Short Communication

Relations between individual cellular motions and proliferative potentials in successive cultures of human keratinocytes

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Abstract

In the successive cultures of human keratinocyte cells, cellular motions of extension and rotation were analyzed based on observation of the individual cells, to evaluate the proliferative potential in a whole cell population. In lag phases of the serial cultures, an extension index of individual cells, R_E , was defined as an average spreading rate divided by initial cell area for each cell. The mean value of R_E was found to relate to prolongation of lag time; namely it decreased with increasing passage number in the successive cultures approaching cellular senescence. During the courses of the cultures, the rotation rate of paired cells was also measured through time-lapse observation. The mean value of rotation rate, $|\bar{\omega}|$, decreased with an increase in doubling time caused by the progress of cellular age, reaching an almost constant value of $|\bar{\omega}| = 40$ degrees h^{-1} in the cultures with prolonged doubling time of over 59 h. It was concluded that the indices determined from the motions of individual cells, R_E and $|\bar{\omega}|$, were correlated with the lag time and doubling time, respectively, which are growth parameters varied with the vitality of the cells approaching cellular senescence.

Nomenclature: A_C – projected area of each cell (μm^2); A_{C0} – initial projected area of each cell (μm^2); n – number of examined cells (cells); N_d – cumulative number of population doublings; N_{df} – final value of cumulative number of population doublings; N_P – number of passages; r_s – spreading rate of each cell ($\mu m^2 h^{-1}$); r_s^{ave} – average spreading rate of each cell ($\mu m^2 h^{-1}$); R_E – cell extension index (h^{-1}); $\bar{R}_E$ – mean value of R_E (h^{-1}); t – observation time (h); t_d – doubling time (h); t_L – lag time (h); $|\omega|$ – absolute value of rotation rate (degree h^{-1}); $|\bar{\omega}|$ – mean value of $|\omega|$, (degree h^{-1})

Introduction

For the production of cultured tissues such as epithelial sheets, a series of monolayer cultures of anchorage-dependent cells is generally performed to expand cell number requisite for tissue reconstruction. In these cultures, the natures of source

cells are of heterogeneity depending on the respective biopsies from which the cells are isolated, and the vitality of cell individuals progressively lowers due to cellular senescence inherent in normal human cells.

From a viewpoint of growth kinetics of anchorage-dependent cells, lag time and doubling time are

important parameters to estimate the cell vitality or activity. In a lag phase, cells extend with morphological alternation to ensure firm adhesion onto culture surface, giving a delay in cell growth, lag time, that changes with culture conditions and/or cell states (Re et al. 1994). In a subsequent growth phase, cells generate their daughters through repeated cell division at an interval of generation time, relying on multiplying ability of each cell.

In previous work (Hirai et al. 2002; Kino-oka et al. 2004), we developed the culture system which facilitated the direct observation of dynamic behaviors of individual cells. It was demonstrated that the spreading rate of each cell found in lag phase could be a criterion for estimating time until the first cell division in the culture of murine fibroblasts prepared under varied conditions of trypsin treatment. Moreover, the rotation rate of paired cells was proposed to evaluate the vitality or latent potential of growth recovery in the culture of human immortal epithelial cells kept under glucose-limited conditions. The aim of the present study is to extend the motion analyses of cell spreading and rotation to evaluating the proliferative potential in the successive cultures of normal human keratinocytes. The growth potential parameters of lag time and doubling time that change with the progress of cellular age will be discussed in terms of the proposed indices determined from observing the dynamic behaviors of individual cells.

Materials and methods

Human keratinocytes (neonatal foreskin origin) were obtained as frozen cells from Kurabo Ind., Osaka, Japan (No.9C0708 in supplier's file). The cells in vials were thawed according to the supplier's instruction, and then cultivated in a 25 cm² T-flask (Nuncclon Delta Flask; Nunc, Roskilde, Denmark) as the first passage ($N_P = 1$) in a series of successive cultures. The cultures were conducted using serum-free medium (HuMedia-KG2; Kurabo Ind., Osaka, Japan) at 37°C under air containing 5% CO₂. The medium was maintained at a depth of 4 mm in the flask and spent medium was renewed every 3 days. On reaching about 80% confluence of the cells on the flask bottom, the cells were harvested by centrifugation (200×g, 25°C) after enzymatic treatment with a mixture of

0.1% trypsin 1:250 and 0.02% EDTA (Sigma Aldrich, St. Louis, MO, USA). The subsequent passages were repeated in a similar manner until the cell growth substantially ceased. In these successive cultures, the initial concentration of viable cells, determined by trypan blue exclusion test, was fixed at 1.0×10^4 cells/cm². Throughout the experiments, all the cultures for the data analyses were carried out in triplicate. During each passage, the remaining number of population doublings, $N_{df} - N_d$, was determined as a measure for cellular senescence, based on the change of adherent cell concentration as shown elsewhere (Umegaki et al. 2002).

As described in a previous study (Kino-oka et al. 2004), an observation tool was constructed by installing a CCD camera in the computer-aided culture system and employed to analyze individual cellular motions during the courses of the cultures. The cell images were captured in a manner of time-lapse observation, every 40 min for 10 h in an early lag phase and every 20 min for 24 h in an early growth phase, for analyzing cell spreading rate and paired cells rotation, respectively, at five positions in each flask. Table 1 indicates the values of N_P and $N_{df} - N_d$ during the successive cultures of keratinocytes (Runs 1–3) employed for the observation of these cellular motions.

Results and discussion

To analyze the spreading rate of the keratinocyte cells, the projected area of each cell, A_C , in lag phase was determined by extracting cellular edge using a line-drawing tool in the software (IMAQ Vision Builder; National Instruments, Austin, TX, USA). The A_C values of individual cells increased

Table 1. Values of N_P and $N_{df} - N_d$ in successive cultures of keratinocyte cells used for experiments.

