조직배양공학을 이용한 인공피부의 개발 및 응용

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Development and Application of Artificial Skin Using Tissue Engineering

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ABSTRACT

An *in vitro* construct of three dimensional artificial skin equivalent has been engineered using human cervical epithelial cells and human foreskin fibroblasts with a matrix of bovine type I collagen. Two cell lines were established from cervical uteri cancer tissues which have the HPV(human papillomavirus)18 genome. These two cell lines came from the same origin but have slight differencies in growth rate and tumorigenicity. The organotypic raft culturing of epithelial cells were accomplished at air-liquid interface. The differentiation related characteristics were examined by immunohistochemistry using monoclonal antibodies against EGFreceptor, cytokeratin 5/6/18 as proliferation markers and against filaggrin, involucrin, and cytokeratin 10/13 as differentiation marker. We have obtained the stratification and the differentiation in the artificial skin equivalent, and differentiation-related proteins were expressed more in the C3-artificial skin, and proteins of proliferation were expressed more in the C3N-artificial skin, relatively. We found that reconstituted artificial skin have the same characteristics of differentiation proteins of original tissue or cells of human body.

INTRODUCTION

Tissue engineering technology includes tissue culture technique, in which cells originated from the various tissues of animal or human body are cultured and reconstituted *in vitro* to make either three dimensional artificial tissue or hybrid type artificial organ(1-4).

Current research investigations have explored tissue engineering for a wide variety of organs and applications. Probably the most advanced case is that of the skin replacement. Several different types of replacement skin tissue have been developed and are currently in clinical trials(5-7).

The evolution of a HPV-viral life cycle is tightly coupled to the differentiation program of keratinocytes in which virion production is limited to differentiating suprabasal cells. The tissue culture system will be useful in studies of the mechanisms whereby latency is maintained and terminated, and in the systhesis and assembly of papillomavirus virions(8-11).

In this work, we have been trying to develop artificial skin *in vitro* using tissue engineering technique for application to set up a system for the development of skin as a model system of HPV-associated carcinogenesis. We have made out two kinds of artificial skin from two cell lines which have slight differences in growth rate and tumorigenicity. And these engineered artificial skin were investigated that have some distinctions in morphology and characteristics of differentiation.

MATERIALS AND METHODS

1. Cell lines

Human cervical cancer cell lines, C3 and C3N epidermal cells which received from Catholic University were routinely maintained in Dulbecco

's modified Eagle's medium containing 10% fetal bovine serum under conditions of 5% CO_2 in air at 37℃. The C3 epithelial cell lines derived and established from untreated initial stage of squamous cell carcinoma of cervix uteri which isolated from patients of cervical cancer neoplasia. The C3 cells of 35th passage are grafted onto nude mice and formed to tumors. The cell lines established from these tumors were named C3N. The C3 and C3N cell lines have slight different growth characteristics including plating efficiencies. The C3N cell line was regulated to give more tumorigenic property than the C3 cell line.

2. Construction of dermal equivalent

Dermal fibroblasts were isolated from a neonatal foreskins, and whole skin split into its constituent parts, epidermis and dermis, using the enzyme 0.9 caseinolytic units/ml of dispase (GIBCO, Grand Island, NY). The fibroblasts were isolated as outgrowth and spreading from these attached pieces of dermis. The *in vitro* production of a unit of dermal equivalent begins by casting a bovine type I collagen(Cell Matrix, Nitta Gelatin, Japan) with isolated and cultured fibroblasts. The cell density was 1.5×10^5 cells/ml, and these mixture inoculated onto the culture plate insert, millicell PC(Millipore, MA, U.S.A.). The insert is consists of 3.0 μm pore size polycarbonate membrane filter with a 0.6 cm^2 effective surface area sealed to a polystyrene cylinder. Human dermal fibroblasts contract the collagen gel into a fibrillar connective tissue-like dermal lattice.

3. Construction of artificial skin equivalent

Epidermal cells were seeded and grown on the dermal equivalent and then induced to differentiate from basal cells to three dimensional artificial skin tissue. The culture medium of dermal equivalent is aspirated to leave the lattice surface exposed, epidermal cells(C3 and C3N) are 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 is exchanged DMEM medium supplemented with hydrocortisone, cholera toxin, insulin, transferrin, triiodothyronine(T3), epidermal growth factor (EGF) and 10% fetal bovine serum. The construct is submerged in medium to allow these cells to spread and cover the surface of the dermal lattice. The developing skin equivalent is then cultured 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 skin equivalent is placed in environment which have similarity to *in vivo*.

