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Difference in Migratory Ability between Human Lung and Skin Fibroblasts

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ABSTRACT—TIG-3 human lung fibroblasts were found to differ in their migratory ability from TIG-3S human skin fibroblasts derived from the same fetus: (1) TIG-3 cells migrated in medium supplemented with 10% fetal bovine serum (FBS) more slowly than TIG-3S cells. (2) TIG-3 cells migrated in serum-free medium as effectively as in medium supplemented with 10% FBS, whereas TIG-3S cells migrated in serum-free medium much more slowly than in medium supplemented with 10% FBS. (3) The migration of TIG-3S cells was changed more markedly by the pH of the culture medium than that of TIG-3 cells. The second was the most striking difference in migratory ability between the TIG-3 and TIG-3S cells, and was also the case when several human fetal lung fibroblasts (TIG-1, TIG-7, WI-38, IMR-90, MRC-5), and skin fibroblasts from adult and elderly donors were tested. The monovalent ionophore, monensin, inhibited the migration of TIG-3 and TIG-3S cells in our experimental system according to monensin concentration, and immunofluorescence staining for fibronectin demonstrated that monensin inhibited the secretion of fibronectin. This implies that secreted substances including fibronectin regulate cell migration. However, the migration of TIG-3S cells was not decreased to the same degree as that of TIG-3 cells after monensin treatment. The role of the extracellular matrix in this difference of migratory ability between human lung and skin fibroblasts is discussed.

INTRODUCTION

Since human fibroblasts have an intrinsic limit of cell division potential, they are often used as a model system of cellular aging *in vitro* [1, 2]. Human fibroblasts derived from different tissues seem to possess similar features. However, it has been reported that human fibroblasts show heterogeneity and tissue differences [3–8]. Our previous study on the effects of serum from human subjects of various ages on cell migration demonstrated a difference in migratory ability between human fetal lung fibroblasts (TIG-1) and skin fibroblasts from adult donors: Adult donor skin fibroblasts migrated in serum-free medium much more slowly than in medium supplemented with 10% FBS, whereas TIG-1 cells migrated in serum-free medium as effectively as in medium supplemented with 10% FBS [9]. There has been no previous report of any tissue difference in the migratory ability of human fibroblasts. The present study was therefore carried out to confirm and generalize the differences in migratory ability between human lung and skin fibroblasts.

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MATERIALS AND METHODS

Cells

Several human fetal lung fibroblast lines (TIG-1, TIG-3, TIG-7, WI-38, IMR-90, MRC-5) were used. TIG-1, TIG-3 and TIG-7 cells were established at a project team of the Tokyo Metropolitan Institute of Gerontology [10, 11]. WI-38 and IMR-90 cells were obtained from the Institute for Medical Research (Camden, USA), and MRC-5 cells were obtained from the American Type Culture Collection (Rockville, USA). Several human skin fibroblast lines (TIG-3S, ASF-4, ASF-5, ASF-3, ASF-2) were also used. Human fetal skin fibroblasts (TIG-3S) were established at a project team of the Tokyo Metropolitan Institute of Gerontology, and skin fibroblasts from adult(ASF-

4, ASF-5) and elderly (ASF-2, ASF-3) donors were kindly supplied by Drs. K. Kaji and M. Matsuo [12]. The cells were cultured in Eagle's basal medium (BME) (GIBCO) supplemented with 10% FBS and antibiotics, as described in a previous paper [13].

Mycoplasma contamination in these cell cultures was measured by the method of Kihara *et al.* [14], but none was detected.

Serum

One lot of FBS (Hyclone, #100394) was used throughout all experiments.

