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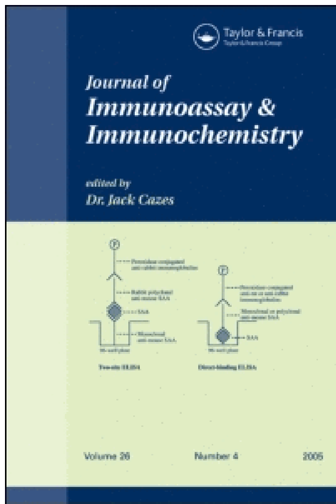

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Determination of Optimal Conditions for the Immobilization of Cells in a Cell Capture Enzyme Immunoassay (CC-EIA) by a Simple Giemsa Assay

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DETERMINATION OF OPTIMAL CONDITIONS FOR THE IMMOBILIZATION OF CELLS IN A CELL CAPTURE ENZYME IMMUNOASSAY (CC-EIA) BY A SIMPLE GIEMSA ASSAY

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ABSTRACT

To determine the optimal conditions for the immobilization of cells in a cell capture enzyme immunoassay (CC-EIA), the most suitable diluent, and the optimal pH, temperature and period of incubation were examined using WI-38, a human embryonic lung fibroblast cell line. For the evaluation, we devised a simple Giemsa assay method, in which immobilized cells on a microplate were stained with Giemsa solution, the stained dye was eluted with ethanol after washing the plate, and the optimal density (O.D.) was measured at wavelength 620 nm. The optimal conditions for the immobilization were determined to be treatment with 5% formalin in phosphate-buffered saline (PBS) (pH 7.2) for 15 minutes at room temperature, which were confirmed to be suitable for the measurement of cell associated collagen by CC-EIA. Additionally, we found that the simple Giemsa staining method was also useful for evaluating the number of immobilized cells on the microplate after CC-EIA. (KEY WORDS: Giemsa assay, chemical antigen immobilization, fibroblast, formalin, cell associated collagen, EIA)

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INTRODUCTION

Synthesis of collagen by various tissue fibroblasts plays a role in wound healing and also in the pathogenesis of many diseases, such as senile cataract (1,2), after cataract (3,4), keloids (5,6), liver cirrhosis (7,8) and Dupuytren's contracture (9). Regulation of the collagen synthesis is important in the treatment of these diseases. Recently, a few drugs have been reported to regulate collagen synthesis in vitro (10-13). To establish a new effective drug for such regulation, further in vitro screening of a variety of candidate chemicals is required.

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At present, immunohistochemical (14) and ^{3}H-proline uptake (15) methods are used for the measurement of collagen. The immunohistochemical methods allow qualitative, but not quantitative evaluation. The ^{3}H-proline uptake method allows quantitative measurements but involves the complicated process of extraction of collagen. Another available method for the measurement of collagen is enzyme immunoassay (EIA). Obata et al. (16) developed a simple sandwich EIA for the measurement of immunoreactive type IV collagen in human sera. The assay is quantitative and simple for the measurement of soluble, but not cell associated collagen. In order to measure cell associated collagen, the collagen should first be extracted and then measured by EIA.

Sirius red staining was reported as another method for the measurement of cell associated collagen. Lopez-De Leon & Rojkind (17) reported a quantitative morphometric measurement method for collagen, involving histochemical staining, applied to paraffin embedded sections of liver (18) and lung tissue (19). Jimenez et al. (18) developed a semiquantitative Sirius red staining method involving elution and measurement of the dye from units of tissue sections. The method is not simple when cell cultures are used.

For the screening of chemicals for the regulation of collagen synthesis, we desire a simple method, such as EIA, for the direct measurement of collagen in cultured cells. To measure the cell associated collagen in cultured cells, we consider that a cell capture enzyme immunoassay (CC-EIA) using a microplate is highly suitable. The target cell can be immobilized on the microplate by treatment with chemicals such as aldehydes. Since detachment of the immobilized cells during the process of CC-EIA could yield inaccurate results, proper immobilization of the cells is essential. In this study, we therefore determine the optimal conditions for whole cell capture on a microplate for CC-EIA, and also describe a method for the evaluation of the number of immobilized cells on the microplate after the immunoassay.

