Construction of Artificial Epithelial Tissues Prepared from Human Normal Fibroblasts and C9 Cervical Epithelial Cancer Cells Carrying Human Papillomavirus Type 18 Genes

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One cervical cancer cell line, C9, carrying human papillomavirus type 18 (HPV18) genes that is one of the major etiologic oncoviruses for cervical cancer was characterized. This cell line was further characterized for its capacity related to the epithelial cell proliferation, stratification and differentiation in reconstituted artificial epithelial tissue. The *in vitro* construction of three dimensional artificial cervical epithelial tissue has been engineered using C9 epithelial cancer cells, human foreskin fibroblasts and a matrix made of type I collagen by organotypic culture of epithelial cells. The morphology of paraffin embedded artificial tissue was examined by histochemical staining. The artificial epithelial tissues were well developed having multilayer. However, the tissue morphology was similar to the cervical tissue having displasia induced by HPV infection. The characteristics of the artificial tissues were examined by determining the expression of specific marker proteins. In the C9 derived artificial tissues, the expression of EGF receptor, an epithelial proliferation marker proteins for stratum basale was observed up to the stratum spinosum. Another epithelial proliferation marker for stratum spinosum, cytokeratins 5/6/18, were observed well over the stratum spinosum. For the differentiation markers, the expression of involucrin and filaggrin were observed while the terminal differentiation marker, cytokeratins 10/13 were not detected at all. Therefore the reconstituted artificial epithelial tissues expressed the same types of differentiation marker proteins that are expressed in normal human cervical epithelial tissues but lacked the final differentiation capacity representing characteristics of C9 cell line as a cancer tissue derived cell line. Expression of HPV18 E6 oncoprotein was also observed in this artificial cervical epithelial tissue though the intensity of the staining was weak. Thus this artificial epithelial tissue could be used as a useful model system to examine the relationship between HPV-induced cervical oncogenesis and epithelial cell differentiation.

Key words: artificial epithelial tissue, differentiation, tissue engineering, human papillomavirus (HPV) oncogenesis

INTRODUCTION

The multistage nature of carcinogenesis was first defined over 50 years ago by the sequential topical application of clinical agents to mouse skin [1]. More than 90% of human cancers derived from epithelial cells of each organ. Therefore, researches on the epithelial tissue have provided remarkable insights into the biology, biochemistry, pharmacology, and genetics of carcinogenesis of human cancers. These studies indicated that the progressive stages were predictable during the clonal evolution of a normal epidermal cell through the benign squamous papilloma stage into a squamous cell carcinoma, and the reproducible genetic and epigenetic events contributed to these changes [1].

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Human papillomaviruses (HPVs) are important human pathogens associated with a variety of neoplasia in skin and genital mucosa. E6 and E7 oncogenes of HPV type 16 and 18, detected in genital carcinomas induce immortality in cultured human foreskin epithelial cells, cervical epithelial cells and breast epithelial cells [2, 3]. The study of the complete life cycle of these viruses has been hampered by the lack of a cell culture system that would permit vegetative viral replication. Researchers have propagated HPVs in rodents by grafting infected tissue, but no efficient reproducible permissive *in vitro* system has yet been developed. This is probably the result of the evolution of a viral life cycle that is tightly coupled to the differentiation program of epithelial cells in which virion production is limited to differentiating suprabasal cells. Recently researchers have been trying to develop artificial epithelial tissue for a system to study the cervical cancer including papillomavirus induced oncogenesis *in vitro*

and also epithelial cancer in general.

Intensive investigations have resulted in developing tissue engineering technologies for the production of epithelial tissues from many different epithelial cell sources for various clinical applications [4-10]. Epithelial tissue is composed of two components, a connective tissue or dermis and a covering epithelium. The extracellular matrix materials in the dermis provide the structural and biological support. The epithelium in turn provides biological feedback to the dermis through the release of cytokines and other cellular biological mediators. The tissue culture system made from cervical epithelial cells carrying HPV genome could be used as an unique system in the studies of the mechanisms how this viral latency is maintained or terminated and how the regulation of the synthesis of viral genes and proteins and the assembly of papillomavirus virions through *in vitro* epithelial tissue development is controlled [11-14].

In this paper, we screened for a cervical cancer cell line, C9 that carries HPV 18 genome and expresses HPV 18 oncoprotein E6. With this cell line, artificial epithelial tissues *in vitro* were constructed using tissue engineering technique to develop a model system to study human papillomavirus-associated cervical carcinogenesis and epithelial cell differentiation. The morphology of artificial epithelial tissues was examined by histochemical staining. The differentiation-related characteristics were examined by immunohistochemical analysis using monoclonal antibodies against marker proteins for proliferation and differentiation of cervical epithelial tissues.

