A NEW WOUND HEALING MODEL USING TISSUE CULTURED CORNEAL ENDOTHELIUM

培養細胞を用いた角膜内皮細胞創傷治癒モデル

宮田和典

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I Introduction

The Wound healing process of the corneal endothelium comprises two different cellular kinetics in general: one is cellular migration, the other is cell proliferation by cell division(1,2). The wound healing mechanism of the corneal endothelium is different among species. Although the human endothelial cell has the capability of cell division, this activity is so weak that migration and enlargement of cells play the main role in wound healing of the corneal endothelium(3). Corneal endothelial cells of other species have a higher capacity for cell division(4,5) than the human endothelial cell. Regarding the wound healing process in rabbit corneal endothelium in vivo, the migration of cells into a denuded cell area begins at three hours after the wounding (6, 7) and the following period between 12 and 24 hours cell migration plays a main role in wound healing mechanism(4,8). After then, cell mitosis occurs at the edge of the wound for the period between 24 and 48 hours after the wounding (4,5,8). When the wound is re-covered by endothelial cells, contact inhibition mechanism occurs and the extracellular matrix is regenerated(5,6,9,10,11). These wound healing processes have been observed in in vivo study using a specular microscope(6, 7,12,13,14). The method of making an endothelial wound in in vivo experiment was either by causing cell denudation by an needle inserted into the anterior chamber(8) or by transcorneal

cryocoagulation of the endothelium(3,4,5,11). However, the latter method has disadvantages in specular microscopic observation due to epithelial damage and stromal opacity. A small wounding technique using a nylon thread introduced into the anterior chamber through a needle enabled a serial follow up observation by specular microscopy and a study of the endothelial wound healing process in vivo(6)(7). However, this method has also disadvantages by causing a long lasting wound and possible damage of Descemet's membrane. A method of organ culture using a sclerocorneal button has been developed for an in vitro study(15, 16). However, this method is inadequate for a long term study because of resulting stromal opacity. These in vitro studies involve problems such as difficulty in a serial observation of cell kinetics in the wound healing process and in quantifying the wound size. Therefore, we developed an in vitro wound healing model using tissue cultured corneal endothelial cells which was feasible for quantitative evaluation of the wound healing process.

II Materials and Methods

1.Primary culture

For the primary culture we used endothelial cells with Descemet's membrane(17). We used eyes taken from either adult albino rabbits weighing 2.3-3.0Kg of both sexes or adult bovine.

The cornea was excised, after rinsing the eyeball with Ringer's solution containing 20μg/ml gentamicin. The corneal endothelial layer was taken together with Descemet's membrane using a corneal forceps under a dissecting microscope. It was cut into small pieces about 1-2mm^2 in size by a pair of corneal scissors. Five or six pieces were placed on the plastic culture dishes which were 35mm in diameter(Iwaki Glass, Tokyo). These cells were incubated in a humidified 5% CO_2 atmosphere at 30°C and cultured in Eagle's MEM Earle's solution(GIBCO, Grand Island, NY, USA) with 10% fetal bovine serum (GIBCO)and 20μg/ml gentamicin. The culture medium was changed three times per week during the primary culture.

2. Endothelial cell sheet

After 7 days of the primary cell culture, a sufficient number of cells extended and proliferated from the original cell blocks. Then, EDTA-trypsin(GIBCO) processing was carried out to separate cells from the culture dish. The cell suspension was centrifugated for 3 minutes at 400rpm and was adjusted to a cell suspension with 2.0 x 10^4 cells/ml.

The secondary cell culture was performed on a 24mm-square cover slip(Matunami Glass, Tokyo) using hydroxyethyl methacrylate (HEMA)(HOYA, Tokyo) to form a culture area of 8 mm in diameter. The HEMA solution was polymerized at 120°C for three hours and the cover slip was sterilized in an autoclave. The cell

suspension with 2.0 x 10^4 cells/ml was placed on the HEMA-free area of the cover slip and cultured immediately. Eight hours after the cell culture, an adequate number of cells attached to the surface of the cover slip, and then 1.5ml of the culture medium was added to the culture system. The cell culture was kept for a further 10 days(Figure 1). During the cell culture, cell density was determined in a random sampling manner. The sampled specimen was fixed with 10% buffered formalin and stained with hematoxylin for the cell count. These data were compared with the number of endothelial cells in vivo which were examined by specular microscopy.

3. Wound formation and quantitative measurement of the wound

The wound was made by a cell denudation using a sterilized rotating silicone tip as shown in Figure 2 after the 10-day culture. Recovering of the wound area was observed using an inverted phase contrast microscope (NIKON, Tokyo) at 0, 6, 12 hours and thereafter every 12 hours after the wounding. Photographs were taken at each time, and the margin of the wound was traced. The area of the wound was measured using a computed image analyzer(Hamamatsu Photonics, Tokyo), and the wound healing rate (healed area per hour) was calculated between each observation period.

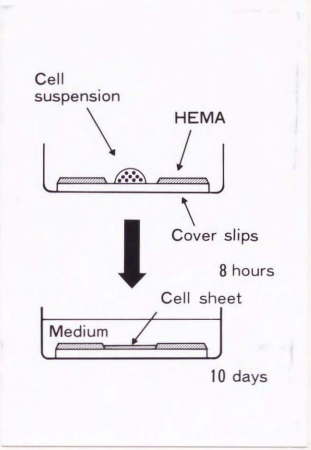

Figure 1. Outline diagram of method for producing corneal endothelial cell sheets.

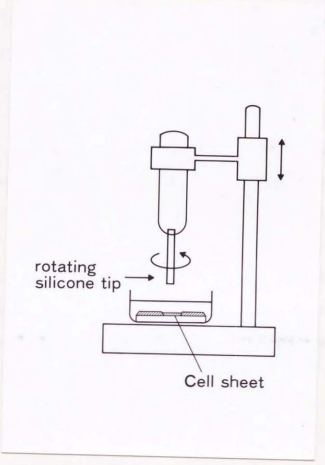

Figure 2 Outline diagram of procedure for creating a wound using a silicone tip.

