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Inhibition of growth of cervical cancer cells using a dominant negative estrogen receptor gene

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Abstract

Objective—Estrogen stimulates human papilloma virus oncogene expression, promotes cervical cancer (CC) cell proliferation and prevents apoptosis. Therefore, blockage of estrogen function may have therapeutic application to CC.

Methods—CasKi CC cells were transfected with an adenovirus expressing a dominant negative estrogen receptor gene (Ad-ER-DN) and their responses were investigated by RT-PCR, Flow Cytometry and Western blot assays.

Result—Transfected cells showed disturbance of cell colony morphology, reduced HPV E6 and E7 mRNA, interruption of cell proliferation, reduced cyclin D1 protein and expression of apoptosis.

Conclusion—We report, for the first time, the use of Ad-ER-DN to block estrogen receptors which led to dramatic changes in CC cells that are consistent with the possible reactivation of cellular p53 and Rb function. Their reactivation most likely allowed the recognition of existing chromosome abnormalities as a serious stress signal and the initiation of a cascade of cellular events in response to the stress, including the activation of the core apoptotic machinery which led to self-destruction of the CC cells.

Keywords

cervical cancer; estrogen receptor; estrogen; molecular intervention; gene therapy; cyclin D1; HPV; dominant negative estrogen receptor gene

Introduction

Cervical cancer (CC) is the second leading cause of cancer morbidity and mortality for women around the world, especially in many developing countries. To reduce the high disease burden, the development of effective prevention and therapeutic procedures is needed. With the advancement of molecular techniques, innovative intervention and therapeutic procedures can be developed for a variety of cancers [1,2].

It is well-known that infection with high risk human papilloma virus (HPV) is the predominant risk factor for CC. Since most infected females do not develop the disease, other factors must contribute to the initiation of the cancer. A contributing risk factor is chronic estrogen exposure

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because the risk for CC is increased 2–4 times for women with extended use of oral contraceptives and 4 times for women having 7 or more children [3,4]. Although estrogen is a human carcinogen for a variety of cancers, its effect on CC has not received much attention. However, in a transgenic mouse model that expresses HPV16, administration of estrogen is needed to cause the initiation of CC and the continued availability of estrogen is essential to the growth of the tumors [5]. Similar observations were reported in another transgenic mouse model, HPV-18 URR E6/E7 [6]. Therefore, the estrogen pathway may be an important etiologic factor and a potential therapeutic target for CC [7].

Anti-estrogen chemotherapy has been used in breast cancer but rarely in CC [8]. In breast cancer, both ER-positive and -negative lesions have been responsive to anti-estrogen chemotherapy [9]. However, anti-estrogen chemotherapy has potential serious side effects as well as drug resistance problems [8]. Besides chemotherapy, novel molecular approaches such as gene therapy are being developed to block different stages of cancer process [10]. Some investigators have attempted to use the interference RNA (siRNA) to block the expression of HPV in CC [11,12]. The approach may, however, require the blockage of multiple targets, e.g. different HPVs and helper viruses [12,13]. The estrogen pathway presents a simple target with broad application to different types of CC and to other estrogen-dependent cancers.

In our study, we have used an adenovirus system to deliver a dominant negative estrogen receptor mutant gene (Ad-ER-DN) into CC cells to investigate their effect on HPV oncogene expression, cell proliferation and apoptosis. Among the different dominant negative ER mutants [14–18], the Ad-ER-DN we used has been shown to inhibit ERE-Luc transactivation in several cancer cell types [16, 17, our unpublished observations]. In addition, the mutated ER forms heterodimers with ER and inactivate both ER α and β [18]. We have also reported that the level of estrogen in the growth medium is quite enough to elucidate the effect of Ad-ER-DN without the need to add exogenous estradiol [19]. In the same study, we showed that Ad-ER-DN has therapeutic efficacy against uterine leiomyoma, an estrogen-dependent tumor of the uterus, in both in vitro and in vivo systems [19]. Here, we report for the first time that transfection of Ad-ER-DN into CC cells caused reduced HPV16 E6 and E7 mRNA and cyclin D1 protein, inhibition of cell proliferation and induction of apoptosis.

Materials and Methods

Cell line, vectors and transfection

CaSki CC cells which contain approximately 600 copies of HPV-16 genome per cell were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained as per ATCC instructions. Cultures were subcultured one day before the transfection, and harvested at 24, 48 and 72 hours later for laboratory analyses. At least two independent assays were conducted for each analysis. We used an adenoviral vector that carries the dominant negative ER mutant ER1-536 under CMV promoter [17], a generous gift from Dr. Eun J. Lee (Northwestern University, Feinberg School of Medicine, Chicago, Illinois). Based on our experience with using different kinds of cancer cells, CaSki was transfected with Ad-ER-DN or Ad-LacZ with approximately 1×10^8 pfu/100 mm plates in 1.5 ml medium with 2% serum. Ad-LacZ contained the LacZ bacterial gene therefore these transfected cells served as transfection controls. In addition, there were un-transfected controls. The transfected cultures were rocked gently in a CO₂ incubator for 4 hours to ensure even transfection to the cells. Then, complete culture medium was added to each of these cultures.

