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Original Article

Comparative Study on Motility of the Cultured Fetal and Neonatal Dermal Fibroblasts in Extracellular Matrix

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One of the differences between fetal and adult skin healing is the ability of fetal wounds heal without contraction and scar formation.

Extracellular matrix (ECM) provides a substratum for cells adhesion, migration, and proliferation and can directly influence the form and function of cells.

As motility is essential for many important biological events, including wound healing, inflammatory response, embryonic development, and tumor metastasis, this study was designed to compare the motilities cultured dermal fetal and neonatal fibroblasts in the extracellular matrix.

The motility of cultured fetal and neonatal fibroblasts was compared using a video-microscopy system that was developed in combination with a self-designed CO_2 mini-incubator. To determine migration speed, cells were viewed with a 4× phase-contrast lens and video recorded. Images were captured using a color CCD camera and saved in 8-bit full-color mode.

We found that cultured fetal fibroblasts move faster than neonatal fibroblast on type I collagen (fetal fibroblast, 15.1 μm/ hr; neonatal fibroblast, 13.7 μm/hr), and in fibronectin (fetal fibroblast, 13.2 μm/hr; neonatal fibroblast, 13.0 μm/hr) and hyaluronic acid (fetal fibroblast, 11 μm/hr; neonatal fibroblast, 9.8 μm/hr).

Key Words: Fetal fibroblast, neonatal fibroblast, migration, extracellular matrix, collagen, fibronectin, hyaluronic acid, video-microscopy system

INTRODUCTION

Advances in the diagnostic tools and the techniques of open fetal surgery for some life threatening congenital anomalies have provided us with an unique opportunity to examine fetal surgical wound healing, which occurs rapidly without scarring.

The different healing responses of fetal and adult tissues could be due to factors caused by different biologic conditions and locations in vivo. In addition, different intrinsic properties of the constituent cells of these tissues may contribute to their different healing abilities. Fibroblasts imbedded in the amorphous healing tissue matrix migrate into damaged sites during the wound healing process that precedes the formation of new connective tissue after tissue injury. The cell motility involved in this migration is strongly influenced by cellular adhesion to proteins of the extracellular matrix (ECM).

Many modern techniques have been used to analyze the roles of different fetal environments, growth factors, cellular regulations, and ECM remodeling, to elucidate the mechanisms of scarless fetal wound healing, but the exact mechanism of fetal scarless healing remain unveiled.

The mechanism of fetal wound healing may provide a blueprint for an ideal tissue repair system in almost every medical specialty such as keloid, scleroderma, gastrointestinal adhesion and stricture, liver cirrhosis, pulmonary fibrosis, rheumatic heart disease, and retrolental fibroplasias.

So we compared the motility of cultured fetal and neonatal fibroblasts on coated with type I collagen, and in culture media with fibronectin (Fn) and hyaluronic acid (HA) using a video-microscope system to examine the unveiling the mechanism of scarless fetal wound healing.

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MATERIALS AND METHODS

Materials

Reagents including fibronectin (Fn) from bovine plasma and hyaluronic acid (HA) for migration assay were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Type I collagen, atelocollagen from calf skin, was purchased from RegenMed Inc. (Seoul, Korea).

The inverted microscope (model IX70) was manufactured by Olympus Optical Co., Ltd. (Tokyo, Japan) and the color charge-coupled device (CCD) camera and the video recording system with a video-tape recorder (VTR) and a color line monitor was purchased from Techsan Medical Co., Ltd. (Seoul, Korea).

Cells & cell cultures

In order to evaluate cell migration, two kinds of human dermal fibroblast cells derived from a fetus and a neonate were used. Dermal fibroblast cells were obtained from a fetus, which was aborted at 28 weeks gestation and a neonate, and outgrown cells were taken from primary culture following established methods. The cells were cultured in a T-75 flask (Nalge Nunc International Corp., Naperville, IL, USA) using Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, and 10% fetal bovine serum (FBS) at 37℃ in a humidified atmosphere (5% CO_2). Cells from passages 2 to 5 were used for the experiments.

Video-microscopy system and image processing software

For tracking and examining single cell migration, a video-microscope system was developed in combination with a self-designed CO_2 mini-incubator. This system consists of an inverted phase-contrast microscope attached to a color CCD camera, a computer system equipped with a frame grabber card, a VTR, software for image processing, and a temperature controller connected to CO_2 mini-incubator and multi-tester monitoring the temperature in the CO_2 mini-incubator.