MATERIALS AND METHODS

Cell Lines and Media

Human skin cancer cell lines (SPF1 and SPF2 were obtained from our laboratory stocks) and cervical cancer epithelial cell lines (C9 and C10 established by Dr. J. Kim at Catholic University) were used. The cervical epithelial cancer cell lines were established from tumor tissues of squamous cell carcinoma of Korean women uterine cervix. These cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) under conditions of 5% CO_2 in air at 37°C.

Screening Cell Lines Carrying HPV 18 E6 Genes

The presence of HPV related genes in the above cell lines was confirmed by the amplification of HPV genes from chromosomal DNA isolated from cells used as target material using DNA polymerase chain reaction (PCR). The procedures used for the amplification of HPV 18 E6 oncogene using specific amplimers were described previously [15].

Construction of Dermal Equivalent

Dermal fibroblasts were isolated from a neonatal human foreskins obtained from routine circumcisions following the published procedures [16]. Whole skin was split into its constituent parts, epidermis and dermis, using the 0.9 caseinolytic units/ml of dispase (GIBCO, Grand Island, NY). The separated dermis was cut into 2×2 mm by scissors, and several pieces were attached on the surface of tissue culture flask and fed with DMEM containing 10% FBS. The fibroblasts were isolated after the extensive outgrowth and spreading was observed from these attached pieces of dermis. The *in vitro* production of dermal equivalent was carried out by casting a bovine type I collagen (Cell Matrix, Nitta Gelatin, Japan) plus concentrated medium on isolated and cultured fibroblasts [17]. The total volume of collagen mixture was 0.2 ml, and the cell density was 1.5×10^5 cells/ml, and this mixture was poured onto the culture plate insert, millicell PC (Millipore, MA, U.S.A.). The insert was made of polycarbonate membrane filter having 3.0 μm pore size with a 0.6 cm^2 effective surface area and a polystyrene cylinder.

Construction of Artificial Epithelial Tissues

The artificial epithelial tissues were developed by organotypic raft culture technique by following the published methods [5, 10]. Epithelial cells were seeded and grown on the dermal equivalent and then induced to proliferate and differentiate from monolayer cells to three dimensional multilayered artificial epithelial tissue. The culture medium of dermal equivalent was aspirated to leave the lattice surface exposed and epithelial cells were seeded onto dermal equivalent at an initial density of 5×10^4 cells for 0.6 cm^2 effective surface area of millicell PC. At that time, the culture medium was exchanged to DMEM supplemented with hydrocortisone (74 μg/l), epidermal growth factor (EGF, 5 μg/ml), triiodothyronine (T3, 6.7 μg/l), insulin (5 mg/l), transferrin (5 μg/ml), cholera toxin (1 mg/ml), and 10% FBS. The construct was submerged under the medium to allow these cells to spread and cover the surface of the dermal lattice for approximately 7 days of incubation. Then the epithelial tissues were cultured by so-called raft culture method that is growing cells at air-liquid interface by floating the culture insert for 2 weeks. Since the nutrients and growth factors are diffused to the epidermis through the prepared dermal equivalent, the *in vitro* artificial epithelial tissues are grown in the environment which is similar to *in vivo* epithelial tissue development.

Morphological Analysis

Raft cultured tissues were fixed in Carnoy fixative (ethanol : acetic acid : chloroform=6 : 1 : 3) for 1 hour at 4°C and dehydrated *via* graded ethanols and then embedded into paraffin (Paraplast, Oxford, MO, U.S.A.) in dry oven at 56°C for 3 days. Paraffin blocks containing tissues were cross-sectioned into 5 μm thick layer by microtome (820 JUNG HISTOCUT, Leica, Nussloch, Germany) with a disposable blade. These tissue sections were mounted on the gelatin coated slide glasses at 45°C on a slide warmer. The tissue sections were deparaffinized by dipping the slides in 100% xylene, and then rehydrated with 100%, 95%, 70%, 50%, 30%-ethanol, and water serially in staining jars. These rehydrated tissue sections were stained with hematoxylin for nuclei-staining of cells and counter-stained with eosin for cytoplasmic staining. To preserve these stained sections, slides were covered with Canadian Balsam mounting solution.