RT-PCR analysis for determination of HPV16 E6 and E7 expression

Untreated cultures and cultures transfected with Ad-ER-DN or Ad-LacZ were trypsinized and RNA was extracted using the RNA isolation kit from Ambion (Ambion Inc., Austin, TX). For

real time RT-PCR analysis, the Applied Biosystems Assays-by-Design 20× assay mix of primers and TaqMan MGB probe (FAMTM dye-labeled) was used for our target genes. The primer and probe sequences were:

HPV16E6: probe—CACGTCGCAGTAACTGT, forward primer—ATGATATAATATTAGAATGTGTGTACTGCAAGCA, reverse primer—GCATAAATCCCGAAAAGCAAAGTCA; HPV16E7: probe—CCAGCTGGACCATCTAT, forward primer—AGCTCAGAGGAGGAGGATGAA, reverse primer—CTCTGTCCGGTTCTGCTTGT

For the relative quantitation of gene expression, separate tubes (singleplex) in one-step RT-PCR were performed with 40ng RNA for both target genes and endogenous control, using the TaqMan one-step RT-PCR master mix reagent kit (P/N 4309169). The cycling parameters for one-step RT-PCR were: reverse transcription 48°C for 30 min, AmpliTaq activation 95 °C for 10 min, denaturation 95 °C for 15 sec and annealing/extension 60 °C for 1 min (repeat 40 times) on ABI7000. Duplicate C_T values were analyzed in Microsoft Excel using the comparative C_T($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta C_T}$) was obtained by normalizing to an endogenous reference (18s) and relative to a calibrator (one of the experimental samples) [20].

Flow cytometric analysis

Culture cells were trypsinized and processed according to published procedure [21]. Cells were washed with 0.1% sodium azide solution in phosphate buffered saline (Ca⁺⁺ and Mg⁺⁺ free) and stained with 0.05 mg/ml propidium iodide solution in hypotonic polyethylene glycol solution for 20 minute at 37°C. Then, the hypotonic solution was normalized by using a hypertonic sodium chloride solution. The latter cell suspension was maintained at 4°C overnight and then analyzed using the Becton-Dickinson FACS Canto instrument and standard programs for determination of cell cycle and apoptosis components in each cell sample (by our the Flow Cytometric Core Laboratory).

Western blot analysis

The procedure for the analysis has been described before [19]. Briefly, culture cells were washed and lysed. Protein was extracted and quantitated. Equal amounts of protein were resolved in 10% polyacrylamide gel, transferred to polyvinylidene fluoride paper and stained with primary and secondary antibodies. The mouse monoclonal anti-cyclin D1, mouse monoclonal anti-actin and rabbit anti-mouse monoclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used. The specificity of the antibody was validated using cyclin D1 positive control cells. Protein bands in the membrane were visualized using the horseradish peroxidase reaction and detected using X-Omat film (Eastman Kodak Co., Rochester, NY). The detected bands were quantitated using a digital image analyzer. The expression of cyclin D1 in each sample was normalized to that of actin in the same sample and standardized to that in the untreated control culture.

Results

Cell response to transfection

The CaSki CC cells responded specifically to Ad-ER-DN transfection. Under the inverted microscope, the boundaries for the cell colonies retracted drastically at 24 hours after transfection (Figure 1, middle left panel). At 48 and 72 hours, some cell divisions occurred again but the cultures did not regain the normal morphology (Figure 1, middle right panel). Such drastic cellular response was distinctly different from the concurrent untreated control (Figure 1, top left and right panels) and not observed in cultures transfected with the control

virus, Ad-LacZ (Figure 1, bottom left and right panels). We also conducted cell counts from the three experimental conditions. The Ad-ER-DN transfected cultures had significantly less cells at 48 and 72 hours than the others (data not shown).

Expression of HPV16 E6 and E7

As shown in Figure 2, Ad-ER-DN consistently caused approximately 50% reduction in both HPV16 E6 and E7 mRNA relative to that of the untreated controls, as determined by Real Time RT-PCR analyses. The inhibition occurred as early as 24 hours post-transfection and persisted up to 72 hours. The expression was normalized to internal 18S rRNA levels and compared to that of untreated control. On the other hand, LacZ transfection had no effect on the HPV E6 and E7 expression.

Cell proliferation and apoptosis

As shown in Figure 3, our Flow Cytometry analyses indicate that Ad-ER-DN blocked cell proliferation at the S-phase at 24 hour (D24 column) compared to the control (C column). The blockage is consistent with the drastic reduction of cells at the G2 phase of cell cycle. The S-phase blockage by Ad-ER-DN continued up to 48 hr (D48 column). During our observation duration, the frequency of G2 cells never reached higher than 2.5% compared to the untreated control of 11.5% and the Ad-LacZ controls of 9.5–14.5% from 24 to 72 hr. An important observation is that the induction of apoptosis was observed only in the Ad-ER-DN transfected cells. The frequencies of apoptotic cells increased from 0.05 to 6.9 and to 7.9% at 24, 48 and 72 hr, respectively (D24, D48 and D72 columns). The data indicate that the ER blockage caused cell cycle inhibition and induction of apoptosis.

