Biochemical and Biophysical Research Communications 405 (2011) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Efficacy of TRAIL treatment against HPV16 infected cervical cancer cells undergoing senescence following siRNA knockdown of E6/E7 genes

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ARTICLE INFO

Article history:
Received 8 December 2010
Available online 16 December 2010

Keywords:
Drug-resistant cervical cancer
Tumor necrosis factor-related apoptosis inducing ligand (TRAIL)
siRNA therapy
Combinatorial cancer treatment
Apoptosis
Senescence

ABSTRACT

In this study we investigated E6 and E7 oncogenes from the Human Papilloma Virus as targets for siRNA knockdown in order to boost the efficacy of the anti-cancer drug 'tumor necrosis factor-related apoptosis inducing ligand' (TRAIL). SiHa cells were treated with TRAIL following transfection with E6/E7 siRNA and the expression of death receptors DR4 and DR5, cell viability, apoptosis, senescence and cell cycle analysis were undertaken using flow cytometry, MTT viability assay and cellular β-galactosidase activity assays. E6/E7 siRNA resulted in significant upregulation of death receptors DR4 and DR5 but did not result in an enhanced sensitivity to TRAIL. Our results indicate that E6/E7-siRNA induces senescence rather than apoptosis in SiHa cells. The occurrence of senescence in drug resistant cervical cancer cells such as the SiHa cell line by E6/E7 siRNA, among other factors, may prevent TRAIL induced activation of extrinsic and intrinsic pathways that lead to apoptotic cell death. Our findings are significant for combinatorial strategies for cancer therapy since the induction of senescence can preclude apoptosis rendering cells to be recalcitrant to TRAIL treatment.

1. Introduction

Every year, about half a million cases of cervical cancer are diagnosed worldwide [1]. According to the CDC, in the United States alone nearly 12,000 women were diagnosed with cervical cancer in 2006 and almost 4000 died [2]. In many developing countries, where 80% of new cases arise, it is the leading female malignancy and a common cause of death for middle-aged women [3,4]. Through epidemiological studies, it has been discovered that the sexually transmitted Human Papilloma Virus (HPV) is the etiological agent for cervical cancer [5,6]. HPV is a double-stranded DNA virus that infects human epithelial tissue resulting in warts, or in extreme cases, cancer. In a worldwide study, it was discovered that HPV DNA was detected in 93% of cervical tumors [7]. HPVs are divided into low-risk and high-risk categories according to their ability to transform epithelial cells and induce cancer. Examples of low-risk HPVs are HPV 6 and 11 which are responsible for 90% of genital warts whereas the high-risk subtypes such as HPV 16 and 18 are responsible for a combined 70% of cervical cancers [8]. HPV 16 is by far the most prevalent HPV that causes cervical cancer, being associated with about 50% of the cases.

The ability of HPV to transform normal epithelial tissue and the causative process have been well studied [9]. The HPV genome encodes for six early expressed genes (E1, E2, E4, E5, E6 and E7) which are associated with viral replication, and two late expressed genes (L1 and L2) for viral capsid proteins. Malignancy occurs through the pleiotropic transforming effects exerted by both E6 and E7 viral proteins. The *de novo* expressed E6 oncoprotein forms a complex with p53 and the ubiquitin-ligase enzyme which leads to the ubiquitination of p53 and its subsequent proteosomal degradation [10]. The removal of p53 allows cells to replicate indefinitely through the impairment of the of cells' ability to initiate apoptosis since p53 is no longer able to detect DNA damage and regulate the G_1/S cell cycle checkpoint. The E7 oncoprotein sequesters hypophosphorylated Retinoblastoma (pRb) which is a negative regulator of the transcription regulator E2F and promotes its eventual proteosomal degradation. As pRb is lost, E2F is free to release transcription factors that promote cell cycle progression and the eventual clonal expansion of transformed cells [1,11]. Expression of either E6 or E7 alone does not guarantee carcinogenesis; however, when they work in unison the chances that a cell will become oncogenic is drastically increased [11].

Among the newer strategies under investigation for targeted treatment of cancer, blocking viral oncogene expression using RNA interference (RNAi) offers the promise of high specificity combined with limited undesired side effects. RNAi is a gene silencing mechanism in which double stranded RNA molecules, known as

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doi:10.1016/j.bbrc.2010.12.056

short interfering RNA (siRNA), can block translation of gene specific mRNA through complementary base pairing [12]. For cervical cancer therapy, inhibition of E6 and E7 expression by siRNA in HPV transformed cells can potentially restore p53 and pRb function leading to restoration of the cell cycle checkpoints. Unfortunately, reports of the induction of apoptosis in E6/E7 siRNA treated cervical cells are inconsistent. RNA interference of E6 and E7 expression has shown some success in cultured cervical cancer cells with a gain of function observed for both p53 and pRb and subsequently, apoptosis [13]. However, in other studies, the siRNA-mediated gene silencing of E6 or E7 did not induce apoptosis, but rather exhibited only reduced cell proliferation [14]. Other cervical cancer cell lines such as the HPV 18 infected HeLa cells, when transfected with E6/E7 siRNA, undergo cellular senescence [13].