Measurement of cell migration

Cell migration was measured by a slight modification of the method of Stenn [15]. Before cell preparation, cover glasses (22×22 mm, No. 1, Matsunami Glass Ind., Ltd, Japan) were cleaned, and coated by dipping them in 1,2-dichloroethane (Dojindo Lab., Japan) solution containing 1% Formvar powder (Polyvinyl Formal, Oken Shoji Co., Japan) and 0.2% Scarlet red (Sudan IV, Chroma-Gesellshaft Schmid GMBH & Co., DDR). The coated cover glasses were placed in 35-mm plastic Petri dishes (Falcon, 3001) and secured to the bottom of the dishes with sterile silicone. Confluent cells, obtained 1 week after subcultivation with a split ratio of 1:4, were harvested from 60-mm plastic culture dishes (Falcon, 3002) by treatment with 0.25% trypsin (Difco, 1:250) in Mg^{2+}-, Ca^{2+}- free phosphate-buffered saline (PBS), pH 7.4. Confluent cells (split ratio 1:1) or 1×10^6 cells per dish were poured into the 35-mm plastic culture dishes containing the previously prepared cover glasses. The cells covered the bottom of the dish as a monolayer. The cell numbers were determined with a Coulter counter (Coulter Electronics, Hialeah, FL). After culture for 1 day at 37°C in a 5% CO_2 incubator, the cultures were washed once with BME. Each cover glass was placed on a sterile glass slide and the cell sheet on the cover glass was marked by cutting off the cell-Formvar coat along the midline of the cover glass with a sterile stainless steel blade (Disposable Dermatome, Feather Ind., Ltd., Japan). Then, the cell-coated cover glass was secured to the bottom of a new 35-mm culture dish containing culture medium with or without 10% FBS. On the second day after re-culture, the outgrowths were stained by removing the culture medium and flooding the cover glass for 2 min with a staining solution composed of 0.73% toluidine blue and 0.27% basic fuchsin in 30% ethanol. Outgrowths were quantified using a calibrated ocular micrometer by measuring the maximal linear distance of cell movement from the cut edge.

Immunofluorescence staining of fibronectin

In order to detect extracellular fibronectin, immunofluorescence staining of human fibroblasts was performed by the method described previously [16]. Cell sheets on Formvar-coated cover glasses were washed three times with PBS, pH 7.2, and incubated with rabbit antibody to human fibronectin (E-Y Laboratories, Inc., USA) at 1:20 dilution without fixation or drying. Control cultures were incubated with nonimmune rabbit serum or PBS. After incubation for 30 min at room temperature, the cell sheets were washed three times with PBS, and then fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (E-Y Laboratories, Inc., USA) as a second antibody at 1:20 dilution was poured over the cell sheets and incubated at room temperature for 30 min. The cell sheets on Formvar-coated cover glasses were washed three times with PBS and then embedded with glycerol solution (glycerol/PBS:9/1).

RESULTS

Migratory ability of human fetal lung and skin fibroblasts derived from the same fetus

The previous study showed that human fetal lung fibroblasts (TIG-1) migrated linearly during 3 days of incubation [9]. Figure 1 also demonstrates that human lung (TIG-3) and skin (TIG-3S) fibroblasts derived from the same fetus migrated linearly for 3 days in culture medium supplemented with 10% FBS. TIG-3 cells migrated in serum-free medium as effectively as in medium supplemented with 10% FBS (Fig. 1A), although the number of migrating cells seemed to be much larger in serum-supplemented medium than in