4. Kinetic study of cell proliferation

The mitotic cell count was performed by the following method using a bovine corneal endothelial cell culture. After the wounding, 10μg/ml of 5-bromo-2'deoxyuridine (BrdU, SIGMA, St Louis, MO, USA) was added to the culture solution every 12 hours. The samples were fixed at 4° C for 24 hours with 10% buffered formalin. After rinsing in 0.01M of phosphate buffered solution (PBS), the cells were hydolyzed with 1N HCl and digested with 0.05% protease type IV (SIGMA) for 15 minutes. The cells were then rinsed with PBS and distilled water again. After an incubation for 20 minutes at 37° C in the normal blood serum, the cells were incubated for 24 hours at 4° C in antiBrdU monoclonal antibody (Cosmobio Tokyo). Then the cells were stained by avidin biotin peroxidase complex method using a Vectastain ABC kit (Vector, Burlingame, CA, USA) and incubated at 37° C for one hour in biotinylated antimouse IgG. Then, the cells were incubated at room temperature for 30 minutes in 0.3% hydrogen peroxide methanol solution. After once rinsing with PBS, the cells were incubated at 37° C for one hour in the avidin biotin peroxidase complex. Following a rinsing with PBS, 0.1% diaminobenzidine tetrachloride(SIGMA) mixed in solution with 0.02% hydrogen peroxide was added and incubated at room temperature for 30 minutes. The treated cells were rinsed with PBS and sealed using a slide glass. The samples were taken every 12 hours after

the wounding until 72 hours.

As controls, one sample was treated by normal non-immune IgG in place of the anti-BrdU monoclonal antibody, and the other was treated according to the method as described above without BrdU.

The stained samples were observed using a microscope and photographed. The photographs were printed in magnification of 100 times. The total number of the labeled cells was counted and their locations were determined to define the relationship between the labeled cells and the wound.

III Results

1 Primary culture

The corneal endothelial cells extended outside of the exoplant cell block by the 3rd culture day. The cells migrated gradually, keeping in touch with other cells and formed a monolayer by the 6th day(Figure 3a, 3b). Many cells demonstrated proliferative figures. The cells close to the exoplant showed a smaller cell size and a nearly hexagonal shape. This cellular proliferation was observed in the same way in both rabbit and bovine cells.

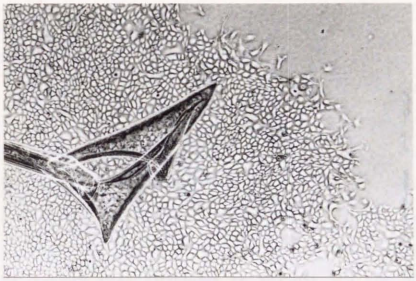

Figure 3a Primary culture 6th culture day; rabbit (magnification x10)

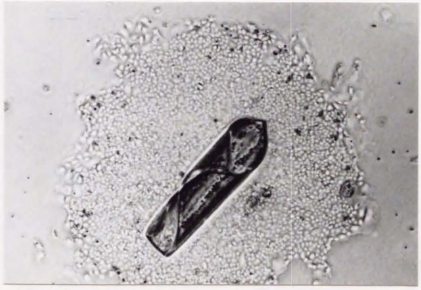

Figure 3b Primary culture 6th culture day; bovine (magnification x10)

2 Endothelial cell sheets

The cultured cells adhered to the cover slip by 3 hours after the seeding. However, the cells did not extend the HEMA-coated surface. The cultured area was completely covered by monolayer corneal endothelial cells by 4 days. After the 4th day, the cell density became stable, about 2.0 x $10^3/mm^2$ in both specimens (Figure 4a,4b). After 10-day-culture the cell sheet became a confluent monolayer and cells did not migrate to the HEMA coated area as shown in Figure 5a and 5b.

The number of corneal endothelial cells in vivo measured by specular microscope were 2265±410 /mm^2 for bovine and 3825± 703/mm^2 for rabbit(mean±SD,n:3). In vivo and in vitro cell counts were more similar for the bovine samples than rabbit samples.

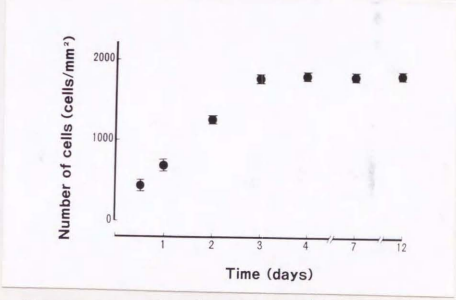

Figure 4a Changes over time in cell density on culture surface; rabbit (Bar means S.D.)

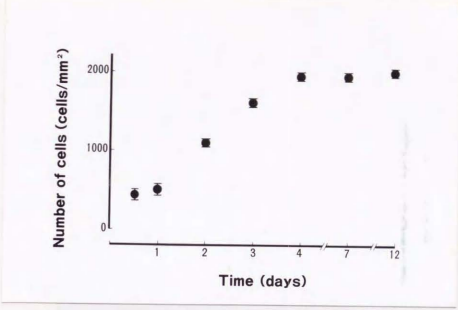

Figure 4b Changes over time in cell density on culture surface; bovine (Bar means S.D.)