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MATERIALS AND METHODS

Cell line and culture

WI-38, a human embryonic lung fibroblast cell line was cultured in Eagle's minimum essential medium (E-MEM; Nissui, Japan) supplemented with 10% fetal bovine serum (FBS) at 37℃ for 5 days. The precultured cells were harvested by treatment with trypsin solutions. The cells were inoculated onto a 96 well microplate (MICROTEST Tissure Culture Plate, Flat Bottom, Falcon ,U.S.A.) at the designated cell densities in 100 μl culture medium and incubated overnight to allow attachment. For some experiments, the cells were cultured for five days on a microplate. The culture supernatant was removed by decantation to aid in cell immobilization. The cells were counted trypan blue dye exclusion method.

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Immobilization of cells

Formalin containing 37% formaldehyde and 8% methanol in distilled water was used as immobilization. To immobilize the cells on the microplate, 100 μl of 5-15% diluted formalin in distilled water and in 150 mM phosphate bufferd saline (PBS) containing 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 at various pHs were added to the microplate on ice, at room temperature and at 37℃.

Room temperature was controlled at approximately 22℃ for this experiment. Immobilization was allowed to occur for 5-60 min. The immobilized cells were then washed five times with 200 μl of 150 mM PBS (pH 7.2) prior to the evaluation of the number of cells immobilized on the microplate. All experiments were performed in triplicate.

Giemsa assay

The immobilized cells on the microplate were stained with 100 μl of Giemsa solution (Merck Japan, Tokyo, Japan) for 30 min at room temperature and washed with water. The residual dye was eluted in 100 μl of 70% ethanol, and the O.D. of the dye solution in ethanol was immediately measured with a microplate reader (NJ-2001, Japan InterMed, Japan) using 405, 450, 490, 540, 620 and 680 nm optical filters. In order to evaluate the amount of residual stain in the immobilized cells after intensive washing, 0-100,000 cells were immobilized and stained as described above, and the O.D.s were measured with the reader using a 620 nm optical filter. All experiments were performed in triplicate.

Cell capture enzyme immunoassay

The cells were cultured on a 96 well microplate at densities of 0-100,000 cells/well overnight at 37℃, and directly immobilized on the

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plate by treatment with 5% formalin at room temperature for 15 min. Collagen associated with the immobilized cells was measured by the following protocol. To avoid nonspecific reaction of the antibodies mentioned below, 300 μl of blocking solution containing 5% bovine serum albumin (BSA) in PBS was added to each well. After removal of BSA solution, Rabbit anti-collagen antibodies against collagen type I-V (Chemicon International Inc., California, U.S.A.) were mixed together and used as the first antibody in the immunoassay. Aliquots of 100 μl of the antibody solution were added to each well at a dilution of 1:50 and the microplate was incubated at 37℃ for 60 min. The wells were washed 5 times with 150 mM PBS (pH 7.2). The second blocking was performed with 300 μl/well of 20% FBS. After removal of FBS, alkaline phosphatase (ALP)-labeled goat anti-rabbit IgG (American Qualex Manufactures, California, U.S.A.) was added to the wells at dilutions of 1:500 and incubated at 37℃ for 60 min. Each well was washed again in the same manner as described before. Commercially available paranitrophenylphosphate solution (ALPopt, Roche Diagnostic, Switzerland) were used as substrate. To block the endogenous alkaline phosphatase, 0.2 mM levamisole at final concentration were added to the substrate solution. One hundred microliters of the substrate solution were added immediately after washing and incubated at 37℃ for 30 min. The O.D. of the color was immediately measured using a

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microplate reader (NJ-200, Japan InterMed, Tokyo, Japan) at the wavelength of 405 nm (O.D. $_{405}$). The O.D.$_{620}$ value of the background was subtracted from direct value of O.D.$_{405}$, and the amount of collagen was expressed as the direct O.D.$_{405\ 620}$ value. All experiments were performed in triplicate.