Software for image processing was designed using the MATLAB V5.3 (The Mathwork Inc., Natick, MA, USA) and Visual BASIC V6.0 languages (Microsoft, Seattle, WA, USA), and tested on a personal computer.

For measuring migration speed, the cells were viewed with a 4× phase-contrast lens and video recordings were made. Images were captured using a color CCD camera and saved in 8-bit full-color mode.

The procedure for image processing was performed as follows (Fig. 1): Captured color images were incorporated into image analysis software,

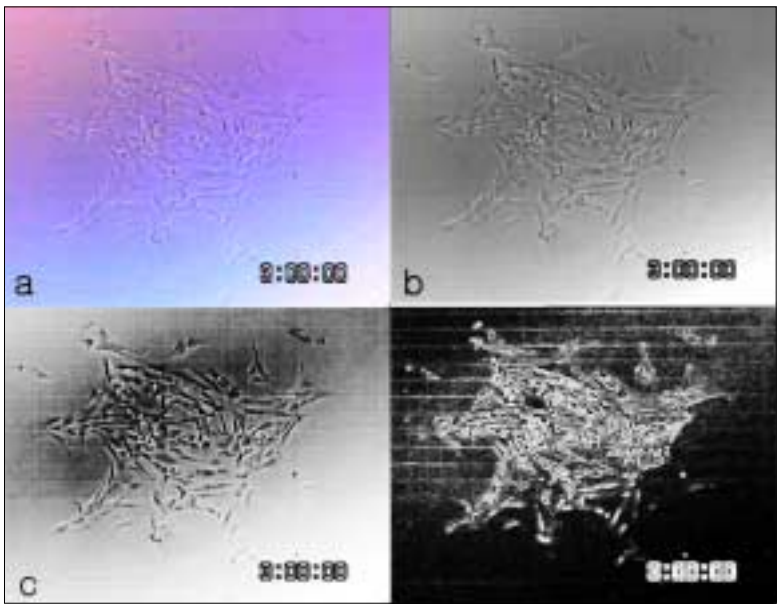

Fig. 1. The procedure used for image-processing of human fetal fibroblasts. Captured color images were incorporated into image analysis software, which was self-designed in our laboratory, and were transformed into gray scale images. To enhance the morphology of the cells, histogram equalization can be applied. From image of histogram equalization, the edge point of each cell was detected by method of edge detection. After edge detection, an appropriate cell was selected for centering, boundary setting, and tracking of each cell. (a) original image, (b) gray scale image, (c) histogram equalization image, (d) edge detection image.

which was self-designed in our laboratory, and were then transformed into gray scale images. Histogram equalization can be applied to enhance the cell morphology. The edge point of each cell was detected by edge detection, and an appropriate cell was selected for centering, boundary setting, and tracking of each cell.

Cell migration assays

Cell migration on type I collagen

To evaluate the migration of fibroblast cells on type I collagen, a glass surface was coated with solution of 10 μg/ml type I collagen in 0.01% acetic acid. The solution was sprayed three times on the surface of glass and dried for 1hr at 37℃. After washing with PBS, the glass surface was blocked with 2% heat-treated bovine serum albumin (BSA) solution, sterile filtered and heat inactivated for 20 min at 60℃, in PBS for 1hr, and finally rinsed twice in PBS.

Silicon rubber (width 0.5 mm) with a hole of 380 μm in diameter, was placed on the glass surface coated with type I collagen, and cells were plated into the hole of silicon rubber at low density (below 2×10^2 cells). Plated cells were cultured at 37℃ in a humidified atmosphere (5% CO_2) for 30 min. Silicon rubber was cautiously removed from the glass surface, and the attached cells (as shown in Fig. 2) were cultured in DMEM on a chambered cover-glass slide in a self-designed CO_2 mini-incubator for 12 hrs, and were observed with video-microscopy.

Cell migration in fibronectin and hyaluronic acid

In order to evaluate the migration of fibroblast cells by Fn and HA, plating of the cells was performed as described above and attached cells were cultured in DMEM media with 10 μg/ml of Fn and 0.1% of HA, respectively, for 12 hrs.

