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EFFECT OF HUMAN PAPILLOMAVIRUS 16 IMMORTALIZATION ON RETINOIC ACID REGULATION OF EPIDERMAL GROWTH FACTOR RESPONSIVENESS AND DIFFERENTIATION OF NORMAL ECTOCERVICAL EPITHELIAL CELLS

by

NYWANA SIZEMORE

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Thesis Advisor: Dr. Ellen A. Rorke

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January, 1995
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GRADUATE STUDIES

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EFFECT OF HUMAN PAPILLOMAVIRUS 16 IMMORTALIZATION ON RETINOIC ACID REGULATION OF EPIDERMAL GROWTH FACTOR RESPONSIVENESS AND DIFFERENTIATION OF NORMAL ECTOCERVICAL EPITHELIAL CELLS

Abstract

by

NYWANA SIZEMORE

This study was based on the hypothesis that human papillomavirus (HPV) type 16 immortalization of normal human ectocervical epithelial (ECE) cells alters the retinoic acid regulation of normal cervical cell differentiation and epidermal growth factor (EGF) stimulated growth. The rational for this study is that it might explain the anti-neoplastic effect of retinoic acid on HPV positive cervical lesions. As a model system to study normal cervical cell function, primary ectocervical epithelial (ECE) cells were cultured from cervical tissue explants on feeder layers. Under these conditions, ECE cells retain in vivo-like keratin expression, hormonal responsiveness, and differentiation status in vitro. Human papillomavirus cell lines are produced from cultures of primary ECE cells using eukaryotic expression vectors containing the HPV type 16 genome. The ECE16-1, ECE16-D1, and ECE16-D2 cell lines are models of an HPV16-immortalized ectocervical cell.

The significant findings were: 1) Immortalization of normal ECE cells by HPV type 16 induces increased EGF receptor expression and raises growth sensitivity
to EGF. 2) Retinoic acid, while not effecting growth of normal ECE cells, inhibits EGF stimulated growth of HPV immortalized cell lines in a dose dependent manner. 3) Retinoic acid growth suppression of ECE16-1 and ECE16-D1 cell lines was preceded by a reduction of epidermal growth factor (EGF) receptor binding, protein levels, mRNA levels, and promoter activity. Suppression of the EGF receptor was not detected in the normal ECE or the ECE16-D2 cell line. 4) Transient cotransfection of the HPV-16 E6/E7 genes with the EGF receptor promoter in normal ECE cells stimulates activity of the promoter two- to three-fold. 5) Retinoids decrease normal ectocervical epithelial cell differentiation by changing transglutaminase activity and keratin gene expression. Immortalization by HPV type 16 does not alter the effect of retinoic acid on transglutaminase or keratin expression, but the immortalized cells seem to have a heightened sensitivity to retinoids.

Based on these results I conclude, the effect of retinoic acid in vivo may be to maintain normal ectocervical epithelial cell physiology while inhibiting the growth and progression of HPV-positive lesions.
DEDICATION

I would like to dedicate my thesis to my mother (Mary Lou Sizemore) and brother (Micheal John Sizemore). This achievement would not have been possible without their love and support. I would like to say that I love you both very much and to thank for your tremendous contributions in my life and my work.
ACKNOWLEDGEMENTS

I would like to thank many people, without whom I would have not made it this far. First and most important I would like to thank my mother and brother, who have always been there and supported me throughout my life. I would also like to thank the rest of my family for their support and encouragement. I want to thank my best friend John P. Biesterfeldt, who is very dear to me and has always been the best friend I ever had. I would like to thank Milita Panin for being my best buddy and for not letting me take myself too seriously. Finally, I would like say thanks to all my friends in at CWRU, whom are too numerous to mention but I had the pleasure of knowing and partying with.

I want to give a special thanks to my advisor Dr. Ellen A. Rorke, who has guided me through my graduate career and without whom this work would have not been possible. I would also like to thank my thesis committee (Dr. G. David McCoy, Dr. Cathy Carlin, Dr. Richard L. Eckert, and Dr. David Sedwick) for their invaluable advise on my project and their encouragement along the way.
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Effect of EGF and retinoic acid on Cell Growth

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Chapter II: The Effect of Human Papillomavirus 16 Immortalization on Retinoid Regulation of Normal Ectocervical Cell Differentiation

Retinoid regulation of transglutaminase activity in normal ectocervical epithelial cells
LIST OF ABBREVIATIONS

DLFCS = delipidized fetal calf serum

DMEM = Dulbecco's modified eagle medium

DMSO = dimethyl sulfoxide

ECE16-1/ECE16-D1/ECE16-D2 = ectocervical epithelial cells immortalized with HPV type 16 DNA

ECE cells = primary ectocervical epithelial cells

EGF = epidermal growth factor

F12 = F12 nutrient mixture (Ham)

FCS = fetal calf serum

HBSS = Hank's Balanced Salt Solution

HC = hydrocortisone

HEPES = N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]

HPV = human papillomavirus

HRE = hormone response element

RA = trans-retinoic acid

RAR = retinoic acid receptor

RB = retinoblastoma

Ro 13-6298 = (p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl) -propenyl]-benzoic acid ethyl ester)
SDS-PAGE = sodium dodecyl polyacrylamide gel electrophoresis

T$_3$ = triiodothyronine

TG = transglutaminase

TGF = transforming growth factor

TPA = phorbol 12-myristate 13-acetate

TRE = thyroid hormone response element
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INTRODUCTION

Overview of cervical cancer

The cervix is a part of the female reproductive tract and is composed of three regions. The endocervix which adjoins the uterus, the transition zone where the endocervix meets the ectocervix, and the ectocervix which adjoins the vagina. The ectocervix consists of a squamous stratifying epithelium and an underlying stromal region (1). The ectocervical epithelial cells normally undergo proliferation and differentiation during the menstrual cycle due the influence of the sex steroid hormones (2). HPV infection of the ectocervical epithelium is strongly associated with development of cervical carcinoma. Infection of ectocervical epithelial cells with HPV is identified with development of benign proliferative lesions that in certain cases may progress to cervical cancer. The finding that over 90% of all cervical cancers contain HPV DNA integrated into their genome, suggests an important role for this virus in the development of cervical cancer (3-6).

Epidermal growth factor and retinoids play important roles in regulating cervical epithelial cell growth and functioning, particularly of cells which have undergone HPV infection and malignant transformation. Increased EGF receptor expression was detected in 100% of the lung, vulval, and cervical carcinomas examined (7,8). Examination of EGF receptor expression in cervical biopsies in which HPV was present revealed elevated EGF receptor expression in dysplastic and neoplastic cervical tissue as compared to adjacent normal cervical tissue (9,10).
Though no direct evidence presently exists, increased growth sensitivity of HPV infected cervical epithelial cells to epidermal growth factor and other EGF receptor ligands due to elevated EGF receptor expression may be an important factor in the genesis of cervical cancer.

Numerous epidemiological studies have demonstrated inverse correlations between dietary intake or blood levels of vitamin A and cervical cancer risk (11-14). Recently retinoic acid has been found to reverse early proliferative dysplastic and neoplastic cervical lesions where HPV is expressed (15-19). Previous in vivo studies confirm the necessity of retinoids in maintaining the normal functioning of the cervical epithelium (41,42). These results indicated that retinoids may reduce the risk of cervical cancer by maintaining normal functioning of the ectocervical epithelial while inhibiting the proliferation of HPV harboring transformed cells.

These results strongly support our hypothesis that human papillomavirus is able to alter normal cell responsiveness to epidermal growth factor and retinoids and that alterations in these responses could be important in the emergence of cervical cancer from HPV infected cells. Despite the importance of alterations in growth factor and hormonal regulation of ectocervical epithelial cell growth and differentiation caused by the presence of HPV DNA, no in depth investigations into this phenomenon have been conducted.
Ectocervical epithelial cell growth and differentiation

The epithelial cells lining the cervix are primarily of two types, the mucus-producing (endocervix) and squamous (ectocervix) phenotypes (1). The endocervical cells secrete mucus which forms a plug at the cervical opening to hinder harmful and infectious agent entry into the uterus. The ectocervical epithelial cells form a stratified epithelium which serves as a protective barrier for the uterus. The ectocervical epithelium is composed mainly of squamous-type cells which are arranged in stratified layers. The lowest layer is composed of proliferating basal cells while the upper layers are composed of cells in various stages of differentiation. The uppermost layer consists of terminally differentiated cells, called cornified envelopes (1,20). This envelope consists of covalently cross-linked protein that is assembled beneath the plasma membrane during the terminal phases of differentiation by the action of type I transglutaminase (21-23). Type I transglutaminase is a membrane-bound, calcium-dependent enzyme which catalyzes the formation of covalent e-(c-glutamyl) lysine cross-links between proteins (21,22). The reaction proceeds via exchange of primary amines at the carboxamide group of peptide-bound glutamine residues (21,22). Transglutaminase levels are regulated by calcium, retinoids and other agents that effect cell differentiation (23,24-27).

During envelope assembly, most cellular structures are destroyed by proteolytic and nucleolytic activity so that the cornified envelope lacks subcellular organelles, nucleic acids and other macromolecules (28,29). However, an important
structure that persists is the network of intermediate filaments which are assembled from keratin monomers (30). There are twenty members of the keratin multi-gene family and each keratin is expressed in a tissue-specific manner during differentiation and development (31-34). *In vivo* and *in vitro* studies indicate that specific keratins are also expressed in ectocervical epithelial cells (35,36). For example, cytokeratin K13 is expressed at a high level in cervical cells (35). The differentiation process of ectocervical epithelial cells can be monitored by specific changes in transglutaminase activity and cytokeratin gene expression. The differentiation process of the ectocervical epithelial is depicted in Table #1.
Table #1. The differentiation process of the ectocervical epithelium. This table depicts the differentiation process of the ectocervical epithelial. The lowest layer of cells is a layer of actively dividing proliferative cells. As the cells move up through the stratification, a differentiation process begins. An enzyme called transglutaminase type I is induced, causing crosslinking of major cytoskeletal proteins including involucrin. Also cytokeratin filaments are assembled from keratin monomers. This protein crosslinking and keratin assembly produces a terminally differentiated cervical cell called the cornified envelope.
Hormonal regulation of ectocervical cell growth and differentiation.

Proliferation and differentiation of the cervical epithelial lining is tightly regulated during the ovarian cycle (1,37). The cells undergo a cycle of proliferation, differentiation and desquamation under the influence of the sex steroids (1,37). Moreover, normal cervical cell function also depends upon appropriate glucocorticoid and retinoid stimulation (35,38). A delicate balance of proliferation and differentiation of these ectocervical cells occurs monthly during menstruation. The changing levels of estrogen and progesterone control cycling of proliferation and differentiation of the cervical epithelial cells during the monthly cycle around a set point (35,38). Glucocorticoids and retinoids (derivatives of vitamin A) play a role in maintaining the proliferation and differentiation set point of the cervical cells (38). These factors usually act by regulating the gene expression of important proliferation and differentiation specific genes (39). In several disease states this regulation is perturbed. In hyper-estrogenic states the cervix can undergo hypertrophy and hyperkeratosis (40). Vitamin A (a retinoid) deficiency leads to transformation of normal mucus-phenotype cells to a metaplastic squamous phenotype which under certain conditions can progress to produce malignancies. This malignant transformation can be reversed by retinoid administration (41,42).
Human papillomavirus and cervical cancer

