Available online at www.sciencedirect.com

SCIENCE DIRECT

Experimental Cell Research 305 (2005) 300–311

Experimental Cell Research

www.elsevier.com/locate/yexcr

Survivin enhances telomerase activity via up-regulation of specificity protein 1- and c-Myc-mediated human telomerase reverse transcriptase gene transcription

Teruo Endoh, Naoki Tsuji, Koichi Asanuma, Atsuhito Yagihashi, Naoki Watanabe*

Department of Clinical Laboratory Medicine, Sapporo Medical University, School of Medicine, South-1, West-16, Chuo-ku, Sapporo 060-8543, Japan

Received 17 September 2004, revised version received 16 December 2004

Abstract

Suppression of apoptosis is thought to contribute to carcinogenesis. Survivin, a member of the inhibitor-of-apoptosis family, blocks apoptotic signaling activated by various cellular stresses. Since elevated expression of survivin observed in human cancers of varied origin was associated with poor patient survival, survivin has attracted growing attention as a potential target for cancer treatment. Immortalization of cells also is required for carcinogenesis; telomere length maintenance by telomerase is required for cancer cells to proliferate indefinitely. Yet how cancer cells activate telomerase remains unclear. We therefore examined possible interrelationships between survivin expression and telomerase activity. Correlation between survivin and human telomerase reverse transcriptase (hTERT) expression was observed in colon cancer tissues, and overexpression of survivin enhanced telomerase activity by up-regulation of hTERT expression in LS180 human colon cancer cells. DNA-binding activities of specificity protein 1 (Sp1) and c-Myc to the hTERT core promoter were increased in survivin gene transfectant cells. Phosphorylation of Sp1 and c-Myc at serine and threonine residues was enhanced by survivin, while total amounts of these proteins were unchanged. Further, "knockdown" of survivin by a small inhibitory RNA decreased Sp1 and c-Myc phosphorylation. Thus survivin participates not only in inhibition of apoptosis, but also in prolonging cellular lifespan.

Keywords: Survivin; Colon cancer cells; Telomerase; Human telomerase reverse transcriptase (hTERT); Specificity protein 1 (Sp1); c-Myc; Phosphatidylinositol 3 kinase (PI3 kinase)

Introduction

Suppression of apoptosis is thought to contribute to carcinogenesis by several mechanisms including aberrant prolongation of the cellular lifespan, which facilitates the accumulation of gene mutations and permits growth factor-independent cell survival [1]. In addition, since the host's immune surveillance system eliminates cancer cells by induction of apoptosis, inhibition of apoptosis is important for cancer cells to survive under immune cell attack. Several proteins involved in inhibition of apoptotic signaling have been identified, including the *bcl-2* family and the inhibitor of apoptosis protein (IAP) family [2,3]. Survivin, a member of the IAP family, has been shown to inhibit activation of downstream effectors of apoptosis, caspase-3 and -7, in cells exposed to apoptotic stimuli [4–7]. Since elevated expression of survivin observed in human cancers of various origins such as breast [8], esophagus [9], stomach [10,11], pancreas [12], and colon [13,14] was associated with poor patient survival [9,11,13,14], survivin has attracted growing attention as a possible point of therapeutic attack in cancer treatment.

An additional process required for carcinogenesis is immortalization of cells. In normal human cells, the nucleoprotein complexes at chromosome ends, called telomeres, shorten by some 50 to 200 bp with each cell division [15–18] as terminal sequences are lost in the course of DNA replication. When telomeres become sufficiently short, cells either enter irreversible cell cycle arrest, termed

* Corresponding author. Fax: +81 11 622 7502.
E-mail address: watanabn@sapmed.ac.jp (N. Watanabe).

0014-4827/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2004.12.014

senescence, or undergo apoptosis. Cancer cells usually can proliferate indefinitely, reflecting the ability to maintain telomeres during ongoing proliferation.

Telomerase, a ribonucleoprotein complex, helps to stabilize telomere length by adding TTAGGG repeats to telomeres [19]. Telomerase activity has been detected in almost all human tumors including those of the stomach [20] and colon [21], but not adjacent normal cells [22,23]. These results suggest that cancer cells stabilize their telomeres primarily through activation of telomerase.