Immunohistochemical Analysis

Differentiation related characteristics of reorganiz-

ed epithelial tissues were examined by indirect immunohistochemistry. To examine the basal layer of epidermis, monoclonal antibody against EGF receptor (TRITON diagnostics, CA) was used as primary antibody. To examine the stratified and differentiated suprabasal layer, monoclonal antibodies against involucrin, filaggrin (Biomedical Technologies, Inc., MA, U.S.A) and cytokeratins 5/6/18, cytokeratins 10/13 (Novocastra Lab., Newcastle, UK) were used as primary antibodies. The ABC kit (Vector Laboratories, Burlingame, CA) for the avidin-biotin conjugate (ABC) reagents was used by following their protocols for dilutions. The Vector Laboratories' diaminobenzidine substrate kit for horseradish peroxidase detection system was also used. The expression of oncoprotein E6 of HPV 18 was determined by immunohistochemistry with antibody that we prepared against this oncoprotein previously [16].

RESULTS AND DISCUSSIONS

Confirmation of the Presence of HPV Genes

Two skin cancer cell lines (SPF1 and SPF2) and two cervical cancer cell lines (C9 and C10) were grown to select for cell lines carrying HPV 18 genes. HPV 18 E6 gene fragment was amplified using HPV 18 E6 specific primers which only amplify the HPV 18 E6 region from these cell lines. The presence of HPV 18 E6 gene was shown in C9, while the amplified band intensity was low in C10 and that was not in SPF1 and SPF2 (Fig. 1). Thus we chose one cell line, C9, for further characterization.

Morphology of Artificial Epithelium

The morphology of the artificial epithelial tissues produced from C9 cell line by raft culture method was examined (Fig. 2). These tissues were fixed for light microscopy after 2 weeks culture at the air-liquid interface. Histologic examination shows epidermal layer with distinct stratification and dermal substrate containing human dermal fibroblasts plus type I collagen. The stratified epidermal layer was composed of approximately 10-15 epithelial cell layers. This artificial tissues look more similar to the cervical tissues having dysplasia than to normal cervical tissue. Therefore, even though this artificial tissue culture methods could induce the stratification and differentiation of epithelial cell line, still the morphological feature was influenced by the characteristics of original cancer cell line.

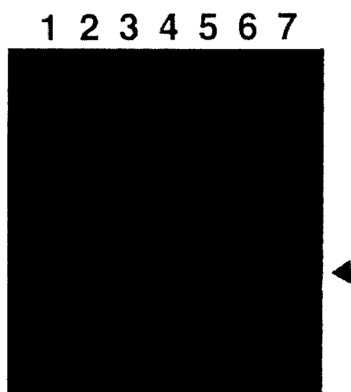

Fig. 1. PCR amplification of HPV 18 E6 genes using HPV 18 E6 specific primers. lanes 1-4; DNA fragments amplified from four different cell lines C10, C9, SPF1, SPF2, lane 5; DNA fragments amplified from HeLa cells used as positive control, lane 6; DNA fragments amplified from SiHa cells used as negative control, lane 7; standard size marker.

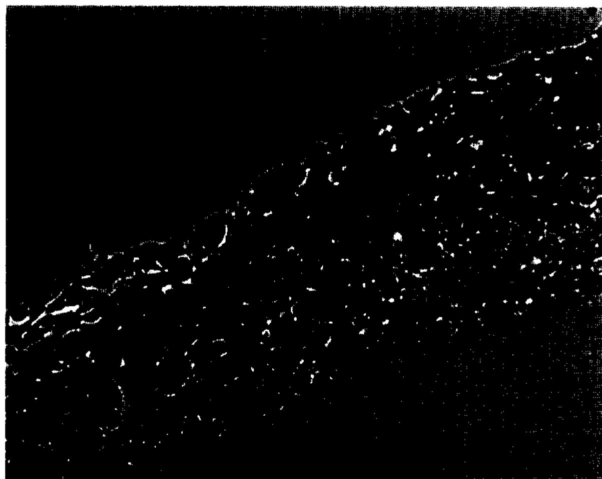

Fig. 2. Morphology of C9-derived artificial epithelial tissue ; paraffin embedded tissue was stained with hematoxylin and eosin.

Differentiation of Artificial Epithelial Tissues

The epidermis consists of stratified squamous epithelium formed by continuous proliferation and differentiation of cells from the basal to suprabasal layers. Cells become flattened and the expression of structural proteins such as cytokeratins, involucrin, and filaggrin occurrs [18]. The stratified epidermis displays a highly coordinated program of sequential changes in gene expression that are coincident with the phenotypic evolution from a proliferating basal cells to the mature, nonviable squame [2]. Tumor tissues are characterized by hyperplasia of the spinous layer and abnormal differentiation. Many tumor cell lines overexpressed the epidermal growth factor (EGF) receptor on their surface [3]. The expression of EGF receptor was observed not only in the epidermal basal layer but in the upper layers of artificial epithelial tissues (Fig. 3). Basal cells contact an epidermis-specific basement membrane and express characteristic proliferation markers including EGF receptor and cytokeratins. While the skin epithelium expresses cytokeratins type 10 and 14, the cervical epithelial stratum basale and stratum spinosum expresses cytokeratin 5/6/18. EGF receptor and cytokeratins 5/6/18 were expressed intensively over the C9 derived epithelial tissues in stratum basale and stratum spinosum (Fig. 3 and 4)

