INCREASE IN NUCLEAR DNase I-SENSITIVE SITES IN COLO-201 CELLS WITH RELATION TO CELL ADHESION TO EXTRA-CELLULAR MATRIX

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ABSTRACT

Using a human colonic adenocarcinoma cell line (COLO-201), we have revealed that cell adhesion to extracellular matrix (ECM) influences morphology and growth, and the expression of nuclear DNase I-sensitive sites, or genome exposure sites, of the cells. The cells which had been plated on basement membrane extract, matrigel, developed duct-like structures *in vitro*. The cells plated on ECM substrata showed a retarded growth pattern in the early stage of the culture, and the nuclear DNase I-sensitive sites of the cells increased markedly. Culture of the cells on ECM in the presence of staurosporine decreased cell adhesion to ECM and suppressed an increase in the nuclear DNase I-sensitive sites of the cells which had remained attached to ECM. These findings indicate that signals from ECM are transmitted into the cell nucleus through pathways involving protein kinase reactions.

Cell adhesion to extracellular matrix (ECM) proteins is not merely a mechanical attachment, but the interaction between integrin adhesion receptors and the ligand that persistently sends the extracellular signals into the cells. The signal brings about a cascade of events within the cells and leads to changes in cell behavior; morphology, motility, growth and differentiation (2, 12, 20, 25). The response of the cells to the signals is perpetual and sometimes the cells appear to be in a terminally differentiated state with characteristic cell shapes and the seemingly ceaseless production of specific proteins (15).

The pathway mediating the adhesion signals toward the cell nucleus is thought to resemble the signal transduction system found in the cell responses to hormones and growth factors. The signal transduction system of these humoral factors, the system in which protein phosphorylation reactions are involved, has been studied in detail (1, 2, 12, 13, 19). As to the signal transduction pathway from integrin adhesion receptors to the cell nucleus, only limited information has been available until now (8, 11).

It has been known that the specific fractions of DNA which are sensitive to hydrolysis by DNase I

correspond to the sites of genome exposure. Weintraub and Groudine (21), and others (3, 4, 24), have directly proved by using a DNA hybridization method, that transcriptionally active genes are exceedingly sensitive to cleavage by DNase I. Using the *in situ* nick translation method, Krystosek and Puck (10, 14) have shown that between normal cells and carcinoma cells there are differences in sensitivity of specific fractions of DNA to hydrolysis by DNase I. The results revealed that normal cells expressed the sites of genome exposure more than did carcinoma cells.

In this paper, using a human colonic adenocarcinoma cell line (COLO-201) (16), we have studied the effects of ECM on growth, morphology and gene expression of the cells.

MATERIALS AND METHODS

Cell Culture

A human colonic adenocarcinoma cell line (COLO-201), and a normal human diploid cell line (TIG-1), were obtained from the Japanese Cancer Research Resources Bank. Cells were grown in Dulbecco's modified minimal essential medium/

Ham's F12 mixture (DMEM/F12) (D-2906, Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL-Life Technologies, Tokyo) and antibiotics.

ECM Proteins and Antibodies

Fibronectin (human; F003, Iwaki Glass, Tokyo) and laminin (mouse; L0001, Iwaki Glass) were dissolved in ice-cold phosphate-buffered saline (PBS), and type IV collagen (porcine; Cellmatrix, Nitta Gellatin, Osaka, Japan) was dissolved in ice-cold distilled water before use. Matrigel (Collaborative Biomedical Products-Nippon Becton Dickinson, Tokyo) and staurosporine (S-4400, Boehringer Mannheim Yamanouchi, Tokyo) were purchased through the suppliers. Monoclonal antibodies against integrins; $\alpha_2\beta_1$ (CDw49b, Immunotech, Marseilles, France), α_5 -subunit (CDw49e, Immunotech), α_6 -subunit (CDw49f, Immunotech), and the corresponding second antibodies were purchased through MBL, Nagoya, Japan.