Human telomerase is composed of human telomerase RNA, telomerase-associated protein 1, and human telomerase reverse transcriptase (hTERT) [19,24]. It has been reported that ectopic expression of hTERT in normal human cells results in an extended lifespan [25], while expression of dominant-negative hTERT in human cancer cells resulted in telomerase inactivation and telomere shortening [26,27]. Accordingly, hTERT is believed to be a major determinant of telomerase activity. Although up-regulation of hTERT gene expression has been observed in various cancer cells [28], mechanisms causing cancer cells to express hTERT are not well defined.

We hypothesized that inhibition of apoptosis and cellular immortalization may be intimately linked, very likely by survivin. We previously demonstrated that survivin enhanced Fas ligand (FasL) expression by up-regulating transcription of the FasL gene [29]. This finding depicts survivin as a multifunctional protein and suggests the possibility that survivin might be involved in regulation of other genes expression including hTERT.

In the present investigation of regulation of telomerase by survivin in human colon cancer cells, telomerase activity proved to be enhanced significantly by survivin, via increased Sp1- and c-Myc-dependent hTERT gene transcription.

Materials and methods

Patients and frozen tissue samples

Colorectal adenoma and cancer tissues, as well as adjacent normal colorectal mucosa counterparts, were obtained from patients undergoing surgery at Sapporo Medical University Hospital or Hokkaido Gastroenterology Hospital (Sapporo, Japan). Before the acquisition of these tissues, informed consent was obtained explaining the investigational nature of the study. Tissues were immediately frozen and stored in liquid nitrogen. Tissues were also stained with hematoxylin/eosin (Merck KGaA, Darmstadt, Germany), and were reviewed by well-experienced gastrointestinal pathologists.

Cell culture

Human colonic adenocarcinoma cell lines, SW480, LS180, HT-29, Caco2, and Colo320HSR were obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and grown at 37°C in a humidified atmosphere of 5% CO_2.

Quantification of survivin and hTERT mRNA

The expression of survivin and hTERT mRNA was determined by a quantitative RT-PCR using an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA). Isolation of total RNA and synthesis of cDNA were performed using ISOGEN reagent (Nippon Gene, Toyama, Japan) and TaqMan Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's protocol, respectively. Gene-specific primers and fluorescent hybridization probes used in quantitative PCR were as follows: survivin forward primer, 5′-AAG AAC TGG CCC TTC TTG GA-3′; reverse primer, 5′-CAA CCG GAC GAA TGC TTT T-3′; and probe, 5′-(FAM) CCA GAT GAC GAC CCC ATA GAG GAA CA (TAMRA)-3′, hTERT forward primer, 5′-ACG GCG ACA TGG AGA ACA A-3′; reverse primer, 5′-CAC TGT CTT CCG CAA GTT CAC-3′; and probe, 5′-(FAM) CTC CTG CGT TTG GTG GAT GAT TTC TTG TTG (TAMRA)-3′. Two splice variants of survivin, survivin-2B (retaining a part of intron 2 as a cryptic exon) and survivin-Δ Ex3 (lacking exon 3), were not detected by this set of primers and probe [30]. Quantitative RT-PCR was performed using a TaqMan Universal PCR Master Mix (Applied Biosystems) as described previously [29]. To compare amounts of mRNA encoding survivin and hTERT in different samples, the quantity of specific mRNA was normalized as a ratio to the amount of 18S ribosomal RNA (18S rRNA) [31], which was determined using TaqMan Ribosomal RNA Control Reagents (Applied Biosystems) according to manufacturer's protocol. PCR products were also confirmed by agarose gel electrophoresis.

Immunostaining

Immunostaining for survivin and hTERT was performed using a standard three-stage indirect immunoperoxidase technique on 5-μm serial tissue sections. Briefly, fixed tissue sections were rehydrated in graded alcohols and then rinsed in a running water bath, with endogenous peroxidase activity quenched by preincubating slides in 3% hydrogen peroxide in a light-impermeable chamber. After washing in phosphate-buffered saline (PBS), slides were incubated for 10 min at 121°C, 20 min in blocking solution (5% skim milk and 0.15% H_2O_2 in deionized water). After washing slides with PBS, rabbit anti-human survivin polyclonal antibody (pAb, Novus Biologicals, Littleton, CO) or mouse anti-human hTERT IgG monoclonal antibody (mAb, Novocastra Laboratories, Newcastle, UK) was applied at a 1:125 dilution, and tissues were incubated for 1 h in a humid chamber. After washing again in