RESULTS

In this study, we have established a computer-aided time-lapse video-microscopy system, which can yield dynamic tracking and rapid examination of single cell migration, which has various advantages that make possible direct observation of whole cell morphology and cell position at first sight, migration patterns such as vector or direction also make possible detection once, for plating of cells as Fig. 2.

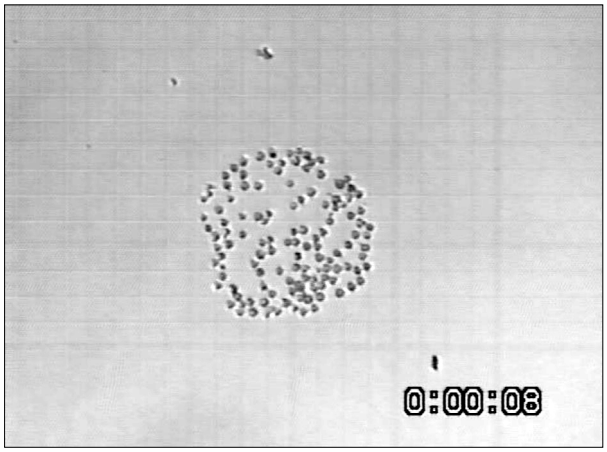

Fig. 2. The morphology of plated human fetal dermal fibroblast cells after 30 min. Cells plating was performed as follow: Silicon rubber (width 0.5 mm) with a hole, 380 μm in diameter, was placed on the surface of glass coated with type I collagen, and cells were plated at a hole in silicon rubber at low density (below 2×10^2 cells). Plated cells were cultured at 37℃ in a humidified atmosphere (5% CO_2) for 30min. After 30min, cautiously removed silicon rubber from the surface of glass, and attached cells were cultured in DMEM at chambered cover-glass slide in a self-designed CO_2 mini-incubator placed on the stage of inverted microscope for 12 hrs.

The migration of human dermal fibroblast cells isolated from fetus and neonate biopsies was evaluated in ECM such as type I collagen, Fn and HA by means of established systems, and the results from this study were compared in order to analyze differences in migration speed between fetal and neonatal fibroblasts.

Fig. 3 shows the morphology of the migrated fetal fibroblast cells versus the time on the surface of glass coated with type I collagen. The migration speed of fetal fibroblast cells was gradually increased from 4 hrs and the migration distance was also extended depending on time.

The average speed of fetal and neonatal fibroblast cells on the glass surface coated with type I collagen was faster than control (fetus, 13.6 μm/hr; neonate, 13.3 μm/hr), non-coated glass. Fetal fibroblast cells (15.1 μm/hr) moved faster than neonatal fibroblast cells (13.7 μm/hr) as shown in Fig. 4.

The migration of fetal and neonatal fibroblast

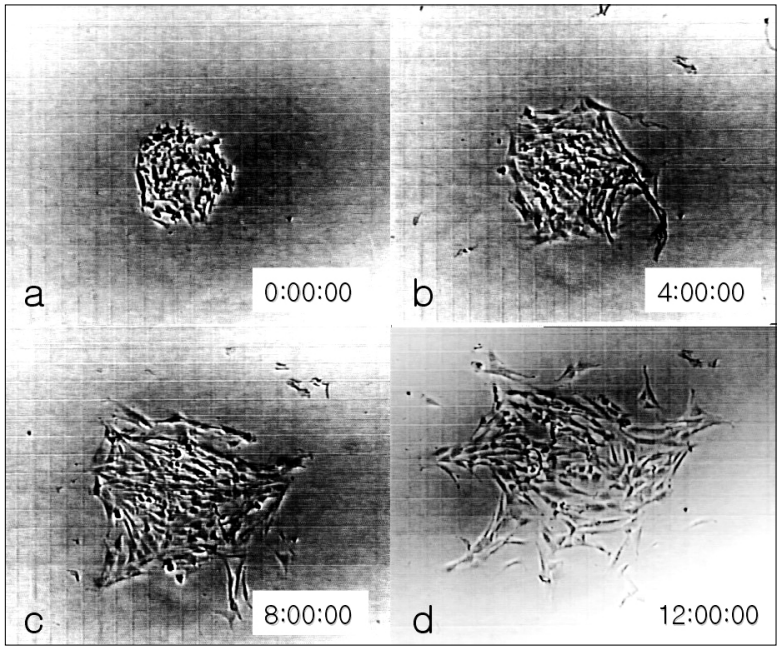

Fig. 3. The morphology of migrated fetal fibroblast cells versus time on the surface of glass coated with type I collagen. This pictures were histogram equalization images. (a) 0hr, (b) 4hrs, (c) 8hrs, (d) 12hrs.