Cervical cancer is another disease state in which the equilibrium of proliferation and differentiation of the ectocervical epithelium is disturbed. Certain human papillomavirus (HPV) infections of the cervical epithelium are causally associated with cervical cancer (3-6). The family of papillomaviruses consists of over 70 distinct types. Human papillomaviruses were originally discovered by clinicians as the etiological agent of infectious warts. The papillomaviruses are small DNA viruses which commonly infect man and other higher vertebrates. Human papillomaviruses are strictly epitheliotropic and depend on the environment of terminally differentiating squamous epithelium for completion of their life cycle (43). Papillomavirus infection of genital cutaneous or mucosal epithelium usually results in benign, self-limiting proliferative lesions known as genital warts or condylomas. During this stage the HPV genome is episomal allowing for full viral genome expression and production of infectious viral particles. The viral genome consist of several open reading frames shown in table #2: The E1 protein is necessary for maintenance of the plasmid state of the viral DNA, the E2 protein is necessary for transactivation of the viral promoter, the E4 protein is necessary for maturation of the virus, the E5 protein can alter cell growth factor receptor recycling, the E6 protein causes degradation of the p53 tumor suppressor protein, the E7 protein inactivates the pRB tumor suppressor protein, E8 whose function is unknown, and the L1,L2 proteins which are the major and minor viral capsid components (43).
Genital condylomas can be caused by a variety of HPVs, including the high risk papillomas (associated with cervical carcinoma) and a second group called low risk HPVs (not associated with cervical carcinoma) (43). However infection of the cervical epithelium with low risk papillomavirus (6, 10 or 11) rarely results in malignant progression in contrast to infection with high risk HPV (16, 18, 31, 33 and 35) which frequently leads to cervical cancer. Little is known about the mechanisms involved in the transformation and progression of HPV infected cervical cells to cancerous cells. It is known, however, that cervical cancer is preceded by benign condylomas induced by infection with high risk human papillomaviruses (43). After a long latency, condylomas can progress to dysplastic and neoplastic lesions in which the viral genome is integrated and incomplete viral expression occurs consisting mainly of the E6 and E7 reading frames (44-48). Table #3 depicts the life-cycle of both low and high risk types of human papillomavirus. The presence of DNA from these high risk papillomaviruses (HPV 16, 18, 31, 33 and 35) in over 90% of intraepithelial neoplasias and carcinomas of the cervix strongly suggests an important role of these viruses in the development of cervical cancer (3-6). It should be noted that infection of cervical epithelium with a high risk HPV in itself is not sufficient for cervical cancer development. Other mutagenic events such as cigarette smoking or herpes simplex virus infection may play a role in the progression of the benign condylomas to malignant lesions (43), see table #3.
However, transfection of normal human keratinocytes and cervical cells with either HPV 16 or 18 DNA results in the establishment of immortalized cell lines (49,50). The immortalization by HPV DNA is accomplished by a specific integration process in which the expression of the E6 and E7 genes is selectively maintained (44-48). The HPV viral proteins, E6 and E7, interact with and disrupt the normal functioning of the protein products of two important tumor suppressor genes, the p53 and retinoblastoma (RB) genes (49,50). The interaction of E6 and E7 with these two cellular oncogenes seems to be essential for in vitro cellular transformation and immortalization (51). The interactions of high risk human papillomavirus E6 and E7 with cellular tumor suppressor proteins, p53 and pRB, are depicted in table #4. The high risk HPV protein, E6, can bind to p53 and promote degradation of the p53 protein. The high risk HPV protein, E7, binds to pRB and interrupts its normal interactions with the important transcription factor E2F. In some but not all cell systems, direct modulation of HPV E6 and E7 levels is associated with proliferation of HPV immortalized cell lines (52,53). However, other HPV alterations in normal cell functions, such as HPV-induced changes of cervical cell responsiveness to endogenous growth factors and hormones could be important in the initiation and progression of cervical cancer.
Table #2. Human papillomavirus gene map. The hatched area is the HPV16 P7 promoter which is the major transcriptional start site. The early and late genes are indicated. The viral genome is shown linearized at the unique BAMHI site.
HPV 16 GENOME
Table #3. The life-cycle of low and high risk human papillomavirus types. This table depicts the life-cycle of both low and high risk HPV types. Initially after infection, both the low and high risk types exist as an episomal DNA in which full viral expression is maintained and infectious viral particles are shed. This initial infection leads to benign proliferative lesions called genital warts or condylomas.

However, the high risk HPV types tend to undergo an integration process in which the viral DNA becomes integrated into the host cell’s genome. This integration process leads to incomplete viral expression in which virus is no longer produced. Following integration, there is a long latency period allowing for further mutational events leading to the development of cervical cancer.
Table #4. The interactions of high risk human papillomavirus E6 and E7 proteins with cellular tumor suppressor proteins, p53 and pRB. The high risk HPV protein, E6, can bind to p53 and promote degradation of the p53 protein. The high risk HPV protein, E7, binds to pRB and interrupts its normal interactions with the important transcription factor E2F.
INTERACTIONS OF HIGH RISK HPV E6 AND E7 WITH CELLULAR PROTEINS
Evidence for HPV alterations in retinoid regulation of ECE cells.

One important hormonal response of normal ectocervical epithelial cells which seems to be altered by human papillomavirus immortalization is the response to retinoids. Keratinocytes and cervical cells which have been immortalized with HPV seem to respond differently than their normal counterparts to retinoids (49,50,52,53). Retinoids are required for vision, reproduction, embryonic development, as well as for the growth and differentiation of a variety of cell types throughout the lifetime (39). Retinoids, including vitamin A and its metabolites, are known to be important regulators of epithelial cell growth and differentiation in vitro and in vivo (54-58). Vitamin A (retinol) is a nutrient derived from carotinoids in plants and retinyl esters in animal fat and must be metabolized to become biologically active. Under proper nutritional conditions, retinol is steadily present at high concentrations in the plasma and therefore is readily available to cells at all times (39). Retinol is released from the liver and transported through the plasma bound to a retinol binding protein (RBP). Retinol uptake by target cells is mediated through this retinol binding protein. Once inside the target cells, retinol is metabolized to its biologically active metabolites including, but not limited to, all-trans retinoic acid (atRA), didehydroretinoic acid (ddRA), and 9-cis-retinoic acid (9cRA). Table #5 depicts retinol (vitamin A) and its metabolites. There are several cellular retinoid binding proteins involved in this metabolism process. The two cellular retinoid binding proteins (CRBPs), CRBP I and II, bind retinol and direct it specifically to retinol
dehydrogenase in the metabolic activation pathway leading to atRA, ddRA, and 9cRA synthesis. Similarly, cellular retinoic acid binding proteins I and II (CRABPI, CRABPII) direct all-trans-retinoic acid to cytochrome P450 for catabolism to more polar and biologically inactive metabolites (39). All-trans-retinoic acid, once produced in the cells, can then exert its biological actions by binding to specific nuclear receptors present in the cell that modulate gene transcription. Table #6 depicts the retinoid metabolism and activation pathway. The nuclear receptors for retinoic acid belong to the steroid hormone receptor / thyroxine receptor family, and seem to effect growth and differentiation in a similar manner. Table #7 illustrates a structure and function analysis of a typical steroid/retinoid receptor family member. Retinoids have been reported to transcriptionally regulate many important cellular genes, including the epidermal growth factor receptor (59-61). Three different retinoic acid receptors (RARs) which bind trans-retinoic acid have been identified, RAR-α, RAR-β, and RAR-γ. These RAR receptors modulate gene expression by binding to specific but diverse hormone response elements (HREs) in the gene's promoter region. Three distinct receptors that bind the 9-cis form of retinoic acid but not trans-retinoic acid have been identified, RXR-α, RXR-β, and RXR-γ. Interactions between the RARs, RXRs, and T3 receptors have been reported. Table #8 give examples and illustrates the homology found between members of the steroid/retinoid receptor family members. Retinoic acid receptors can also have an antagonistic activity on the AP-1 pathway, possibly by occupying AP-1 response
elements or forming inactive complexes with AP-1. AP-1, a transcription complex of c-fos and c-jun, is activated by growth factors and the tumor promoter phorbol 12-myristate 13-acetate thereby activating gene transcription leading to proliferation (39). These interactions of the retinoic acid receptors with other nuclear transcription factors indicate complex interplays between these nuclear receptors in vivo, see table #6. Retinoids not only regulate growth and differentiation of normal epithelial cells, but they have also been shown to have an anti-neoplastic effect on many virally and chemically induced neoplasias (62-65). Several epidemiological studies suggest an increased cervical cancer risk with low dietary intake of retinoids (11-14). Recently retinoic acid has been found to reverse some pre-neoplastic cervical lesions and dysplasias (15-19), demonstrating that retinoic acid may be effective in preventing and treating cervical cancer. Retinoids have recently been shown to inhibit proliferation of HPV immortalized cervical and keratinocyte cell lines (52,53,66). In some studies, retinoid growth inhibition was correlated with a decline in E6 and E7 viral RNA expression (52,53). However, levels of E6 and E7 do not always correlate with the retinoid inhibition of HPV immortalized cell proliferation (66). Although less extensively investigated, retinoid inhibition of HPV positive cell proliferation may be mediated by altering responsiveness to important epithelial cell growth factors.
Table #5. Retinol (vitamin A) and it’s metabolites. This table depicts retinol (ROH) which is metabolized to retinal (RAL). Retinal is then further metabolized to all-trans retinoic acid (atRA), didehydroretinoic acid (ddRA), and 9-cis-retinoic acid (9cRA).
Table #6. The retinoid metabolic and activation pathway. This table illustrates the metabolism of retinol (vitamin A) to its biologically active metabolites and the interaction of the atRA and 9cRA with their nuclear receptors, RARs and RXRs, respectively. Also depicted is the complex interplay between steroid/retinoid nuclear receptor family members and their interaction with other nuclear transcription factors.
Table #7. A structural and functional analysis of an typical member of the steroid/retinoid receptor family. The N-terminus of these receptors, tends to effect the cell specific and promoter specific activation properties of these receptors. The middle portion of the receptors provide DNA binding and dimerization capacities, while the C-terminus is responsible for the ligand binding and trans-activating capacities of the receptors.
Table #8. Examples and homology of several members of the steroid/retinoid receptor family. This table pictures an all-trans retinoic acid receptor (RARα), a 9-cis retinoic acid receptor (RXRα), a thyroid hormone receptor (T3Rβ), and a vitamin D receptor (VD3R).
Evidence for HPV alterations of EGF responsiveness in ECE cells

An important epithelial cell growth factor whose responsiveness may be altered by human papillomavirus infection and modulated by retinoic acid is epidermal growth factor (EGF). Epidermal growth factor is a potent mitogen for a variety of cultured epithelial cells, including normal cervical cells (67). EGF is an important regulator of normal ectocervical cell growth and function (67). EGF is a small protein which interacts with a transmembrane glycoprotein receptor on the surface of certain cell types. The receptor for EGF is an 170-kilodalton cell surface glycoprotein present in many tissues and cell types. The EGF receptor binds epidermal growth factor at the receptor’s N-terminus leading to activation of the EGF receptor’s C-terminal tyrosine kinase. The activation of the tyrosine kinase leads to autophosphorylation of the EGF receptor itself, as well as initiating intracellular signal transduction through a cascade of second messages (68,69). This intracellular signalling transmits EGF’s mitogenic signal to the nucleus. Alterations in epidermal growth factor (EGF) receptor expression or function can cause aberrant mitogenic signaling possibly contributing to the initiation and progression of several malignant cancers (70-72). Overexpression of the EGF receptor is a common characteristic in many tumors (73-76). Though overexpression of the EGF receptor results from gene amplification in some tumors (77-79), overexpression in other tumors has been reported to result from transcriptional or post-transcriptional events (80). Therefore, modifications in transcriptional control of the EGF receptor promoter may be
important events in the genesis of some tumors. The EGF receptor promoter lacks CAAT and TATA promoter elements, but has multiple start sites and is GC-rich (81,82). This promoter region contains binding sites for many transcription factors and hormone receptors, including retinoic acid receptors (83-85). Increased EGF receptor expression was detected in 100% of the lung, vulval, and cervical carcinomas examined (7,8). Examination of EGF receptor expression in cervical biopsies in which HPV was present revealed uniformly elevated EGF receptor expression associated with dysplastic and neoplastic changes of cervical tissue as compared to adjacent normal cervical tissue (9,10). Though no direct evidence presently exists, elevated EGF receptor expression induced by HPV in dysplastic changes of the cervix may be an important factor in the genesis of cervical cancer.

**Objective of thesis research**

From these studies it is clear that high risk papillomavirus infection of the ectocervical epithelium is important to the development of cervical cancer. Therefore since ectocervical epithelial cells in culture retain in vivo-like function, the effect of human papillomavirus type 16 immortalization on normal ectocervical epithelial cell growth and differentiation can be assessed in vitro. It is clear that both epidermal growth factor and retinoic acid have profound effects on the reproductive tract. Interactions between epidermal growth factor and retinoic acid are probably important in regulation of normal cervical epithelial cell growth and differentiation. Alterations in cervical cell responsiveness to these compounds induced by human papillomavirus
infection may contribute to the cervical cancer progression. Despite the importance of this question, few studies have focused on alterations in retinoic acid regulation of epidermal growth factor responsiveness and differentiation of normal ectocervical epithelial cells induced by HPV immortalization. The evidence of elevated EGF receptor expression in dysplastic and neoplastic changes of cervical tissue induced by HPV infection, supports the thesis that HPV induced alterations in the EGF receptor pathway of cervical epithelial cells may be important for the initiation and progression of cervical cancer. The fact that keratinocytes and cervical cells which have been immortalized with HPV seem to respond differently than their normal counterparts to retinoids (clinically used to regress cervical dysplasia and neoplasia) (49,50,52,53), supports the thesis that retinoid responsiveness of ectocervical epithelial cells is also altered by immortalization with HPV.

Our results suggest that immortalization of normal ectocervical epithelial cells may modulate the effects of these compounds on the cervical epithelium growth and differentiation (49,52,126). Therefore the objective of this thesis is to investigate the effect of HPV immortalization on normal ectocervical epithelial cell growth and differentiation responsiveness to retinoids.
HYPOTHESIS AND SPECIFIC AIMS OF THESIS RESEARCH

Our hypothesis is that human papillomavirus (HPV) immortalization specifically alters retinoic acid regulation of epidermal growth factor response and differentiation of normal human ectocervical epithelial cells and that this process may be important in development of cervical cancer. Therefore, to determine whether HPV immortalization alters retinoic acid regulation of ectocervical cell responsiveness to epidermal growth factor and differentiation, the specific aims of the proposal are:

(1) Determine if HPV immortalization alters the growth responsiveness of normal ectocervical epithelial cells to epidermal growth factor and/or retinoic acid. This will be accomplished by studying the growth of both normal and immortalized ectocervical epithelial cells, under the influence of EGF and retinoic acid.