The targeted induction of apoptosis in cancer cells can be achieved using tumor necrosis factor-related apoptosis inducing ligand (TRAIL). TRAIL is a membrane bound and soluble ligand from the tumor necrosis factor family that has been the focus of basic as well as clinical research for cancer therapy due to its ability to promote apoptosis in cancerous cells while leaving healthy cells relatively unaffected [15,16]. TRAIL acts by binding to its own set of cellular receptors and is capable of inducing apoptosis through both an extrinsic and intrinsic mechanism [17]. There are two functional death receptors (DR), DR4 and DR5, that become active when TRAIL binds the receptors resulting in caspase-dependent apoptosis [18,19]. TRAIL can also activates the intrinsic pathway of apoptosis after binding the death receptors by promoting the Bcl2 family dependent release of cytochrome C from the mitochondria, formation of the apoptosome and subsequent caspase cascade, complementing p53 apoptotic activity [20,21]. The discovery of the presence of DR4 and DR5 in cervical neoplasia specimens suggests that therapy using TRAIL may be efficacious [22]. The *in vitro* effects of TRAIL on cultured cervical cancer cells have been mixed; while HeLa and CaSki cells show varying degrees of cytotoxicity, SiHa cells are not responsive [16,23].

In this study, we explore the strategy of a synergistic approach for the treatment of non-responsive SiHa cervical cancer cells involving induction of apoptosis specifically using a combination of siRNA and TRAIL. Our initial hypothesis was that the siRNA knockdown of E6 and/or E7 would prevent proteosomal degradation of p53 in SiHa cells and lead to the transactivation of the death receptor expression on the cell surface, subsequently sensitizing these cells to TRAIL and leading to induction of apoptosis. Contrary to our premise, we report here that the knockdown of E6/E7 genes in SiHa cervical cancer cells by siRNA leads to senescence rather than apoptosis and does not enhance sensitivity to TRAIL. To understand the mechanisms of this phenomenon, we compared our results to the effects of MG132, a proteosome inhibitor which can recover p53, increase DR5 expression, and lead to sensitization of SiHa cells to TRAIL [23]. Our results indicate that during combinatorial approaches for cancer therapy, the induction of senescence may preclude apoptosis, thus rendering cells recalcitrant to TRAIL treatment.

2. Materials and methods

2.1. Transfection of SiHa Cells

The human cervical cell line SiHa was obtained from the American Type Culture Collection (Manassas, VA) and cultured according the supplier's instructions. Transfections were performed with 10 nM of E6/E7 siRNA using HiPerfect Transfection Reagent (Qiagen, Valencia, CA). The guide strand sequence for E6 siRNA was 5′-AGACAUACAUCGACCGGUCCCA-3′ (base pair position 397, Naito et al. [24,25]). The guide strand sequence for E7 siRNA was 5′-CAUUCGUACUUUGGAAGACCUGUUA-3′ (base pair position 225) and its design is based on Stealth technology (Invitrogen, Carlsbad, CA). Two rounds of transfections were performed for each experiment; 1 and 4 days after plating the cells (See Table 1, Supplementary material). A medium-content GC (MGC) scrambled siRNA (Invitrogen) was used as the negative transfection control throughout the experiments. Western Blot Analysis of p53 levels was undertaken as mentioned in the Supplementary material section.

2.2. Flow cytometry analysis

Cells were analyzed for the expression of death receptors DR4 and DR5 by flow cytometry following transfection with E6/E7 or treatment with 10 μM MG132 according to Hougardy et al. [23]. For the analysis of apoptosis, cells were treated with siRNA, MG132 and TRAIL (see below) and stained with Annexin V (eBiosciences, San Diego, CA) and propidium iodide (Roche, Basel, Switzerland). For the analysis of cell cycle, siRNA, MG132 and TRAIL treated cells were fixed and stained with propidium iodide (Roche). The methods used are described in detail in the Supplementary material.

2.3. TRAIL treatment and analysis of cell viability by MTT assay

SiHa cells were plated 2500 cells per well in a 96 well cell culture plate in triplicates and transfected. Twenty-four hours after the second transfection, 1 μg/ml human recombinant TRAIL (Calbiochem/EMD Biochemicals, Gibbstown, NJ) was added to the wells and incubated overnight. For the MG132 treatment, cells were pretreated with 10 μM MG132 for 2 h and then TRAIL was added. Following a 24 h incubation, viability of the cells was analyzed with an MTT cell proliferation kit (Trevigen, Gaithersburg, MD).

2.4. Senescence activated β-galactosidase staining

Cells were stained for β-galactosidase activity using the Senescent Cells Histochemical Staining Kit (Sigma–Aldrich, St. Louis, MO). Cells were transfected as mentioned before and analyzed for β-galactosidase activity on the sixth day. Brightfield images (10×) were scored based on percentages of stained/unstained cells.

3. Results and discussion

3.1. Expression of death receptors in response to knockdown of E6 and E7 genes

Transfection of SiHa cells with 10 nM of E6 and E7 siRNA results in a marginal decrease in cell viability over a 4 day period, as measured by MTT assay, in comparison to controls which were transfected with control siRNA or incubated with the transfection reagent alone. A recovery of cell viability is observed for E6/E7 siRNA treated cells from day five onwards (data not shown). Previous studies have shown that multiple siRNA transfections over a week are necessary to obtain a robust suppression of E6/E7 gene expression in SiHA cells [26]. Based on these findings and our results, a second transfection was carried out on day 3 to provide a sustained knockdown of E6 and E7 proteins. Expression of cell surface DR4 and DR5 proteins were analyzed by flow cytometry 1 day after the second transfection. A significant increase in the levels of death receptors was observed for cells transfected with E6/E7 siRNA or treated with the proteosomal inhibitor MG132 as compared to control siRNA transfected cells (Fig. 1A). The percentage shifts in the geometric mean of the flow cytometry profiles indicate that