4. Morphological analysis

Raft cultured tissues were fixed and dehydrated via graded ethanols and then embedded into paraffin. Paraffin blocks containing tissues were cross sectioned into 5 ㎛ thickness layer by microtome. These tissue sections were stained with hematoxylin for nuclei-staining of epidermal layer cells and counter-stained with eosin for cytoplasmic staining.

5. Immunohistochemical analysis

Differentiation related characteristics of reorganized skin equivalent were examined by indirect immunohistochemistry. We have tried avidin-biotin conjugate(ABC) immunoperoxidase method. To examine the basal layer of epidermis, we used monoclonal antibody against EGFreceptor (TRITON diagnostics, CA) as primary antibody. To examine the differentiated suprabasal layer, we used monoclonal antibodies against filaggrin, involucrin, cytokeratin 5/6/18, and cytokeratin10/13 by Novocastra(Newcastle, UK) as primary antibodies. We have confirmed that C3 and C3N cell lines were infected with human papillomavirus type18 (HPV18) and have carried out immunohistochemistry with antibody against E6 and E7 of HPV 18.

RESULTS AND DISCUSSION

To compare the growth rate of two cell lines in the monolayer culture, doubling time and specific growth rate of C3 cells are 2.25 day and 0.31 day^{-1} and those of C3N are 1.5 day and

0.46^{-1}. It was found that the growth rate of C3N cell line is higher than C3 cell line.

The morphology of the artificial skin equivalents produced by this process bears the form after the pattern of human epidermal neoplasia(Fig.1-a,b). The stratification of epidermal layer is composed of 10-15 cells, and C3N-skin equivalent is composed of more number of cell layers than C3-skin equivalent. This result means that C3N-skin equivalent is more proliferative than C3-skin equivalent.

The epidermis consists of stratified squamous epithelium formed by movement of cells from the basal to superficial layers. Cell flattening and expression of genes for structural proteins such as cytokeratins, involucrin, and filaggrin are occurred(12). Ordinary tumors are characterized by hyperplasia of the spinous layer and abnormal differentiation, and overexpressed the EGFreceptor on their surface(13). As the characteristic markers of proliferation of basal epidermal cell, EGF receptor and cytokeratin 5/6/18 were expressed intensively over the C3N-skin equivalent (Fig. 2-b,d), and expressed weakly on the C3-skin equivalent (Fig. 2-a,c).

The migration of basal cells into the more superficial spinous layer and granular layer is associated with the loss of proliferative capability, suppression of cytokeratin 5/6/18 gene expression, and the up-regulation of transcripts for involucrin and filaggrin, markers for an stage of epidermal differentiation. Involucrin and filaggrin were expressed intensively in the C3-skin equivalent (Fig. 3-a,c), and expressed weakly on the C3N-skin equivalent (Fig. 3-b,d). Many tumor cell lines overexpressed the proliferation markers and restricted normal differentiation. That is to say, C3N-skin equivalent is more tumorigenic relative to C3-skin equivalent.

Upon further maturation and migration into the stratum corneum compartment, involucrin and filaggrin are suppressed and cytokeratin 10/13 is up-regulated. These unique proteins are essential components for the terminal phase of differentiation in normal skin tissues. On the other hand, reorganized tissues of C3 and C3N cell lines have a reduced capacity for terminal differentiation, and then cytokeratin 10/13 was not produced (Fig. 3-e,f). And we have carried out immunohistochemistry with antibodies against E6 and E7-genomic proteins of HPV18 which were made in our laboratory. As presented in Fig. 4, the E6 and E7 proteins were expressed over these tissues but not so much.

In conclusion, we have obtained artificial skin equivalent of HPV-associated cervical cancer cell lines by tissue engineering, and examined expression of differentiation, and the results of the above mentioned are summerized to Table 1. The C3-skin equivalent expressed more differentiation markers, that is involucrin and filaggrin, and C3N-skin equivalent expressed more proliferation markers, that is EGF receptor and cytokeratin 5/6/18, relatively. And the cytokeratin10/13 of the terminal differentiation marker in stratum layer of normal skin are not expressed. Two cell lines which have distinction in ability to form the tumor, and also have distinction in expression of differentiation factors.

Table 1. Immunohistochemical analysis of the artificial skin equivalent prepared from cervical carcinoma epithelial cells.