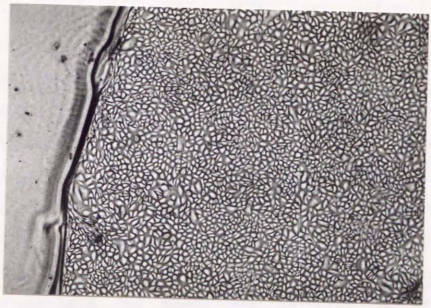

Figure 5a Cultured corneal endothelial cell sheet delimited by HEMA; rabbit (magnification x10)

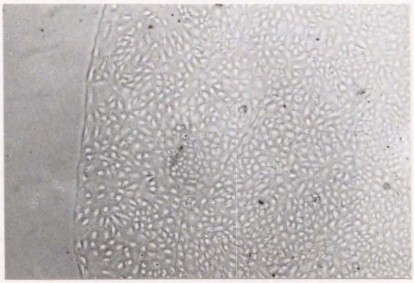

Figure 5b Cultured corneal endothelial cell sheet delimited by HEMA; bovine (magnification x10)

3 Quantitative measurement of the wound healing

Regarding the wound size, mechanical cell denudation using a rotating silicone tip could produce fairly a consistent wound size as shown in Figures 8a and 9a; $3.00 \pm 0.21 mm^2$ (mean$\pm$SD, :n=5)for rabbit and $3.18 \pm 0.28\ mm^2$ for bovine. The cells at the wound margin grew into the wound up by 6 hours after the wounding in rabbit(Figure 6b), but no remarkable cell movement was observed in bovine (Figure 7b). In both species, the cell migration into the wound was observed at 24 hours after the wounding(Figures 6c, 7c). At 60 hours, the wound was completely recovered in rabbit(Figure 6e), but still incompletely recovered

in bovine. The wound in bovine was recovered completely at 84 hours as shown in Figure 7e.

A time course study of the wound size are shown in Figure 8. The wound healing rate was $22.0 \pm 4.9 \times 10^3 \mu m^2/h$ for the first 6 hours in rabbit. However, no remarkable wound healing was observed in bovine at that time with the wound healing rate of about $0.8 \pm 0.6 \times 10^3 \mu m^2/h$. By 12 hours, in both species, the wound area decreased linearly. The wound healing rates were fairly consistent between 12-24 and 48-36 hour periods (Figures 8b, 9b). According to the wound recovery after 48 hours for rabbit and after 60 hours for bovine respectively, the wound healing rate decreased.

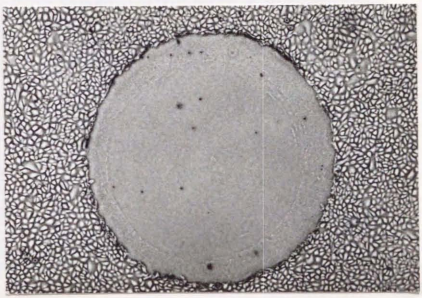

Figure 6a Phase contact micrographs of wound immediately after the wounding; rabbit. (magnification x10)

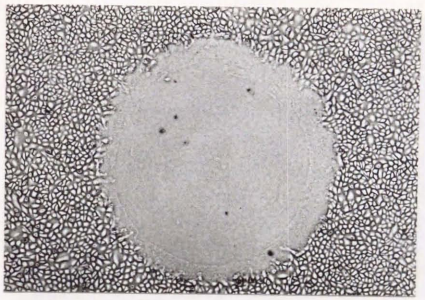

Figure 6b Phase contact micrographs of wound 6 hours after the wounding; rabbit. (magnification x10)

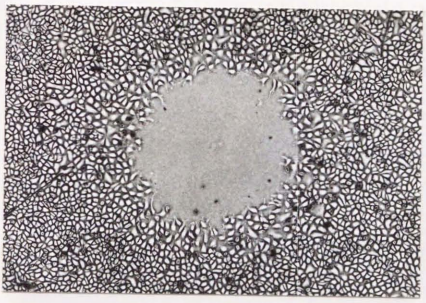

Figure 6c Phase contact micrographs of wound 24 hours after the wounding; rabbit. (magnification x10)

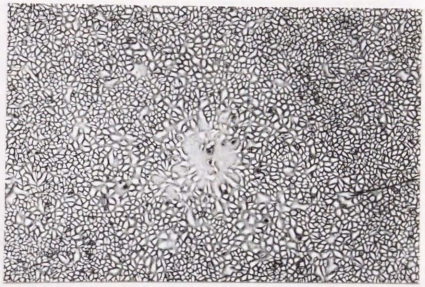

Figure 6d Phase contact micrographs of wound 48 hours after the wounding; rabbit. (magnification x10)

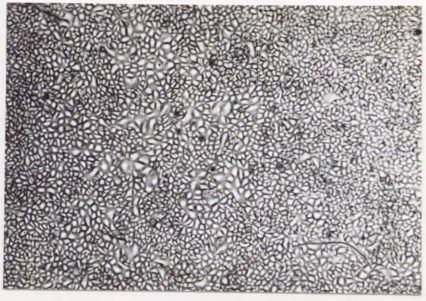

Figure 6e Phase contact micrographs of wound 60 hours after the wounding; rabbit. (magnification x10)

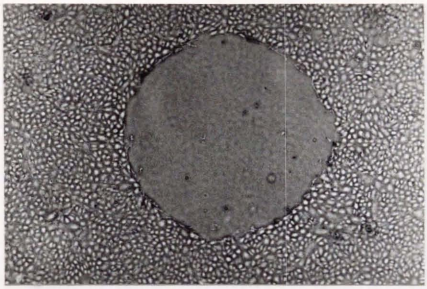

Figure 7a Phase contrast micrographs of wound immediately after the wounding; bovine (magnification x10)

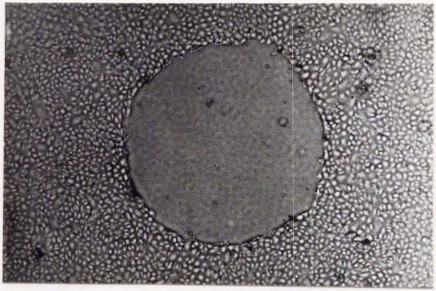

Figure 7b Phase contrast micrographs of wound 6 hours after the wounding; bovine (magnification x10)

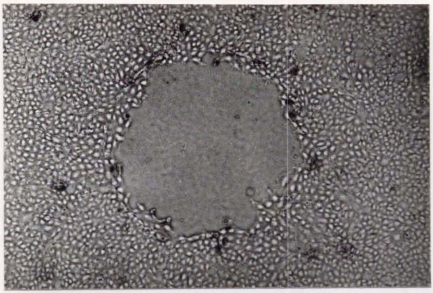

Figure 7c Phase contrast micrographs of wound 24 hours after the wounding; bovine (magnification x10)