(2) Determine if retinoic acid can modulate EGF growth responsiveness of either normal or HPV immortalized cervical epithelial cells. This will be accomplished by measuring the effect of retinoic acid on EGF stimulated growth of normal and HPV immortalized cervical epithelial cells.

(3) To determine if HPV immortalization alters EGF receptor number or activity of normal cervical epithelial cells and if retinoic acid has any effect on EGF receptor number and activity of either normal or HPV immortalized cells. Changes in EGF receptor number will be measured by determination of EGF receptor protein levels, mRNA, and promoter activity in control or retinoic acid treated normal and HPV immortalized cervical cells. Changes in EGF receptor activity will be measured
by determination of EGF receptor affinity, tyrosine kinase activity, metabolism, and EGF binding of control or retinoic acid treated normal and HPV immortalized cells.

4) To determine if certain HPV-16 genes are able to modulate transcription of the EGF receptor promoter by performing transient cotransfection studies in normal ectocervical epithelial cells using the HPV 16 E6/E7 genes to determine the effect on EGF receptor promoter activity and retinoid responsiveness of the promoter.

5) To determine if HPV immortalization alters the effects of retinoic acid on differentiation specific genes in normal ectocervical epithelial cells. Under retinoid-free conditions, the effect of retinoic acid on transglutaminase and keratin expression will be evaluated in normal ectocervical epithelial cells. These results will be compared to the effects of retinoic acid on transglutaminase and keratin expression in the HPV immortalized cell line ECE16-1 obtained previously (49).

Table #9 depicts the experimental flow chart that will be followed to achieve the specific aims.
Table #9. Experimental flow chart. This table depicts the experimental flow chart that will be followed to achieve the specific aims of this study.
EXPERIMENTAL FLOW CHART: THE EFFECT OF HPV IMMORTALIZATION ON RETINOIC ACID REGULATION OF NORMAL ECE EGF STIMULATED GROWTH AND DIFFERENTIATION

A comparison of normal versus HPV immortalized ectocervical epithelial cells.

GROWTH

Characterize growth responsiveness to EGF and RA

Determine effect of RA EGF receptor protein and binding levels affinity and function

Determine effect of HPV E6 and E7 expression on EGF receptor promoter

Determine effect of RA EGF receptor mRNA and promoter activity

Determine effect of RA EGF receptor protein metabolism

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DIFFERENTIATION

Determine effect of RA on transglutaminase enzyme activity and gene expression

Determine effect of RA on cytokeratin protein and gene expression
EXPERIMENTAL PROCEDURES

Materials

Cysteine-free RPMI-1640, Dulbecco’s Modified Eagles, Ham’s F12 medium and fetal bovine serum were purchased from Gibco (Gaithersburg, MD). Tissue culture plastic was obtained from Falcon (Lincoln Park, NJ). The antiphosphotyrosine antibody PY-20 and human recombinant EGF used in cell culture were obtained from UBI (Lake Placid, NY). $^{35}$S cysteine and carrier-free Na$^{125}$I were obtained from Amersham (Arlington Heights, IL). Goat-anti-mouse secondary antibody was obtained from NEN (Wilmington, DE). Antiserum for the EGF receptor, EGF-R1 originally described by Waterfield et al. (86), was the purchased from Amersham (Arlington Heights, IL). Trans-retinoic acid (tRA), retinol (ROH), phorbol 12-myristate 13-acetate (TPA), and all hormonal medium additives were obtained from Sigma (St. Louis, MO). The potent synthetic retinoid, Ro 13-6298 (p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-propenyl]-benzoicacid ethyl ester) (87), was kindly provided by Dr. Stanley Shapiro (Hoffman-La Roche Inc.).
Method of tissue collection.

Human subjects involved in this study were women in good general health between the ages of 20 and 45 of mixed, but predominantly Caucasian background. Discarded cervical tissue samples were obtained from women undergoing routine gynecological procedures, initially screened by a pathologist. Records of the patients history remained with the patient’s physician. Only the patients initials were used to track tissue derived from each patient and the tissue was obtained solely for research purposes. All samples were discarded tissue samples provided by staff physicians in the Division of Reproductive Biology at University Hospitals. No contact with the patient or the patient’s family was required. These experiments have been reviewed and approved without reservation by the hospital internal review boards. No patients were actively recruited to participate in these studies. There were no physical, psychological, social, legal or other risks involved in these studies. The names of participating patients were retained by the physician of record.

Ectocervical samples were only taken from regions well separated from the endocervix and transition zone. The tissue was washed in Hank’s balanced salt solution (HBSS) and the underlying stromal cell layer was removed. The ectocervical epithelium was then cut into 1mm squares and placed on dishes of irradiated mouse 3T3 feeders.
Preparation of mouse 3T3 feeder cells.

Murine embryonic fibroblasts were grown as described by Rhienwald et al. (88). The cells were removed with Hank's EDTA and irradiated with 6000 Rads of Cobalt-60 and plated at a density of 2 x 10^4 cells/cm^2.

Cell Culture.

Normal human ectocervical epithelial cells (ECE cells), ECE16-D1, and ECE16-D2 cells were grown using ^60^Co-irradiated 3T3 feeder cell layers by the method of Rheinwald and Green (88). The ECE16-1 cells were grown in the same growth medium but do not require the use of feeders. The growth medium was DMEM/F12 (3:1 ratio) containing nonessential amino acids, penicillin (100U/ml), L-glutamine (2mM), streptomycin (100ug/ml), insulin (5ug/ml), hydrocortisone (1 x 10^-5 M), adenine (1.8 X 10^-4 M), transferrin (5ug/ml), triiodothyronine (2 x 10^-9 M), EGF (10ng/ml), and 10% FCS. Trans-Retinoic acid and TPA was prepared as 1000-fold stocks in dimethyl sulfoxide (DMSO). For experiments, all cultures were shifted to growth medium lacking EGF containing 0.4% delipidized fetal calf serum (DLFCS). Delipidation removes endogenous retinoids from the serum.

Normal ectocervical epithelial cells are grown on mouse 3T3 feeder layers. Under these conditions, ECE cells retain in vivo-like keratin expression, hormonal responsiveness, and differentiation status in vitro. Human papillomavirus cell lines are produced from cultures of primary ECE cells using eukaryotic expression vectors
containing the HPV type 16 genome. The ECE16-1 cell line was immortalized with a single copy of the HPV 16 genome while the ECE16-D1 and ECE16-D2 cell lines were immortalized with a head-to-tail dimer of the HPV 16 genome. The ECE16-1, ECE16-D1, and ECE16-D2 cell lines are models of an HPV16-immortalized ectocervical cell. Since these HPV immortalized cell lines do not form tumors in nude mice, they can be used to study progression from initiation to malignant transformation in a cervical system.

**Growth Experiments.**

To quantify growth factor mediated changes in cell growth, ectocervical cells were plated in 24-well plates at a density of $1 \times 10^4$ cells/well in normal growth medium. Two days after plating, cells were switched to 0.4% DLFCS containing medium including the indicated concentrations of human recombinant EGF and/or trans-retinoic acid. Control cultures were treated with vehicle alone. Fresh growth factor and/or hormone containing medium was added on alternative days. Duplicate wells were harvested with trypsin after a seven day treatment and counted using a Coulter cell counter (model ZBI).
EGF binding assays

Purified human EGF used in these experiments, was labeled with $^{125}$I by the chloramine-T method of Aharonov (89); 5ug EGF in 5ul of 0.25M sodium phosphate buffer (pH 7.4), 5ul of 20 mCi/ml solution of Na$^{125}$I, and 10ul of 1mg/ml chloramine-T solution 0.25M sodium phosphate buffer (pH 7.4) were mixed and incubated for 30 seconds at room temperature. The reaction of adding the radioisotope to the EGF protein, was stopped by further addition of 10ul of 1mg/ml Na$_2$S$_2$O$_3$ solution and 10ul of 1mg/ml NaI 0.25M sodium phosphate buffer. The reaction was then put through a G25 sephadex column to purify the $^{125}$I labeled EGF.

Ectocervical cells were plated in 24-well plates at a density of 1 X 10$^4$ cells/well in normal growth medium. After 2 days, the cell were treated with the indicated concentrations of retinoic acid. Two days after treatment, EGF binding was measured by adding $^{125}$I-EGF to 0.5ml medium lacking EGF and incubating for 3 hours at 4°C. Then the cells were washed extensively with HBSS, solubilized in 0.2N NaOH, and the cell associated radioactivity counted in a gamma radiation counter. Nonspecific binding, determined by adding a 1000-fold excess of unlabeled EGF, was 3-6% of the total binding and was subtracted from all points. Determination of nonspecific binding was done on duplicate wells and total binding was measured on triplicate wells. The average differences between duplicate and triplicate wells was less than 10%. Two duplicate wells were counted on a Coulter cell counter (model ZBI) for normalization of the binding data to cell number.
To distinguish surface bound from internalized EGF for the receptor internalization studies, we used a technique described by Haigler et al. (90). Briefly cells were incubated with $^{125}$EGF and washed to remove unbound radioactivity. Then externally bound EGF was collected by an acid-salt wash (0.2M acetic acid, 0.5M NaCl) and the internalized EGF was collected by solubilization in 0.2N NaOH. The $^{125}$I was counted on a gamma counter (39).

$^{35}$S cysteine metabolic labeling and EGF receptor immunoprecipitation

Preconfluent cells (80% of dish area) were treated with either vehicle or the specified concentration of trans-retinoic acid in medium containing 5ng/ml EGF. After 2 days, cell were labeled for 2 hours in cysteine-free RPMI-1640 containing 100uC/ml $^{35}$S cysteine, which was incorporated into cysteine-containing proteins. Cell number was quantified in replicate dishes not containing isotope. Cell extractions and EGF receptor immunoprecipitation were preformed as previously described by Hailger et al. (90). Cell extracts were prepared as follows. Cells were washed 3X in PBS and scraped into cell harvesting PBS (PBS, 0.002M EDTA, and 0.005M EGTA) which contained 340 ug/ml PMSF, 10 ug/ml Pepstatin A, and 2.5 ug/ml Leupeptin. The cells were then collected by centrifugation and solubilized by resuspension in solubilizing buffer (15% glycerol, 0.1M Tris Ph 6.8, 0.002M EDTA, and 0.001M EGTA) which contained the same concentration of protease inhibitors as the cell harvesting PBS. Then 10% NP-40 was added to a final concentration of
1% and the cell extracts were incubated on ice for 1 hour. The contents were transferred to microfuge tubes and insoluble material was pelleted by centrifuging for 15 minutes at top speed in a refrigerated microfuge. The NP-40 extracts were precleared for 1 hour with Pansorbin and subsequently incubated with 10ul of the EGF receptor antibody (R1), which bound the EGF receptor protein present in the cell extracts. The immune complexes were precipitated by protein A, electrophoresed in a 7.5% SDS-PAGE gel corrected for cell number, dried by a Bio-Rad gel dryer and then exposed to film at -70°C. The relative density of the bands produced by the radio-labeled EGF receptor protein was determined on a Sci-Scan 5000 densitometer. The density of the bands corresponded to the amount of EGF receptor protein synthesized by the cell during the metabolic labeling.

**Immunoblotting for EGF receptor autophosphorylation assay**

To measure autophosphorylation of the EGF receptor, preconfluent cells (80% of dish area) were treated with medium used for cell growth curves containing vehicle or the specified concentration of trans-retinoic acid. After 2 days, the cells were pulsed for 5 minutes with 150ng/ml EGF and immediately solubilized in 1X Laemmli buffer. Cell number was determined in replicate wells. The solubilized cell proteins were corrected for cell number, separated by electrophoresed in a 7.5% SDS-PAGE gel, and transferred onto nitrocellulose membranes. The nitrocellulose membranes were incubated with the PY-20 antiphosphotyrosine antibody which
detected autophosphorylation of the EGF receptor induced by EGF binding, incubated with $^{125}$I labeled secondary antibody and exposed to film at room temperature after the membrane was washed extensively. The relative density of bands produced by the radiolabeled antibody was determined on a Sci-Scan 5000 densitometer. The relative density of the bands was proportional to the amount of autophosphorylation occurring on the receptors present. The level of autophosphorylation was normalized to EGF binding in replicate wells and expressed as relative autophos unit/fmol EGF bound/cell.

Isolation and quantification of EGF receptor mRNA

EGF receptor mRNA was isolated by poly-A agarose chromatography of guanidine thiocyanate extracted total RNA (91,92). Next, 2 ug of each mRNA sample was subjected to alkaline denaturation and slot blotting onto nylon membranes. The nylon membranes were baked in a vacuum oven for 2 hours and hybridized to $^{32}$P-labeled actin or EGF receptor cDNAs and washed under stringent conditions. Films were exposed to XAR film at -70 °C and relative density of the bands was determined on a Sci-Scan 5000 densitometer.
EGF Receptor Promoter Studies.