Cell Attachment Assay

Plates (96 wells; #25801, Corning, Corning, NY) were coated with 100 μ l each of various concentrations of type IV collagen, laminin or fibronectin, and incubated overnight at 4°C. The residual binding sites of the plastic wells were blocked with 2% bovine serum albumin (BSA). Cell suspension (100 μ l each) containing 2×10^4 cells in serum-free DMEM/F12 were seeded in the wells, and then the plates were incubated in a CO₂-incubator for 2 h. After discarding the medium, each well was soaked gently in PBS to remove non-adherent cells. Attached cells were fixed with 10% formalin, stained with 0.2% crystal violet with care not to blot the well-walls, and then washed with tap water. The stained cells were lysed with 2% sodium dodecyl sulfate and then absorbance at 595 nm for each lysate was read using a Microplate Reader (Model 450, Bio-Rad, Richmond, CA). The adherence activities of the cells were thus expressed in terms of absorbance.

Cell Growth

COLO-201 cells in DMEM/F12 with 10% FBS were plated at 1×10^4 cells/well in 4-well Multi-dishes (#176740, Nunc, Kamstrup, Denmark) which had been precoated with ECM proteins; type IV collagen, laminin or fibronectin followed by

blocking with 2% BSA. They were incubated in a CO₂-incubator for 5 days. The cells in the wells were harvested with trypsinization and were counted using a Coulter counter at 24 h-intervals.

Immunohistochemical Staining

COLO-201 cells were plated in 8-chamber slides (#177402, Nunc) which had been precoated with ECM proteins and blocked with 2% BSA. The cells were cultured in a CO₂-incubator for 2 days. The cells, washed three times with PBS and fixed with acetone, were then stained immunohistochemically with monoclonal antibodies; CDw49b, CDw49e and CDw49f, using the avidin-biotin-peroxidase complex (ABC) method (Histofine-SAB-PO(M) kit; Nichirei, Tokyo). The cells were also stained with hematoxylin and eosin for general morphological study.

Electron Microscopy

COLO-201 cells were seeded onto 4-chamber slides (#177399, Nunc) which had been precoated with ECM proteins and blocked with 2% BSA. The cells were cultured for 5 days. After discarding the spent medium, the cells were washed with PBS and prefixed in 2.5% glutaraldehyde, followed by postfixation in 1% OsO₄. The cells were then dehydrated in ethanol and quickly embedded in Epon capsules. Ultrathin sections were cut vertically to the slide surface and then stained with uranyl acetate and lead citrate. The specimens were examined with a Hitachi H-800 electron microscope (Hitachi, Tokyo).

Detection of the Nuclear DNase I-Sensitive Sites

DNase I treatment Round glass coverslips (22 mm, No. 2 thickness) placed in 35 mm-tissue culture dishes were coated with 200 μ l each of ECM solution (50 μ g/ml); type IV collagen, laminin, or fibronectin. The coverslips were rinsed twice in PBS and blocked with 2% BSA at room temperature for 2 h. COLO-201 cells (4×10^4 cells) were then seeded on the coverslips in 2 ml each of DMEM/F12 medium and incubated in a CO₂-incubator. A normal human diploid cell line, TIG-1, was used for the positive control. The cells were rinsed three times in PBS and fixed in cold methanol/acetic acid (20:1, v/v) for 30 min, followed by rinses with PBS to obtain the pH around 9. DNase I (50 ng/ml) (Takara, Kyoto, Japan) diluted with DN

Fig. 1 COLO-201 cells cultured on matrigel for 5 days. A: Formation of cell aggregate (hematoxylin and eosin stain). $\times 200$. B: Vertical section of an aggregate. The cells developed duct-like structure (hematoxylin and eosin stain). $\times 200$

Fig. 2 Attachment activities of COLO-201 to ECM. $\circ$, laminin; $\square$, type IV collagen; and $\diamond$, fibronectin. Points represent the mean $\pm$ SEM.

buffer (7.5 mM NaCl; 100 mM Na-acetate; 5 mM MgSO_4 , pH 5.2) was added to the cells on the coverslips, incubated for 10 min at room temperature, washed with distilled water and immersed in 20 mM EDTA solution (pH 8.0). The cells were then washed with distilled water and processed for DNA nick end labeling.