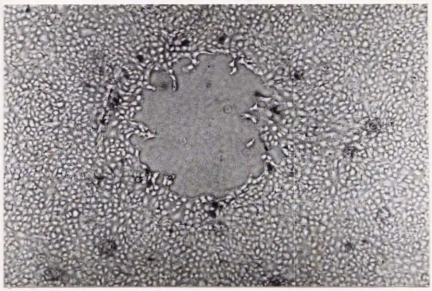

Figure 7d Phase contrast micrographs of wound 48hours after the wounding; bovine (magnification x10)

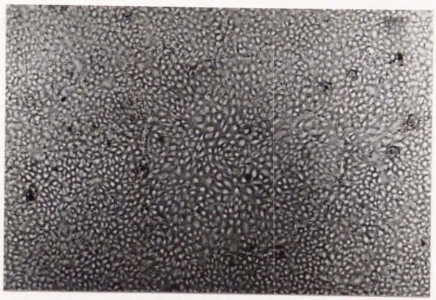

Figure 7e Phase contrast micrographs of wound 84 hours after the wounding; bovine (magnification x10)

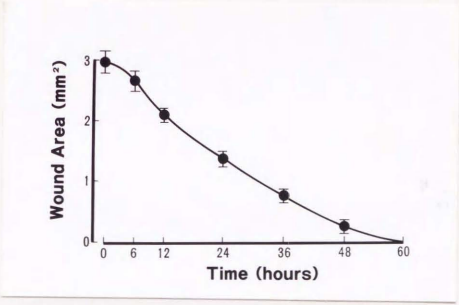

Figure 8a Changes over time in area of wound; rabbit. (Bar means S.D.)

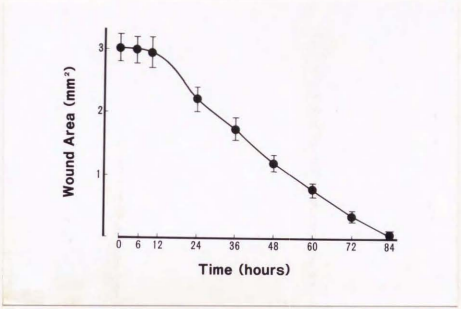

Figure 8b Changes over time in area of wound; bovine.
(Bar means S.D.)

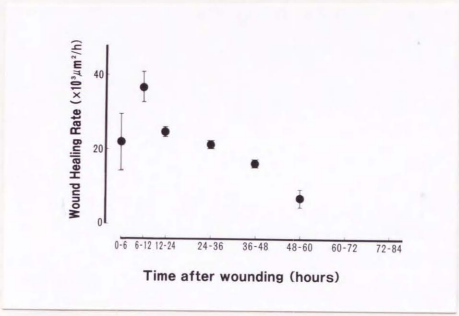

Figure 9a Wound healing rate over various periods; rabbit.
(Bar means S.D.)

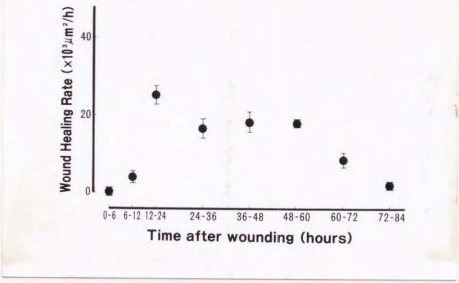

Figure 9b Wound healing rate over various periods; bovine. (Bar means S.D.)

4 a. Kinetic study of cell proliferation in bovine endothelium

During 0-12 hours after the wound, no cell division was found(Figures 10a, 13). During 12-24 hours when the cells started to migrate, some cell division along the wound edge was observed(Figure 10b, 11a); 14.6±4.0 cells(Figure 13). During 24-36 hours, the cells located in the second and third rows along the wound edge were stained for cell division (Figures 10c, 11b), and the number of the cells increased up to 198.0±2.0 cells (Figure 13). During 36-48 hours and 48-60 hours, the cells in the fifth and sixth rows along the wound edge were stained (Figures 10d,e, 11c,d); the number of labeled cells showed a

peak 201.7±16.1 cells and 226.3±24.9 cells respectively (Figure 13). During 60-72 hours, the number of labeled cells decreased (Figure 10f). No labeled cell was observed in the two control groups (Figure12).

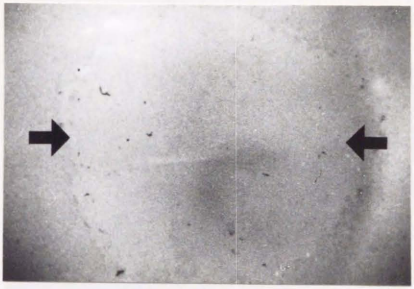

Figure 10a Light micrograph of labeled cells and area of wound after wounding during 0-12 hours; bovine. Arrows indicate edges of wound. (magnification x10)

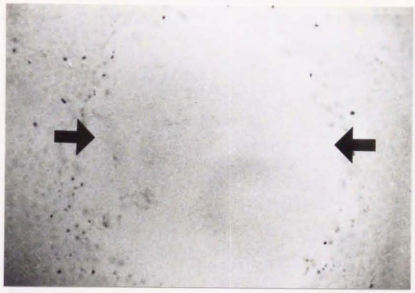

Figure 10b Light micrograph of labeled cells and area of wound after wounding during 12-24 hours; bovine. Arrows indicate edges of wound. (magnification x10)

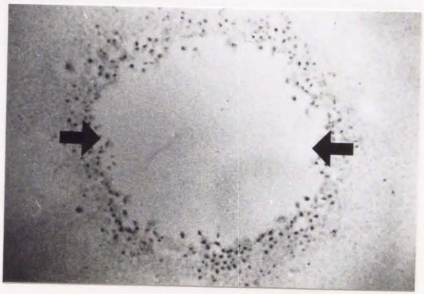

Figure 10c Light micrograph of labeled cells and area of wound after wounding during 24-36 hours; bovine. Arrows indicate edges of wound. (magnification x10)

