inhibitor or ERK inhibitor + Akt inhibitor) suppressed telomerase activity to a level similar to the suppression elicited by the EGFR inhibitor, suggesting that the simultaneous activation of several downstream signaling components of the EGFR is involved in the full activation of telomerase in HSC-1 cells. Consistent with this notion, AG 1478 was able to inhibit the phosphorylation of ERK, Src, and Akt.

A previous study showed that the induction of telomerase elicited by progesterone could be suppressed by an ERK inhibitor, indicating the crucial role of ERK signaling for this effect (Wang *et al*, 2000). The hypoxia-induced increase in telomerase in A2780 and in HT-29 cells could also be repressed by an ERK inhibitor (Seimiya *et al*, 1999). Furthermore, the upregulation of telomerase in regenerating hepatocytes after partial hepatectomy was repressed by an ERK inhibitor but not by a p38 MAPK inhibitor (Inui *et al*, 2002). Regarding Src, it has been known to be activated by EGF (Osherov and Levitzki, 1994; Xian *et al*, 1997) and Src activation has been shown to be crucial for tumor promotion during skin carcinogenesis (Xian *et al*, 1997). Our finding that an Src inhibitor reduced telomerase activity in HSC-1 cells contradicts a previous study that showed that chicken neuroretina cells transfected with v-src did not have increased activity of telomerase (Falchetti *et al*, 1999). This discrepancy might be due to differences in the types of cells studied. Our results showed that an ERK or an Src inhibitor suppressed hTERT mRNA expression in HSC-1 cells. In contrast, we found that an Akt inhibitor suppressed telomerase activity but not hTERT mRNA expression. This is consistent with previous studies, which revealed that Akt increased telomerase activity not through transcription of the hTERT gene, but through phosphorylation of the hTERT

Figure 6. Inhibitors for ERK, Src, or Akt suppressed telomerase activity in HSC-1 cells. (*a*) Immunoprecipitation using the EGFR antibody, following western blotting with a phosphotyrosine antibody or an EGFR antibody, demonstrated that AG 1478 suppressed the phosphorylation of EGFR. Western blots using phospho-ERK1/2 (p-ERK), phospho-Src (p-Src), and phospho-Akt (p-Akt) showed that AG 1478 also suppressed the phosphorylation of ERK1/2, Src, and Akt. (*b*) Western blots using phospho-ERK1/2 (p-ERK), phospho-Src (p-Src), and phospho-Akt (p-Akt) antibodies show the specificity of the ERK inhibitor (PD 98059), the Src inhibitor (PP2), and the PI3K/Akt inhibitor (LY 294002), respectively. (*c*) RT-PCR analysis shows the effects of those inhibitors on hTERT mRNA expression in HSC-1 cells. HSC-1 cells were treated with the inhibitors, and the treated cells were then extracted for RNA at different times along with control solvent-treated cells and RT-PCR assays were performed using LT5/LT6 primers for the expression of hTERT mRNA. An actin mRNA was used as an internal control. *Lane 1*, control; *lane 2*, AG 1478; *lane 3*, ERK inhibitor; *lane 4*, Akt inhibitor; *lane 5*, Src inhibitor. (*d*) TRAP assays show the effect of the inhibitors on telomerase activity in HSC-1 cells. HSC-1 cells were treated with the inhibitors as noted. Treated cells were then extracted at day 5 along with control solvent-treated cells and TRAP assays were performed to measure telomerase activity. *Lanes 1–7*, control solvent-treated cells, 2 μM AG 1478, ERK inhibitor, Src inhibitor, ERK + Src inhibitor, Akt inhibitor, and ERK + Akt inhibitor, respectively. IC, internal control.

