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In Vitro Culture of Human Epithelial Cells on a Modified Xenogenic Collagen Support

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Abstract. Human epithelial cells (HeLa, HaCaT, NHK) were cultured *in vitro* on chemically modified collagen membranes. Adhesion to the support was measured by estimation of the percentage of adhering ^{51}Cr-labeled cells. Proliferation was estimated with the XTT test. Morphological observations of cells growing on HCl-treated collagen were performed using histological and electron microscopic techniques. HCl and trypsin-modified xenogenic collagen was found to be a good support for human cells *in vitro*. EDTA-incubated collagen enhanced neither adhesion nor proliferation. The best adhesion and proliferation were found on HCl-treated collagen, depending, however, on the kind of cells.

Key words: collagen modification; keratinocytes growth.

Introduction

Biological material such as collagen is conducive to the promotion of cellular adhesion, proliferation, migration and other cellular functions[2,5,10,13,17,21,22,24,25,30,31]. The assessment of cell adhesion to biological materials plays an important role in many cell culture models and has been described earlier[9, 16, 23]. Some proteins play an important role in adhesion, e.g. the family of integrins[1,29], cadherins[7] and selectins[8], and the proliferation of cells can be stimulated, e.g. by growth factors[3,27], calcium and a calcimimetic compound[14] and hyaluronic acid[6], but it can also be inhibited, e.g. by vit D_3, $1,25(OH)_2D_3$[26], pirfenidone[11] or melatonin[15].

In a previous paper[12] we described a possibility of collagen film modification. We have found that a high concentration of the free carboxyl group is present after incubation of collagen film with trypsin. Morphological changes, such as delamination of the collagen structure, are seen after incubation with HCl and trypsin. This study compares the adhesion and growth of various types of epithelial cells with premodified collagen membranes.

Materials and Methods

Collagen substrata. For the cell culture, the collagen film was modified as follows: the collagen disks were treated with a 0.5% solution of EDTA or 0.1 N HCl or 0.25% trypsin[12]. The collagen disks prepared thus served as the support for cell culture *in vitro*.

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Cell cultures. Three types of epithelial cells were used:

1. Normal human keratinocytes (NHK) were cultured as described by RHEINWALD and GREEN[19] or REID et al.[18]. Skin fragments, removed from women patients about forty years old were obtained. The skin was cut into small slices and suspended for 24 h at 4°C in a 0.25% dispase solution. The epidermis was peeled from the dermis and subsequently treated with a trypsin/EDTA solution (5 : 2 w/w). To obtain the cell suspension, the cells were washed with 1 mg/ml of soybean trypsin inhibitor (Sigma), centrifuged for 5 min at 1000 rpm, and resuspended in keratinocyte growth medium (KGM), supplemented with 30 μg/ml bovine pituitary extract (BPE), 0.5 μg/ml hydrocortisone (HC), 0.1 μg/ml human epidermal growth factor, 25.0 μg/ml amphotericin and 25.0 μg/ml gentamicin. The suspension was dispensed into tissue culture flasks and incubated at 37°C in 5% CO_2 atmosphere. The cells were cultured to obtain 60–70% confluency, then trypsinized and reseeded on the experimental support.

2. HeLa (Ohio) cells, which are human cervical carcinoma epithelial from the European Collection of Animal Cell Cultures (UK cat. no. 84121901), were prepared by passaging. The cells were cultured, centrifuged at 1000 rpm for 5 min and washed in culture medium: minimal essential medium (MEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 25.0 μg/ml gentamicin. HeLa cells were incubated in a medium in an atmosphere of 5% CO_2, 95% air at 37°C for 2–14 days. Medium was changed every second day of culture.

3. Human HaCaT keratinocytes used in this study are a spontaneously transformed cell line which is phenotypically simlar to normally, cultured keratinocytes in its pattern of growth and differentiation (gift from Heidelberg, Germany). The HaCaT cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS, Life Technologies, Paisley, Scotland), 25 μg/ml gentamicin and 0.25 μg/ml amphotericin.

The NHK, HeLa and HaCaT cells were seeded on the collagen-disks in culture dishes (4×10^4 cells/ml/ /well) and incubated at 37°C for observation of adhesion and proliferation.

