Original article

Development of Dynamic Cell Adhesion Assay under Rotary Shaking

Jun KOBAYASHI[1,2], Hiroshi TERADA[2], Keiichi IKEDA[3] and Hideo SUGIYAMA[4]

[1] School of Veterinary Nursing and Technology, Faculty of Veterinary Medicine, Nippon Veterinary and Life Science University, Musashino, Tokyo, Japan
[2] Department of Environmental Health, National Institute of Public Health, Wako, Saitama, Japan
[3] Department of Health Science, Juntendo University School of Health and Sports Sciences, Inzai, Chiba, Japan
[4] Department of Health and Dietetics, Faculty of Health and Medical Science, Teikyo Heisei University, Toshima-ku, Tokyo, Japan

Abstract

The purpose of this study was to develop a method for determining the adhesive ability of cultured cells. The method differs from the conventional methods, and considers the blood stream on the cell adhesion. Also, to keep the cell suspension to be uniform, the procedure was conducted by rotary shaking (120 rpm) the 1×10^6 cultured cells/10 mL in a 10 cm diameter laboratory dish at 37℃ using a bio-shaker. Measurements of the number of cells remaining in the supernatant were performed over time by using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) method. The adhesive ability of the cells was determined as the time when 50% of the cells had been floating in the supernatant. Four cell lines (8505C, human thyroid carcinoma ; 8305C, human thyroid carcinoma ; A431, human epidermoid carcinoma ; CHO-K1, Chinese hamster ovary) were distinguishable for the adhesive ability (113.1, 131.6, 33.2 and 221.1 min, respectively). For example, by trypsin treatment to harvest nearly all the cells from the laboratory dish bottoms, general reaction times (8505C, 3 min ; 8305C, 1.5 min ; A431, 7 min ; CHO-K1, 1.5 min) are reverse order above. These results do not contradict the conventional methods. This method is simple and does not need skilled techniques unlike the conventional methods.

Key words : Adhesion assay, Cultured cell, Rotary shaking

Bull. Nippon Vet. Life Sci. Univ., **60**, 67–72, 2011.

Introduction

Adhesion to extracellular matrices, such as collagen and fibronectin, is indispensable in the growth, migration and proliferation of almost cells[1–3]. Especially in solid cancer cells, multiplication, permeation and transition occur only after adhesion. In recent years, various studies have been conducted with regards to cancer prevention and cures using cultured cells[4]. It is thought that the treatment of cancer with medicines is possible by reducing or intercepting adhesion functions at the cell level. The development of artificial matrices, such as peptides, for antineoplastic drugs has been performed and screened using cultured cells[5,6]. Experimental methods have been developed to evaluate adhesion, migration and proliferation among different cells *in vitro* using cultured cells[7]. By these methods, attempts have been made to predict the permeability and transition of cancer cells *in vivo*, and determine the effect of previous artificial matrices. However, most of them require skilled techniques to obtain precise data.

With the conventional methods[8–13], cells are scattered on plural culture plates or laboratory dishes at once,

and then left statically in a CO_2 incubator. The supernatants including the non-adhering cells are then removed from separate containers at each time interval, and the attaching cell concentrations are measured using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) or other methods, or by dyeing with trypan blue after removal with trypsin treatment. With these methods, the washing stages require skill and if they are not completed thoroughly the data will be faulty. Also, it is necessary to take the cells in and out of the incubator many times. Therefore, data variation occurs as a result of changes in incubation time and temperature ; only experimental data measured simultaneously can be comparable.

When the cancer cells are carried to other organs by blood flows, the dynamic adhesion has occurred in the blood stream. To reproduce the state that the cells were thrown into culture medium, rotary shaking was used to be done while adhesion in the present study. It is a special point of this study different from the conventional methods. To accurately measure differences of adhesion abilities among cells in a prepared suspension, the ability was taken as the time when the supernatant cell density had reached half that what it was at the start. Moreover, we tried to measure the adhesion abilities of some cultured cells and both accuracy and reproducibility were compared with a conventional method.

Materials and Methods

Instruments and Equipment

A bio-shaker (BR-15 ; TAITEC, Tokyo, Japan ; shaking width, 4 cm ; speed, 0-210 min^{-1}) was used to shake the cell suspensions. Laboratory dishes (Iwaki, Tokyo, Japan ; 10 cm ϕ) were used to directly contain the cell suspensions. All other instruments were used after autoclave sterilization processing.