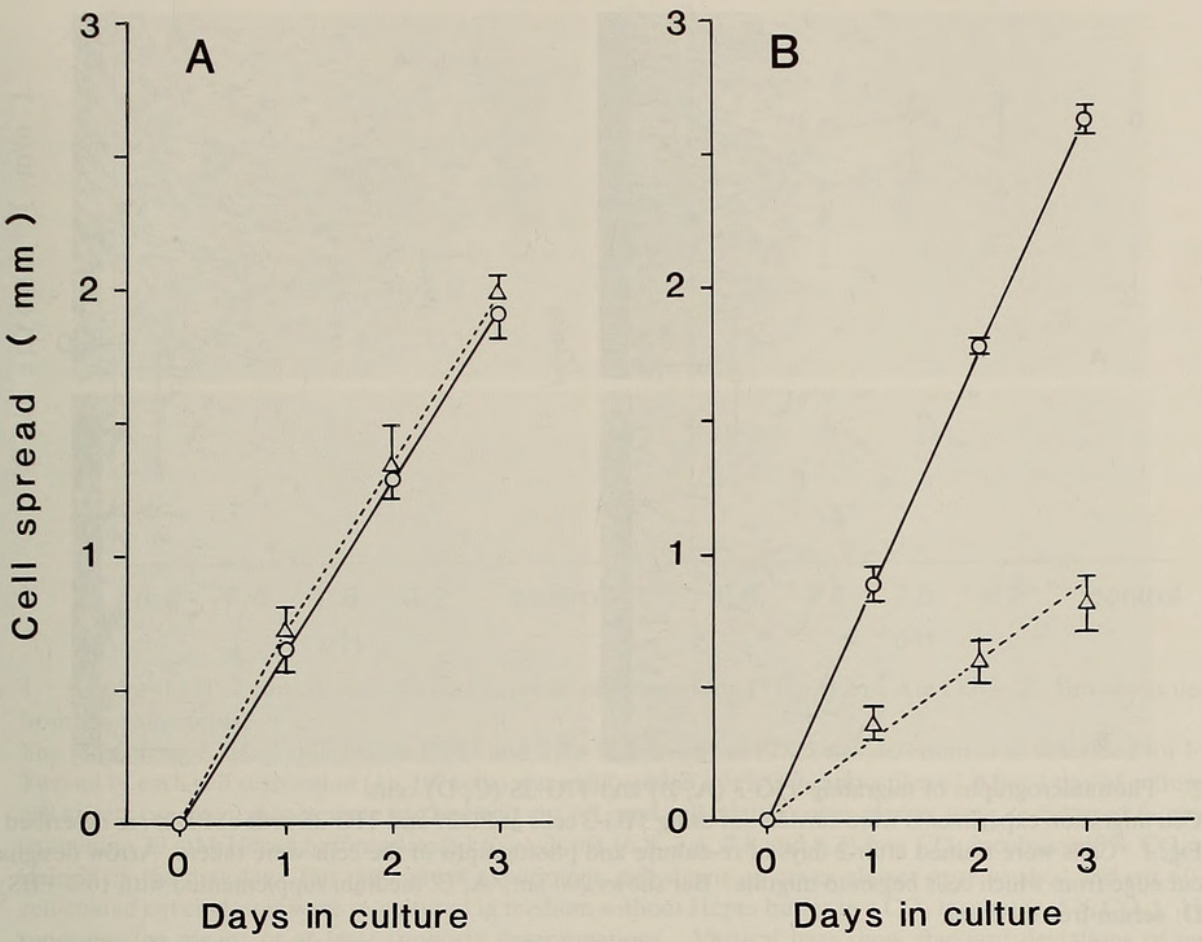

FIG. 1. Time course of migration of human lung (TIG-3) and skin (TIG-3S) fibroblasts derived from the same fetus. TIG-3 (A) and TIG-3S (B) cells were cultured in BME supplemented with 10% FBS in a humidified CO_2 incubator (5% CO_2) at 37°C and cell migration experiments were carried out as described in Materials & Methods. TIG-3 cells at PD29 and TIG-3S cells at PD25 were treated with 0.25% trypsin and suspended in culture medium. Two ml (1×10^6 cells) of each cell suspension was inoculated into a 35-mm plastic culture dish containing a Formvar-coated cover glass. After culture for 1 day, each culture was washed once with BME. The cell sheets on cover glasses were cut off, and the cell-coated cover glasses were re-cultured in BME with or without 10% FBS and stained at regular time intervals. Values represent the means of at least triplicate determinations. Vertical bars show standard deviations of means. ○——○, medium supplemented with 10% FBS; △·····△, serum-free medium.

serum-free medium (Fig. 2). On the other hand, the migration rate of TIG-3S cells decreased remarkably when serum was removed from the culture medium (Figs. 1B, 2). TIG-3 cells migrated more rapidly than TIG-3 cells.