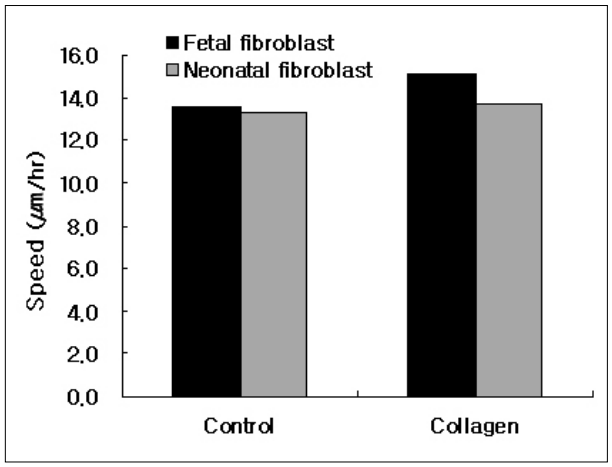

Fig. 4. Migration speed of fetal and neonatal fibroblast cells on type I collagen in 0.01% acetic acid.

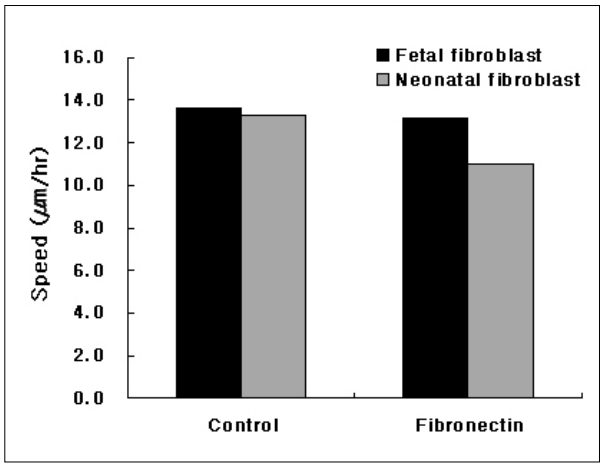

Fig. 5. Migration speed of fetal and neonatal fibroblast cells in 10 μg/ml of fibronectin (Fn).

cells in culture media with each 10 μg/ml of Fn and 0.1% of HA, respectively, was also evaluated by established system. The migration speed of fetal and neonatal fibroblast cells in culture media with Fn is shown in Fig. 5. The migration speed (fetus, 13.2 μm/hr; neonate, 13.0 μm/hr) of fetal and neonatal fibroblast was slower than that of control. The migration speed (fetus, 11 μm/hr; neonate, 9.8 μm/hr) of fetal and neonatal fibroblast in HA culture was also slower than that of control (Fig. 6).

In culture media with Fn and HA the migration speed was slower than control, and the migration speed of fetal fibroblast cells was slower than that of neonate.

In conclusion, this study confirmed that the migration speed of fetal and neonatal fibroblast cells on the surface of glass coated with type I collagen was faster than control and in culture media with Fn and HA, and fetal fibroblast cells were moved faster than neonatal fibroblast.

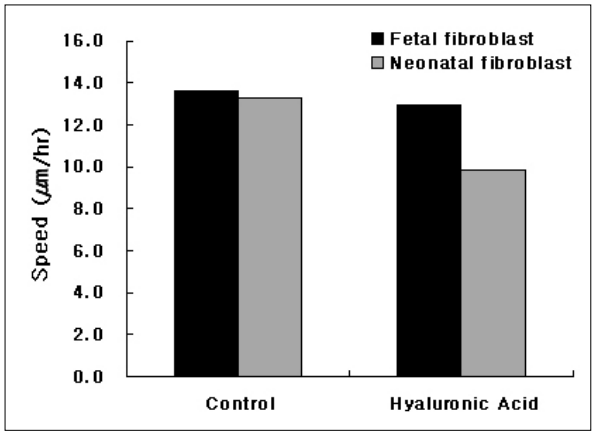

Fig. 6. Migration speed of fetal and neonatal fibroblast cells in 0.1% hyaluronic acid (HA).