Reagents

The MTT was purchased from Dojindo Laboratories (Kumamoto, Japan)[14-16], and special grade dimethyl sulfoxide (DMSO) was purchased from Wako (Tokyo, Japan). RPMI-1640 medium supplemented with 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (Sigma, St. Louis, MO ; filter-sterilized ; pH 7.4 ; abbreviated to "Modified RPMI"), and 100 μg/ml streptomycin and 100 U/ml penicillin were used as the adhesion medium. All other reagents used were commercial biochemical grade.

Cultured Cells

Four adhesive cell lines, Chinese hamster ovary cells (CHO-K1), human epidermoid carcinoma cells (A431), and two human thyroid carcinoma cell lines (8505C and 8305C) were used. The cells were subcultured in medium (Ham's F-12, MEM, and RPMI-1640, respectively), which were considered optimal in the presence of 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). To optimize the MTT method, human stomach carcinoma cells (KATO-III), which are floating cells, were also cultured under optimal conditions (data not shown) and evaluated separately.

Procedures

After culturing the adhesive cells in a CO_2 incubator, they were washed with PBS and then removed from the laboratory dishes by acting 0.25% trypsin in 1 mM EDTA. After suspending the samples in PBS, centrifugation was carried out at 800 rpm for 5 min. The supernatant was then removed and once again suspended in Modified RPMI at 1×10^5 cells/ml. Rotary shaking (120 rpm) was immediately begun on 10 ml suspension samples at 37℃ using the bio-shaker (an air-bath equipped with a shaking and thermostat device, but no CO_2 supply). On every 15 min, 0.5 ml of supernatant was removed and transferred to 1.5 ml microtubes. To measure cell density, 50 μl of MTT reagent (0.4 mg/ml) and 25 μl of FBS were added to the samples and left at 37℃ for 2 h. Centrifugation was then carried out at 15,000 rpm for 10 min. Then the supernatant was immediately removed, 250 μl of DMSO was added and the MTT cell metabolites were dissolved into it. Absorbance at 550 nm was measured with a plate reader using 200 μl of solution[15,16]. In each experiment, time zero (when the cell suspension was first added) and time infinity (when the cells were excluded from the suspension ; equal to the medium only) were measured simultaneously, and then a calibration curve of the two points was created. The adhesive abilities of each cell type were computed as the time when 50% of the cells had been floating in the supernatant.

Results

Although the adhesive speed of the cells increased with slow shaking, a flocculation phenomenon was also observed at a speed of less than 50 rpm. At below 75 rpm cell diffusion is thought to become uneven thus causing data variation and making comparisons among cell types difficult. On the other hand, if shaking is too quick, adhesion takes too long or the cell suspension escapes from the container. Considering these results, a speed of 120 rpm was chosen.

Firstly, the conditions of the MTT method were

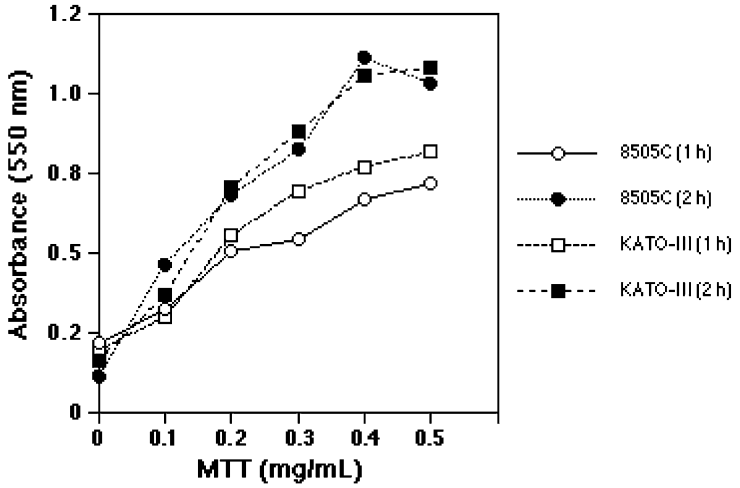

Fig. 1. Effect of MTT Concentration on Absorbance
Each 500 μl sample contained 1×10^5 8505C or KATO-III cells/ml.
After adding 50 μl of MTT and 25 μl of FBS, the samples were left at 37℃.
Centrifugation of the MTT reactant was then carried out at 15,000 rpm and the residue was dissolved in 250 μl of DMSO.
Absorbance was measured at 550 nm using 200 μl of solution and a plate reader.
Reaction time is shown on the right side.

optimized using the KATO-III and 8505C cell lines. By examining the sensitivity of the cell counts, the concentration of MTT and reaction time chosen were 0.4 mg/ml and 2 hr, respectively, because the reaction reached nearly a plateau (Fig. 1).