RESULTS

The O.D.s of the eluted dye from the Giemsa stained cells measured using each optical filter were examined. Since the major O.D. peak was observed at the wavelength of 620 nm, the number of immobilized cells was evaluated by measuring the O.D. value using the 620 nm optical filter (O.D.$_{620}$). The Giemsa assay provided visible semiquantitative results (Figure 1a) and allowed quantitative detection of 1,000 - 100,000 cells on a microplate (Figure 1b; R^2=0.997). In order to clarify whether the Giemsa assay can be used to evaluate the number of immobilized cells in CC-EIA, the amount of cell associated collagen and the number of immobilized cells were measured by EIA and Giemsa assay, respectively. The O.D.$_{405\text{-}620}$ in the CC-EIA correlated well with the O.D.$_{620}$ in the Giemsa assay (Figure 2; R^2=0.963).

Since EIA using a microplate requires washing of the microplate, the cells immobilized under various conditions were intensively

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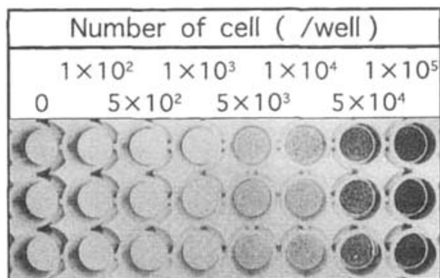

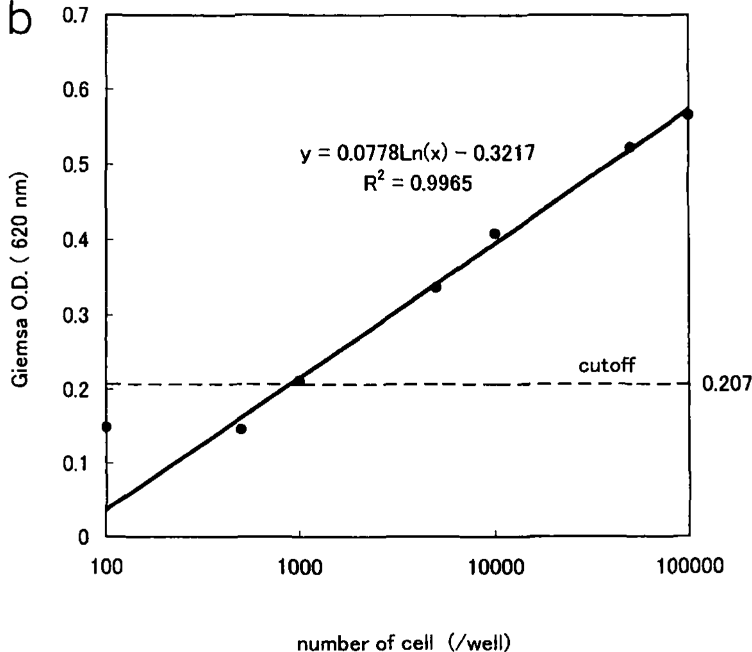

FIGURE 1. Giemsa stained microplate and correlation between the number of inoculated cells and the O.D. values. Panel A: Photograph of a Giemsa stained microplate. Zero to 100,000 cells/well were inoculated onto a microplate and incubated overnight to allow attachment. The cells were immobilized in the presence of 5% formalin solution in PBS at room temperature for 15 min, washed with water and stained with Giemsa solution. Panel B: Correlation between the number of inoculated cells and the O.D. values. The O.D. values were obtained from the microplate and plotted on a graph. Logarithmic correlation was noted in the plot with an R^2 value of 0.997.