Cells were plated at a density of 5 X 10^5 cells per 100 cm dish in normal growth medium and allowed to adhere overnight. Each dish was transfected using 3 ug of an EGF receptor promoter Luciferase reporter construct (kindly provided by Dr. Gordon Gill, University of California-Davis). For cotransfections 3 ug of the EGF receptor promoter plasmid plus the indicated concentration of the HPV E6/E7 construct or the E6/E7 control plasmid (same plasmid as HPV E6/E7 but minus the E6/E7 gene) was transfected using 10 ug Lipofectin reagent (Gibco-BRL) per 3ug DNA transfected for all transfections. The transfection was performed in serum free DMEM medium for 5 hours. The transfection medium was removed and the cells were washed. The cells were then treated for 2 days with DMSO or the indicated concentration of retinoic acid in medium containing 0.4% DLFCS. After two days the cells were washed and extracted. The cell extractions and Luciferase activity measurement was performed using a standard Luciferase assay system (Promega), light activity was measured for 10 seconds following the initiation of the reaction. The data was normalized to protein content and equal transfection efficiency was determined by cotransfection with the SV/β-gal plasmid (Promega).
**Transglutaminase Assay**

Cells were washed twice with Tris-buffered saline (TBS; 10 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl) and homogenized in 20 mM HEPES (pH 7.2) containing 2 mM EDTA, 30 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 2 mM dithiothreitol. The homogenate was centrifuged at 100,000 x g for 30 minutes to yield the "soluble" TG fraction. Membrane bound (particulate) TG was isolated by extracting the pellet for 60 min in homogenization buffer containing 0.2% Triton X-100 and centrifuging the extract at 100,000 x g to yield a supernatant containing the particulate TG. Particulate and soluble fractions were assayed in triplicate for TG activity as measured by incorporation of 3H-putrescine (40 μM, 450 mCi/mmol) into reductively methylated casein (2 mg/ml) in buffer containing 100 mM Tris-HCl (pH 7.8), 2 mM CaCl₂, 2 mM dithiothreitol, 30 μg/ml PMSF. Reactions were incubated at 37 C for 1 hour, terminated by the addition of 4 volumes of 12.5% trichloroacetic acid, washed three times and counted in a scintillation counter. Enzyme activity was normalized to protein content.

**Immunoprecipitation of transglutaminase**

To measure type I TG protein concentration in the fractions prepared for TG enzyme activity assays (particulate and soluble), NaCl was added to each sample at a final concentration of 137 mM prior to immunoprecipitation. Type I transglutaminase was measured by incubating extracts for 1 h at 4 C with a type I
transglutaminase-specific monoclonal antibody (93) followed by incubation with protein A sepharose for 1 h at 4 C. The protein A-antibody-antigen complex was collected by centrifugation, washed three times with PBS and resuspended in Laemmli sample buffer (94).

Detection of cytokeratins

The cytokeratin fraction was prepared for characterization by two dimensional electrophoresis (95) from 35S-methionine labeled cells as described by Fuchs et al. (96).

Detection of transglutaminase and cytokeratin mRNA

Total RNA was prepared by lysis of ectocervical cells in guanidine isothiocyanate followed by centrifugation through a cushion of cesium chloride (91). Poly (A)+ RNA was collected by oligo (dT) cellulose chromatography (92), electrophoresed on RNA denaturing gels, transferred to Biodyne A membrane and hybridized using plasmids specific for cytokeratin K5, K13 and K19 (97), involucrin (98) and type I transglutaminase (99) that were labeled by random primer extension in the presence of 32P-dCTP.
RESULTS

Chapter I: Effect of Human Papillomavirus 16 Immortalization on Retinoic Acid Regulation of Normal Ectocervical Epithelial Cell Epidermal Growth Factor Responsiveness

Effect of EGF and retinoic acid (RA) on Cell Growth

EGF and RA are important regulators of cervical cell growth and squamous differentiation. In order to determine if HPV immortalization alters ectocervical cell responsiveness to EGF and if retinoic acid is able to modulate this responsiveness, normal ECE and ECE16-1 cells were plated at a low density and the effect of increasing concentrations of EGF, RA, or EGF + RA on cell proliferation was determined following a 7 day treatment. EGF was found to stimulate cell proliferation in both cell types, but the ECE16-1 cells were responsive to higher doses of EGF (Fig.1). The maximum responsive dose for the normal ECE cells was approximately 20 ng/ml whereas ECE16-1 cells were further growth stimulated by 50 ng/ml EGF. The effect of retinoic acid on cell growth in both cell types was determined in a similar manner as for EGF except cell were treated with increasing concentration of retinoic acid at a constant concentration of 5ng/ml EGF and is shown in figure #2. Retinoic acid had no or a slightly positive effect on normal ECE cell growth, whereas a dramatic dose dependent decrease in cell growth was observed in the immortalized ECE16-1 cells with a maximum reduction of 40-50% with $10^{-7}$ M retinoic acid.
Figure #1. Effect of EGF on normal ECE and ECE16-1 cell growth. Normal ECE(■) and ECE16-1(●) cells were plated in 24 well plates at a density of 1 X 10^4 cells/well. Two days after plating, cell were shifted to medium containing 0.4% DLFCS and treated with indicated concentrations of EGF. Fresh growth factor containing medium was added every 48 hours. The cells were harvested with trypsin and cell number determined on a Coulter Counter after 7 days. The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of three separate determinations.
Figure #2. Effect RA on normal ECE and ECE16-1 cell growth. Normal ECE(■) and ECE16-1(○) cells were plated in 24 well plates at a density of 1 X 10⁴ cells/well. Two days after plating, cell were shifted to medium containing 0.4% DLFCS and 5ng/ml EGF then treated with indicated concentrations of retinoic acid. Fresh retinoid containing medium was added every 48 hours. The cells were harvested with trypsin and cell number determined on a Coulter Counter after 7 days. The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of three separate determinations.
These results indicated that ECE16-1 cells had a heightened growth response at lower physiologic concentrations of EGF as well as being capable of responding to higher concentrations of EGF than normal ectocervical epithelial cells. Also retinoic acid, at a single concentration of EGF, was able to inhibit ECE16-1 proliferation while not effecting normal ectocervical epithelial cells. Therefore we decided to investigate the effect of retinoic acid on EGF stimulated growth in both normal and ECE16-1 ectocervical epithelial cells.

The effect of retinoic acid on EGF stimulated cell proliferation

The effect of retinoic acid on EGF stimulated growth in normal ECE and ECE16-1 cells was analyzed as described above. Cells were treated for 7 days with increasing concentrations of EGF +/- 10^-7 M retinoic acid. Retinoic acid treatment of ECE16-1 cells attenuated EGF stimulated growth even at concentrations as high as 100 ng/ml EGF, with greater attenuation occurring at lower EGF concentrations (Fig.3). In contrast, retinoic acid treatment had no effect on the responsiveness of normal ECE cells to EGF (Fig.4). These experiments revealed that retinoic acid while having no effect on normal ectocervical epithelial cell growth, was able to inhibit EGF stimulated growth of an HPV type 16 immortalized cell line (ECE16-1).
Figure #3. Effect retinoic acid on EGF stimulated ECE16-1 cell growth.

ECE16-1 cells were plated in 24 well plates at a density of $1 \times 10^4$ cells/well. Two days after plating, cell were shifted to medium containing 0.4% DLFCS and treated with indicated concentrations of EGF +/- $10^{-7}$M RA (EGF ALONE = solid symbols, EGF + $10^{-7}$M RA = open symbols). Fresh growth factor and retinoid containing medium was added every 48 hours. The cells were harvested with trypsin and cell number determined on a Coulter Counter after 7 days. The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of three separate determinations.
Figure #4. Effect retinoic acid on EGF stimulated normal ECE cell growth. Normal ECE cells were plated in 24 well plates at a density of 1 X 10⁴ cells/well. Two days after plating, cell were shifted to medium containing 0.4% DLFCS and treated with indicated concentrations of EGF +/- 10⁻⁷M RA (EGF ALONE = solid symbols, EGF + 10⁻⁷M RA = open symbols). Fresh growth factor and retinoid containing medium was added every 48 hours. The cells were harvested with trypsin and cell number determined on a Coulter Counter after 7 days. The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of three separate determinations.
This is an important observation since retinoic acid is used clinically to slow and regress the growth of cervical dysplastic and neoplastic lesion positive for human papillomavirus. Therefore we tested two more HPV immortalized cell lines for retinoic acid growth inhibition at 5ng/ml EGF. Since maximal growth inhibition of the ECE16-1 cell line occurred at 10⁻⁷M retinoic acid, this was the dose we used for measuring the effects of RA on the new cell lines. Normal ECE cells (R37 and R38) and HPV immortalized cell lines (ECE16-1, ECE16-D1, and ECE16-D2) were plated at a low density and the effect of 10⁻⁷M RA on cell proliferation was determined following a seven day treatment. Retinoic acid was found to decrease cell proliferation in all three HPV immortalized cell lines while having no significant effect on normal ECE cells (Fig. 5). The degree of growth inhibition in the immortalized cell lines varied from approximately 65% for ECE16-1 to 30% for ECE16-D1 to only 15% for ECE16-D2 (Fig. 5). Since the inhibition of HPV immortalized cell growth by retinoic acid could possibly explain its therapeutic effect in vivo, we decided to investigate the mechanism of retinoic acid mediated inhibition of EGF stimulated cell growth of HPV immortalized cervical cells.
Figure #5. Effect of RA on HPV 16 immortalized and normal ECE cell growth.
ECE16-1, ECE16-D1, ECE16-D2 and normal ECE cells (R37 and R38) were plated in 24 well plates at a density of 1 X 10^4 cells/well. Two days after plating, cell were shifted to medium containing 0.4% DLFCS and 5ng/ml EGF then treated with 10^-7M retinoic acid. Fresh retinoid containing medium was added every 48 hours. The cells were harvested with trypsin and cell number determined on a Coulter Counter after 7 days. The graph represents retinoic acid treated cell number as a percent of control, DMSO treated, for each cell type. The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of three separate determinations.
Regulation of EGF Receptor Protein and EGF Binding by Retinoic Acid

Since EGF stimulated growth was modulated by RA in ECE16-1 cells and not in normal ECE cells, retinoic acid's effect on EGF receptor protein levels and EGF binding of the cells was examined. Retinoic acid reduces immunoprecipitable EGF receptor protein levels by 50-60% in ECE16-1 cells but not in normal ECE cells (Fig 6). This reduction in EGF receptor protein levels is associated with a 40-50% reduction in EGF binding in ECE16-1 cells following RA treatment. Retinoic acid treatment had no effect on EGF binding in normal ECE cells (Fig.6). EGF binding was significantly decreased in a dose dependent manner only in the ECE16-1 cells, where half maximal reduction in EGF binding was observed around $10^{-10}$M RA (Fig.7). The same treatment resulted in no detectable change in EGF binding in normal ECE cells, Fig. 8. EGF binding also decreased in the ECE16-D1 cell line but not the ECE16-D2 cell line (Fig. 9). The reduction in EGF binding was not as dramatic in the ECE16-D1 cell line (25% reduction) as it was for the ECE16-1 cell line (50% reduction) (Fig. 9). The immunoprecipitable EGF receptor protein levels were reduced by retinoic acid in the ECE16-1 and ECE16-D1 cell lines but retinoic acid had no effect on EGF receptor protein levels in normal ECE cells and the ECE16-D2 cell line (Fig. 9).
Figure #6. Regulation of EGF receptor protein levels and EGF peptide binding in normal ECE and ECE16-1 cells by RA. Preconfluent cell were treated for 2 days with DMSO (clear bars) or 10⁻⁷M RA (hatched bars) in medium containing 0.4% DLFCS. To quantify EGF binding, the treated cells were incubated with ¹²⁵I-EGF ± 1000 fold excess unlabeled EGF for 3 hours at 4°C and specific EGF binding was determined as described in "Experimental Procedures". The results are expressed as the mean ± SEM of triplicate wells.

For immunoprecipitation, cells were prelabeled with S³⁵-cysteine followed by solubilization, immunoprecipitation, electrophoresis, and autoradiography as described under "Experimental Procedures." The immunoprecipitates are shown above their corresponding EGF binding data. These experiments are representative of at least three separate determinations.
Figure #7. EGF binding dose response to RA in ECE16-1 cells. Preconfluent ECE16-1 cells were treated for 2 days with vehicle (DMSO) or specified concentration of RA for 2 days. EGF binding assays were preformed as described in Fig. 6. The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of at least three separate determinations.
Figure #8. EGF binding dose response to RA in normal ECE cells. Preconfluent ECE cells were treated for 2 days with vehicle (DMSO) or specified concentration of RA for 2 days. EGF binding assays were preformed a described in Fig. 6. The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of at least three separate determinations.
Figure #9. Regulation of EGF peptide binding and EGF receptor protein by retinoic acid in HPV 16 immortalized and normal ECE cells. Preconfluent cells were treated for 2 days with DMSO (solid bars) or $10^{-7}$M RA (hatched bars) in medium containing 0.4% DLFCS. For the EGF binding determinations, the treated cells were incubated with $^{125}$I-EGF ± 1000 fold excess unlabeled EGF for 3 hours at 4°C and specific EGF binding was determined as described in "Experimental Procedures". The results are expressed as the mean ± SEM of triplicate wells.

