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Inhibition of the epidermal growth factor receptor by erlotinib prevents immortalization of human cervical cells by Human Papillomavirus type 16

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ABSTRACT

The Human Papillomavirus type-16 (HPV-16) E6 and E7 oncogenes are selectively retained and expressed in cervical carcinomas, and expression of E6 and E7 is sufficient to immortalize human cervical epithelial cells. Expression of the epidermal growth factor receptor (EGFR) is often increased in cervical dysplasia and carcinoma, and HPV oncoproteins stimulate cell growth via the EGFR pathway. We found that erlotinib, a specific inhibitor of EGFR tyrosine kinase activity, prevented immortalization of cultured human cervical epithelial cells by the complete HPV-16 genome or the E6/E7 oncogenes. Erlotinib stimulated apoptosis in cells that expressed HPV-16 E6/E7 proteins and induced senescence in a subpopulation of cells that did not undergo apoptosis. Since immortalization by HPV E6/E7 is an important early event in cervical carcinogenesis, the EGFR is a potential target for chemoprevention or therapy in women who have a high risk for cervical cancer.

Introduction

Cervical cancer is a major cause of death in women worldwide. The incidence is high in underdeveloped countries and in individuals who have low socioeconomic status or are immune compromised (Schiffman and Brinton, 1995). The major risk factor for cervical cancer is persistent infection with high-risk types of human papillomavirus (HPV) (Schiffman and Brinton, 1995). The HPV E6 and E7 genes from high-risk HPV types (HPV-16 and -18) are sufficient to immortalize human epithelial cells (Hawley-Nelson et al., 1989; Munger et al., 1989), and these genes are selectively retained and expressed in most cervical cancers (von Knebel Doeberitz et al., 1992). The majority of HPV infections are eliminated by the host's immune response, but individuals who have compromised immunity (AIDS patients or transplant recipients) develop persistent infections with multiple HPV types (Palefsky, 2009). Persistent infection with high risk HPVs and immortalization of infected cells are important early events in the development of cervical cancer.

Therapy for cervical cancer involves surgery, chemotherapy or radiation, and the 5-year survival for women with invasive cervical cancer is poor. In contrast, if the disease is detected early by PAP screening or HPV testing, the prognosis is good (Arbyn et al., 2009). Chemoprevention of cervical cancer has been explored in several clinical trials, but most agents have not been effective (Vlastos et al., 2003). Recently, prophylactic vaccines have been shown to prevent HPV infection (Campo and Roden, 2010). These vaccines induce type-specific immunity to the two most common high risk HPVs (HPV-16 and HPV-18) which account for approximately 70% of cervical cancers. However, they are not effective in women who have existing HPV infections. Due to the long latency of cervical carcinogenesis, the prophylactic vaccine is unlikely to have a major impact on the incidence of cervical cancer for many years. In addition, the vaccine may be less effective in women who are immune deficient and infected with multiple high-risk types (AIDS patients and transplant recipients) (McKenzie et al., 2010). Thus, women who have a high risk for cervical cancer would benefit from improved methods for therapy or chemoprevention that target signal pathways critical for cervical cancer development.

The epidermal growth factor receptor (EGFR) is a membrane tyrosine kinase expressed by most epithelial cells. Cervical epithelial cells secrete several EGF-like growth factors that activate the EGFR by autocrine and paracrine pathways (Woodworth et al., 1995). Ligand binding leads to receptor dimerization, tyrosine kinase activation and stimulation of intracellular signal pathways that regulate growth,

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survival, motility, and angiogenesis. EGFR signaling is important for normal cell function, but inappropriate activation or over expression can contribute to malignant development (Normanno et al., 2005). Over expression of the EGFR or mutations in the EGFR gene have been implicated in the pathogenesis of multiple types of human cancer. The EGFR is frequently over expressed in cervical dysplasia and cervical cancer, and patients who have high levels of EGFR in their tumors have a poor prognosis (Kersemaekers et al., 1999). The HPV-16 E6 and E7 proteins stimulate EGFR expression on epithelial cells (Akerman et al., 2001; Sizemore et al., 1998) and the HPV E5 protein increases recycling of the EGFR to the cell surface (Straight et al., 1995) and alters EGF endocytic trafficking (Suprynowicz et al., 2010). Targeted disruption of the EGFR gene inhibits development of papillomas and carcinomas from HPV-immortalized epithelial cells in mice (Woodworth et al., 2000). Together, these observations suggest that activation of the EGFR is important for the HPV life cycle and progression to cervical cancer.

Inhibitors of the EGFR have been used for therapy of several types of human cancer (Chen et al., 2009). The two major classes of inhibitors are monoclonal antibodies that interfere with ligand binding and small molecule kinase inhibitors that selectively block receptor activation. Erlotinib is a small molecule inhibitor that competes reversibly with ATP for binding to the tyrosine kinase domain of the EGFR. Erlotinib is administered to patients orally and the side effects are usually well tolerated (Li and Perez-Soler, 2009). Erlotinib has been approved by the FDA for treatment of recurrent non-small cell lung cancer and for first-line treatment of advanced pancreatic cancer with gemcitabine (Iyer and Bharthuar, 2010). Given the importance of EGFR signaling to the HPV life cycle, we investigated whether erlotinib had activity against HPV-infected cervical cells. We observed that erlotinib prevented immortalization of cultured human cervical epithelial cells by the complete HPV-16 genome or the E6/E7 genes. This response was associated with induction of apoptosis in cells that express E6/E7 and stimulation of senescence in surviving cells. These results suggest that the EGFR might be an effective target for chemoprevention in women who have a high risk of cervical cancer.