A study was carried out to determine whether the difference in the migratory ability between TIG-3 and TIG-3S cells was observed in culture media containing various concentrations of FBS (Fig. 3). TIG-3S cells migrated in media containing 2.5–50% FBS much more rapidly than TIG-3 cells, although the opposite result was obtained in serum-free medium. The migratory ability of TIG-3 and TIG-3S cells gradually decreased according to increased serum concentration, and the slope of the decline in migration rate was more rapid for TIG-3S cells than for TIG-3 cells. Next, the effects of culture medium pH on the migration of TIG-3 and TIG-3S cells were measured (Fig. 4). When culture medium containing 10% FBS was used, the migration rate of TIG-3 and TIG-3S cells was lower at pH 6.8 than at pH 7.4–8.2. The migration rates of TIG-3 cells were the same within a pH range of 6.8–8.2, even when serum was removed from the culture medium. On the other hand, the migration rate of TIG-3S cells at

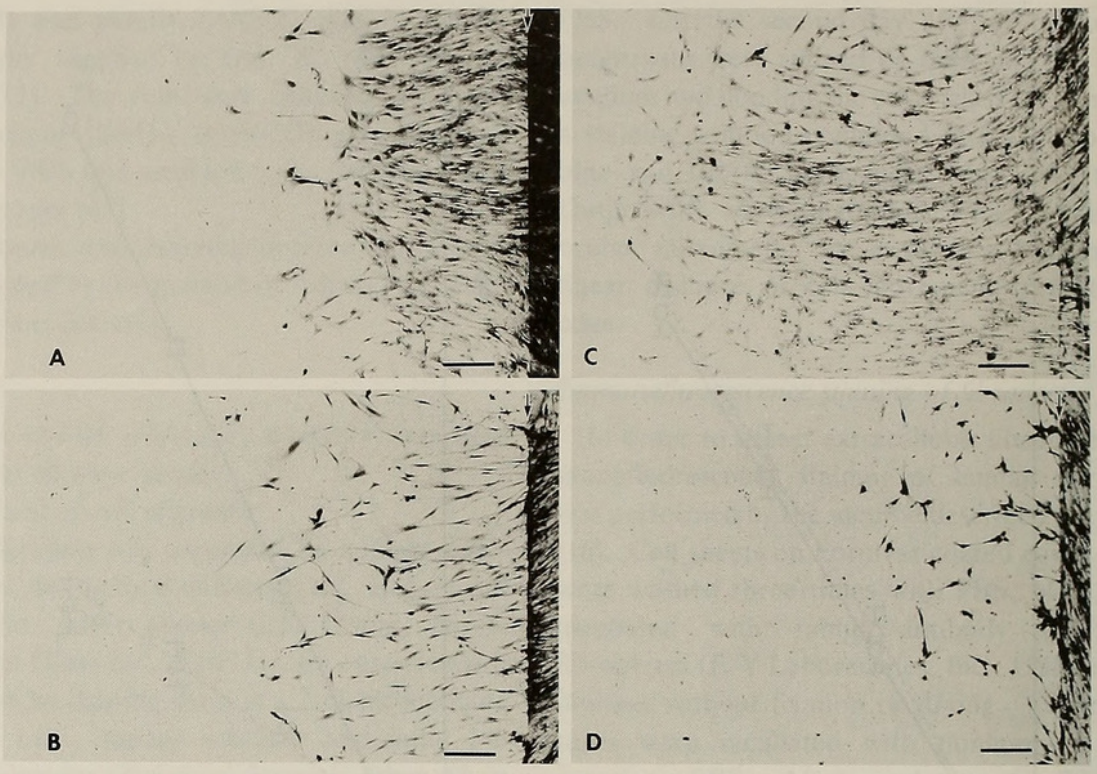

FIG. 2. Photomicrographs of migrating TIG-3 (A, B) and TIG-3S (C, D) cells.
Cell migration experiments were carried out using TIG-3 cells at PD29 and TIG-3S cells at PD25, as described for Fig. 1. Cells were stained after 2 days of re-culture and photographs of the cells were taken. Arrow designates cut edge from which cells began to migrate. Bar shows 200 μm. A, C: medium supplemented with 10% FBS; B, D: serum-free medium.