Adhesion assay. Cells adhesion was evaluated with ^{51}Cr-labeled NHK, HaCaT and HeLa cells. The cells were incubated with 0.1 μCi/ml of $Na_2{}^{51}CrO_4$ (Radioisotope Research Development Centre Świerk, Poland) in a culture medium for 1.5 h at 37°C, as described elsewhere[28]. 4×10^4 of the labeled cells were then seeded onto collagen films placed in the wells of a 96 flat-bottom multiwell plate in a total volume of 200 μl culture medium. The plates were then incubated for 0, 1, 3 and 6 h at 37°C. After incubation non-adherent cells were removed. Adherent cells were then lysed with 0.5% Triton X-100. The radioactivity of the cell lysate was then measured with a gamma counter (Beckman). The percentage of adherent cells was calculated as follows:

$$\% \text{ adhesion} = \frac{\text{RAC}}{\text{TRC}} \times 100,$$

where: RAC – mean radioactivity of adherent cells, TRC – total radioactivity of cells.

The test was repeated 3 times for 8 samples from each group.

Proliferation assay. Cell viability and proliferation rate was tested by XTT[20]. At 1, 2, 3, 4 and 5 days of incubation, 100 μl of XTT/PMS fresh solution of 1 mg XTT/ml in PBS + 1.53 mg PMS/ml in PBS was added to each well. After 4 h at 37°C incubation, the plates were mixed in a mechanical plate shaker and the solution optical density was measured with Labsystems AUTO EIA automatic plate reader at 450 nm. A direct increase of the extinction value in comparison with the control was the measure of cell proliferation. Each experiment was repeated 3 times for 8 samples.

Histological examination. Cultures of cells on the modified collagen film were fixed for 1 h in 10% buffered formalin (pH 7.4), dehydrated in ethyl alcohol and embedded in paraffin. The paraffin blocks were cut (8–10 μm) using a Leitz 513 microtome and the sections were stained with hematoxylin for observation in an Axiovert 100 Zeiss (Germany) light microscope (original magnification × 400).

Electron microscope. After 5 days of culture, the specimens of collagen film with cells were fixed in 3% glutaraldehyde, postfixed in a 1% osmium tetroxide solution, washed in water, dehydrated through a graded series of ethanol, embedded in Epon-812, and ultra-thin sections were obtained. The sections were contrasted in 2% uranyl acetate and 0.1% lead citrate and examined using a JEM 100S Japan electron microscope.

Statistical test. The differences in the adhesion and in the proliferation rates of cells cultured on various collagen were evaluated using a non-parametric Wilcoxon test. A value of $p<0.05$ was considered significant.

Results

Adhesion of cells to the collagen film incubated with HCl

The highest values of adhesion obtained in the experiments are presented in Fig. 1. The collagen film

incubated with HCl appeared to be the best for adhesion.

Figure 1A shows that the adhesion of cells depends on the cell type and differs with time. After 6 h of observation it was found that 65% of the tumor cells (HeLa) 25% of the spontaneously-transformed keratinocyte (HaCaT), and 35% of the normal human keratinocyte (NHK) adhered to the support.

Adhesion of cells to the collagen film incubated with trypsin

After trypsinization of the collagen support, 44% of the tumor cells (HeLa) adhered to the surface, while the remaining cell lines demonstrated less adhesion: HaCaT – 17% and NHK – 22% (Fig. 1B).

Adhesion of cells to the collagen film incubated with EDTA

A low percentage of cells demonstrated adhesion to the collagen film treated with EDTA:

HeLa – 20%, HaCaT – 12%, NHK – 14% (Fig. 1C).