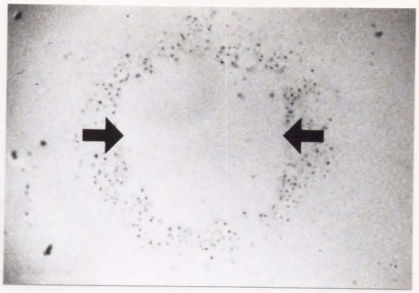

Figure 10d Light micrograph of labeled cells and area of wound after wounding during 36-48 hours; bovine. Arrows indicate edges of wound. (magnification x10)

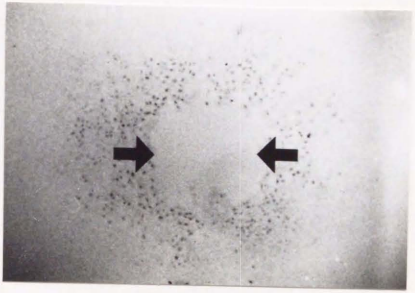

Figure 10e Light micrograph of labeled cells and area of wound after wounding during 48-60 hours; bovine. Arrows indicate edges of wound. (magnification x10)

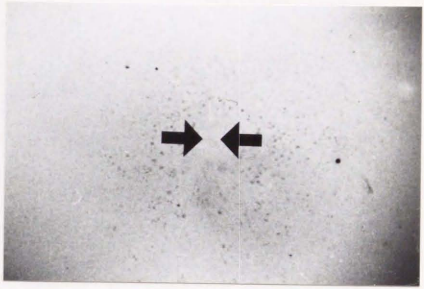

Figure 10f Light micrograph of labeled cells and area of wound after wounding during 60-72 hours; bovine. Arrows indicate edges of wound. (magnification x10)

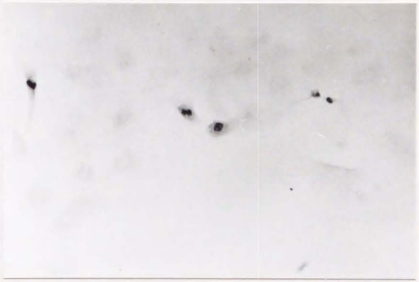

Figure 11a Light micrograph of labeled cells at area of wound edge during 12-24 hours; bovine. (magnificaiton x50)

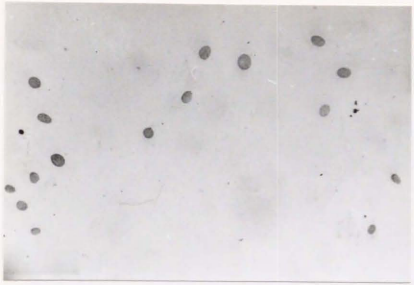

Figure 11b Light micrograph of labeled cells at area of wound edge during 24-36 hours; bovine. (magnificaiton x50)

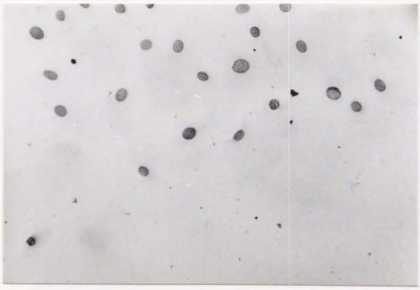

Figure 11c Light micrograph of labeled cells at area of wound edge during 36-48 hours; bovine. (magnificaiton x50)

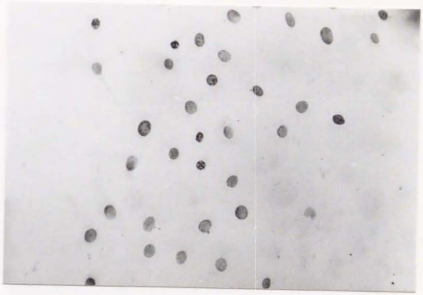

Figure 11d Light micrograph of labeled cells at area of wound edge during 48-60 hours; bovine. (magnificaiton x50)

Figure 12a Control study for BrdU immunohistochemistry using normal non-immune IgG. (magnification x10)

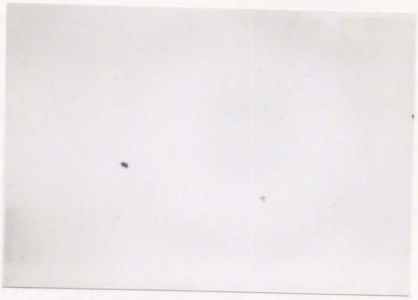

Figure 12b Control study for BrdU immunohistochemistry without BrdU. (magnification x10)

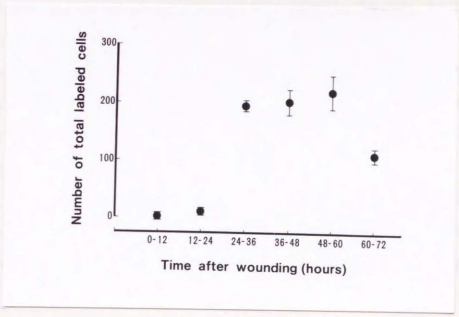

Figure 13 Changes over time in the total number of labeled cells. (Bar means S.D.)

4 b. Localization of the labeled cells in bovine endothelium

The relationship between the labeled cells and the wound at each time period is shown in Figure 14. At the 12th hour after the wounding, the average edge of the wounds was observed at the distance of 0.90±0.10 mm from the center of the wound, but no labeled cell was seen. At the 24th hour, the average edge of the wound was at 0.74±0.04 mm from its center, and a few labeled cells were discerned in the area between 0.6 and 1.0 mm from the center of the wound. At the 36th hour, the average edge of the wounds was at 0.62±0.08 mm from the center, and the number of the labeled cells showed a peak value of 176.9±28.0 cells/mm^2 at 0.6-0.8 mm from the center. No labeled cell was observed beyond 1.2 mm from the center of the wound. At the 48th and 60th hours, the average edges of the wound were at 0.50±0.80 mm and 0.36±0.05 mm from its center, respectively, and the number of the cells was 142±66.8 cells/mm^2 and 228.8±15.6 cells/mm^2, respectively, in the area between 0.4 and 0.6 mm from the center of the wound. At the 72nd hour, the edge of the wound was less than 0.2 mm from its center. The number of the labeled cells decreased and scattered in the area within 0.8 mm from the center of the wound. No labeled cells were seen beyond 1.0mm from the center.