For immunoprecipitation, cells were prelabeled with S$^{35}$-cysteine followed by solubilization, immunoprecipitation, electrophoresis, and autoradiography as described under "Experimental Procedures." A graph of corresponding density of the RA treated EGF receptor band as a percent of the control treated EGF receptor band of the immunoprecipitates is shown as an insert of the EGF binding graph. The immunoprecipitates are shown in the lower panel. These experiments are representative of at least three separate determinations.
These experiments revealed that: 1) HPV immortalized cell lines have elevated EGF receptor levels, higher EGF binding capacity and EGF growth sensitivity as compared to normal ectocervical epithelial cells. 2) Retinoic acid, while not effecting the growth of normal ectocervical epithelial cells, inhibited EGF stimulated cell proliferation of the HPV immortalized cell lines and the growth inhibition of two of the HPV cell lines was associated with a dramatic decrease in EGF binding and receptor levels. Therefore we decided to continue our investigation by seeing if retinoic acid could alter the activity of the EGF receptors in the HPV immortalized cell lines.

Effect of retinoic acid on EGF receptor affinity

Saturation analysis, performed in both cell types after a 2 day treatment with vehicle or 10⁻⁷M retinoic acid, revealed that the affinity of the EGF receptor was not significantly altered following retinoic acid treatment in either the ECE16-1 cells (Fig.10) or the normal ECE cells (Fig.11). A slight increase in the number of EGF binding sites in normal ECE cells was detected in this analysis, however this slight increase was not detected in two other normal ECE cell strains analyzed. The number of EGF binding sites on four normal ECE cell strains tested ranged from 0.13 X 10⁶ to 0.63 X 10⁶ sites/cell (Fig.11). The analysis of the ECE16-1 cells, however, did confirm the 40-50% reduction in the number of EGF binding sites from 3.71 X 10⁵ sites/cell in vehicle treated cells to 1.92 X 10⁵ sites/cell following a 2 day treatment with 10⁻⁷M retinoic acid (Fig.10).
Figure #10. Saturation analysis of EGF binding in vehicle (●) and RA (▲) treated ECE16-1 cells. The EGF binding assay was performed as described in Fig. 6. and the data analyzed by the method of Scatchard. The Kd of the EGF receptor was similar for control and RA treated cells, 5.46 nM (correlation coefficient of 0.8732) versus 5.01 nM (correlation coefficient of 0.8780) respectively. The number of binding sites was decreased by approximately 50% from $3.71 \times 10^3$ sites/cell for control treated cells to $1.92 \times 10^3$ sites/cell for RA treated cells. This experiment is representative of at least two separate determinations.
Figure #11. Saturation analysis of EGF binding in vehicle(●) and RA(▲) treated normal ECE cells. The EGF binding assay was performed as described in Fig. 6. and the data analyzed by the method of Scatchard. The Kd of control and RA treated cells were not significantly different, 4.54 nM (correlation coefficient of 0.9166) and 4.82 nM (correlation coefficient of 0.8601) respectively. The number of binding sites was not significantly altered with 0.616 X 10^9 sites/cell for control treated cells and 0.787 X10^9 sites/cell for RA treated cells. This experiment is representative of at least two separate determinations.
The Effect of Retinoic Acid on EGF Receptor Autophosphorylation.

In order to determine if RA altered EGF receptor activity as well as number, the effect of retinoic acid on EGF receptor autophosphorylation was determined. After the two day treatment both control and RA treated ECE16-1 cells were stimulated with a saturating dose of EGF (150 ng/ml) for 5 minutes, to fully activate tyrosine autophosphorylation. Western blotting using PY20, an antibody specific for phosphotyrosine residues, revealed a 170 KD band which was absent in extracts not stimulated with EGF. This band was found to be significantly decreased in the RA treated ECE16-1 cells; this decrease paralleled the RA induced decrease of $^{125}$I-EGF binding. When the autophosphorylation data was corrected for the number of EGF binding sites (which correlates directly to loss of EGF receptor protein), there was no significant change in the autophosphorylation capacity of the EGF receptor (Fig.12).
Figure #12. Autophosphorylation of the EGF receptor in control and RA treated ECE16-1 cells. Preconfluent ECE16-1 cells were treated for 2 days with either control or $10^{-7}$ M RA. The cells were then stimulated with 150 ng/ml EGF for 5 minutes followed by solubilization, electrophoresis, and western blotting as described in "Experimental Procedures." The EGF receptor autophosphorylation was detected with PY20 antibody, and visualized by a $^{125}$I labeled secondary antibody and subsequent autoradiography. Densitometric measuring of the bands was used to determine relative units of EGF receptor autophosphorylation. The level of autophosphorylation was normalized to EGF binding in replicate wells. The EGF binding was determined as described in Fig.6. This experiment is representative of two separate determinations.
0.77

0.77

AUTOPIOS UNITS/PMOL EGF BOUND/10^9 CELLS

17.90

8.97

RELATIVE OD UNITS

10 M RA

CONTROL

TREATMENT
Effect of retinoic acid on EGF receptor metabolism

Possible explanations for a reduction in EGF binding and EGF receptor protein other than decreased synthesis of the EGF receptor protein could include, RA induced secretion of a ligand for the EGF receptor, altered EGF receptor internalization or stability of the EGF receptor. Therefore, the effects of RA on these processes was investigated.

Retinoic acid induction of a secreted EGF receptor ligand was tested using RA conditioned medium from ECE16-1 cells for an EGF binding assay. If retinoic acid were inducing the ECE16-1 cells to secrete a ligand for the EGF receptor then it would be present in the RA conditioned medium. This medium when used for the EGF binding assay would inhibit the $^{125}$I EGF binding to both control and RA treated cells equally. However in the presence of RA conditioned medium, EGF binding still decreases only in the RA treated ECE16-1 cells (Fig.13).

To analyze cells for altered internalization, EGF receptor internalization was measured in control and RA treated cells. EGF binding sites were saturated at 4°C and external versus internalized $^{125}$I-EGF was measured over time at 37°C. The internalization of the EGF receptor is not affected by RA treatment as shown in Fig.14. The half-time for internalization of the EGF receptor is approximately 18 minutes for both the control and RA treated cells.
To examine the possibility that the reduction in EGF binding was due to altered EGF receptor stability, pulse-chase experiments were conducted. The EGF receptor protein’s half-life in the absence of EGF is 17-20 hours (100). Control and RA treated ECE16-1 and normal ECE cells were labeled with $^{35}$S-cysteine for 1 hour and chased for 15 hours with non-isotopic cysteine. Immunoprecipitations were preformed at the beginning of the chase and at the end of the 15 hour period. The immunoprecipitates were electrophoresised on SDS-PAGE gels, the gels were then dried, autoradiographed, and the relative OD measured by densitometry. No changes in stability following retinoic acid treatment were detected in the normal ECE cells (Table 10). The stability of the EGF receptor in the RA treated ECE16-1 cells was not diminished but actually increased over that of the control treated cells, see Table 10. This may indicate that not only is retinoic acid decreasing the amount EGF receptor protein but may be inhibiting the production of a membrane bound ligand for the EGF receptor in ECE16-1 cells. This experiment indicates that the observed decrease in EGF receptor protein in ECE16-1 cells following retinoic acid treatment is not due to decreased stability of the EGF receptor.

These experiments demonstrate that retinoic acid in the HPV immortalized cells seems only to reduce the levels of the EGF receptor protein and not to decrease the activity or stability of the EGF receptor.
Figure #13. Effect of RA conditioned medium on EGF binding in control (○) and RA (▲) treated ECE16-1 cells. Preconfluent cell were treated for 2 days with DMSO (○) or the indicated concentrations of RA (▲). An EGF binding assay which was preformed on the treated cell monolayers as described in Fig. 6, except that medium from ECE16-1 cells that had been treated with the indicated concentration of RA for 2 days was used as the binding medium for each EGF binding determination. The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of at least three separate determinations.
Figure #14. Distribution of ECE16-1 cell-associated $^{125}$I-EGF over time in control (○) and RA (●) treated cells. Preconfluent ECE16-1 cells were treated with vehicle (DMSO) or 10$^{-7}$ M RA for 2 days. The cells were incubated with $^{125}$I-EGF ± 1000 fold excess unlabeled EGF for 3 hours at 4°C. Cells were washed extensively with Hank's balanced salt solution to remove unbound $^{125}$I-EGF. Fresh binding medium at 37°C was added to the wells. Following the indicated incubation periods at 37°C, the medium was removed and counted in a gamma counter. The $^{125}$I-EGF binding only to surface receptors was measured by acid-salt washes (solid symbols) and then NaOH was used to solubilize the cells to measure $^{125}$I-EGF that was resistant to acid-salt washes (open symbols). The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of at least two separate determinations.
Table #10. Pulse-chase immunoprecipitations of the EGF receptor in control and RA treated normal ECE and ECE16-1 cells. Preconfluent cell were treated with DMSO (vehicle) and 10⁻⁷M RA for 2 days. The cells were then metabolically labeled with ³⁵S-cysteine for 1 hour followed by a 15 hour chase with non-isotopic cysteine. Immunoprecipitations were preformed at the beginning and the end of the chase period as described in "Experimental Procedures." The immunoprecipitates were electrophoresed on a SDS-PAGE gel which was subsequently dried and autoradiographed. The intensity of the 170 Kd bands were measured by densitometry. The data is presented as the OD of 15 hour band ÷ the OD of the corresponding 0 hour band. This experiment is representative of at least two separate determinations.
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Effect of retinoic acid on EGF receptor mRNA and promoter activity.

To further define the nature of retinoid regulation of the EGF receptor protein levels in normal ectocervical epithelial cells and the HPV immortalized cell lines, EGF receptor mRNA and promoter activity following retinoic acid treatment was measured. Cells were treated for 48 hours with either DMSO (control) or $10^{-7}$M retinoic acid (RA). The mRNA was extracted and subjected to alkaline denaturation and slot blotting onto nylon membranes. The nylon membranes were hybridized with β-actin or EGF receptor P32-labeled c-DNA. The amount of EGF receptor mRNA was normalized to the amount of actin mRNA. The amount of EGF receptor mRNA decreased approximately 50% in ECE16-1 cells and 25% in ECE16-D1 cells, no changes in EGF receptor mRNA levels were detected in normal ECE or the ECE16-D2 cells (Fig. 15).

EGF receptor promoter activity was measured by transfecting cell with a luciferase reporter plasmid containing 1081 basepairs 5’ to ATG translation start site of the EGF receptor gene as reported by Hudson et al. 1989 (101). Table #11 shows a representation of the EGF receptor promoter plasmid with all the known transcriptional factor binding sites indicated (101). This EGF receptor promoter plasmid (kindly provided by Dr. Gordon N. Gill, University of California-San Diego) was transfected using lipofectin into each cell type, cells were treated with either vehicle or $10^{-7}$M retinoic acid for two days, extracted and luciferase activity was measured. The activity of the EGF receptor promoter for DMSO (solid) and $10^{-7}$M
retinoic acid (hatched) treated cells is shown in Fig. 16. Activity of the EGF receptor promoter is decreased by approximately 50% in both the ECE16-1 and ECE16-D1 cell lines. No significant changes in promoter activity is detected following retinoic acid treatment in normal ECE or ECE16-D2 cells (Fig. 16).

To insure that the lack of retinoid responsiveness of the EGF receptor promoter transfected in the normal ECE and ECE16-D2 cells was not due to inactivity, each cell type was transfected as above and then treated with phorbol 12-myristate 13-acetate (TPA) for two days. TPA has been previously shown to induce this promoter plasmid (101). Fig. 17 shows that TPA is very efficient at inducing EGF receptor promoter activity in all of the cells types.
Figure #15. Regulation of EGF receptor mRNA levels by retinoic acid in HPV 16 immortalized and normal ECE cells. mRNA was isolated by poly-A agarose chromatography of guanidine thiocyanate extracted total RNA. 2 μg of each mRNA sample was subjected to alkaline denaturation and slot blotting onto nylon membranes. The nylon membranes were then hybridized and washed as in Experimental Procedures. The mRNA bands for β-Actin and EGF receptor are shown above a graph of corresponding to the resulting density of the RA treated EGF receptor mRNA band expressed as a percent of the control treated EGF receptor mRNA band. This experiment is representative of at least two separate determinations.
16-1 Control
16-1 Ra
D-1 Control
D-1 Ra
D-2 Control
D-2 Ra
R37 Control
R37 Ra

A   E

% Control Band Density

0   25   50   75   100   125   150

16-1  D-1  D-2  R37

Cell Type
Table #11. The EGF receptor promoter plasmid structure.

The EGF receptor promoter contains many important transcriptional regulatory sites. This 5' upstream region of the EGF receptor gene (positions -1100 to -19) is linked to the gene encoding luciferase terminated by the SV40 terminator. This promoter lacks TATA and CAAT box sequences (112). The major in vivo start site indicated by the solid downward arrow is at position -48. There are sites for uncharacterized transcriptional regulators such as the positive transcriptional regulator, EGFR-TF (transcription factor) around position -250 (118). There are also sites for the negative transcription factor EGFR-TR (transcriptional repressors) (119) and for the transcription factor E2F (114) around position -900 (119). There is an enhancer region from positions -485 to -134 containing several transcriptional activating sites (TCC)n and binding sites for the transcription factor SP1 (112,116). The region closest to the start site (positions -124 to -19) contains response elements that mediate responsiveness to phorbol ester, retinoic acid, glucocorticoids, and cAMP (101). All the above sites are indicated in the key.
THE EGF RECEPTOR PROMOTER
Figure #16. Regulation of EGF receptor promoter activity by retinoic acid in HPV 16 immortalized and normal ECE cells. Transfection of cells with the EGF receptor/luciferase reporter plasmid was carried out as described in experimental procedures. Cells were then treated for 2 days with DMSO (solid bars) or $10^{-7}$M RA (hatched bars) in medium containing 0.4% DLFCS. Cell were extracted and luciferase activity determined. The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of at least 3 separate experiments.
Figure #17. Induction of the EGF receptor promoter by TPA in each cell type.