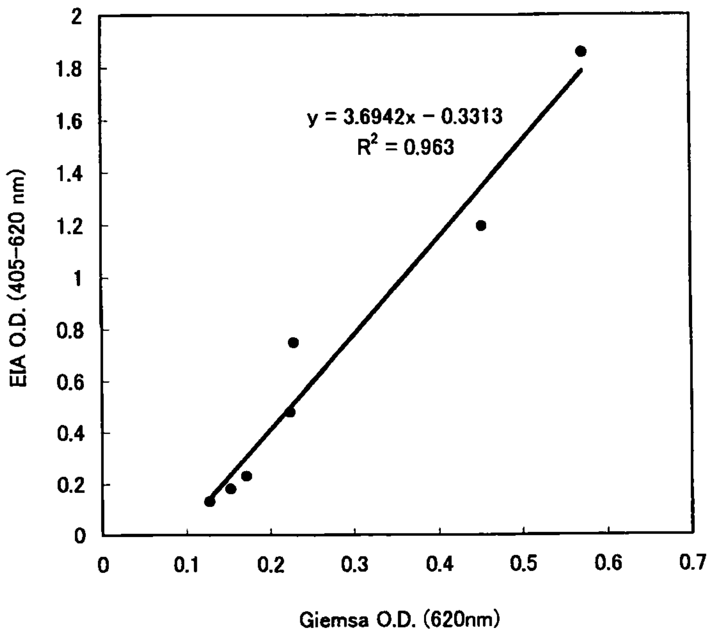

FIGURE 2. Correlation between Giemsa assay and EIA for collagen. The O.D. values determined from the Giemsa assay and EIA are linearly correlated with an R^2 value of 0.963.

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washed prior to the Giemsa assay. Immobilization of the cells in the presence of formalin solutions in distilled water resulted in a slight increase of $O.D._{620}$ in a time dependent manner (Figure 3a). Immobilization of the cells in the presence of formalin solution in PBS also increased the $O.D._{620}$ in a time dependent manner (Figure 3b). We found that higher concentrations of formalin in PBS resulted in a lower $O.D._{620}$ and that effective immobilization was obtained with treatment using 5% formalin solution in PBS for 15 min. In order to

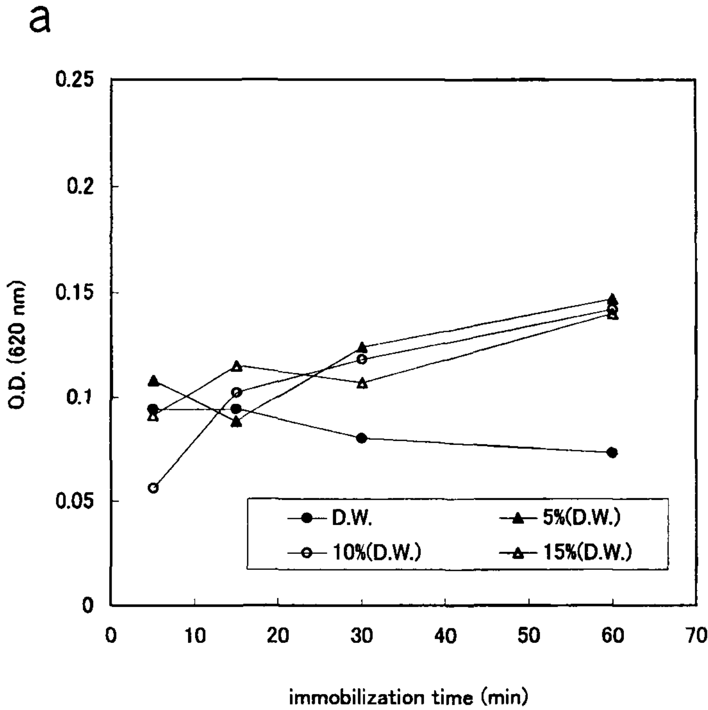

FIGURE 3. Residual cells immobilized on a microplate. WI-38 cells were cultured for five days and immobilized in the presence of various concentrations of formalin solutions in distilled water (a) and phosphate-buffered saline (b) at room temperature for the designated times. The cells were stained with Giemsa solution for 30 min and washed with water. The O.D. of the dye eluted from the stained cells was measured using a 620 nm optical filter, with a microplate reader.

determine the optimal pH of the PBS, cells were immobilized in the presence of 5% formalin in PBS at pHs of 5.9, 7.2, 8.0 and 9.2. The highest $O.D._{620}$ for Giemsa stained cells was obtained when the formalin solution in PBS with a pH of 7.2 was used. To determine the