Adhesion of cells to the untreated (control) collagen film

Adhesion of cells to the standard collagen was simi-

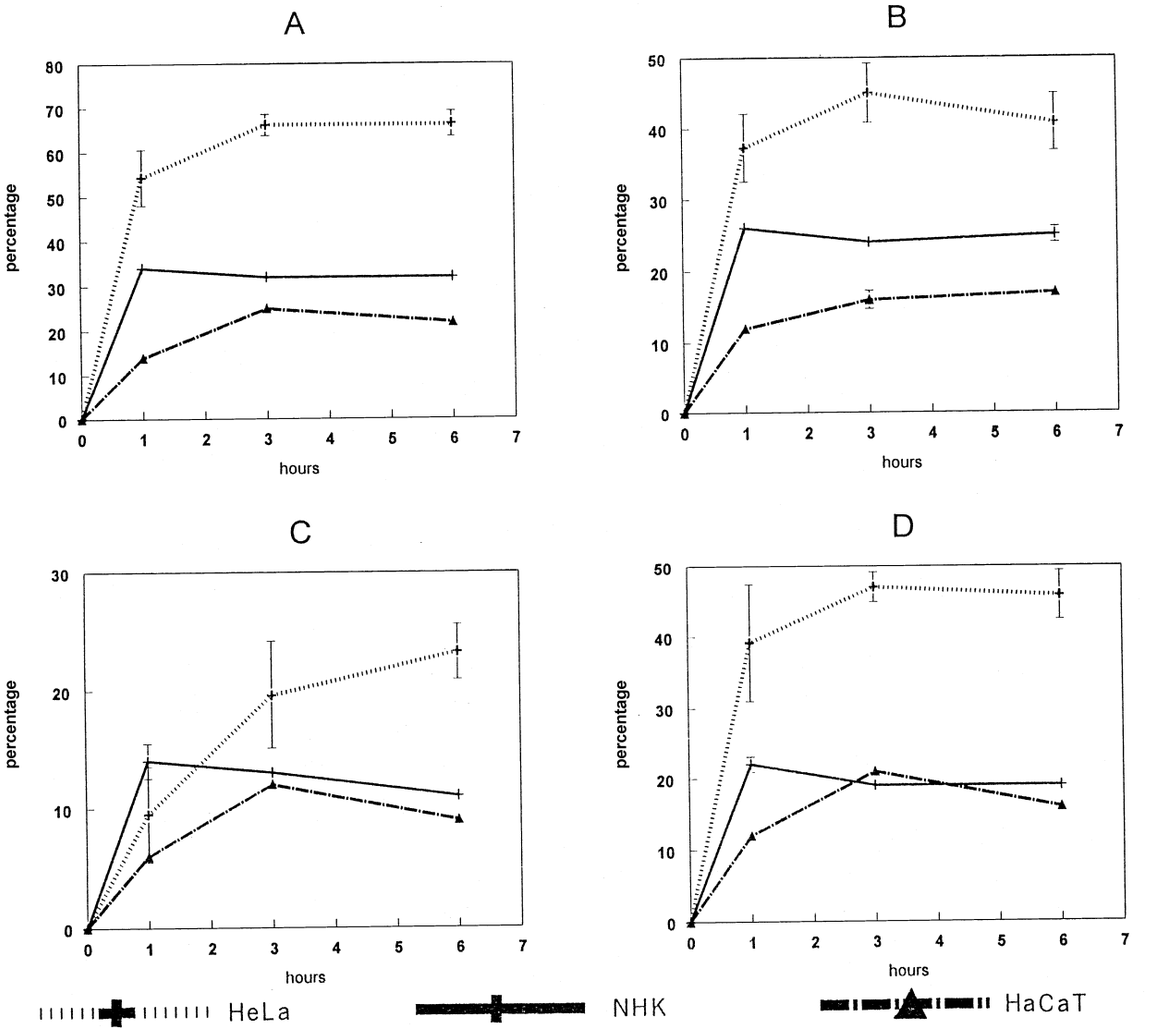

Fig. 1. Adhesion of cells to collagen support: in % of adhered cells. Mean values of 24 measurements. A – HCL-treated, B – trypsinized, C – EDTA-treated, D – untreated (control)

lar to the adhesion to the trypsinized collagen: Hela cells – 45%, HaCaT – 21%, NHK – 26%, (Fig. 1D).