The cell density used for the experiments was set at 10^5 cells/ml as this matched the conditions of the MTT method measurements, and because linearity was almost always acquired between the number of cells used and absorbance at 10^4–10^6 cells/ml (Fig. 2). Although the results showed that the linearity of the calibration curve was good, absorbance varied in the between-run assay. In each experiment, the calibration curve was drawn.

For the adhesion process, FBS was not added into the medium but needed for the MTT method. Furthermore, when a mixture of FBS and MTT is left longer, it proceeds to deteriorate more in response to the influence of light or temperature (data not shown). Therefore, FBS and the MTT reagent were added separately. A concentration of 5% was chosen after testing FBS concentrations ranging from 0–10%.

Usually culture plates and laboratory dishes are used as containers. It is easy to vary the amount of supernatant removed and to mistake when removing the reaction product from the containers, absorbance was varied. In this method, the sample was removed from a 10 cm laboratory dish and was transferred to a 1.5 ml microtube. Consequently, high-speed centrifugation at 15,000 rpm could be conducted, the unreacted reagent could be partitioned simply, and reproducibility improved. The calibration curve created using the above conditions showed r = 0.995 or more (Fig. 2).

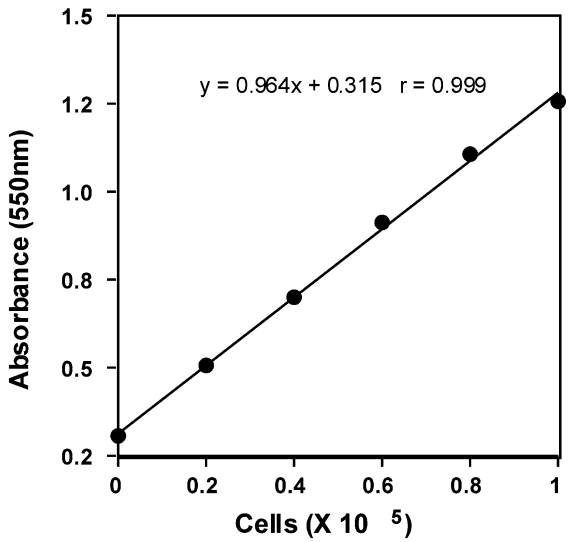

Fig. 2. Calibration Curve of the 8505C Cells
Each 500 μl sample contained 0–1×10^5 8505C cells/ml.
The other procedures were conducted as indicated in the legend of Fig. 1.

The results of the adhesive ability of the four cell

lines are shown in Fig. 3 and Table 1.

Discussion

In this study, there is the possibility that a few cells are killed by trypsin treatment, and thus be included in the analysis irrespective of whether they have adhered or not. It is therefore better that these cells are neglected, and that the MTT method, which measures only living cells, is used in the present study.

Although unlike conventional methods this method does not need two or more sample containers, two or more samples were obtained from each container. Therefore, although there is a risk of environmental contamination (invasion of some bacteria from outside), the cells were only used once. Because the experiment was completed within the same day, the addi-