Results

Erlotinib prevents immortalization by the complete HPV-16 genome

Primary cultures of human cervical epithelial cells were transfected with a plasmid containing the complete HPV-16 genome plus the neomycin resistance gene. After selection in medium with G418, stably transfected cells were maintained in keratinocyte serum-free medium (KSFM) until they became senescent or grew continuously. As a negative control for immortalization, cultures were transfected with the plasmid containing only the neomycin resistance gene (no HPV). Cells transfected with HPV-16 grew continuously and became immortalized (Table 1, untreated), although there was evidence of crisis (slow growth and cell lysis) in some dishes. Cells transfected with only the neo gene became senescent after 2 to 3 passages (data not shown). Senescence occurred after a total of 40–60 population doublings and 20–40 population doublings were required to establish and transfect cell cultures. Treatment with erlotinib significantly ($p \leq 0.05$) decreased the percentage of cultures that contained immortal cells in 3 independent experiments using cells from different individuals (Table 1). Erlotinib inhibited immortalization in a dose-dependent manner over a range of drug concentrations between 0.1 and 1.0 μM, and 0.2 μM of the drug inhibited immortalization by 50% (ID_{50}).

The serum-free medium used to culture primary cervical cells contains relatively high levels of recombinant EGF (5 ng/ml) which raised the possibility that cells maintained in this medium might become dependent on exogenous EGF for growth. Cultured cervical cells produce multiple growth factors (including the EGF-family members amphiregulin and TGF-α) that stimulate cells by autocrine and paracrine methods (Woodworth et al., 1995). Therefore, we investigated whether erlotinib prevented immortalization when cells were maintained in medium without exogenous EGF. Immortalization assays were repeated in KSFM lacking EGF and bovine pituitary extract (which contains EGF). Under these conditions, HPV-16 transfected cells grew more slowly but continued to form immortal colonies (Table 1). In 3 independent experiments, erlotinib completely prevented immortalization by HPV-16. Immortalization was prevented by low concentrations of erlotinib (0.1 μM) that were not effective when assays were performed in complete KSFM with exogenous EGF (Table 1). Because erlotinib inhibits the EGFR in a reversible manner, high concentrations of EGF might compete more effectively with the drug for receptor activation. These results suggest that paracrine or autocrine growth stimulation by endogenously produced EGF-family growth factors is important for immortalization of cervical epithelial cells, and that erlotinib prevented immortalization more effectively in the absence of high levels of recombinant EGF.

Erlotinib prevents immortalization by HPV-16 E6 and E7 genes

The complete HPV-16 genome encodes 3 oncoproteins including E5, E6, and E7. The E6 and E7 proteins are sufficient for immortalization (Munger et al., 1989), but E5 increases signaling through the EGFR (Straight et al., 1995) and alters epithelial growth via an EGFR dependent pathway (Genther Williams et al., 2005). Therefore, we examined whether erlotinib could prevent immortalization in the absence of E5. Cervical cells were infected with retroviruses encoding the HPV-16 E6 and E7 genes but lacking the E5 gene. All viruses contained the neomycin resistance gene (Halbert et al., 1991). As a negative control for immortalization, cultures were infected with retroviruses containing only the neo gene. Infected cultures were selected in KSFM containing G418 and stably infected cells were plated at clonal density (500 cells/60 mm dish). Cultures were fed every 2 days with KSFM or KSFM containing various concentrations of erlotinib. We found that cultures infected with HPV-16 E6/E7 retrovirus grew rapidly and became immortalized (Table 2, untreated). In contrast, cultures infected with only the neo gene became senescent after 2 to 3 passages (data not shown). In 3 independent experiments

Table 1
Frequency of immortalization of human cervical cells by the complete HPV-16 genome after treatment with erlotinib.

Erlotinib	KSFM[a]			W/o EGF		
	HCX-1	HCX-2	HCX-3	HCX-1	HCX-2	HCX-3
Untreated	6/6[b]	6/6	5/5	6/6	6/6	6/6
0.1 μM	5/6	3/6	5/6	0/5	0/6	0/6
0.3 μM	2/5	0/5	0/6	0/5	0/6	0/6
1.0 μM	0/6	0/6	0/6	0/6	0/5	0/6

[a] Immortalization assays were performed in KSFM with 5 ng/ml EGF (3 left columns) or KSFM without EGF (3 right columns). Each column represents an independent experiment using cells from a different sample of cervix.
[b] Number of wells with immortal cells/the total number of wells examined.

Table 2
Frequency of immortalization of human cervical cells by HPV-16 E6/E7 genes or by SV40 after treatment with erlotinib.

Erlotinib	HCX4–E6/E7	HCX5–E6/E7	HCX6–E6/E7	HCX7–SV40
untreated	5/5[a]	6/6	6/6	6/6
0.1 μM	4/6	4/6	5/6	5/6
0.3 μM	3/6	2/6	2/6	4/6
1.0 μM	0/6	0/6	0/5	0/5
3.0 μM	0/4	0/6	0/6	0/6

[a] Number of wells with immortal cells/ total number of wells examined. Each column represents an independent experiment using cells from a different sample of cervix. All experiments were performed in KSFM plus 5 ng/ml EGF.