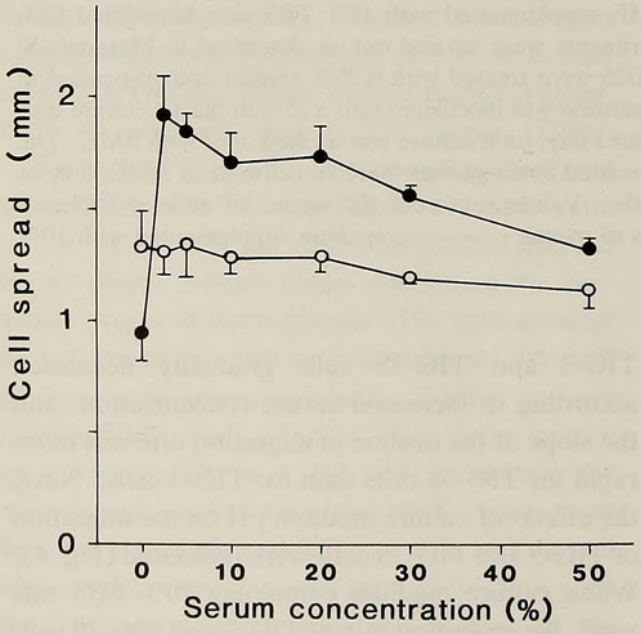

FIG. 3. Effects of serum concentration on migration of human lung (TIG-3) and skin (TIG-3S) fibroblasts derived from the same fetus.
The migration of TIG-3 cells at PD29 and TIG-3S cells at PD25 was determined as described for Fig. 1. Two ml of each cell suspension (1×10^6 cells) was inoculated. After 1 day of culture, the cell sheets on cover glasses were washed with BME and cut off, and cell-coated cover glasses were re-cultured in BME containing various concentrations of FBS and stained on the second day. Values represent the means of at least triplicate determinations. Vertical bars show standard deviations of means. ○——○, TIG-3 cells; ●——●, TIG-3S cells.

pH 6.8 was much lower in serum-free medium than in medium supplemented with 10% FBS. However, TIG-3S cells migrated rapidly at pH 7.8 and 8.2, although the migration of TIG-3S cells was lower in serum-free medium than in medium supplemented with 10% FBS.

Comparison of migratory ability of several fibroblast cell lines cultured from lung and skin

When FBS was removed from the culture

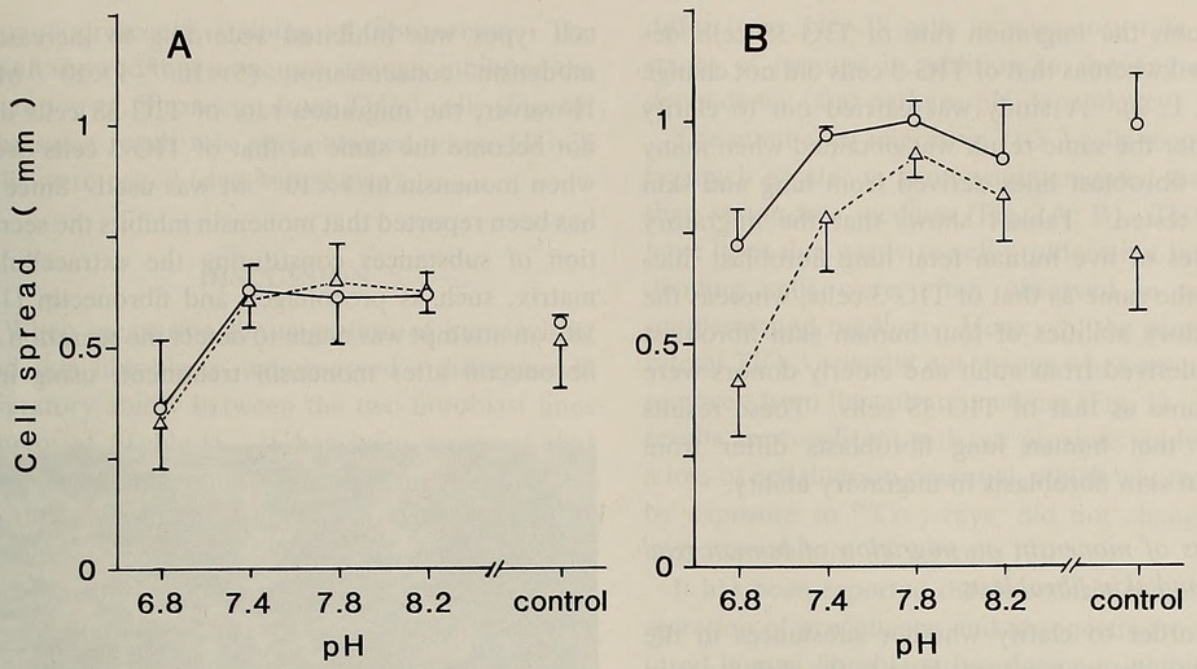

FIG. 4. Effects of pH of culture medium on migration of human lung (TIG-3) and skin (TIG-3S) fibroblasts derived from the same fetus.