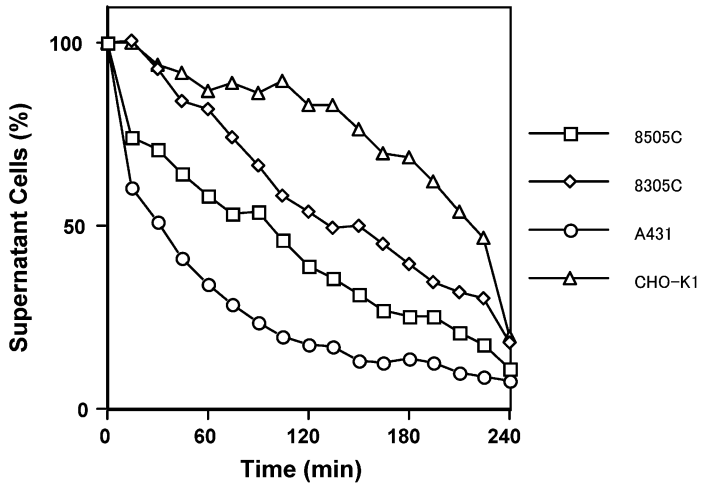

Fig. 3. Typical Cell Adhesion Profiles
Each cell type sample (10 ml×10^5 cells/ml) was added to a 10 cm in diameter dish and rotary shaken.
Every 15 min, 0.5 ml samples were transferred to a 1.5 ml microtube.
The cell density of each sample was measured by the MTT method and the time taken to reach the mean absorbance between the original cell suspension and medium was calculated.
The Y-axis shows the cell residue (%) in the supernatant when the time of an experiment start is made into 100%.
The other procedures were as in Fig. 1.
n＝3.

Table 1. Adhesive Ability of Several Cells

Cell	Origin	Adhesion ability ($T_{1/2}$)	CV (%)
within-run assay			
8505C	human thyroid carcinoma	113.1 ± 2.3	2.0
8305C	human thyroid carcinoma	131.6 ± 3.1	
A431	human epidermoid carcinoma	33.2 ± 5.6	
CHO-K1	Chinese hamster ovary	221.1 ± 6.2	
between-run assay			
8505C		105.5 ± 9.6	9.1

Each adhesive ability of cells was measured independently above the 3 experiments at one day (within-run assay) and during 3 days period.
The other conditions are as Fig. 3.
n = 3.
On the conventional methods, the CVs of within-run assays become nearly 10%, and the ones of between-run assays aren't computable (too bad).

tion of antibiotics was only necessary.

We think that the strength of adhesive ability was related to those cells matter easily stripped from the dishes. In cell culture, to harvest nearly all the cells from the laboratory dish bottoms, general reaction time of trypsin was as follows : CHO-K1, 1.5 min ; A431, 7 min ; 8505C, 3 min ; 8305C, 1.5 min[17]. Four cultured cells adhesion ability obtained by the present study do not contradict previous knowledge. When this method was applied to the CHO-K1 cells that integrin expression plasmids (mock, wild and mutant types) had transfected stably, and the order of their adhesion abilities was wild＞mutant＞mock types by using with a conventional method (the remaining adhered cells were detected by the MTT method). The order of ability of the three transformed CHO-K1 cells was corresponding (data not shown).

Other groups have reported various adhesive methods using more sensitive methods such as a peroxidase fluorescence[9,10,13], autoradiography[11] and flow cytometry[8,12]. So the kinds of detectable cells used are restricted in many cases, these methods do not improve adhesion like that presented here. In the future, we would compare this method with other adhesive methods and reveal data concerning the adhesive ability of other cells. Also, we would consider the influence of medicines. Moreover, it seems that the higher reproducibility can be obtained if a peristaltic pump is used for consequent sampling instead of pipettes.

Acknowledgements

We would like to thank the Institute of Development, Aging and Cancer, Tohoku University, for supplying the cultured cells (8505C and 8305C).