Proliferation of epithelial cells on modified collagen films

The increasing numbers of cells in the course of the experiments are shown in the Fig. 2. Over 5 days of observation the increase of cell number is nearly linear in the cases of the standard and HCl-treated collagen (Fig. 2A), trypsin (Fig. 2B) and untreated (control) collagen (Fig. 2D). Growth of the cells on EDTA-treated film is also marked, with the exception of NHK, which do not show any proliferation over 5 days (Fig. 2C). There are some differences depending on the type of cells. The highest proliferation rate was seen in the HeLa cells, followed by normal (NHK) and transformate keratinocyte (HaCaT). The proliferation curves presented in the figure for cell growth on HCl-treated films seem to show the best condition for proliferation. Normal human keratinocytes do not proliferate on the EDTA-treated collagen, however, the tumor cells (HeLa) and transformed keratinocytes (HaCaT) grow on it and show an increased number of cells.

Morphology of the cell cultures

Histological examination. Observation of histologi-

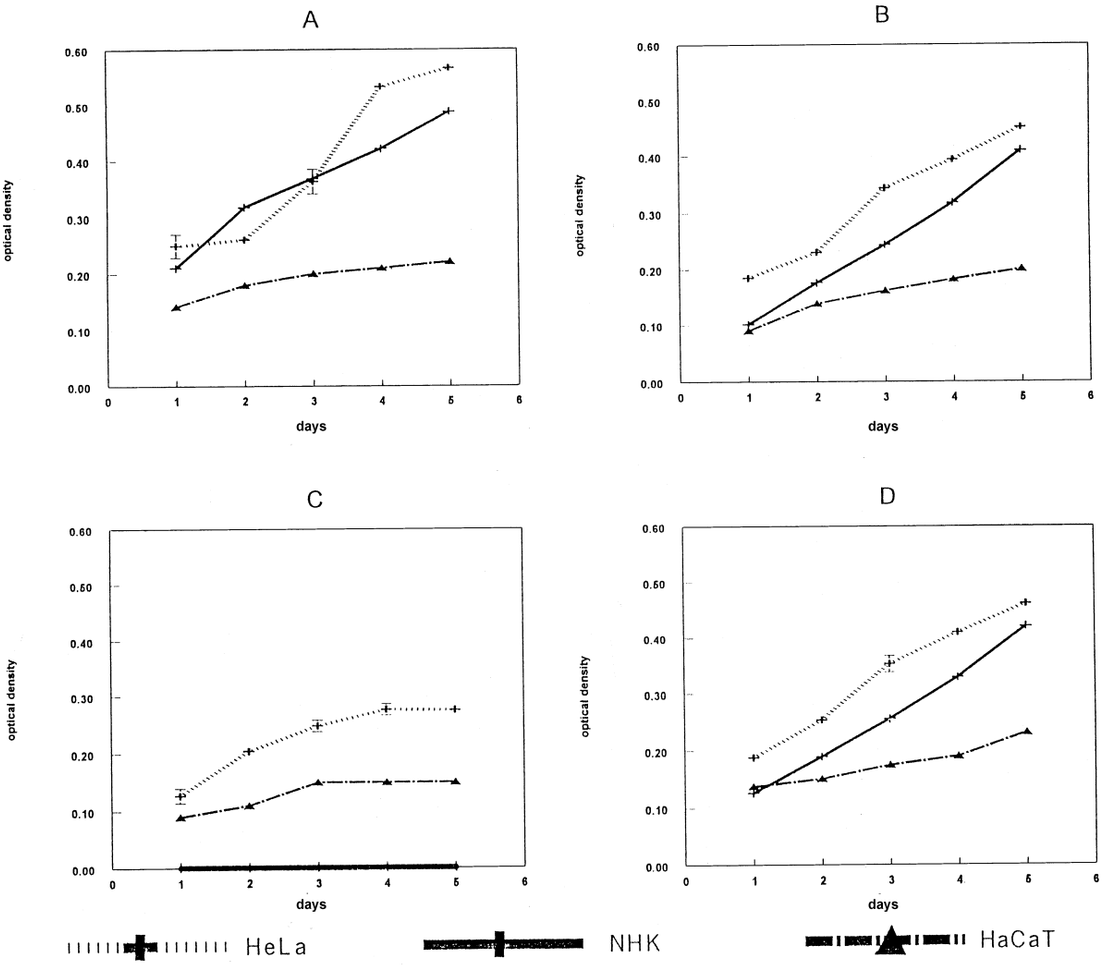

Fig. 2. Proliferation of cells on modified support; based on 24 measurements for each point. A – HCl-treated, B – trypsinased, C – EDTA--treated, D – untreated (control)