Transfection of cells with the EGF receptor/luciferase reporter plasmid was carried out as in Fig. 16. Cells were then treated for 2 days with DMSO (control) or $10^{-7}$M TPA in medium containing 0.4% DLFCS. Cell were extracted and luciferase activity determined. The graph represents the activity of the EGF receptor promoter under the control of TPA as a fold induction over control activity of the EGF receptor promoter when treated with vehicle (DMSO). The results are expressed as the mean ± SEM of triplicate wells. This experiment is representative of at least 3 separate experiments.
Effect of HPV E6/E7 gene expression on EGF receptor promoter activity in normal ECE cells.

To assess the effect of Human papillomavirus type 16 E6 and E7 expression on the EGF receptor promoter in normal ECE cells, normal ECE cells were cotransfected with the EGF receptor promoter plasmid plus an equal concentration of a plasmid containing the HPV 16's E6 and E7 gene. The cells were treated with DMSO (control) or 10^{-7}M retinoic acid (RA) and EGF receptor promoter activity was measured as above. The cotransfection of HPV 16 E6 and E7 genes with the EGF receptor gene cause a 2-3 fold induction of EGF receptor promoter activity over control plasmid transfected cells but does not induce retinoic acid down regulation of the promoter activity (Fig. 18). A dose dependent increase in EGF receptor promoter expression is elicited when normal ectocervical epithelial cells are cotransfected with increasing concentrations of the HPV 16 E6/E7 plasmid (Fig. 19).
Figures #18 and 19. Effect of HPV 16 E6 and E7 expression on the EGF receptor promoter activity in normal ECE cells. Cotransfection of cells with the EGF receptor/luciferase reporter plasmid and the HPV E6 and E7 plasmid was carried out as in experimental procedures. Cells were then treated for 2 days with DMSO (solid bars) or 10^{-7}M RA (hatched bars) in medium containing 0.4% DLFCS (Fig. 18). Cell were extracted and luciferase activity determined. For Fig. 19, cells were cotransfected with 3ug of the EGF receptor promoter plasmid plus increasing amounts of the HPV E6/E7 plasmid. The results are expressed as the mean ± SEM of triplicate wells. Both experiments are representative of at least 3 separate experiments.
These results, along with in vivo data indicating that EGF receptor levels are elevated in cervical cancer, suggest that HPV is able to elevate EGF receptor levels in ectocervical cells via HPV type 16 E6 and E7 protein expression. In addition, the results indicate that retinoic acid's therapeutic effect on HPV involved cervical lesions may be mediated by down regulating elevated EGF receptor levels and decreasing growth sensitivity to EGF. To test this hypothesis, we screened known HPV positive cervical cancer cell lines, along with other normal and virus immortalized cell lines, to determine whether this phenomenon is associated with HPV presence. 

**Screening of Retinoic Acid Effects on EGF Binding in Other Virus Immortalized, Normal, and Cervical Carcinoma Cell Lines.**

The effect of retinoic acid on EGF binding was measured in normal ectocervical epithelial cells and HPV immortalized cell lines. Several HPV positive cervical carcinoma cell lines including the CaSki, ME180, and HeLa cell lines were also included in the study for comparison to the HPV immortalized cell lines. Two HPV negative cervical carcinoma cell lines, HT3 and C33A, were also measured. Several non-cervical cell types not containing HPV DNA were measured including normal human foreskin keratinocytes, KER-1 an SV40 large T-antigen immortalized human keratinocyte cell line (102), A431 an squamous carcinoma of the vulva.

Cells were treated with vehicle and 10^{-7}M retinoic acid for 2 days and EGF binding assays were performed. Normal ectocervical and ECE16-D2 cells exhibit lower EGF binding levels and no retinoic acid regulation is observed. Normal human
foreskin keratinocytes and the SV40 large T-antigen immortalized KER-1 cell line were chosen because they represent an analogous system of virus immortalization to that of the normal ECE and HPV 16 immortalized ECE16-1 cells. Both the normal and the immortalized keratinocytes had similar EGF binding levels and no significant change in EGF binding was detected following retinoic acid treatment (Table 12). A431 cells were found to have elevated levels of EGF receptors, even 2-3 times higher than that of the HPV positive cervical cell lines but the level of EGF receptor was not regulated by retinoic acid (Table 12). This result is not unexpected since the EGF receptor gene is highly amplified in this cell line (103). The HPV immortalized cell lines, ECE16-1 and ECE-D1, demonstrated elevated EGF binding levels that were significantly decreased by retinoic acid treatment (Table #12). Of the three HPV positive cell lines tested two, Caski (HPV16) and ME180 (HPV18 related HPV type), were found to have elevated levels of EGF binding and similar retinoic acid regulation as observed for ECE16-1 cells (Table 12). Although the EGF binding levels of the ME180 and the Caski cell lines were not as dramatically decreased at 10^{-7}M RA as were the ECE16-1 cells, EGF binding decreased by 70% and 45% respectively following treatment with 10^{-5}M RA (Table 12). These results indicated that HPV positive cervical cancer cell lines, having undergone malignant progression can become increasing resistant to the inhibitory effect of retinoic acid. HeLa cells (HPV18) were found to have lower EGF binding levels than the other HPV positive cell lines and significant alteration of EGF binding levels by retinoic
acid was not detected (Table 12). The variation between the HeLa cells and the other HPV positive cell lines may be due to the fact that HeLa cells do not contain functional receptors for retinoic acid (61) indicating that escape from retinoic acid regulation may be important in the progression of HPV positive cervical cancer cells toward malignancy. The two HPV negative cervical carcinoma cell lines, HT3 and C33A, exhibited different levels of EGF binding and neither demonstrated significant loss of EGF binding following retinoic acid treatment (Table #12). This result indicates that although elevation of EGF receptor levels can occur as a result of malignant progression that it can also occur specifically by the process of immortalization by the HPV genome. This result also suggests that the ability of retinoic acid to suppress EGF receptor levels and possibly EGF growth capacity is unique for HPV positive cells.
Table #12. Effect of RA on ¹²⁵I-EGF binding in other normal, virus immortalized, and cervical carcinoma cell lines. The ¹²⁵I-EGF binding was determined as described in "Experimental Procedures," following treatment with either vehicle or 10⁻⁷ M RA for 2 days. ‡ = Further EGF binding decreased following treatment with 10⁻³ M RA (Caski = 45% decrease, ME180= 70% decrease in EGF binding).

Each value is the mean ± SD of three determinations. This table is representative of at least 3 separate experiments.
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Since HPV immortalization had produced such a dramatic effect on retinoic acid regulation of normal ectocervical epithelial cell epidermal growth factor responsiveness, we decided to see if another retinoid regulated response of normal ectocervical cell was altered by HPV immortalization. Previous work had revealed that retinoids were potent regulators of ectocervical cell differentiation (38). Therefore we investigated retinoic acid regulation of genes which control the differentiation process, the transglutaminase and cytokeratin genes.

Chapter II: The Effect of Human Papillomavirus 16 Immortalization on Retinoid Regulation of Normal Ectocervical Cell Differentiation.

Retinoid regulation of transglutaminase activity in normal ectocervical epithelial cells. Transglutaminase (TG) is an enzyme present in stratifying epithelial cell types that catalyzes peptide cross-links thereby forming the cornified envelope (21-23). Moreover, TG activity is frequently regulated by agents that regulate cell differentiation (23, 24-27). We have previously shown that hydrocortisone stimulates and retinoic acid suppresses ECE cell terminal differentiation as measured by cornified envelope production (35,38). We therefore assayed the effects of these agents on TG activity (Fig. 20). Hydrocortisone (HC) at 1 μM only slightly increases TG activity while 10 nM retinoic acid (RA) suppresses activity by 9 to 10-fold (Fig. 20). In addition, when the cells are simultaneously treated with hydrocortisone and retinoic acid (HC+RA), the suppressive effect of retinoic acid predominates (Fig. 20).
Figure #20. Retinoid and glucocorticoid regulation of transglutaminase activity. ECE cultures were grown until near-confluent in normal growth medium and then shifted to the identical medium except that the fetal calf serum was replaced with 5% DLFCS and the medium was supplemented with 1 μM hydrocortisone (HC), 10 nM retinoic acid (RA), hydrocortisone and retinoic acid (HC+RA) or vehicle alone (C). After seven days of treatment, the cells were harvested and total TG activity was measured. The results are presented as the mean ± SEM for three experiments.
The dissociation constant (Kd) for binding of retinoic acid to the retinoic acid receptor has been estimated in the range of 0.1 to 10 nM (104,105). As shown in Fig. 21, retinoic acid regulates transglutaminase activity in a range consistent with an interaction with the retinoid receptor(s). Half-maximal suppression of transglutaminase activity by RA is observed at 1 nM with maximal suppression at 10 nM. Ro 13-6298, an unhydrolyzable analogue of retinoic acid, is 10 times more effective with half-maximal and maximal suppression at 0.1 and 1 nM, respectively.

Because soluble and plasma membrane bound forms of transglutaminase have been described that may have different functions (93), we examined the subcellular localization of TG activity in ECE cells. As shown in Fig. 22, in cells grown in retinoid-free medium, 70% and 30% of the TG activity is localized in the membrane and soluble fractions, respectively. Addition of increasing concentrations of RA resulted in a dose-dependent decrease in soluble and particulate TG with maximal suppression of 6-fold (membranous) and 2.5-fold (soluble) at 10 nM RA.
Figure #21. Concentration dependent suppression of TG activity by retinoids. ECE cells were grown until near-confluent in normal growth medium and then shifted to the identical medium in which the fetal calf serum was replaced by 5% DLFCS. The medium was further supplemented with various concentrations of the synthetic retinoid, Ro 13-6298 (O) or the natural retinoid, trans-retinoic acid (●). After seven days of treatment, extracts were prepared and assayed for total transglutaminase activity. Each curve was generated from a separate experiment and the data points represent the means ± SEM of triplicate determinations.
Figure #22. Subcellular localization of transglutaminase activity. Near-confluent cultures of ECE cells growing in medium supplemented with 5% DLFCS were treated with the indicated concentration of trans-retinoic acid for 7 days. The cells were then harvested and assayed for TG activity in the membrane bound (O) and soluble (●) fractions as outlined in experimental procedures. The results are expressed as the mean ± SEM of triplicate cultures.
Retinoid regulation of transglutaminase protein and RNA levels in normal ectocervical epithelial cells.

There are several transglutaminase enzymes (21,26,27,93). Keratinocyte type I transglutaminase is a 90 kDa membrane bound protein that is responsible for cornified envelope formation in epidermal keratinocytes (93). An antibody specific for type I TG immunoprecipitates a 90 kDa protein from soluble and membrane fractions prepared from ECE cells. The level of this immunoprecipitable material decreases 90% in cells treated with 10 nM retinoic acid (Fig 23). Moreover, as shown in Fig. 24, ECE cells grown in retinoid free medium (-) express high levels of the 3.6 kb mRNA that hybridizes to a type I TG specific cDNA, while cells grown in the presence of 10 nM retinoic acid for 5 days (+) express low or undetectable levels of this transcript. Actin mRNA levels (A) are not effected by retinoid treatment.
Figure #23. Immunoprecipitation of transglutaminase. To measure type I TG protein concentration in the fractions prepared for TG enzyme activity assays (particulate), NaCl was added to each sample at a final concentration of 137 mM prior to immunoprecipitation. Type I transglutaminase was immunoprecipitated by incubating extracts with a type I transglutaminase-specific monoclonal antibody (93) followed by incubation with protein A sepharose. The protein A-antibody-antigen complex was collected by centrifugation, washed three times with PBS and resuspended in Laemmli sample buffer. M = molecular weight markers, C = vehicle treated cultures, RA = 10 nM RA treated cultures, and tg = transglutaminase.
Figure #24. Retinoid regulation of transglutaminase mRNA levels. Poly (A) + RNA was prepared from post-confluent cultures of ECE cells grown for 5 days in the presence (+) or absence (-) of 10 nM trans-retinoic acid. The RNA was electrophoresed on an agarose gel, transferred to Biodyne A membrane and parallel sets of lanes were hybridized with 32P-labeled cDNAs encoding type I transglutaminase (TG) (pTG-7) or actin (A) (pA-1). The blots were then washed and exposed on x-ray film.
Regulation of cytokeratin protein and mRNA levels of normal ectocervical epithelial cells.

The cytokeratins are a large gene family of proteins that form the intermediate filaments in epithelial cells and are regulated during terminal differentiation in stratifying epithelial cell types. As shown in Fig. 25, ECE cell cytokeratin levels are dramatically altered by treatment with 10 nM RA. When cells are grown in retinoid-free medium (panel A), cytokeratins K5, K6, K13, K14, K16, K17 and K19 are detected. When cells are treated for 4 days with 10 nM RA, the levels of cytokeratins K5, K6, K13, K14 and K17 are unchanged relative to K13. The level of K16 is dramatically reduced (>20-fold) (panel B). In contrast, cytokeratin K19 is increased (5 to 10-fold). Cytokeratins K7 and K8 are also increased by retinoid treatment (Panel B).