The migration of TIG-3 cells (A) at PD29 and TIG-3S cells (B) at PD25 was determined as described for Fig. 3. Two ml of each cell suspension (1×10^6 cells) was incubated in a 5% CO_2 atmosphere. After 1 day of culture, the cell sheets on cover glasses were washed and cut off, and cell-coated cover glasses were re-cultured in medium containing 30 mM Hepes buffer adjusted to each pH (6.8, 7.4, 7.8 and 8.2) in a CO_2 incubator (0% CO_2), and stained on the first day. For the control experiment, cell sheets on cover glasses were washed and cut off, and cell-coated cover glasses were re-cultured in medium without Hepes buffer in a CO_2 incubator (5% CO_2). Values represent the means of at least triplicate determinations. Vertical bars show standard deviations of means. ○——○, medium supplemented with 10% FBS; △·····△, serum-free medium.

TABLE 1.. Migration of human lung and skin fibroblast lines in media with or without FBS

Cells (Donor age, Sex)	PD used/ Total PD	(No.)	Cell spread (μm)		10% FBS/ No serum
			No serum	10% FBS	
Lung					
TIG-1 (Fetus, F)	(26/67)	(7)	1126±99	1223±84	(+ 9%)
TIG-3 (Fetus, M)	(25/75)	(1)	1026±198	1212±84	(+ 18%)
TIG-7 (Fetus, M)	(23/63)	(1)	912±90	939±85	(+ 3%)
WI-38 (Fetus, F)	(39–41/52)	(2)	902±7	1159±1	(+ 28%)
IMR-90(Fetus, F)	(24/62)	(1)	758±85	1052±81	(+ 39%)
MRC-5(Fetus, M)	(42–44/55)	(2)	928±53	1047±61	(+ 13%)
Skin					
TIG-3S(Fetus, M)	(25–29/69)	(2)	677±162	1723±60	(+155%)
ASF-5 (21Y, M)	(26/66)	(1)	519±11	1420±64	(+174%)
ASF-4 (36Y, M)	(32–34/74)	(2)	450±7	1330±33	(+196%)
ASF-2 (65Y, F)	(25/56)	(1)	587±74	1642±140	(+180%)
ASF-3 (77Y, M)	(24–26/43)	(2)	407±37	1050±75	(+158%)

Migration experiments were carried out as described for Fig. 1. The numbers of cells inoculated onto each 35-mm culture dish were 1×10^6 cells for TIG-1 cells and confluent cells (split ratio 1 : 1) for other cell lines. After 1 day of culture, the cell sheets on cover glasses were washed and cut off. The cell-coated cover glasses were re-cultured in BME with or without 10% FBS, and stained on the second day. Values represent the means (±S.D.) of at least triplicate determinations.

medium, the migration rate of TIG-3S cells decreased whereas that of TIG-3 cells did not change (Figs. 1, 2). A study was carried out to clarify whether the same result was obtained when many other fibroblast lines derived from lung and skin were tested. Table 1 shows that the migratory abilities of five human fetal lung fibroblast lines were the same as that of TIG-3 cells, whereas the migratory abilities of four human skin fibroblast lines derived from adult and elderly donors were the same as that of TIG-3S cells. These results imply that human lung fibroblasts differ from human skin fibroblasts in migratory ability.