cal slides of cultured cells with collagen support (HCl--treated) showed that the cells used in the experiments (HeLa, HaCaT and NHK) demonstrated multilayer growth after seven days (Fig. 3A, B, C). Hela cells seem to demonstrate the most effective proliferation; however, HaCaT cells and NHK cells proliferate well.

Electron microscope observation. The interaction of cells with the HCl-treated support was observed. NHK cells (Fig. 4) demonstrate a good contact to the collagen. Many cytoplasmic processes penetrate from the cell body into the substrate (Fig. 4A). Exocitotic and phagocytotic vacuoles are seen in the cytoplasm of the cells. Sometimes, desmosomes are developed between the cells. On the surfaces of cells in close contact with the support, phagocytic vacuoles are formed with dense membrane fragments, similar to the clatrin-coated vesicles (Fig. 4B). HaCaT cells (Fig. 4C) in contact with the collagen develop numerous very thin processes. HaCaT cells, more often than other cells, demonstrate the presence of lipid droplets and numerous dense lysosomes.

HeLa cells (Fig. 4D) develop large cytoplasmic processes penetrating into the collagen membranes. Typical cell structures are distinctly visible.

Discussion

A xenogenic collagen in the form of a thin film was used as the support for epithelial cells cultured *in vitro*. Numerous papers have shown that the support not only plays a mechanical role, but can also influence cell proliferation, differentiation and stability[17]. The main task of this experiment was to find out if modification of collagen membranes by means of various chemical factors may influence adhesion and proliferation of cells. The role of other chemical agents has been described earlier[4].

From the previous paper we know that collagen treated with 0.1 N HCl demonstrates a change in the structure of the membrane. The membrane becomes loose and the concentration of the free carboxyl groups is lower (LESIAK-CYGANOWSKA et al., in print). The adhesion of HeLa, HaCaT and NHK to this support was better than that to untreated collagen. The proliferation of cells in 5-day culture was found to be very effective.

The growth of the cells on HCl-treated collagen, observed on histological slides and by use of an electron microscope, is evident. In Fig. 3 the multilayer epithelium is seen. All cell types used in the experiment showed good proliferation. In the electron microscopic observation (Fig. 4), close contact between collagen and the cell membranes is seen. Cytoplasmic structures i.e. processes penetrating into the collagen, phagocytotic and exocitotic vacuoles, and invaginations of membranes, are developed by all kinds of cells. Normal human keratinocytes developed desmosomes. These phenomena indicate that the xenogenic support for human epithelial cells provides good conditions for adhesion and proliferation.

The collagen membranes treated with trypsin show morphological and chemical changes. Stratification and areas of low density were observed and a very high concentration of free carboxyl groups are seen. Adhesion of cells to this support, highest in HeLa cells and lowest in HaCaT, is rather similar to that observed in the control collagen. The proliferation of all the cell types demonstrates high dynamics, but lower than that observed on the HCl-treated support.

EDTA-treated collagen does not demonstrate any morphological changes and the concentration of the free carboxyl groups is lower than in the controls. All the types demonstrate low adhesion and proliferation. Normal Human Keratinocytes do not proliferate on it during 5 days of culture.

The adhesion and proliferation of epithelial cells are enhanced or the HCl and trypsin-treated support; however, they differ, depending on the strain of cells. It seems that chemically modified, xenogenic collagen is a good support for epithelial cells *in vitro*. We expect that it may be used as a scaffold materials in tissue engineering.

In conclusion: 1). Measurements of adhesion and proliferation of epithelial cells as well as morphological observation confirmed that xenogenic, chemically modified collagen is a good support for these cells cultivated *in vitro*, 2). Adhesion and proliferation of cells to the support depend on the kind of cells and type of collagen modification.

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