References

1) Coraux, C., Delplanque, A., Hinnrasky, J., Peault, B., Puchelle, E. and Gaillard, D. (1998) Distribution of integrins during human fetal lung development. *J. Histochem. Cytochem.,* **46**, 803–810.
2) Germer, M., Kanse, S.M. , Kirkegaard, T., Kjoller, L., Felding-Habermann, B., Goodman, S. and Preissner, K.T. (1998) Kinetic analysis of integrin-dependent cell adhesion on vitronectin : the inhibitory potential of plasminogen activator inhibitor-1 and RGD peptides. *Eur. J. Biochem.,* **253**, 669–674.
3) Li, E., Brown, S.L., Stupack, D.G., Puente, X.S., Cheresh, D.A. and Nemerow, G.R. (2001) Integrin alpha(V)beta1 is an adenovirus coreceptor. *J. Virology,* **75,** 5405–5409.
4) Kawahara, E., Nakada, N., Hikichi, T., Kobayashi, J. and Nakanishi, I. (2002) EGF and beta1 integrin convergently regulate migration of A431 carcinoma cell through MAP kinase activation. *Exp. Cell Res.,* **272**, 84–91.
5) Retta, S.F., Balzac, F., Ferraris, P., Belkin, A.M., Fassler, R., Humphries, M.J., Leo, G.D., Silengo, L. and Tarone, G. (1998) Beta1-integrin cytoplasmic subdomains involved in dominant negative function. *Mol. Biol. Cell,* **9**, 715–731.
6) Retta, S.F., Cassara, G., D'Amato, M., Alessandro, R., Pellegrino, M., Degani, S., Leo, G.D., Silengo, L. and Tarone, G. (2001) Crosstalk between beta(1) and alpha(V) integrins : beta(1) affects beta(3) mRNA stability. *Mol. Biol. Cell,* **12**, 3126–3138.
7) Goto, M., Tsukahara, T., Sato, K., Konno, T., Ishihara, K., Sato, K. and Kitamori, T. (2007) Nanometer-scale patterned surfaces for control of cell adhesion. *Anal. Sci.,* **23**, 245–247.
8) Ostrowski, K., Watson, J.V., Barnard, P.J., Barnard, E.A., Thomas, K., Freedman, L. and de Stavola, B. (1986) The application of flow cytophotometry in measurements of cell adhesion. *Histochemistry,* **85**, 423–429.
9) Akeson, A.L. and Woods, C.W. (1993) A fluorometric assay for the quantitation of cell adherence to endothelial cells. *J. Immunol. Methods,* **163**, 181–185.
10) Goodwin, A.E. and Pauli, B.U. (1995) A new adhesion assay using buoyancy to remove non-adherent cells. *J. Immunol. Methods,* **187**, 213–219.
11) Dalton, B.A., Dziegielewski, M., Johnson, G., Underwood, P.A. and Steele, J.G. (1996) Measurement of cell adhesion and migration using phosphor-screen autoradiography. *BioTechniques,* **21**, 298–303.
12) Visser, M.J.T., van Lennep, A.C.D., van Bockel, J.H., van Hinsbergh, V.W.M., van der Keur, M. and Hermans, J. (1996) Evaluation of various methods to quantify endothelial cells attached to vascular prostheses : comparison with a new "gold standard" FACS method. *J. Surg. Res.,* **61**, 237–243.
13) Mendis, D., Ginon, I., Louis, H., McGregor, J.L. and Poston, R.N. (2001) Cell biotinylation provides a sensitive and effective detection technique for cellular adhesion assays : comparison with existing methods. *J. Immunol. Methods,* **253**, 57–68.
14) Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival : application to proliferation and cytotoxicity assays. *J. Immunol. Methods,* **65**, 55–63.
15) Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H. and Boyd, M.R. (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.,* **48**, 589–601.
16) Stevens, M.G. and Olsen., S.C. (1993) Comparative

analysis of using MTT and XTT in colorimetric assays for quantitating bovine neutrophil bactericidal activity. *J. Immunol. Methods,* **157**, 225–231.
17） Saito, A. and Kobayashi J. (2006) Our private data (not published).

回転振盪下での動的細胞接着能測定法の開発

小林 淳[1,2]・寺田 宙[2]・池田啓一[3]・杉山英男[4]

[1] 日本獣医生命科学大学獣医学部獣医保健看護学科
[2] 国立保健医療科学院生活環境研究部
[3] 順天堂大学スポーツ健康科学部健康学科
[4] 帝京平成大学健康メディカル学部健康栄養学科

要 約 本研究の目的は，培養細胞の接着能を測定する為の新しい方法を開発することである。血流に乗ってガン細胞は，他の臓器に転移することを考慮し，本研究では従来の静置条件ではなく，動的状態で細胞外マトリックスと細胞との接着を行わせるという新たな試みを行った。そのための装置として，1×10^6 個の培養細胞を含む 10 cm 径の細胞培養シャーレと 37℃ に加温した回転振盪機能付きのインキュベータを使用した。反応後，未接着の浮遊細胞を，MTT（3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide）法により検出した。接着能は，浮遊の細胞濃度が 1/2 になる時間として求めた。この方法を 4 種の細胞（8505C：ヒト甲状腺ガン細胞；8305C：ヒト甲状腺ガン細胞；CHO-K1：チャイニーズハムスター卵巣細胞；A431：ヒト上皮ガン細胞）に適用した結果，接着強度は数字的に区別され，結果が従来法と矛盾することもなかった。この方法は従来法と異なり，特別な手技を必要としないため，有用であると思われる。

キーワード：接着能測定法，培養細胞，回転振盪

日獣生大研報 **60**，67–72，2011.