DISCUSSION

ECM is secreted locally and is assembled into a network in the spaces surrounding cells. It forms a significant proportion of the volume of any tissue and is composed extracellular macromolecules. ECM provides a substratum for cells to adhere, migrate, and proliferate and can directly influence the form and function of cells.

The extracellular macromolecules of the ECM are comprised of a wide variety of different species, which contain distinct domains with, defined structural and biological activities. Cells are known to interact with these molecules via specific receptors. Following activation, these receptors transduce signals either directly to the intracellular cytoskeleton or via different signaling cascade. Cell to ECM interactions not only control the shape and orientation of cells but also directly regulate cellular functions, including migration, differentiation, and proliferation, as well as expression of different genes.

As collagen is the most common protein in the animal world, providing the extracellular frame work of all multicellular organisms and early ECM is rich in HA and Fn, which facilitate cellular migration,[1,2] We select these three ECMs to compare the motility of fetal and neonatal fibroblasts.

Collagen is particular interesting in terms of fetal wound healing because the quantity and pattern of deposited collagen dictates scar formation. Fetal animal wounds demonstrate more orderly and rapid collagen deposition than adult wounds, with reticular patterns which are indistinguishable from the surrounding intact skin.[3]

Fn is more abundant in fetal than in neonatal or adult skin, and this may create a more favorable environment for cell migration and proliferation in fetal wounds and regulate fibroblast migration by integrating the process of membrane extension, attachment, and detachment, with each of these processes being rate-limiting for locomotion in sequential regimes of increasing adhesivity.[4]

But the adhesiveness of fibroblasts to the ECM can be changed by seeding time, confluency, dimension of culture, phenotype of the fibroblasts, and the concentration of each ECM.[5-9] Therefore, to objectively evaluate the motility of cells, the correct seeding time, cell generation, and adequate ECM concentration were considered.

The presence of HA seems to favor scarless healing, whereas HA degradation products promote scar formation. Even in children and adults, HA concentration in the early wound fluid peaks and decrease significantly 3 to 24 hours after surgery, but the fetal wound maintains higher HA concentrations than the adult wound because of the hyaluronic acid stimulating activity (HASA), as HA is abundant in amniotic fluid and urine.[10-12]

HA is maintained at a significant higher level in incisional wound than excisional wounds 3 days after injury. HA receptor expression increased in fetal excisional wounds and is correlated with fibroplasia and a reduced HA contents, Moreover, HA is not the sole species for accelerating the velocity of some cells, such as chondrocytes but the cell motility is accelerated by simultaneously treating bFGF. Nevertheless, the velocity of synovial cells migration was increased by HA, but not by bFGF.[13,14]

As the reaction between cells and the ECM is complex, it is difficult to determine the reason why the motility of fetal and neonatal fibroblasts in Fn and HA are higher than in control media. However, we presume that Fn and HA show only increased adhesiveness and not cytoskeletal contraction nor releasing of the tail of lamellipodia of fibroblasts and variant reaction by different concentration.

Phenotypic differences may represent potentially important ways of explaining the mechanistic differences of motility in the ECM observed in the present study. The activity of fibroblasts depends in various ways upon the presence of ECM molecules, which modulate fibroblasts activity by growth factors and cytokines, as well as by the mechanical load on the cells which are embedded in the matrix, and upon the metabolism via certain integrin receptors.[15] Fibroblast cells form hypertrophic scars or keloid produces more Fn/total protein over time than the normal fibroblast cells. Oral mucosal fibroblasts exhibit phenotypic differences in the production of matrix metalloproteinase-2 and tissue inhibitors of metalloproteinase, which may contribute directly to the observed preferential remodeling of an oral wound.[16,17]

Fibroblast phenotype expression in the fetal lamb demonstrated that the onset of the expression of alpha smooth muscle actin (ASMA) coincides with the onset of scar formation, and that it produces higher collagen protein and mRNA than most types of collagen (particular type III). Difference in the alpha-SM actin and integrin expression and express much greater prolyl hydroxylase, which is controlled by poly-adenosine-diphosphate-ribose, an enzyme that controls the rate limiting step in collagen production, than adult cells patterns represent potent important mechanism used by fetal fibroblast to regulate their response to cytokines, and likely contribute to the resultant different in the quality of wound repair.[2,18,19] Therefore, we can postulate that factors that effect the motilities of fetal and neonatal fibroblasts stem from the different cellular phenotypes.