Expression of cyclin D1

Recent reports suggested that the expression of cyclin D1, a major cell proliferation protein, is associated with estrogen-stimulation of growth in cervical tumors [22]. From our study (Figures 4a and b), there was a dramatic down-regulation of Cyclin D1 protein expression at 24, 48 and 72 hr after transfection with Ad-ER-DN but not after Ad-LacZ, indicating specific blockage of Cyclin D1 expression by Ad-ER-DN. The cyclin D1 expression data were normalized to that of internal actin and compared with concurrent untreated controls.

Discussion

The primary oncogenic mechanism of HPV is the release of viral E6 and E7 proteins that predominantly bind and inhibit the function of cellular tumor suppressor proteins, p53 and pRb, respectively. Their inhibition allows cells harboring genomic damage to survive and to evolve into cancer cells, instead of a fate of elimination and eradication [23,24]. Recent studies indicate that the cancer process is enhanced by estrogen (see Discussion below).

Estrogen activates soluble intracellular receptors (ER α and ER β) which stimulate transcription of specific genes [25]. The cellular consequences include alteration of cell adhesion and migration, increased cell proliferation and resistance to drug-induced apoptosis [25,26]. The estrogen effect may also be mediated via “crosstalk” with p53: “the overall effects of p53-ER crosstalk are negative, leading to the inactivation of p53 as well as ER”, and thus lack of cell cycle control [8,27]. In addition, estrogen interacts with HPV expression in infected cells. For example, exposure of HPV-positive CC cells to estrogen stimulates the expression of HPV E6 and E7 mRNA and cell proliferation, and prevents the induction of apoptosis [26,28,29]. A novel observation recently provides strong evidence that increased estrogen signaling is essential to the growth of cervical tumor, i.e. increased expression of aromatase, estrogen receptors and cyclin D1 are significantly associated with growth of tumors [22]. Hence it is conceivable that blocking the estrogen pathway, specifically the activity of ER, might be a

rational approach to reduce the expression of HPV E6 and E7, and alleviating the inhibition of p53 and pRb. Subsequently, one would speculate that the ER-blocked CC cells would stop proliferating and undergo apoptosis.

We report, for the first time, the use of a dominant negative gene (Ad-ER-DN) to block ER and thus, potentially, estrogen function in CC cells. The specific blockage caused coordinated and wide-spread cellular changes: disturbance of cell colony morphology, reduction of HPV E6 and E7 mRNA, interruption of cell proliferation, reduction of cyclin D1 protein and expression of apoptosis. These changes are consistent with the possible reactivation of p53 and Rb function. Their reactivation most likely allowed the CC cells to recognize existing chromosome alterations and genetic instability as a serious stress signal and to mount a cascade of cellular events in response to the stress. A crucial response is the activation of the core apoptotic machinery that leads to the self-destruction of the CC cells.

The observations may be useful in understanding the crucial molecular pathways that can be exploited for the development of gene therapy for cervical cancer and other estrogen-dependent cancers. Future studies may be designed to document the precise pathway to apoptosis in CC, enhance the induction of apoptosis and deliver the dominant negative gene to tumor cells in vivo.

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Figure 1.
Cell morphologies with and without transfection

Figure 2.

Expression of HPV E6 and E7 after transfection with Ad-ER-DN or Ad-LacZ.

D24, D48 and D72 = data collected at 24, 48 and 72 hours after transfection with Ad-ER-DN; LZ24, L48 and LZ72 = after transfection with the Ad-LacZ gene. Mean and standard deviation data were from 2 independent experiments.

Figure 3.

Cell cycle proliferation and apoptosis after transfection with Ad-ER-DN or Ad-LacZ. C = untreated control; L24, L48 and L72 = analyses conducted at 24, 48 and 72 hours after transfection with Ad-LacZ; D24, D48 and D72 = after transfection with Ad-ER-DN. G1, S and G2 represent cells in different phases of cell cycle. Apo = apoptosis. Mean and standard deviation data were from 2 independent experiments.

Figure 4. Western blot determination of Cyclin D expression after transfection with Ad-ER-DN or Ad-LacZ.

a. *Lanes 1 = untreated positive control at 72 hour; 2 = Ad-LacZ transfected after 24 hr; 3 = Ad-ER-DN after 24 hr; 4 = Ad-LacZ after 48 hr; 5 = Ad-ER-DN after 48 hr; 6 = Ad-LacZ after 72 hr; 7 = Ad-ER-DN after 72 hr

b. D = dominant negative estrogen receptor gene, L = LacZ gene. Mean and standard deviation data were from 2 independent experiments.