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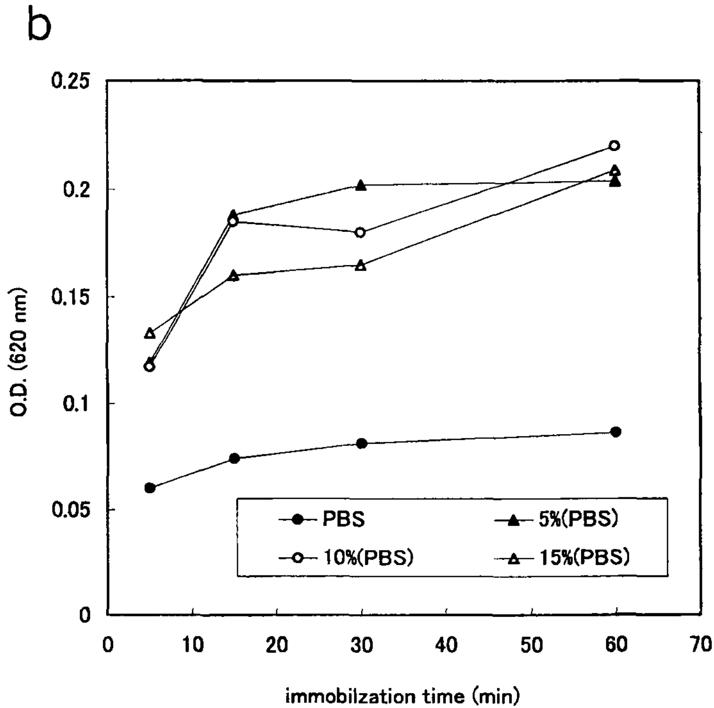

FIG. 3. continued

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optimal temperature for the chemical immobilization, the cells were immobilized in the presence of 5% formalin solution in PBS (pH7.2) on ice, at room temperature and at 37℃. The $O.D._{620}$ of the immobilized cells at room temperature was greater as compared with that of the cells immobilized on ice and at 37℃. We finally determined that the optimal conditions for the immobilization were treatment with 5% formalin in PBS (pH 7.2) at room temperature for 15 min.

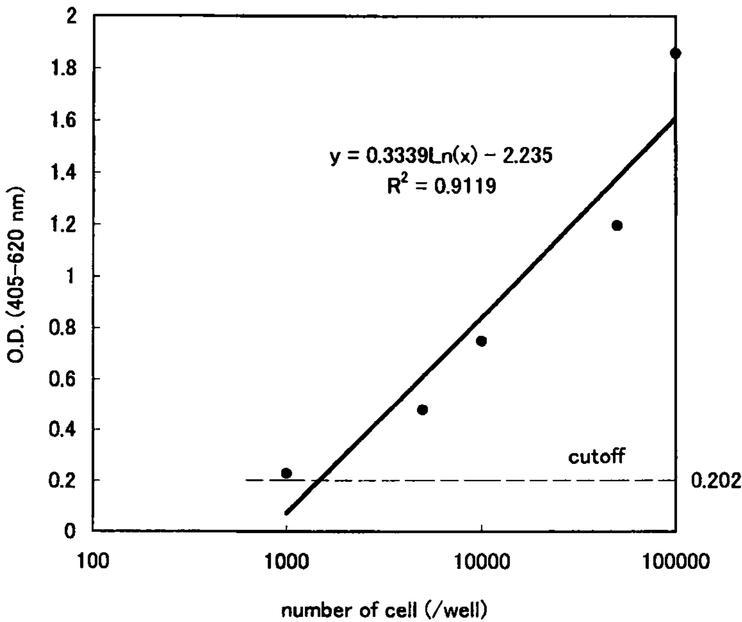

FIGURE 4. Correlation between the number of inoculated cells and the O.D. values in CC-EIA. Various numbers of cells were inoculated onto a microplate and the amount of cell associated collagen was measured by EIA.

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To examine whether the determined conditions provided effective immobilization of cells in CC-EIA, we immobilized cells on a microplate for CC-EIA under the aforementioned conditions, measured the amount of cell associated collagen by CC-EIA and plotted the $O.D._{405\ 620}$ values as a function of the number of cells inoculated. The value of $O.D._{405\ 620}$ in CC-EIA correlated well with the inoculum density between 1,000-100,000 cells/well (Figure 4; R^2=0.912). The coefficients of variation (CV) in each experiment with different number of cell

ranged from 0.058-0.196. We thus confirmed that the conditions were suitable for the immobilization of cells in CC-EIA.