As shown in Fig. 26, changes in cytokeratin levels are directly reflected at the mRNA level. ECE cells were grown in the retinoid-free medium (-) or in the presence of 100 nM RA (+). Retinoid treatment resulted in a 5-fold increase in mRNA encoding K19 and a greater than 20-fold decrease in the mRNA encoding K16. Actin (A) and involucrin (Inv) mRNA levels were not significantly changed by retinoid treatment.
Figure #25. Retinoid regulation of cytokeratin protein levels. ECE cells were grown until near-confluent in normal growth medium and then shifted to medium lacking fetal calf serum and supplemented with 5% DLFCS. The cells were grown for an additional 6 days in this medium without retinoid (A) or supplemented with 20 nM Ro 13-6298 (B). The cells were then labeled with 35S-methionine for 20 h and the cytokeratins were prepared, electrophoresed on two dimensional gels and fluorographed. The bands were visualized by exposure on x-ray film and relative intensity was estimated by densitometry. All changes were determined relative to K13 which does not change with retinoid treatment and serves as an internal control.
Figure #26. Retinoid regulation of RNAs encoding cytokeratins. ECE cells were grown as described in the legend to Fig. 7 in the presence (+) or absence (-) of 20 nM Ro 13-6298. Parallel blots were hybridized with 32P-labeled cDNAs encoding K5, K13, K16, K19, involucrin (Inv) and actin (A). The blots were then washed, exposed on x-ray film and the relative difference in intensity estimated by densitometry. The size of each mRNA species is K19, 1.6 kb; K13, 2.1 kb; K5, 2.4 kb; Inv, 2.4 kb; K16, 2.1 kb and actin, 2.1 kb.
Comparison of retinoic acid's effect on differentiation on normal and HPV type 16 immortalized ectocervical epithelial cells.

In summary the effect of retinoic acid on normal ectocervical epithelial cell differentiation is to reduce differentiation by decreasing transglutaminase activity, protein and mRNA thus inhibiting production of this important enzyme involved in differentiation. Also retinoids change the cytokeratin profile of the ectocervical epithelial cells to a less differentiated phenotype by reducing cytokeratin 16 and increasing cytokeratins 7,8 and 19. Previous results for retinoid regulation of differentiation in the HPV immortalized cell line ECE16-1 revealed an almost identical pattern of regulation. Retinoids were found to decrease transglutaminase activity in ECE16-1 cells and retinoids reduced cytokeratin 16 and increased cytokeratins 7, 8 and 19.

A few differences were found. One difference was that retinoids also reduce cytokeratins 5, 6, 14 and 17 in ECE 16-1 cells which were not changed following retinoid treatment of normal ectocervical epithelial cells (49). Also ECE16-1 cells were sensitive to retinoids in the serum of the medium under normal growth conditions while normal ectocervical epithelial cells were not affected by serum concentration of retinoids. In general a larger fraction of the HPV cells remain in a proliferative state and do not undergo differentiation as well as normal ectocervical epithelial cells. These results indicate that the HPV immortalized cell line, ECE16-1, may be more sensitive to retinoids than the normal ectocervical epithelial cells.
DISCUSSION

Cervical cancer is a leading cause of death for women worldwide (106). Human papillomavirus type 16 is an important etiological agent in the emergence of cervical cancer (43, 48). Since retinoic acid has been used clinically to treat HPV-involved cervical lesions and cancers (14-19), understanding the effects of HPV immortalization and retinoic acid regulation of normal ectocervical epithelial cell biology is of great importance. Understanding HPV alterations in retinoic acid regulation of ectocervical epithelial cells, could lead to the discovery of the mechanisms of retinoic acid’s anti-neoplastic effects on cervical cancer. This study attempts to investigate the alterations in retinoic acid control of growth and differentiation of normal ectocervical epithelial cells induce by HPV immortalization.

Our results indicate that HPV immortalized cell lines have elevated EGF receptor levels, higher EGF binding and EGF growth capacity as compared to normal ectocervical epithelial cells in culture (ECE16-1 > ECE16-D1 > ECE16-D2 cells > normal ectocervical epithelial cells). This result is important in that epidermal growth factor is an important mitogen for many cell types in culture including epithelial cells (67). Many growth factor receptors, including the EGF receptor, can function as oncogenes, that when aberrantly expressed allow for malignant development (70-72). There are many examples of cancer cells obtaining a growth advantage over their normal counter parts through induction of a autocrine growth factor pathway (71,76,79). Often cancer cells have been found to over expresses
both the growth factor and its receptor providing cancer cells with a decreased need for growth factor and increased growth factor autonomy. Alterations in epidermal growth factor (EGF) receptor expression or function can cause aberrant mitogenic signaling possibly contributing to the initiation and progression of several malignant cancers (70-72).

Overexpression of the EGF receptor is a common characteristic in many tumors and was detected in 100% of the squamous lung, vulval, and cervical carcinomas (7,8,73-76). Examination of EGF receptor expression in cervical biopsies in which HPV was present revealed elevated EGF receptor expression associated with dysplastic and neoplastic changes of cervical tissue (9,10). This suggests that ectocervical epithelial cells which express higher levels of EGF receptors have a growth advantage over their normal counterparts. Our results indicate that human papillomavirus immortalization increases EGF receptor protein expression, possibly keeping the HPV immortalized cell lines in a highly proliferative state by increasing epidermal growth factor responsiveness and EGF stimulated growth capacity. A significant implication of these studies is that the increase in EGF receptor levels, seen in HPV positive cervical cancer and cervical cancer cell lines, can occur specifically and as an early event in HPV positive cells.

Our investigation also revealed that retinoic acid, while not effecting the growth of normal ectocervical epithelial cells, inhibited EGF-stimulated cell proliferation in HPV immortalized cell lines although to varying degrees (ECE16-
1 > ECE16-D1 > ECE16-D2). The degree of growth inhibition by retinoic acid was modest in the ECE16-D2 cell line which demonstrated the smallest elevation of EGF receptors over normal ectocervical epithelial cells of the three HPV 16 immortalized cell lines. The growth inhibition of two of the HPV cell lines (ECE16-1 and ECE16-D1) was associated with a dramatic decrease in EGF binding and EGF receptor protein levels, while the lower EGF receptor levels in normal ectocervical epithelial cells and the ECE16-D2 cell line are unaltered by retinoic acid treatment. This observation suggests that although retinoic acid is able to slightly suppress growth of the ECE16-D2 cell line, direct regulation of EGF receptor levels is not an important mechanism of growth control in this HPV 16 immortalized cell line. The susceptibility of the HPV immortalized cell lines to retinoic acid is not unexpected because the initial characterization of the ECE16-1 cells revealed heightened sensitivity to retinoids (49). These results are consistent with previous reports on growth regulation by retinoic acid in HPV positive cell lines. In some studies, growth inhibition was also associated with a decrease in E6 and E7 expression (52,53). Other studies support our results. For example, retinoic acid growth suppression of HPV positive ME180 cells is associated with decreased EGF receptor levels and not with a decrease E6 and E7 expression levels (110). In ECE16-1 cells and ECE16-D2, appreciable retinoic acid suppression of E6 and E7 transcription also does not occur (66, Eckert unpublished results). Our results are the first to indicate that retinoic acid growth inhibition of HPV immortalized cells could occur by
regulation of the EGF receptor pathway. Previous studies of retinoic acid regulation of EGF receptor expression has yielded heterologous results depending on cell type and culture conditions (107-110). However it has been shown that the EGF receptor promoter contains a thyroid (T₃) hormone response element (TRE) to which the receptor for retinoic acid is able to bind and regulate transcription, both alone and in concert with thyroid hormone receptor (61). Modulation of the level of EGF receptors could subsequently increase or decrease the sensitivity of the cell to EGF, which could be an important factor in growth regulation. Therefore, transcriptional down-regulation of elevated EGF receptor levels may be a means by which retinoic acid can decrease proliferation in some HPV immortalized ectocervical cell lines while not effecting the growth and function of normal ectocervical epithelial cells.

If retinoic acid is able to down regulate elevated EGF receptor levels while not affecting normal EGF receptor levels or impairing normal EGF receptor function, then retinoic acid would make an excellent candidate for slowing the growth of malignancies that over express the epidermal growth factor receptor. In our studies retinoic acid did not to interfere with EGF receptor kinase function or metabolism in either normal or HPV immortalized ectocervical epithelial cells. Scatchard analysis confirmed these results and indicated no change in the affinity of the EGF receptor after retinoic acid treatment. The number of EGF binding sites per cell found in the normal cells are comparable to those found on epidermal keratinocytes (111). The number of EGF binding sites in the immortalized ECE16-1 cells is about six times
higher than the normal ECE cells, but around four times less than reported for A431 cells to which normal concentrations of EGF is toxic (111). Retinoic acid treatment of ECE16-1 cells caused no change in EGF receptor autophosphorylation or internalization. However retinoic acid did increase EGF receptor stability in the HPV immortalized cell line ECE16-1. The binding of a ligand to the EGF receptor would be expected to decrease the stability of the receptor. Under control conditions, the ECE16-1 cells might be producing a ligand for the EGF receptor causing rapid turnover of the receptor. The increased stability with retinoic acid treatment may indicate that not only can RA reduce EGF receptor number but it may also interfere with the production of this putative ligand in the ECE16-1 cell line. Retinoic acid treatment did not induce the secretion of soluble ligand for the EGF receptor. Although retinoic acid induction of a membrane bound EGF receptor ligand has not been excluded, it is unlikely based on the stability data. Therefore we postulate that retinoic acid is directly regulating the level of EGF receptor protein expression.

Further investigation of the mechanism of HPV-induced alterations in retinoic acid regulation of EGF receptor levels in normal ectocervical epithelial cells examined EGF receptor mRNA and EGF receptor promoter regulation. These studies revealed that the EGF receptor mRNA levels decreased approximately 50% in ECE16-1 cells and 25% in ECE16-D1 cells. However, no changes in EGF receptor mRNA levels were detected in normal ECE or the ECE16-D2 cells. EGF
receptor promoter activity was measured by transfecting in a luciferase reporter plasmid containing the 5' region of the EGF receptor gene containing 1081 basepairs 5' to ATG translation start site as reported by Hudson et al. 1989 (101). Table #11 shows a reproduction of the map of this EGF receptor promoter plasmid (101). Activity of the EGF receptor promoter is decreased by approximately 50% in both the ECE16-1 and ECE16-D1 cell lines. However, no significant changes in promoter activity is detected following retinoic acid treatment in normal ECE or ECE16-D2 cells. This lack of response was not due to inactivity of EGF receptor promoter in these cell lines because TPA, a known activator of the EGF receptor promoter, is very efficient in inducing EGF receptor promoter activity in all of the cells types. These results indicate that the elevated EGF receptor levels and retinoic acid downregulation of the EGF receptor protein the ECE16-1 and ECE16-D1 cell lines is most likely due to altered transcriptional control of the EGF receptor promoter.

This EGF receptor promoter plasmid contains many important transcriptional regulatory sites. This 5' upstream region of the EGF receptor gene (positions -1100 to -19) lacks TATA and CAAT box sequences (112). The major transcriptional start site is at position -48. Also, there are sites for uncharacterized transcriptional regulators such as the positive transcriptional regulator, EGFR-TF (transcription factor) around position -250 (118). There are sites for the negative transcription factor EGFR-TR (transcriptional repressor) (119) and for the transcription factor E2F (114) around position -900 (119). There is an enhancer region from positions -485
to -134 containing several transcriptional activating sites (TCC)n and binding sites for the transcription factor SP1 (112,116). Moreover, the region closest to the start site (positions -124 to -19) contains response elements that mediate responsiveness to phorbol ester, retinoic acid, glucocorticoids, and cAMP (101). Some of these sites may be important in mediating the altered retinoid transcriptional regulation of the EGF receptor gene induced by HPV immortalization. Our results suggest that human papillomavirus type 16 proteins are capable of modulating EGF receptor promoter activity. Expression of HPV 16 E6 and E7 genes in normal ECE cells increases EGF receptor promoter activity 2-3 fold. This is important since all our HPV immortalized cell lines and many cervical cancer cell lines demonstrated elevated expression of EGF receptors which probably increases their growth sensitivity to EGF as compared to normal cells.

These two HPV gene products can interact with factors that bind to EGF receptor promoter regulatory elements. For example the HPV type 16 E7 protein can compete with the E2F transcriptional factor for binding to the unphosphorylated form of the retinoblastoma protein (RB). This interaction is important because binding of unphosphorylated RB to E2F, inactivates the E2F transcription factor and prevents recruitment the proper transcription complex to the promoter (51,120). However HPV 16 E7 binds RB and liberates E2F thus facilitating transcriptional activation by E2F, a transcription factor known to regulate the EGF receptor promoter. The interaction of HPV E6 with p53 is also interesting because p53 has been shown to
have transcriptional regulatory capabilities. Finally it may also be possible that yet unknown interactions of HPV proteins with transcriptional response site or other transcriptional factors may be important. Also the inactivation of p53 and pRB by HPV E6 and E7 could lead to inhibition of normal ectocervical cell responsiveness to DNA damage. The p53 protein has known necessary functions in regulating normal cell responsiveness to DNA damaging events (122). This inactivation of these two important tumor suppressor proteins could allow for further mutation events necessary for cervical cancer development.