Effects of monensin on migration of human fetal lung and skin fibroblasts

In order to clarify whether substances in the extracellular matrix contribute to the difference in migratory ability between human lung and skin fibroblasts, the effects of monensin on cell migration were measured (Fig. 5). Migration of both cell types was inhibited according to increased monensin concentration (5×10^{-8}–1×10^{-6} M). However, the migration rate of TIG-3S cells did not become the same as that of TIG-3 cells even when monensin at 1×10^{-6} M was used. Since it has been reported that monensin inhibits the secretion of substances constituting the extracellular matrix, such as procollagen and fibronectin [17, 18], an attempt was made to detect the secretion of fibronectin after monensin treatment, using im-

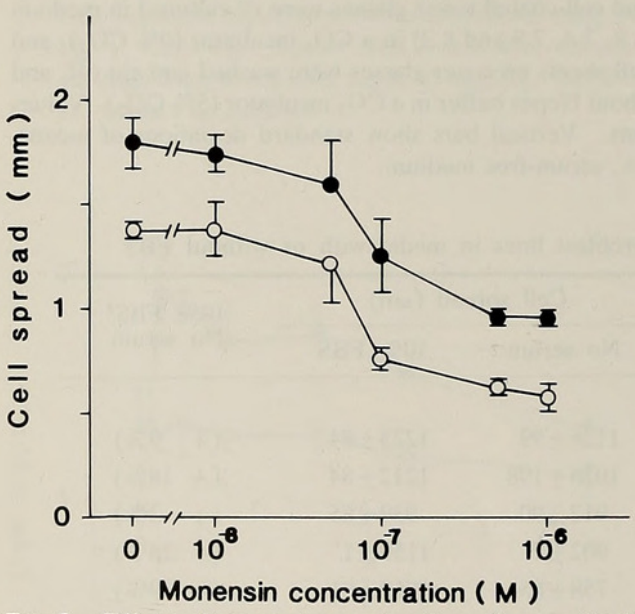

FIG. 5. Effects of monensin on migration of human fetal lung (TIG-3) and skin (TIG-3S) fibroblasts.
The migration of TIG-3 cells (A) at PD35 and TIG-3S cells (B) at PD25 was determined as described for Fig. 3. Two ml of cell suspension (1×10^6 cells) was inoculated. After 1 day of culture, the cell sheets on cover glasses were washed and cut off, and cell-coated cover glasses were re-cultured in BME containing 10% FBS and various concentrations of monensin, and stained on the second day. Values represent the means of duplicate determinations. Vertical bars show standard deviations of means. ○——○, TIG-3 cells; ●——●, TIG-3S cells.

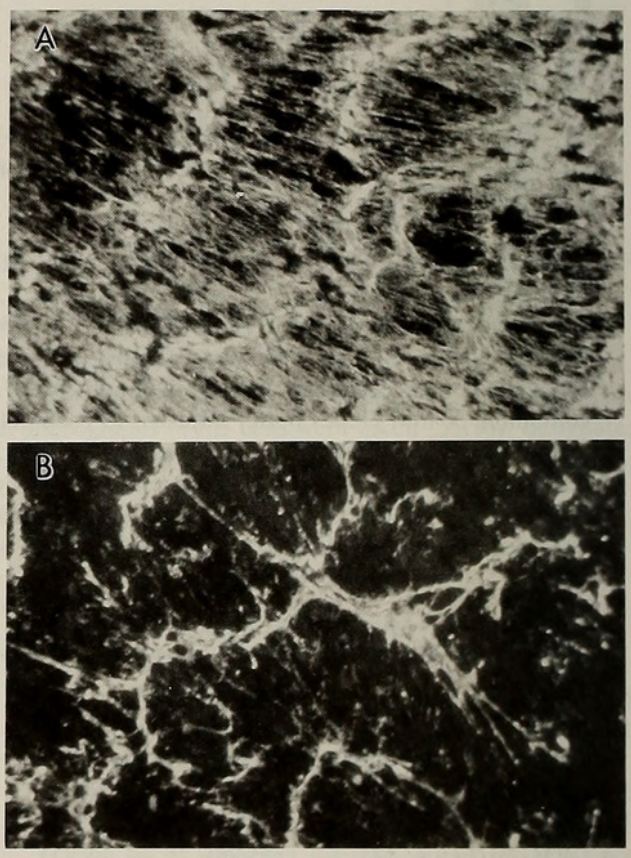

FIG. 6. Immunofluorescence staining of the monolayers of TIG-3 human fetal lung fibroblasts with antibodies to human fibronectin.
Cell migration experiments were carried out using TIG-3 cells at PD37, as described for Fig. 5. Two ml of cell suspension (1×10^6 cells) was inoculated. After 1 day of culture, the cell sheets on cover glasses were washed and cut off, and the cell-coated cover glasses were re-cultured in culture medium with (B) or without (A) 5×10^{-7} M monensin. On the second day, the cell sheets on cover glasses were incubated with antibodies to human fibronectin, and extracellular fibronectin was stained, as described in Materials and Methods. Monolayer cells were microscopically photographed. Control cultures which were incubated with nonimmune rabbit serum or PBS were not stained (data not shown). ×50.