To clarify whether the Giemsa assay is useful for CC-EIA, cells were washed after measurement in CC-EIA and the number of residual cells on the microplate was measured by Giemsa assay. The relative unit amounts of collagen per cell on a microplate for various numbers of inoculated cells were calculated. The ratio, [O.D.$_{405-620}$ value obtained from EIA] divided by [O.D.$_{620}$ value obtained from Giemsa assay] was calculated and expressed as a relative unit. The relative unit values were between 1.33-3.51 and the difference between the values were not found to be statistically significant ($p>0.01$, by one-way ANOVA).

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DISCUSSION

We devised a Giemsa assay method using a microplate reader to evaluate the number of immobilized cells on a microplate. The method afforded simple and convenient evaluation of this parameter. The Giemsa assay technique was also found to be suitable for the evaluation of the number of immobilized cells on a microplate after the performance of CC-EIA. Detachment of immobilized cells from a microplate during the process of CC-EIA could be monitored by the number of residual cells on the microplate by Giemsa assay. Such

monitoring is considered to be beneficial for improving the accuracy of CC-EIA.

In antigen detection by EIA, the target substance can be captured by either chemicals or antibodies. For such capture, the target substances are usually extracted. Since this extraction step requires enzymatic treatment of the target cells and harvesting of the cells by centrifugation, the target materials may be partially lost or degraded, leading to inaccurate results. To measure cell associated collagen, therefore, whole cell immobilization is considered to provide a simple method yielding more accurate results in CC-EIA. Since it is difficult to select the appropriate antibodies for capture of the target cells, chemical immobilization is considered more suitable for the CC-EIA of cell associated collagen. In this immunohistochemical technique, formaldehyde (20), acetone (21) and alcohol (22) are usually applied for the immobilization of antigens. Our assay was performed on a plastic microplate, and accordingly an organic catalyst which could affect the transparency of plastics is considered as not being suitable for such chemical immobilization. Another reason why formalin is suitable in the assay is that in general, the chemical induces permeability of cell membrane by breaking lipid bilayer. Therefore, we chose formalin, which contains 35% formaldehyde and only 8% methanol, in this study.

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In CC-EIA, immobilization of the target substance is essential for the reliability and accuracy of the assay. Fox et al. (20) indicated that aqueous formalin is not suitable for the fixation of cultured cells in morphologic studies; however, immobilization for CC-EIA does not require morphological preservation. Immobilization of antigen and sufficient preservation of its immunoreactivity are pH dependent (23). In this study, we determined that a nearly neutral pH, and room temperature, approximately 22℃, are optimal for immobilization in CC-EIA. To preserve the immunoreactivity of collagen, we used a lower concentration of formalin and shorter incubation period for immobilization, although 5-10% formalin and 15-60 min incubation result in better immobilization.

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Finally, we propose the following conditions as being optimal for whole cell immobilization in CC-EIA: i) addition of 5% formalin in 150 mM PBS (pH 7.2), ii) incubation at room temperature, approximately 22℃, for 15 min, iii) rinsing with PBS. We confirmed that the conditions are suitable for quantitative evaluation of cell associated collagen in cultured cells in CC-EIA.

The relative unit amounts of collagen per cell obtained from the measurement of collagen ($O.D._{405\ 620}$) and immobilized cells ($O.D._{620}$) were similar for various numbers of inoculated cells. Accordingly, we estimate that the Giemsa assay technique could be used for determination of the amounts of cell associated collagen in CC-EIA.

REFERENCES

1. Font, R.L. and Brownstein, S. A light electron microscopic study of anterior subcapsular cataract. Am. J. ophthalmol. 1974; 78: 972-984.

2. Henkind , P., and Prose, P. Anterior Polar Cataract. Am. J. Ophthalmol. 1967; 63: 768-771.

3. Cobo, L.M.,Ohsawa, E., Chandler, D., Arguello, R., and George, G. Pathogenesis of capsular opacification after extracapsular cataract extraction. Ophthalmology 1984; 91: 857-863.

4. Mcdonnell, P.J.,,Zarbin, M.A., and Green, W.R. Posterior capsule opacification in pseudophakic eyes. Ophthalmology 1983; 90: 1548-1553.

5. Abergel, RP., Pizzurro, D., Meeker, CA. and et al. Biochemical composition of the connective tissue in keloids and analysis of collagen metabolism in keloid fibroblast cultures. J. Invest. Dermatol. 1985; 84: 384-390.