The proliferation of epithelial cells up to spinous and granular layer is associated with the loss of proliferative capability, the suppression of cytokeratins 5/6/18 gene expression, and the up-regulation of transcripts for involucrin in stratum spinosum and filaggrin in stratum granulosum, markers for epidermal differentiation. Generally, many tumor cell lines express the proliferation markers strongly while the expression of differentiation markers is inhibited or reduced. The differentiation marker, involucrin was expressed intensively in the C9 derived epithelium while the filagg-

Fig. 3. Immunohistochemical analysis of C9-derived artificial epithelial tissue using monoclonal antibody against EGF receptor protein.

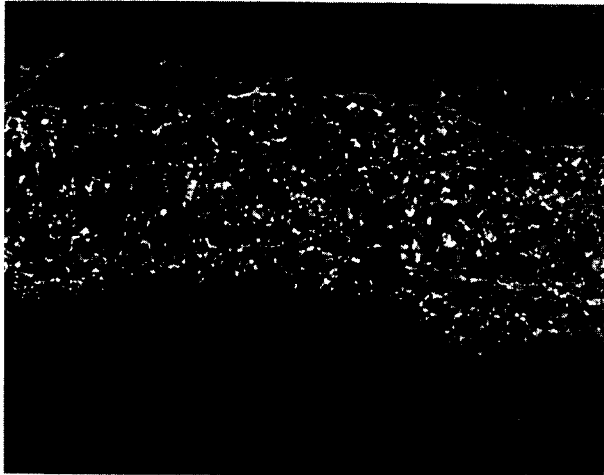

Fig. 4. Immunohistochemical analysis of C9-derived artificial epithelial tissue using monoclonal antibody against cytokeratins 5/6/18.

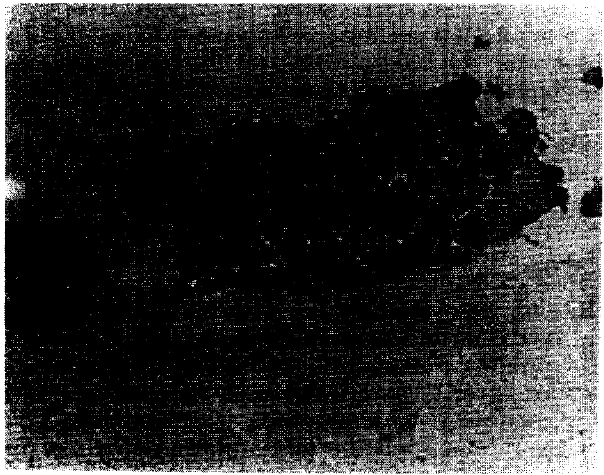

Fig. 5. Immunohistochemical analysis of C9-derived artificial epithelial tissues using monoclonal antibody against involucrin.

rin was expressed less strongly than involucrin did (Fig. 5, 6).

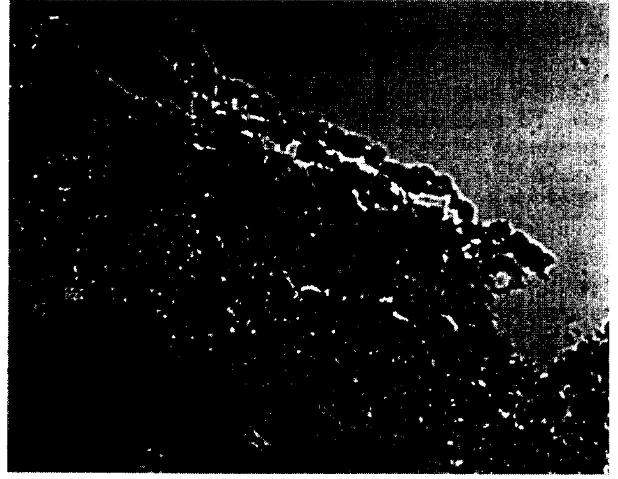

Fig. 6. Immunohistochemical analysis of C9-derived artificial epithelial tissues using monoclonal antibody against filaggrin.