munofluorescence staining of fibronectin. The result showed that monensin strongly inhibited the secretion of fibronectin from TIG-3 cells (Fig. 6). The same result was also obtained when TIG-3S cells were tested (data not shown).

DISCUSSION

When measuring the migration of human lung and skin fibroblasts, we observed a difference in migratory ability between the two fibroblast lines employed (Table 1). It has been reported that human lung fibroblasts differ from human skin fibroblasts in cell morphology, growth rate and cell density at confluence [6], the capacity to change cortisone into hydrocortisone [7], and the Kd value for the binding reaction of dexamethasone to cells [8]. However, a difference in migratory ability between these two fibroblast lines has not been reported. Therefore the findings of our present study seem to be the first evidence of a tissue difference in the migration of human fibroblasts.

The migration rate of human skin fibroblasts (TIG-3S) changed greatly upon removal of FBS from the culture medium, range of pH of serum-free medium and change in the serum concentration of culture medium, unlike the case of human lung fibroblasts (TIG-3) derived from the same fetus (Figs. 1, 3, 4). This result was also obtained when skin fibroblasts from adult and elderly donors were used. This implies that human skin fibroblasts seem to be sensitive to stimuli or environmental change. For skin fibroblasts to perform their role in wound healing, a high sensitivity to many forms of stimulus may be an essential feature. However, the mechanism by which human skin fibroblasts migrate more rapidly than human lung fibroblasts is unclear.

Using relatively early passaged cells, we demonstrated that human lung fibroblasts (TIG-3) differ from human skin fibroblasts (TIG-3S) with regard to migration rate and pH and serum dependency. A study was therefore carried out to clarify whether this phenomenon changed during the *in vitro* aging of human fibroblasts. The same result was obtained, although the migratory ability of cells decreased with successive passages (unpublished data). These results show that TIG-3 cells differ from TIG-3S cells in migratory rate at all stages of passage in addition to showing serum dependency (and perhaps pH dependency).

The number of migrating TIG-3 cells seemed to be much greater in serum-supplemented medium than serum-free medium (Fig. 2A, B). This may have been due partly to cell proliferation because dividing cells were often observed in serum-supplemented medium. However, the migration rate of TIG-3 cells did not change when serum was removed from the culture medium (Fig. 1). These results are consistent with our previous study, i.e., a loss of cell division potential, which was induced by exposure to ^{60}Co-γ-rays, did not change the migration rate of TIG-1 cells [9].

It has been reported that monensin inhibits the secretion of procollagen and fibronectin from cultured human fibroblasts but does not inhibit protein synthesis [17, 18]. Also, since monensin inhibits the spreading of human fibroblasts [19–21], we determined the effects of monensin on cell migration using our present experimental system. Monensin inhibited the migration of TIG-3 and TIG-3S cells according to its conecentration (Fig. 5). Immunofluorescence staining for extracellular fibronectin revealed that monensin strongly inhibited the secretion of fibronectin from TIG-3 (Fig. 6) and TIG-3S cells (data not shown). This implies that monensin inhibits the secretion of secretory proteins including fibronectin. The finding that a relatively low concentration of monensin produced an effect is compatible with the action of monensin on cell attachment and spreading [17, 18]. The monensin concentration effective for inhibition of cell migration was the same in both TIG-3 and TIG-3S cells. However, the migration of TIG-3S did not decrease to the same extent as that of TIG-3 cells even when 1×10^{-6} M monensin was used. In other words, the difference in migratory ability between TIG-3 and TIG-3S cells could not be explained by the quantitative and qualitative differences in extracellular matrix secreted from the two cell types. Rather, it may reflect differences in the cell-specific events necessary for cell migration.

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