Culture	N_P [–]	$N_{df} - N_d$ [–]
Run 1	3	6.6 to 9.6
	4	3.8 to 6.6
	5	0.9 to 3.8
	6	0 to 0.9
Run 2	4	3.5 to 6.8
	5	0.9 to 3.5
	6	0 to 0.9
Run 3	5	2.7 to 5.8
	6	0.1 to 2.7
	7	0 to 0.1

gradually with elapsed time after seeding. Then the spreading rate of each cell, $r_s = dA_C/dt$, was traced and an average r_s of values, r_s^{ave} , was determined in each culture. The determined r_s^{ave} value was standardized in terms of initial cell area, A_{C0} , to define a cell extension index, R_E , as follows.

$$R_E = r_s^{\text{ave}} / A_{C0} \quad (1)$$

Figure 1 shows the histograms of R_E values of the examined cells during the successive cultures (Run 1). In the culture of $N_P = 3$, a broad distribution in the R_E values was recognized, ranging from $R_E = 0.04$ to 0.30 h^{-1} . Afterwards, the distribution shifted to small values with an increase in the N_P value. The frequency of cells with $R_E < 0.10 \text{ h}^{-1}$ was approximately 80% in the culture of $N_P = 6$ with $N_{\text{df}} - N_d = 0$ to 0.9 as

Figure 1. Histograms of cell extension index, R_E , in successive cultures of keratinocyte cells (Run 1). The R_E values were determined for early 10 h in each culture ($n = 15$ to 20 cells).

Figure 2. Histograms of rotation rate, $|\omega|$, in successive cultures of keratinocyte cells (Runs 2 and 3). The $|\omega|$ values were determined for 1 h from $t = 24$ h in each culture ($n = 5$ to 15 cells).

shown in Table 1, and its frequency pattern was different from that in the culture of $N_P = 3$ with $N_{df}-N_d = 6.6$ to 9.6 ($p < 0.05$). These results indicated that the extension potential of the keratinocyte cells in lag phase deteriorated in response to the progress of cellular age, as evaluated by the $N_{df}-N_d$ value.

Next, the rotation of paired cells was observed by capturing cell images during the successive cultures of the keratinocyte cells (Runs 2 and 3). This pairwise rotation was quantified as an absolute value of rotation rate, $|\omega|$, which was determined according to the procedure reported earlier (Kino-oka et al. 2004). Figure 2 shows the histograms of the $|\omega|$ values of the examined cells during the successive cultures. In the culture of $N_P = 4$ (Run 2), a broad distribution in the $|\omega|$ values was observed in the range of $|\omega| = 4$ to 254

degrees h^{-1} , which indicated that the culture comprised of heterogeneous population of cells with varied rotational motions. With an increase in the passage number, the distribution in the $|\omega|$ value narrowed and almost the cells entered a region of $|\omega| < 70$ degrees h^{-1} in the culture of $N_P = 6$ with $N_{df}-N_d = 0$ to 0.9 (see Table 1). A similar tendency was also recognized in the separately conducted successive cultures (Run 3).

Then we intended to correlate these cellular motions of extension and rotation with proliferative potentials of cell population in the successive cultures. As indices for the proliferative potentials of cell population, lag time, t_L , and doubling time, t_d , were determined from the growth profile of cells in each passage as described in previous work (Kino-oka et al. 2000). Figure 3a shows the plot of the mean $\bar{R}_E$ value, $\bar{R}$ against lag time in the

Figure 3. Plots of (a) mean R_E value, $\bar{R}$, against lag time, t_L , and (b) mean $|\omega|$ value, $|\bar{\omega}|$, against doubling time, t_d , in successive cultures of keratinocyte cells. The numerals in the parentheses indicate run number.

successive cultures (Run 1). In these cultures, the cells exhibited substantial proliferation with $t_d = 17.3, 26.5$ and 33.2 h, respectively, at $N_P = 3, 4$ and 5 , while the t_d value extremely prolonged up to 82.2 h at $N_P = 6$. As seen in Figure 3a, lag time elongated along with decreasing $\bar{R}$ from 0.16 to 0.08 h^{-1} and reached $t_L \cong 23$ h at the last two passages of the successive cultures ($N_P = 5$ and 6). This result suggests that cellular senescence causes the deterioration of adhesive potential of anchorage-dependent cells, including the attachment and extension of the cells on culture surface that are critical events in lag phase to render the cells ready for cell division in growth phase, as pointed out in a literature (Gumbiner 1996).

Figure 3b shows the relation between the mean value of $|\omega|$, $|\bar{\omega}|$, and doubling time of the cells in each passage of the keratinocyte cultures. The $|\bar{\omega}|$ value was at low levels of $30\text{--}40$ degrees h^{-1} in a region of $t_d = 59$ to 573 h ($N_P = 6$ and 7), and it exhibited a gradual increase with shortening t_d value observed in the early passages ($N_P = 4$ and 5), indicating that the growth potential is high in the culture containing the cells with the elevated rotation rate. It was thus demonstrated that the rotational motion of the cells can be a parameter to evaluate the multiplying ability of the anchorage-dependent cells.

In conclusion, the individual cellular motions of extension and pairwise rotation were examined in the successive cultures of keratinocytes associated with the progress of cellular age. The indices determined from the motions of individual cells, R_E and $|\omega|$, were correlated with the conventional

proliferative potentials estimated from a whole cell population, lag time and doubling time, respectively. The cellular motion analyses based on observing cell individuals can offer useful tools to know the state of the cells approaching cellular senescence along with the serial passages encountered in the culture of normal human cells.

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