DNA nick end labeling Reaction mixture (25

μl each) was applied to the cells, followed by sandwiching them between the coverslip and a clean glass slide. The reaction mixture was composed of terminal deoxynucleotidyl transferase (TdT) (0.8 e.u./ μl ; GIBCO BRL) and biotin-16-dUTP (0.02 nM; Boehringer Mannheim Yamanouchi) in TdT-buffer (0.1 M potassium cacodylate, pH 7.2; 2 mM CoCl_2 ; 0.2 mM dithiothreitol; GIBCO BRL). Reaction was allowed to proceed in a humid atmosphere at 37°C for 60 min. The coverslips were then washed three times with PBS, and the incorporated biotin was detected using the ABC method.

RESULTS

COLO-201 cells were cultured for 5 days on the basement membrane extract, matrigel, to see if it would bring about morphological changes in the cells. In the early stage of the culture on matrigel, it was observed that COLO-201 cells were embedded in matrigel and formed a network-like structure of cell aggregates, followed by the segregation of the individual aggregates from the network-like structure. Finally, on the 5th day, there was an increase in the size of the aggregates, of which morphology is shown in Fig. 1A. A vertical section of the aggregate revealed that the cells which were cultured on matrigel for 5 days developed duct-like structures *in vitro* (Fig. 1B).

As matrigel is a mixture of the constituents of ECM, adhesion activity of COLO-201 cells to ECM proteins (type IV collagen, laminin and fibronectin) was studied using the attachment assay method, and the result is shown in Fig. 2. COLO-

Fig. 3 Immunohistochemical staining of integrin adhesion receptors of COLO-201. The cells are positively stained with the corresponding antibodies. $\times 200$. A, integrin α_6 -subunit; B, integrin $\alpha_2\beta_1$; C, integrin α_5 -subunit

Fig. 4 Electron micrograph of COLO-201 cells. A: COLO-201 cells cultured for 5 days on the control uncoated glass slide. Cell-cell adhesion looks rather loose and the cytoplasmic structures are poorly developed. $\times 5,500$. B: COLO-201 cells cultured for 5 days on laminin-coated glass slide. Cell-cell adhesions look tight, and many desmosomes (arrows) are observed (inset, $\times 17,500$). Well-developed mitochondria (M) are seen in the cytoplasm, and numerous microvilli-like protrusions are observed on the cells facing the inner lumen (L) of the glandular structure. $\times 7,500$

201 cells showed positive adherence activity to all three ECM proteins tested in a concentration-dependent manner. The cell adhesion to ECM proteins attained a maximum level with concentrations of each ECM protein at $5 \mu\text{g}/\text{well}$. The adhesion activity of COLO-201 cells to fibronectin was the highest and that of the cells to laminin was the lowest.

Fig. 3 shows the immunohistochemical staining of COLO-201 cells with monoclonal antibodies against integrins; α_6 -subunit (Fig. 3A), $\alpha_2\beta_1$ (Fig. 3B), and α_5 -subunit (Fig. 3C). COLO-201 cells were stained positively with all three monoclonal antibodies, and no marked differences in stain intensities were detected among them.

Ultrastructure of COLO-201 cells after 5 days of

culture on glass slides which had been precoated with laminin is shown in Fig. 4. Cells on the control uncoated glass slide showed rather loose cell-to-cell adhesion with adjacent cells, all of which had a large nucleus with clear nucleoli and poorly developed cytoplasmic structures (Fig. 4A). In contrast, the cells on laminin-coated glass slide were tightly connected with adjacent cells with many desmosomes. The cells showed well-developed cytoplasmic structures, i.e. many mitochondria and rough endoplasmic reticulum. Furthermore, numerous microvilli-like protrusions were observed on the cells facing the inner lumen of the glandular structure (Fig. 4B).