Measurements of the expression of the integrin subunit by many modern flow techniques, has reveled different integrin subunit expression in fibroblasts of different origins and their attachment and adherence to extracellular matrix, but advancing age, increased stress, gravity, and seeding times are associated with the phenotypic expression of collagen, Fn, and integrin is capable of changing the regulatory signals to the fibroblasts.[20-30]

Motility is essential for many important biological events, including wound healing, inflammatory response, embryonic development, and tumor metastasis. The process of cell migration can be thought of as a spatially and temporally coordinated process of events that includes lamellipodial extension, the formation of lamellipod-substratum attachments, cytoskeleton contraction, and the release of cell-substratum attachment at the rear of the cell. The cycle of cell movement can be divided into five steps: extension of the leading edge, adhesion to matrix contacts, contraction of the cytoplasm, release from contact sites and recycling of membrane receptors from the rear to the front of the cell. The ability of a cell to migrate is dependent on the cooperative effects of many intracellular signaling events triggered via cell surface receptor (integrins), internal cellular mechanics, and the extracellular matrix. Many of the proteins involved in cell migration remains to be elucidated.[31-34]

Cell migration is regulated simultaneously by growth factor and the ECM. And, the migration speed of cells is determined by the adhesive interactions between a cell and its environment, maximum cell speed occurs at intermediate degrees of adhesiveness.[35,36]

Under many circumstances, cell migration speed is limited by the rate of cell-substratum detachment at the rear of the cell. During rapid detachment cells leave little integrin on the substratum, whereas during slow detachment a large fraction in integrin rips from the membrane. Leukocytes or keratinocytes are able to detach easily and move very quickly while other cell types, such as fibroblast, tend to migrate more slowly and release many more integrins during detachments, Moreover, ECM binding to integrins in the front of lamellipodia causes those integrins to attach to the rearward-moving cytoskeleton. Integrin-cytoskeleton attachments in the front are strengthened as a result of ECM rigidity, enabling the cell to pull itself forward. The reduced contact area at the rear compared with that at the lamellipodium concentrates the traction forces in the rear on fewer integrin-ECM bonds, facilitating release. In such model, cell pathfinding and motility can be influenced by ECM rigidity.[37]

The integrins of cells and ECM relationships can be modified by many methods such as monoclonal antibodys (mAb) to talin, vinculin, alpha-

actinin, exogenous tropomodulin, dexamethasone, PGE2, and even cigarette smoking can modify the proliferation and migration of fibroblasts.[38-41]

Several methods are currently used for in vitro studies upon cell migration. These may be divided into two principally different experimental approaches: the cell-population assay, and the individual-cell assays. Most published cell migration studies are based on cell-population assays, since individual-cell assays generally are more laborious and require more sophisticated and expensive equipment. However, the revolution in computer technology and advances in microscope technology within the last decade have now made large-scale individual-cell assays a realistic task. Individual-cell assays, based on the tracking of individual cells, have typically used an automated image approach and to track the movement of single cells.[41-44]

In this study, we established a computer-aided time-lapse video-microscopy system, which can yield dynamic measurements of cell position and morphology, for rapid and precise analysis of single cell migration, and also have developed a new method of plating cells for cell migration assay. This system provides tools for the analysis of migration and the velocities of cells under various conditions.

CONCLUSION

The motility of fetal and neonatal dermal fibroblast cells was evaluated using a computer-aided time-lapse video-microscopy system. Cultured fetal fibroblast cells moved faster than neonatal fibroblast cells on the surface of glass coated with collagen type I (fetus, 15.1 μm/hr; neonate, 13.7 μm/hr), and in culture media with Fn (fetus, 13.2 μm/hr; neonate, 13.0 μm/hr) and HA (fetus, 11 μm/hr; neonate, 9.8 μm/hr).

We believe that the motility of cells can be evaluated using this video-microscopic system.

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