In some systems, retinoid growth suppression occurs concurrently with a reduction in E6/E7 levels (52,53). However retinoic acid failed to downregulate the EGF receptor promoter when the HPV 16 E6 and E7 genes were expressed in normal ectocervical epithelial cells. Also in ECE16-1 and ECE16-D1 no appreciable decrease in E6 and E7 mRNA following retinoic acid treatment is detected (66, Eckert unpublished results). This data also suggest that direct modulation of HPV E6 and E7 levels is not responsible for the altered retinoid regulation, seen in our system. These data do indicate that other mechanisms of retinoid growth control besides EGF receptor modulation exist in some HPV immortalized cell lines, as is probably the case for our HPV immortalized ectocervical cell line, ECE16-D2. Therefore although HPV E6 and E7 is able to increase EGF receptor promoter expression, direct modulation of the levels of these two HPV proteins is probably not responsible for the retinoic acid regulation of the EGF receptor promoter in our two
HPV immortalized cell lines.

It remains to be seen whether the decrease of EGF receptor promoter activity by retinoic acid in the two HPV immortalized cell lines, is a direct action of one of the HPV 16 proteins on the EGF receptor promoter or an indirect effect caused by interactions of HPV proteins with cellular proteins. Three different retinoic acid receptors (RARs) which bind trans-retinoic acid have been identified RAR-α, RAR-β, and RAR-γ. These RAR receptors modulate gene expression by binding to specific but diverse hormone response elements (HREs) in the gene’s promoter region (39). All three RARs are present in normal cervical epithelial cells (Rorke unpublished results). The nuclear receptors for retinoic acid belong to the steroid hormone receptor / thyroxine (T₃) receptor family and seem to effect growth and differentiation in a similar manner. Three distinct receptors that bind the 9-cis form of retinoic acid but not trans-retinoic acid have been identified, RXR-α, RXR-β, and RXR-γ. Interactions between the RARs, RXRs, and T₃ receptors have been reported, indicating a complex interplay between these nuclear receptors in vivo. Retinoic acid receptors can also have an antagonistic activity on the AP-1 pathway in which a complex of c-fos and c-jun is activated by growth factors and the tumor promoter phorbol 12-myristate 13-acetate (39). Therefore any of the HPV 16 proteins could be involved in the altered retinoic acid regulation of the EGF receptor promoter by their interactions with cellular transcription factors.
In a previous study, retinoic acid decreased EGF receptor levels in ME180 cells (110), a HPV positive cervical carcinoma cell. This decrease in EGF receptor protein levels was found to be caused by decreased transcription of the EGF receptor gene (60). Our screening of other HPV positive cell lines revealed that ME180 cells as well as CaSki cells had elevated EGF binding capacity similar to ECE16-1 cells and that binding decreased similarly with retinoic acid treatment. A second HPV 16 immortalized cell line, ECE16-D1 also demonstrated elevated EGF binding levels and altered RA regulation as compared to the normal ECE cells. Other normal cells (human foreskin keratinocytes) and an SV40 large T-antigen immortalized keratinocyte (KER-1) screened, revealed no significant regulation by retinoic acid. Although both the SV40 large-T antigen and the immortalizing HPV E6 and E7 proteins interact with p53 and RB, they do so in a different manner. For example SV40 Large-T antigen binds to p53 and increases p53’s stability and half-life in cells (122). In contrast, HPV E6 interacts with p53 and in vitro causes p53 to be degraded possibly depleting the cell of p53 (121). Therefore it is not surprising that the HPV 16 and SV40 immortalized cell lines respond differently to retinoic acid. These observations and the finding that the two HPV negative cell lines do not demonstrate significant loss of EGF binding following retinoic acid treatment, support the hypothesis that HPV is directly altering retinoid regulation of EGF receptor expression in normal ectocervical epithelial cells.
Since retinoic acid is able to reverse some cervical lesions and has been used clinically to treat cervical cancers of which a large percentage are HPV positive (14-18), understanding the effects of retinoic acid on normal and HPV immortalized cervical epithelial cells is of great importance. The observation that the HPV immortalized cell lines have higher levels of EGF receptors than their normal counterparts and that retinoic acid is able to inhibit growth of the ECE16-1 and ECE16-D1 cell lines by decreasing these elevated levels of EGF receptor, may in part explain why retinoids are effective agents in treating HPV positive cervical lesions. Also the EGF receptor levels and retinoid regulation observed in two HPV positive cervical carcinoma lines indicates that the EGF receptor levels and retinoid regulation of these levels in these cells is similar to that of the HPV immortalized cell lines, ECE16-1 and ECE16-D1. These data indicate that the elevation of EGF receptor levels in the HPV type 16 immortalized cell lines as compared to normal ectocervical cells and the altered regulation of EGF receptor levels by retinoic acid in ECE16-1 and ECE16-D1 cells could be of importance in understanding the initiation and progression of cervical cancer. Retinoic acid, although somewhat able to mediate growth inhibition in the HPV 16 immortalized cell line ECE16-D2 and the HPV positive cervical cancer cell line Hela (53), did not reduce EGF receptor levels. Hela cells do not contain receptors for retinoic acid (61), which may explain their lack of responsiveness. This may indicate that release from retinoic acid control of proliferation could be an important mechanism in malignant progression
of HPV positive cervical cancer cell lines.

Retinoids are potent regulators of normal ectocervical epithelial cell differentiation (67). We studied retinoid regulation of differentiation dependent genes, transglutaminase and cytokeratins, to determine if HPV 16 immortalization altered other retinoid responses of normal ectocervical epithelial cells. Therefore we investigated retinoid regulation of differentiation specific genes, transglutaminase and cytokeratins and compared these results to results obtained previously for the HPV immortalized ECE16-1 cell line.

The ectocervix is lined by a stratifying squamous, non-keratinizing epithelium that includes three layers: a basal cell layer that consists of proliferating cells, the suprabasal layer composed of differentiated cells and the terminal layer consisting of terminally differentiated cells (called envelopes or superficial cells)(1,2). Differentiation can be monitored by determining the number of envelopes which is a marker of increased cell differentiation (35,38). The cervical cell cornified envelope is comprised of membrane bound and soluble precursors that are covalently cross-linked via iso-peptide bonds by the enzyme transglutaminase. In a previous study we showed that retinoids dramatically suppress ECE cell envelope formation (35,38). However, in spite of this suppression, the level of one of the envelope precursor proteins, involucrin, did not change (38). This result suggests that ECE cell envelope formation may not be regulated by the availability of envelope precursors, at least as measured by involucrin content.
An alternative mechanism is regulation of the level of transglutaminase, the enzyme that catalyzes formation of the covalent cross-links in the envelope (21-23). In ECE cells trans-retinoic acid and the synthetic retinoid, Ro 13-6298 both reduce transglutaminase activity by nine to ten fold. Hydrocortisone, which has been shown to antagonize retinoids in other systems (38), can antagonize the retinoid-dependent suppression of envelope formation in ECE cells (35). However at 10 nM trans-retinoic acid, hydrocortisone (1 uM) is unable to antagonize retinoid suppression of ECE cell transglutaminase activity. ECE cell TG activity is half-maximally inhibited at 1 nM RA and 0.1 nM Ro 13-6298. This is consistent with our previously reported half-maximal inhibition of cornified envelope formation at 0.07 nM Ro 13-6298 (38). Maximal (85-90%) inhibition of envelope production and TG activity is observed at 10 nM RA and 1nM Ro 13-6298. Thus, envelope formation is suppressed in conjunction with suppression of the TG level, indicating that TG activity is likely to be the major regulator of envelope formation in ECE cells.

The magnitude of reduction in level of mRNA encoding type I transglutaminase is similar to the reduction in TG enzyme activity. This result indicates that at steady state, the level of TG enzyme activity is largely determined by changes in the level of TG mRNA. At the present time we have not yet determined whether this is mediated by a change in transcription of the TG gene or regulation of RNA stability/turnover. Thus in human cervical epithelial cells, TG activity and protein appears to be regulated directly by the level of TG mRNA.
The cytokeratins are a family of proteins that form the intermediate filaments in epithelial cells (31-34). Because they are expressed in a tissue-specific manner, keratin expression can be used to identify the tissue of origin of cultured cells (31, 34,36). Cultured ectocervical epithelial cells express cytokeratins K5, K6, K8, K13, K14, K16, K17 and K19 (35), an expression pattern consistent with that observed in the ectocervix in vivo (34). Moreover, keratin expression is regulated in a differentiation-dependent manner by agents that effect epithelial cell differentiation, including retinoic acid (24,25). Because of this, keratin expression is a sensitive barometer of the extent of cell differentiation (25, 31-36). Our present experiments show that ECE cell keratin levels are also regulated by retinoids. Retinoid treatment of ECE cells does not change the levels of cytokeratins K5, K6, K13 and K14, while K16 decreases. In contrast, retinoid treatment increases K7, K8 and K19 levels. The changes in keratin protein expression is reflected by a parallel change in the level of the corresponding mRNAs. Thus, as is the case with transglutaminase (above), keratins are regulated by the transcription rate of each gene and/or the stability of the keratin transcript.

Retinoid regulation of differentiation in the normal ectocervical epithelial cells can be compared with results obtained previously for retinoid regulation in the HPV immortalized cell line ECE16-1 (49). Previous results for retinoid regulation of differentiation in the HPV immortalized cell line ECE16-1 revealed an almost identical pattern of regulation. Retinoids were found to decrease transglutaminase
activity in ECE16-1 cells and to reduce cytokeratin K16 and to increase cytokeratins K7, K8 and K19. This result shows that the effect of retinoic acid on the transcriptional regulation of these genes is unaltered by HPV immortalization. However a few differences were found indicating that HPV immortalization had slightly alter retinoid regulation of differentiation. One difference was that under normal growth conditions cytokeratins K7 and K8 are expressed in the ECE16-1 cell line and retinoid treatment also reduced cytokeratins K5, K6, K14 and K17 in ECE 16-1 cells. The expression of these cytokeratins was unaffected by retinoid treatment of normal ectocervical epithelial cells (49). Also ECE16-1 cells were sensitive to retinoids present in the serum of the medium under normal growth conditions while normal ectocervical epithelial cells were not affected by serum retinoids. In general a larger fraction of the cells remain in a proliferative state and do not undergo differentiation as well as normal ectocervical epithelial cells. These results indicates that HPV immortalization of normal ectocervical epithelial cells interferes with the ability to undergo terminal cell differentiation and causes increased sensitivity to retinoids.

In conclusion, HPV type 16 immortalization of human ectocervical epithelial cells seems to alter retinoid regulation of EGF stimulated proliferation, while not dramatically affecting retinoid regulation of differentiation in normal ectocervical epithelial cells. Human papillomavirus immortalization of normal ectocervical epithelial cells causes a specific increase in the level of EGF receptor, higher EGF
binding and EGF growth capacity as compared to normal ectocervical epithelial cells in culture. Retinoic acid, while not effecting growth of normal ECE cells, inhibits EGF stimulated growth of HPV immortalized cell lines in a dose dependent manner. Retinoic acid growth suppression of ECE16-1 and ECE16-D1 cell lines was preceded by a reduction of epidermal growth factor (EGF) receptor binding, protein levels, mRNA levels, and promoter activity. Suppression of the EGF receptor was not detected in the normal ECE or the ECE16-D2 cell line. Transient cotransfection of the HPV-16 E6/E7 genes with the EGF receptor promoter in normal ECE cells stimulates activity of the promoter two- to three-fold. Retinoids decrease normal ectocervical epithelial cell differentiation by changing transglutaminase activity and keratin gene expression. Immortalization by HPV type 16 does not alter the level of expression of transglutaminase or keratins or the effect of retinoic acid on transglutaminase or keratin expression. However, the immortalized cells seem to have a heightened sensitivity to retinoids.

Therefore, HPV type 16 immortalization induces retinoid regulation of normal ectocervical epithelial cell proliferation, while not dramatically altering retinoid regulation of differentiation. Immortalization of normal ectocervical epithelial cells by HPV increases EGF growth capacity and EGF receptor levels possibly by increasing transcription of the EGF receptor gene through HPV E6 and E7 expression. This is the first report to indicate that the overexpression of the EGF receptor occurring in many HPV positive cervical cancers and cervical cancer cell
lines may be a specific and early event in malignant progression. HPV immortalization of normal ectocervical epithelial cells seems to recast retinoic acid's role as a regulator of differentiation, to a modulator cervical cell growth. This novel growth inhibition by retinoic acid occurring in HPV immortalized cells seems to occur by down-regulation of elevated EGF receptor levels of HPV immortalized cells. This finding is novel and significant in that it is the first report to indicate that HPV immortalization may alter the effect of retinoic acid on normal cervical cell growth by inhibiting an heightened growth factor responsiveness by reducing elevated growth factor receptor levels. This observation suggests a mechanistic basis for retinoid therapy in the treatment of cervical cancer.
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