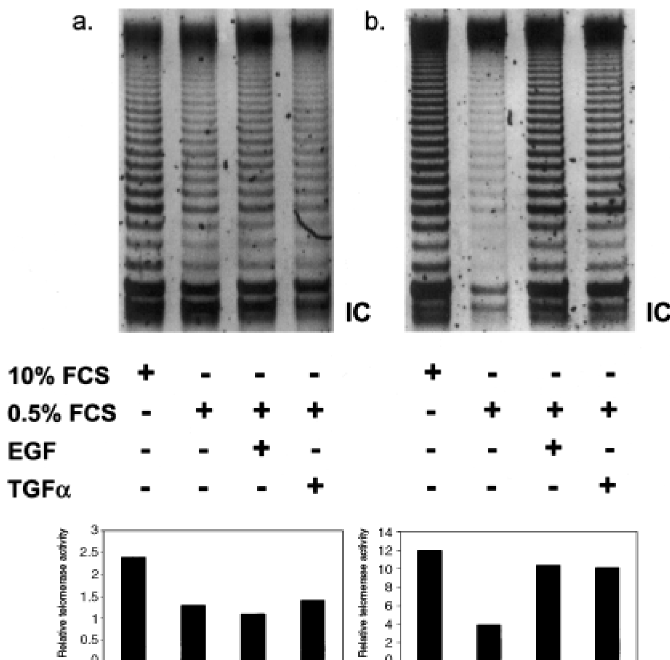


Figure 7. EGF and TGF-α, ligands for the EGFR, did not increase telomerase activity in HSC-1 cells. In contrast, these EGFR ligands increased telomerase activity in HaCaT cells. HSC-1 cells (*a*) or HaCaT cells (*b*) were incubated with low serum medium (DMEM supplemented with 0.5% FBS) for 3 d. The cells were then treated with 10 ng per ml EGF or TGF-α and were extracted at day 2 along with control cells incubated with normal serum medium (DMEM supplemented with 10% FBS). TRAP assays were performed to measure telomerase activity. Relative telomerase activity was assessed by determining the ratio of the entire telomerase ladder to the internal control using NIH image analysis software (version 1.62f). IC, internal control.

protein. Furthermore, an Akt inhibitor was shown to downregulate both hTERT peptide phosphorylation and telomerase activity (Kang *et al*, 1999; Akiyama *et al*, 2002). Taken together, the inhibition of telomerase activity in HSC-1 cells elicited by AG 1478 was due to the accumulating effects of inhibiting ERK, Src, and Akt, but the inhibition of hTERT mRNA expression in these cells by AG 1478 was possibly due to the inhibition of ERK and Src.

The introduction of $p21^{CIP1/WAF1}$ into cells, including keratinocytes, inhibits telomerase activity (Kallassy *et al*, 1998; Harada *et al*, 2000; Wang *et al*, 2000). The introduction of $p16^{INK4a}$ into glioma cells also inhibits telomerase (Fuxe *et al*, 2000), and inactivation of $p16^{INK4a}$ is crucial for the immortalization of keratinocytes (Kiyono *et al*, 1998). We examined whether the suppression of telomerase by AG 1478 is accompanied by an increase of $p21^{CIP1/WAF1}$ and/or $p16^{INK4a}$ protein but we found no significant increases, which indicates that these proteins are not involved in the inhibitory effect of AG 1478 on telomerase activity in HSC-1 cells.

Our results showed that serum starvation in the culture medium suppressed telomerase activity to about 50% the level in control HSC-1 cells. Addition of EGF or TGF-α to the medium did not increase telomerase activity, suggesting that other ligands of the EGFR, such as heparin-binding EGF-like growth factor, amphiregulin, and/or epiregulin, might be involved in activating the EGFR in these cells (Hashimoto, 2000). In contrast, premalignant HaCaT keratinocytes showed a decrease of telomerase activity (about 30% of the control) by serum starvation. Furthermore, EGF or TGF-α restored the activity, indicating that signaling through the EGFR is also important for activating telomerase in HaCaT cells and that EGF and TGF-α can function as ligands for the EGFR in these cells. Less of a reduction in telomerase activity by low serum levels in HSC-1 cells compared with HaCaT cells might indicate the presence of a constitutive mechanism for activation of the EGFR in HSC-1 cells. A mutant EGFR, such as EGFRvIII (Lorimer and Lavictoire, 2001), that has constitutive tyrosine kinase activity, was detected in glioblastoma cells. In HSC-1 cells, the molecular weight of the EGFR detected by immunoprecipitation was that of normal EGF (175 kDa), not of EGFRvIII (two bands of 150 and 140 kDa), which rules out the possibility that these cells have EGFRvIII. The different degree of telomerase suppression by the low serum level may also reflect the different malignant potentials of HSC-1 cells (fully malignant) and HaCaT cells (premalignant). The reversible telomerase activation in HSC-1 cells and in HaCaT cells cultured in low serum may provide a suitable experimental model to detect growth factors and/or environmental factors that regulate telomerase and might allow their molecular mechanisms to be unveiled. We conclude that drugs that block EGFR signaling will show antitumor effects for cutaneous SCC by decreasing telomerase activity.

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