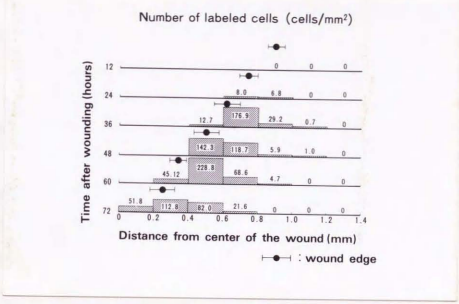

Figure 14 Relationship between labeled cells and wound location. (Bar means S.D.)

IV Discussion

Endothelial cell density of the cell sheet and homogeneity of the original wound size are important in the model for quantitatively evaluation of the wound healing process. The cultured cells migrate toward the denuded area and demonstrate the irregular cell distribution pattern in the conventional culture method. Although the cells at the central part of the confluent cell sheet demonstrate small and homogeneous shape, the cells at the peripheral part showed proliferative activity. We developed the method of encircling the cell culture space with HEMA on the cover slip thereby restriction the culture space and

making a confluent cell sheet.

The wound size is another important factor in quantitative analysis of the wound healing process, since the wound healing rate depends on the initial wound size(7). However, it has been difficult to produce the wounds with a consistent size and shape in the previous methods such as cryocoagulation (5,10,15,16), needles (8), or nylon thread (6). Therefore, we developed a method for wounding a cultured confluent cell sheet using a rotating silicone tip with a fixed diameter. This method enabled us to produce a consistent size of wound.

The difference between rabbit and bovine wound healing process in this model was the kinetics of the cell migration at the early stage. The rabbit cells migrated actively in the early period after the wounding and showed a high wound healing rate. However, the bovine cells showed no remarkable movement and low wound healing rate at the early stage. The rabbit corneal endothelial cells in vivo were reported to migrate actively from the early period in its wound healing process(6,7). The human corneal endothelial cells were reported to have a time lag to start migration in in vivo study(22,23). There is a species difference in the wound healing process at the early stage. The wound healing process of bovine cultured corneal endothelial cells at the early stage is more similar to that of man than that of rabbit. In vivo and in vitro cell counts were more

similar for the bovine samples than for the rabbit samples. Therefore, we used bovine cultured corneal endothelium as a wound healing model for the cell kinetic study using BrdU immunohistochemistry.

A useful method to investigate cell kinetics is to label cells in their division phase. The conventional method for cell labeling is autoradiography using ^{3}H-thymidine (5,18-23). However, it is necessary to use a radioactive isotope and a special equipment and involves a disadvantage in radiation hazard. In the present study, the method was to use the thymidine-analog BrdU which is incorporated by cells in their S-phase, and then to stain the cells using BrdU monoclonal antibody (24).
It is reported that this method with enzyme antibody staining techniques has a good correlation with autoradiography using ^{3}H-thymidine(25). Since the nuclei incorporating BrdU are positively stained in this method, it is considered that the method has an advantage in distinguishing the positively stained cells in a monolayer on a cover slip as shown in this model.

By 24 hours after the wounding in this study, there were very few labeled cells despite the fact that healing of the wound area was seen. The number of labeled cells increased rapidly after 36 hours and reached a its peak between 48 and 60 hours. After then, they decreased rapidly, and none was seen at the time when the wound was closed. These results indicates

that cell migration and cell mitosis play different roles in the wound healing process.

According to localization of labeled cells, it is concluded that the cell division occurs around the wound margin. This result agrees with previous in vivo study(5). It is reported that rabbit corneal endothelial cells have a high mitotic activity, and the incidence of cell mitosis peaks between 24 to 48 hours after the wounding with mitotic cells localizing within 1 mm of the wound edge(5). The cell mitosis decreases rapidly after the wound closure and contact inhibition occurs(5,9). Although there are differences in the time course of the wound healing among reports, the wound healing process demonstrate a similar pattern. In monkeys, the cell mitotic phenomenon has been rarely observed by morphological study (26), but experiments with autoradiography reported that 12 to 19% of the cells around the wound incorporated ^{3}H-thymidine(20). In man, Doughman et al. reported, using the organ culture method, that the wound was healed entirely by cell migration, and that the role of cell proliferation was extremely limited (15,16). However, Treffer reported that human corneal endothelial cells incorporated ^{3}H-thymidine by 48 hours after the wounding, and the number of divided cells reached its peak at 72 hours(15).

Regarding the bovine corneal endothelium, the wound healing process can be divided into four phases; latent phase, migration

phase, phase with concurrent migration and mitosis, and contact inhibition phase(Figure 15).

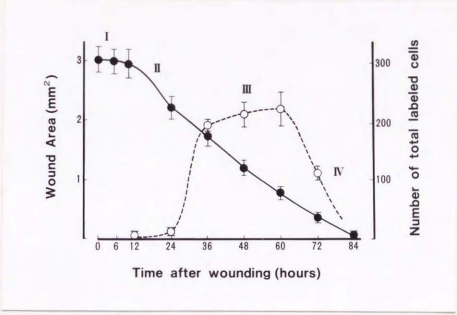

Figure 15 Relationship between wound area and cell proliferation in bovine cornea. I. Latent phase II. Migration phase III. Migration and mitosis phase IV. Contact inhibition phase (Bar means S.D.) ● wound area ○ total labeled cells

The latent phase is the period in which the cells around the wound do not migrate. The duration of this period was different depending on species; this phase was very short in rabbit in vivo(7) and about 24 hours in man using the organ culture method(19).