To investigate the effects of ECM on growth of COLO-201, the cells were cultured in multi-well

Fig. 5 Growth curves of COLO-201 on ECM. $\circ$, Laminin; $\square$, type IV collagen; $\diamond$, fibronectin; and $\triangle$, plastic substratum

plates which had been precoated with type IV collagen, laminin or fibronectin, and cell numbers were counted at 24 h-intervals. Fig. 5 shows the biphasic growth curves of the cells plated on each ECM protein. The cells on ECM showed a retarded growth pattern in the early stage of the culture. However, in the later stage the cells started to grow more rapidly than the control cells that had been plated on the plastic substratum. The doubling time of the cells on ECM was approximately 60 h and 23 h, in the early and in the later stages of the culture, respectively. The growth of the control cells was constant throughout the culturing period and the doubling time was approximately 27 h.

As it has been known that the specific fractions of DNA that are sensitive to hydrolysis by DNase I correspond to the sites of genome exposure, changes in the gene expression of COLO-201 cells cultured for 2 days on ECM-precoated coverslips were examined by applying the DNase I digestion and DNA nick end labeling method, followed by the ABC method (Fig. 6). COLO-201 cells which had been plated on uncoated coverslips showed sparse and weak staining over the nuclei (Fig. 6A). In contrast, the staining intensity was markedly increased on the nuclei of the cells which had been plated on laminin (Fig. 6B). Similar results were

seen in cells plated on type IV collagen and fibronectin (Fig. 6, C and D).

In order to investigate whether protein phosphorylation reactions would be involved in the signal transduction pathway from ECM to the cell nucleus, we examined the effects of staurosporine, the potent inhibitor of protein kinases, on both the adherence activities of COLO-201 cells to ECM and the changes in the nuclear DNase I-sensitive sites of the cells attached to ECM.

Cell attachment assay was performed after incubating the cells for 2 days with various concentrations of staurosporine in the culture medium. Fig. 7 shows that very low concentrations of staurosporine slightly stimulated adhesion of COLO-201 to ECM. At concentrations over 50 nM, staurosporine markedly inhibited cell adhesion to ECM, and 100 nM staurosporine decreased cell adhesion to about 50% of the control.

To investigate the effects of staurosporine on the changes in the nuclear DNase I-sensitive sites of COLO-201, staurosporine (100 nM) was added to the cells and the cells were cultured on coverslips for 2 days. Each coverslip had been precoated with ECM proteins; type IV collagen, laminin or fibronectin. The attached cells were fixed, treated with DNase I, and the resulting nicks were end-labeled as described in the previous section. As a representative picture in Fig. 8C indicates, staurosporine suppressed an increase in the nuclear DNase I-sensitive sites of COLO-201 cells plated on ECM substrata.

DISCUSSION

COLO-201 cells showed morphological change with signs of having undergone cell differentiation after plating the cells on ECM proteins. Walling *et al.* (20) reported that plating murine colonic adenocarcinoma cells on type I collagen had induced columnar and polarized epithelial shaped cells with significantly retarded cell growth. Their results and our present data indicate that the signals for morphological change and differentiation was transmitted to the cell interior from ECM proteins.

COLO-201 cells plated on ECM showed a biphasic growth pattern, while the control cells showed a linear growth pattern. Growth retardation in the early stage of the culture seems to be resulted from cell adhesion to ECM. The recovered cell growth in the later stage of the culture seems to be due to a loss of contact between COLO-201 and ECM, caused by the formation of cell aggregates in which

Fig. 6 Detection of the DNase I-sensitive sites in COLO-201 cells. A: COLO-201 cells on the control uncoated coverslip. The cells show sparse and weak staining over the nucleus. B: COLO-201 cells on laminin. C: COLO-201 cells on type IV collagen. D: COLO-201 cells on fibronectin. The cells on ECM show markedly increased stain intensity over the nuclei.

cells positioned higher up received little influence from ECM.

Adhesion-induced changes in gene expression have been observed in a variety of cell types, including peritoneal macrophages (18), cultured hepatocytes (2) and myoblasts (12). For example, Dipersio *et al.* (2) showed that in the hepatocyte-derived cell line, H2.35, transcription of a liver-specific albumin gene was induced, and albumin enhancer was activated selectively in accordance with adhesion of the cells to type I collagen.