	Proliferation markers		Differentiation markers			HPV	
	EGFR	K5	K10	INV	FIL	E6	E7
C3	-	+	-	++	+	+	-
C3N	+	++	-	+	-	-	+

* EGFR : epidermal growth factor receptor
K5 : cytokeratin 5/6/18, K10 : cytokeratin 10/13
INV : involucrin, FIL : filaggrin

(Reference)

1. Stephen M. Edgington, "A Novel Force In Biotech: Tissue Engineering",Bio/Technology,Vol.44, 38-44, 1994
2. Leon M. Wilkins, "Development of a bilayerd living skin construct for clinical applications", Biotechnology and Bioengineering, Vol. 43, Pp. 747-756, 1994
3. Vicki Glaser, "In vitro systems getting a close look by industry and FDA", Genetic Engeneering News, April 15, 1995
4. Joel S. McClure, "Skinethic Laboratory Perfects Human Epidermal Cell Culture Technique", Genetic Engeneering News, April 15, 1995
5. H.A. Navsaria,"Culturing skin in vitro for wound therapy", *trends in* Biotechnology, Vol. 13, No. 3, Pp. 91-100, March, 1995
6. M. Yamazaki, et. al., "A novel method to prepare size-regulated spheroids composed of human dermal fibroblasts", Biotechnology and Bioengineering, Vol. 44, Pp. 38-44, 1994
7. C.R. Halberstadt, et. al., "The in vitro growth of a three dimensional human dermal replacement using a single-pass perfusion system", Biotechnology and Bioengineering, Vol. 43, Pp. 740-746, 1994
8. S.H. Yuspa, "the Pathogenesis of Squamous Cell Cancer : Lessons Learned from Studies of Skin Carcinogenesis", Cancer Research,Vol. 54, 1994
9. C.D. Woodworth, et. al.,"Human cervical and foreskin epithelial cells immortalized by human papillomavirus DNAs exhibit dysplastic differentiaion in vitro", Cancer Resesrch, Vol. 50, Pp. 3709-3715, 1990
10. Andrea Vambutas, et. al., "Laryngeal papilloma cells have high levels of epidermal growth factor receptor and respond to epidermal growth factor by a decrease in epithelial differentiation", Cancer Research, Vol. 53, Pp. 910-914, 1993
11. Craig Meyers, et. al., "Biosynthesis of human papillomavirus from continuous cell line upon epithelial differentiation", Science, Vol. 257, Pp. 971-973, August 1992
12. Michel Bossens, "In vitro infection of normal human keratinocytes by human papillomavirus type 1 followed by amplification of the virial genome in reconstructed epidermis", Journal of General Virology, Vol.73, 3269-3273, 1992
13. Xu Fang Pei, et. al., "Two strains of human keratinocytes transfected with HPV 16 DNA : comparison with the transfected with HPV 16 DNA : comparison with the normal parental cell", Carcinogenesis, Vol. 12, No. 2, Pp. 277-284, 1991

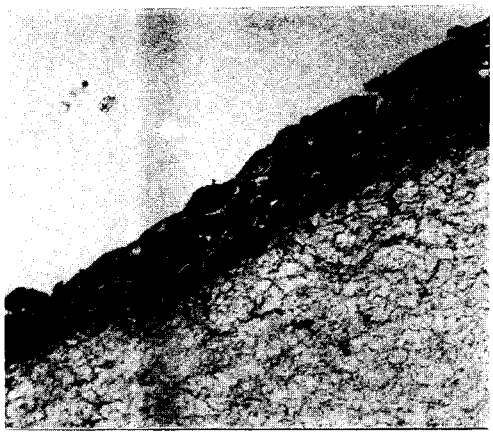

Fig. 1-a. Morphology of artificial skin equivalent-C3 ; paraffin embedded tissue was stained with hematoxylin and eosin.

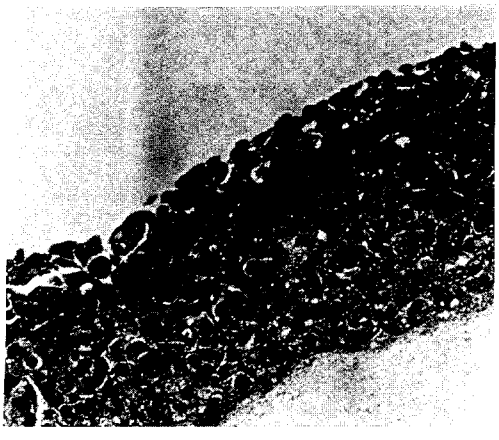

Fig. 1-b. Morphology of artificial skin equivalent-C3N ; paraffin embedded tissue was stained with hematoxylin and eosin.