The migration phase is the period in which the wound is healed mainly by cell migration and its healing speed is almost constant. The cell migration around the wound occurs within the first 6 hours after the wounding in rabbit, but this is not the case in bovine. The duration of this phase in this model was between 12 and 24 hours after the wounding.

The phase with concurrent migration and mitosis is a period in which the cell migration and proliferation occur concurrently. According to in vivo study using rabbit, this phase was observed 16 hours after the wounding(5). While mitosis in our model was observed frequently during the period between 24 and 60 hours after the wounding, it was thought that this phase started 24 hours after the wounding.

The contact inhibition phase is the period in which the wound is almost closed. The cell migration and mitosis are no more observed and rearrangement of cell distribution occurs. It was reported that extracellular matrix structures regenerated during this period(5,9). However it is difficult to identify the time when the phase starts and ends its process. As far as this wound healing model was concerned, it was thought to be 72 hours after the wounding when cell proliferation decreased rapidly.

Conclusion

We developed a new wound healing model using cultured cells to evaluate the corneal endothelial wound healing process quantitatively and time sequentially. This in vitro model has a similar pattern of wound healing processes to the previous reports regarding to the relationship between cell migration and mitosis. Using this new model, we can investigate and clarify the pathophysiology of the corneal endothelium more easily and reliably. We can also determine the effects of various drugs on the corneal endothelium wound healing.

Supplement Effect of drugs on wound healing rate

Various drugs which have been reported to have an effect on cell kinetics were studied in this wound healing model.

Mateirals and Methods

Fibronectin (FN), cytochalacin B (CtB), epidermal growth factor (EGF) and 5-fluorouracil (5-FU). Each drug was dissolved using the basic culture medium and adjusted to the final concentrations: 0.3, 1, 3, 10 ug/ml for FN; 10, 30, 100, 300, 1000 ng/ml for CtB; 3, 10, 30, 100 ng/ml for EGF; and 0.3, 1, 3, 10 ug/ml for 5-FU. Eagle's MEM Earle's solution with 10% fetal bovine serum and 20 ug/ml gentamicin was used as the basic medium. The control was the model studied using only the basic medium. After the wound was made, cell samples were immersed in the basic culture medium, as control, or in the basic medium with one of each of the above drugs being tested in each of the concentrations listed. The wound healing process was observed using an inverted phase contrast microscope. Then the wound healing rates between 12 and 24 hours, which corresponded to the migration phase, and between 36 and 48 hours, which corresponded to the concurrent migration and mitosis phase, were calculated for each group. The wound healing rates of each drug were compared with the control group.

Results

a. Fibronectin (FN)

With concentrations higher than 0.3 ug/ml in the FN group, the wound healing rate was significantly higher than in the control during the migration phase. The concentration of 0.3 ug/ml did not induce a healing rate significantly different than the control. ($P<0.05$ for 1 and 3 ug/ml, $P<0.01$ for 10 ug/ml, Figure 15a).

On the other hand, no significant differences in the wound healing rates were observed among the tested media with various FN concentrations in the concurrent migration and mitosis phase (Figure 15b).

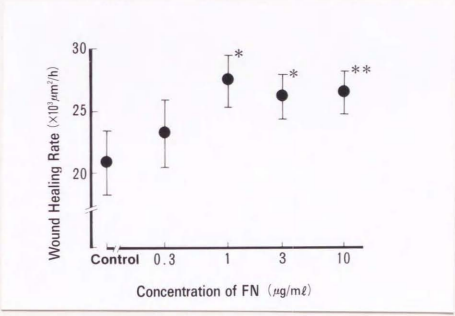

Figure 15a The effect of FN on the wound healing rate 12-24 hours after making the wound (migration phase). (*P<0.05, **P<0.01)

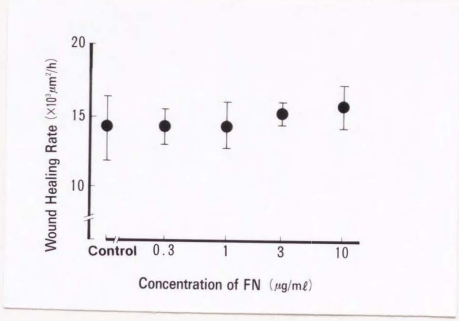

Figure 15b The effect of FN on the wound healing rate 36-48 hours after making the wound (migration and mitosis phase).

b. Cytochalacin B (CtB)

In the CtB groups, wound healing rates were reduced with increasing concentrations in both time periods, 12-24 hours and 36-48 hours after the wounding ($P<0.001$ for 300 and 1,000 ng/ml in the migration phase, and $P<0.05$ for 300 ng/ml and $P<0.001$ for 1,000 ng/ml in the migration and mitosis phase (Figures 16a,b).

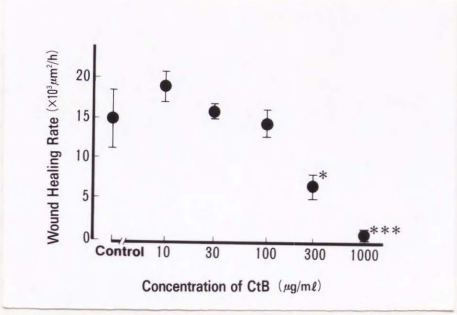

Figure 16a The effect of CtB on the wound healing rate 12-24 hours after making the wound (migration phase). (*P<0.05, ***P<0.001)

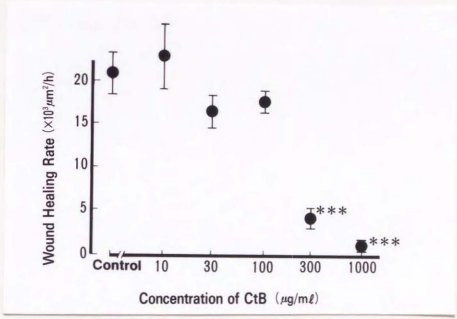

Figure 16 b The effect of CtB on the wound healing rate 36-48 hours after making the wound (migration and mitosis phase). (***P<0.001)

c. Epidermal growth factor (EGF)

There were no significant differences in the wound healing rate in the time period 12-24 hours after the wounding between the control and each of the EGF groups (Figure 17a). However, in the time period 36-48 hours after the wounding the EGF groups showed significantly higher wound healing rates than the control regardless of concentrations. ($P<0.05$ for 30 ng/ml, $P<0.01$ for 3, 10 and 100 ng/ml, Figure 17b).