6. Diegelmann, RF., Cohen, IK. and McCoy, BJ. Growth kinetics and collagen synthesis of normal skin, normal scar and keloid fibroblasts in vitro. J. Cell Physiol. 1979; 98: 341-346.

7. Ala-Kokko, L., Stenback, F. and Ryhanen, L. Preventive effect of malotilate on carbon tetrachloride-induced liver damage and collagen accumulation in the rat. Biochem. J. 1987; 246: 503-509.

8. Soto, H., Masso, F., Cano, S. and Diaz-de-Leon, L. Effects of mebendazole on protein biosynthesis and secretion in human-derived fibroblast cultures. Biochem-Pharmacol. 1996; 52: 289-299.

9. Murrell, G.A., Francis, M.J. and Bromley, L. The collagen changes of Dupuytrens contracture. J. Hand. Surg. Br. 1991; 16: 263-266.

10. Choi, E.T., Callow, A.D., Sehgal, N.L., Brown, D.M. and Ryan, U.S. Halofuginone, a specific collagen type I inhibitor, reduce anastomotic intimal hyperplasia. Arch. Surg. 1995; 130: 257-261.

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11. Ikeda, H., Inano, M.and Fujiwara, K. Inhibitory effect of tranilast on activation and transforming growth factor β 1 expression in cultured rat stellate cells. Biochem. Biophys. Res. Commun. 1996; 227: 322-327.

12. Isaji, M., Aruga, N., Naito, J. and Miyata, H. Inhibition by tranilast of collagen accumulation in hypersensitive granulomatous inflammation in vivo and of morphological changes and functions of fibroblasts in vitro. Life Sci. 1994; 55: PL 287-292.

13. Yamada, H., Tajima, S. and Nishikawa, T. Tranilast, inhibits collgen synthesis in normal, scleroderma and keloid fibroblasts at a late passage culture but not at an early passage culture. J. Dermatol. Sci. 1995; 9: 45-47.

14. Arita, T., Murata, Y.,Lin, L.R., Tsuji, T., and Reddy, V.N. Synthesis of lens capsule in long- term culture of human lens epithelial cells. Invest. Ophthalmol. Vis. Sci. 1993; 34: 355-362.

15. Laurent, M., Kern, P., Courtois, Y., and Regnault, F. Synthesis of types I, III and IV collagen by bovine lens epithelial cells in long-term culture. Exp. Cell Res. 1981; 134: 23-31.

16. Obata, K., Iwata, K., Ichida, T. et al. One step sandwich enzyme immunoassay for human type IV collagen using monoclonal antibodies. Clin. Chim. Acta. 1989; 181: 293-303.

17. Lopez de Leon, A and Rojkind, M.A. A simple micromethod for collagen and total protein determination in formalin-fixed paraffin embedded sections. J. Histochem.Cytochem. 1985; 7: 267-270.

18. Jimenez, W., Pares, A., Caballeria, J. et al. Measurement of fibrosis in needle liver biopsies: evaluation of a colorimetric method. Hepatology 1985; 5: 815-818.

19. Malkusch, W., Rehn, B. and Bruch, J. Advantage of sirius red staining for quantitative morphometric collagen measurements in lungs. Exp. Lung Res. 1995; 21: 67-77.

20. Fox, C.H., Johnson, F.B., Whiting, J. and Roller, P.P. Formaldehyde fixation. J. Histochem. 1985; 33: 845-853.

21. Kaku,T., Ekem,J.K., Lindayen,C., Bailey,D.J., Van-Nostrand, A.W. and Farber, E. Comparison of formalin-and acetone-fixation for

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immunohistochemical detection of carcinoembryonic antigen (CEA) and keratin. Am.J. Clin. Pathol. 1983; 80: 806-815.

22. Altmannsberger, M., Osborn, M., Schauer, A. and Weber, K. Antibodies to different intermediate filament proteins. Cell type-specific markers on paraffin-embedded human tissues. Lab Invest. 1981; 45: 427-434.

23. Berod, A., Hartman, B.K., Pujor, J.F. Importance of fixation in immunohistochemistry : Use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. J. Histochem. Cytochem. 1981; 29: 844-850.

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