It has been known that the active chromatin detectable with DNase I are classified into two categories: as DNase I-hypersensitive sites (5) which are correlated with transcription factor binding sites, and as DNase I-sensitive domain (21, 22) which is thought to render a chromatin domain

accessible to regulatory factors.

Recently, a molecular mechanism that exerts differentiation-associated growth arrest of cells has been reported by several authors (6, 17). It is shown to be correlated with the activated expression of Cdk inhibitor proteins, p21 and/or p27. Although it is probable that the gene for Cdk inhibitors was expressed in COLO-201 cells in the early stage of culture after cell adhesion to ECM, it remains to be elucidated.

Using a nick end labeling method, we showed in this paper how the nuclear DNase I-sensitive sites of COLO-201 cells were influenced by adhesion of the cells to ECM proteins. Krystosek and Puck (10, 14) have shown the location of DNase I-sensitive chromatin in cells using the *in situ* nick translation method. Instead, we used the nick end labeling

Fig. 7 Effects of staurosporine on adhesion of COLO-201 to ECM. Each point represents the percentage of the control absorbance without staurosporine. ○, Laminin; □, type IV collagen; and ◇, fibronectin

method to elucidate the changes in the nuclear DNase I-sensitive sites of COLO-201 cells, because the efficiency of incorporation of the labeled deoxy-nucleotide by the nick translation method had been found to be too low to perform the experiments.

Our results that active chromatin is increased in COLO-201 cells after cell adhesion to ECM imply that the signals from ECM are transmitted to the cell nucleus resulting in changes in gene

expression of the tumor cells. Although, the data have indicated no direct relationship between morphological change and increase in the nuclear DNase I-sensitive sites of the cells, the probable relationship between them is suggested.

The precise mechanism whereby signals from ECM proteins are transmitted via integrins to the cell nucleus remains poorly defined. Juliano and Haskill (8) presented a model of an intracellular signaling pathway from ECM to monocyte nuclei: In focal adhesion where clustering of integrins takes place, focal adhesion tyrosine kinase, pp125fak, is activated, the cytoskeletal elements are reorganized, and then, either negative or positive signals are transmitted to the nuclei resulting in the regulation of gene expression. Several reports have indicated that signal transduction cascades mediated by integrins involve protein kinase C (23) and protein tyrosine kinase (7, 9) in focal adhesion formation.

We have shown that staurosporine, a potent inhibitor of protein kinase C, protein kinase A and tyrosine kinase, reduces cell adhesion to ECM and suppresses an increase in the nuclear DNase I-sensitive sites in COLO-201 cells. These results seem to indicate that protein kinases are involved in cell adhesion and signal transduction from ECM.

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REFERENCES

1. CANTLEY L. C., AUGER K. R., CARPENTER C., DUCKWORTH B., GRAZIANI A., KAPPELLER R. and SOLTOFF S. (1991) Oncogenes and signal transduction. *Cell* 64, 281-302
2. DIPERSIO C. M., JACKSON D. A. and ZARET K. S. (1991) The

Fig. 8 Effects of staurosporine on the changes in nuclear DNase I-sensitive sites. A: The control cells on uncoated coverslip. B: The cells on laminin-coated coverslip. C: The cells cultured in the presence of staurosporine on laminin-coated coverslip show decreased intensity of staining over the nuclei.