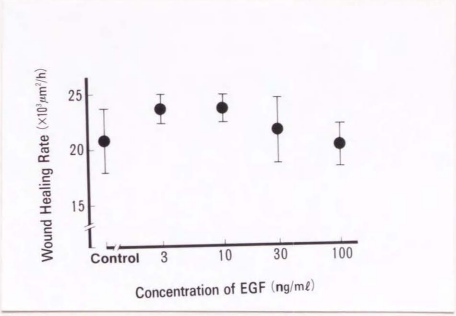

Figure 17a The effect of EGF on the wound healing rate 12-24 hours after making the wound (migration phase).

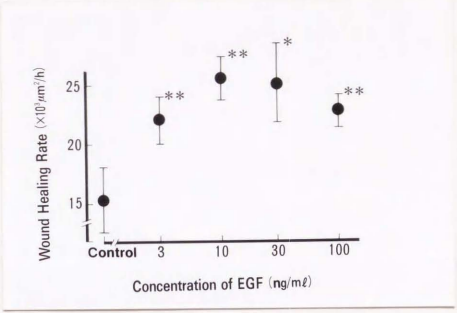

Figure 17b The effect of EGF on the wound healing rate 36-48 hours after making the wound (migration and mitosis phase). (*P<0.05, **P<0.01)

d. 5-Fluorouracil (5-FU)

Among the control and the 5-FU groups, there were no significant differences in the wound healing rate in the time period which corresponded with the migration phase (Figure 18a). However, in the time period which corresponded to the concurrent migration and mitosis phase the wound healing rate in the 5-FU groups with 10 and 30 ug/ml showed a significantly lower wound healing rate than the control (P<0.01 for 10 ug/ml and P<0.001 for 30 ug/ml, Figure 18b).

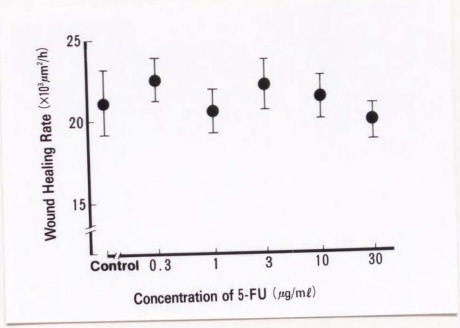

Figure 18a The effect of 5-FU on the wound healing rate 12-24 hours after making the wound (migration phase).

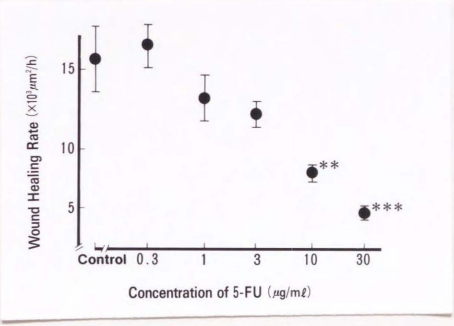

Figure 18b The effect of 5-FU on the wound healing rate 36-48 hours after making the wound (migration and mitosis phase). (**P<0.01, ***P<0.001)

Discussion

Various drugs which have been reported to have an effect on cell kinetics were studied in this wound healing model. FN accelerates the migration of the cells (27). CtB suppresses cellular migration due to the inhibition of actin polymerization (28). EGF promotes cellular mitosis (29) but 5-FU has an inhibiting effect thereon (30). Table 1 summarizes the effects of these drugs on the wound healing rates in the two time periods.

Table 1. Summary of the effect of drugs tested

	Migration phase (12-24 hours)	Migration and mitosis phase (36-48 hours)
FN	increase	no effect
CtB	decrease	decrease
EGF	no effect	increase
5-FU	no effect	decrease

The results of this study demonstrated that the drugs tested have different effects on the wound healing depending on the time period during the wound healing and the concentration of drug used. The different effects of the drugs on cell kinetics confirmed that the time periods 12-24 hours and 36-48 hours correspond to the migration phase and concurrent migrating and mitosis phase, respectively. Therefore, this wound healing model makes it possible not only to analyze the wound healing process but also to evaluate the effects of drugs thereon. It is suggested that EGF and FN which have been introduced

clinically in the treatment of ocular surface disorders show different effects on the wound healing process and duration due to their pharmacological effects.

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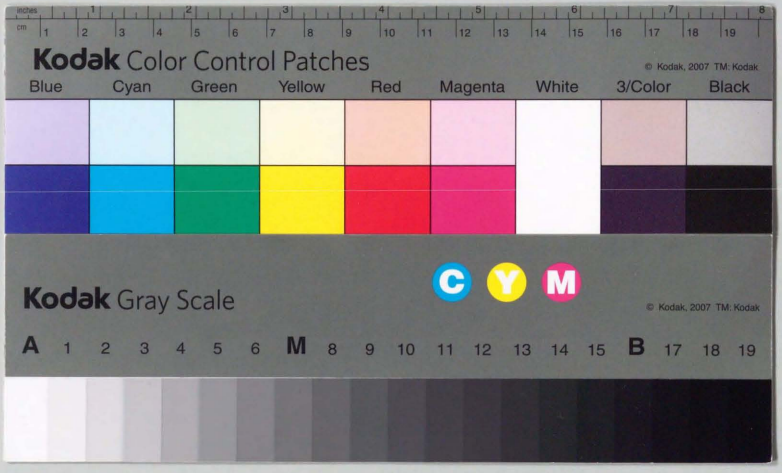
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