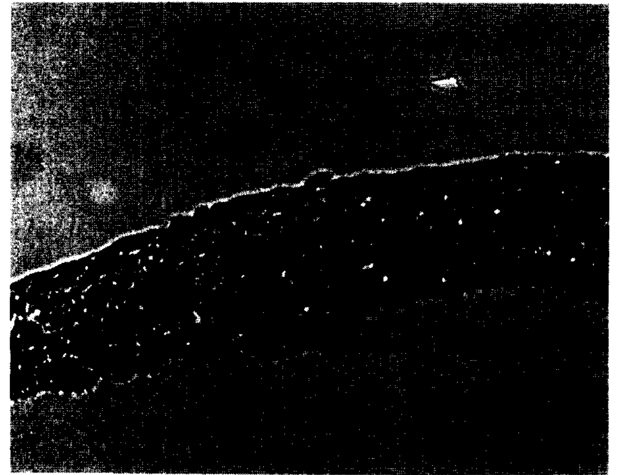

Fig. 7. Immunohistochemical analysis of C9-derived artificial epithelial tissue using monoclonal antibody against cytokeratins 10/13.

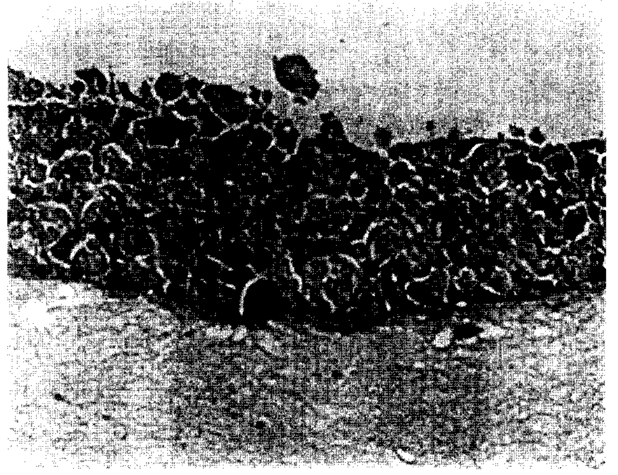

Fig. 8. Immunohistochemical analysis of C9-derived artificial epithelial tissue using antibody against HPV18-E6.

Upon further proliferation and maturation of epithelial cells into the stratum corneum compartment, the expression of involucrin and filaggrin is suppress-

Table 1. Immunohistochemical analysis of the artificial epithelial tissues prepared from cervical carcinoma epithelial cell line C9

Proliferation markers		Differentiation markers			HPV 18 Protein
EGF-receptor	cytokeratin 5/6/18	involucrin	filaggrin	cytokeratin 10/13	E6
+	+	++	+	−	+

ed while the expression of cytokeratins 10/13 is up-regulated. Cytokeratins 10/13 are essential components for the terminal phase of differentiation in normal epithelial tissues. In the reorganized epithelial tissues derived from C9 cell line, these cytokeratins 10/13 were not produced at all implying this cell line lacked the capacity for terminal differentiation (Fig. 7). Immunohistochemical analysis of artificial epithelial tissues was also carried with antibody against E6 proteins of HPV18, which was made in our laboratory. As presented in Fig. 8, E6 proteins were expressed in C9 derived epithelial tissues even though the expression level of these proteins was not so high. This low level expression of oncoprotein might result from the induction of differentiation by coculturing epithelial cancer cells with human normal fibroblasts that would supply differentiation-inducing growth factors and by culturing in highly differentiation inducing air-liquid culture condition.

In conclusion, we prepared artificial epithelial tissues from HPV-gene carrying cervical cancer cell line C9 by tissue engineering techniques, and examined the expression of epithelial proliferation and differentiation marker proteins. The results described above are summerized in Table 1. The C9 derived epithelial tissues expressed proliferation markers, EGF receptor and cytokeratins 5/6/18 strongly. The differentiation marker for stratum spinosum, involucrin was expressed strongly while differentiation marker for stratum granulosum, filaggrin was weakly expressed. However the cytokeratins 10/13, the terminal differentiation markers in stratum corneum layer of normal epthelial tissue were not expressed at all. This implies that this cell line lacked the capacity to follow the final differentiation program since this cell line was derived from cervical cancer tissue infected with HPV 18. These engineered artificial epithelial tissues will be useful in the studies of relation of HPV-associated carcinogenesis and epithelial cell differentiation.

Acknowledgement This work was supported in part by grants from Ministry of Science and Technology (FG1090 to S. Park) and Ministry of National Health and Welfare (BG150M to S. Park). We would like to thank Dr. Young Sook Son, Kyung Soo Hahm and Tae Ik Min for their technical advice and helps in many aspects.

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