- extracellular matrix coordinately modulates liver transcription factors and hepatocyte morphology. *Mol. Cell Biol.* **11**, 4405-4414
3. GAREL A. and AXEL R. (1976) Selective digestion of transcriptionally active ovalbumin genes from oviduct nuclei. *Proc. Natl. Acad. Sci. USA* **73**, 3966-3970
 4. GAREL A., ZOLAN M. and AXEL R. (1977) Genes transcribed at diverse rates have a similar conformation in chromatin. *Proc. Natl. Acad. Sci. USA* **74**, 4867-4871
 5. GROSS D. S. and GARRARD W. T. (1988) Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* **57**, 159-197
 6. HALEVY O., NOVITCH B. G., SPICER D. B., SKAPEK S. X., RHEE J., HANNON G. J., BEACH D. and LASSAR A. B. (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**, 1018-1021
 7. HANKS S. K., CALALB M. B., HARPER M. C. and PATEL S. K. (1992) Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc. Natl. Acad. Sci. USA* **89**, 8487-8491
 8. JULIANO R. L. and HASKILL S. (1993) Signal transduction from the extracellular matrix. *J. Cell Biol.* **120**, 577-585
 9. KORNBERG L., EARP H. S., PARSONS J. T., SCHALLER M. and JULIANO R. L. (1992) Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* **267**, 23439-23442
 10. KRYSOSEK A. and PUCK T. T. (1990) The spatial distribution of exposed nuclear DNA in normal, cancer, and reverse-transformed cell. *Proc. Natl. Acad. Sci. USA* **87**, 6560-6564
 11. LIPPERT L., HAIMOVICH B., SCHALLER M. D., COBB B. S., PARSONS J. T. and BRUGGE J. S. (1992) Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{FAK} in platelets. *J. Cell Biol.* **119**, 905-912
 12. MENKO A. S. and BOETTIGER D. (1987) Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. *Cell* **51**, 51-57
 13. NISHIBE S., WAHL M. I., HERNÁNDEZ-SOTOMYOR S. M. T., TONKS N. K., RHEE S. G. and CARPENTER G. (1990) Increase of the catalytic activity of phospholipase C- γ 1 by tyrosine phosphorylation. *Science* **250**, 1253-1256
 14. PUCK T. T. and KRYSOSEK A. (1992) Role of the cytoskeleton in genome regulation and cancer. *Int. Rev. Cytol.* **132**, 75-108
 15. ROSKELLEY C. D., DESPREZ P. Y. and BISSELL M. J. (1994) Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. *Proc. Natl. Acad. Sci. USA* **91**, 12378-12382
 16. SEMPLE T. U., QUINN L. A., WOODS L. K. and MOORE G. E. (1978) Tumor and lymphoid cell lines from a patient with carcinoma of the colon for a cytotoxicity model. *Cancer Res.* **38**, 1345-1355
 17. SKAPEK S. X., RHEE J., SPICER D. B. and LASSAR A. B. (1995) Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science* **267**, 1022-1024
 18. THORENS B., MERMOD J.-J. and VASSALLI P. (1987) Phagocytosis and inflammatory stimuli induce GM-CSF mRNA in macrophages through posttranscriptional regulation. *Cell* **48**, 671-679
 19. ULLRICH A. and SCHLESSINGER J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212
 20. WALLING J. M., BLACKMORE M., HICKMAN J. A. and TOWNSEND K. M. S. (1991) Role of the extracellular matrix on the growth and differentiated phenotype of murine colonic adenocarcinoma cells *in vitro*. *Int. J. Cancer* **47**, 776-783
 21. WEINTRAUB H. and GROUDINE M. (1976) Chromosomal subunits in active genes have an altered conformation. *Science* **193**, 848-856
 22. WOOD W. I. and FELSENFELD G. (1982) Chromatin structure of the chicken β -globin gene region. *J. Biol. Chem.* **257**, 7730-7736
 23. WOODS A. and COUCHMAN J. R. (1992) Protein kinase C involvement in focal adhesion formation. *J. Cell Sci.* **101**, 277-290
 24. WU C., WONG Y.-C. and ELGIN S. C. R. (1979) The chromatin structure of specific genes: II. Disruption of chromatin structure during gene activity. *Cell* **16**, 807-814
 25. YAMAMOTO R., TATSUTA M., NAKAMURA H., MATSUSAKA T., TERADA N. and TAMURA H. (1988) Morphological changes in a human scirrhous gastric carcinoma cell line (KATO-III) when cultured in collagen-coated dishes. *Virchows Arch. (B)* **55**, 247-253