Korean J. Zool. 38:542-549, 1995

Analyses of the Neurite Outgrowth and Signal Transduction in IMR-32 and SK-N-SH Cells by ECM Proteins

Yoon Jeong Choe, Chul Woo Kim[1], and Kyu Chung Hur*

Department of Biology, Ewha Womans University, Seoul 120-750; [1]Department of Pathology, Seoul National University Medical School, Seoul 110-799, Korea

The effect of extracellular matrix (ECM) protein on the neuronal differentiation of SK-N-SH and IMR-32 human neuroblastoma cell lines was examined. When cells were cultured on the laminin/collagen coated plate for 7 days, the extensive neurite outgrowth was observed in IMR-32. To address the reason why IMR-32 cell line did not respond to ECM proteins, the ECM mediated early signalling mechanisms were analysed in both SK-N-SH and IMR-32. When cells were plated on the laminin/collagen coated plates, tyrosine phosphorylated proteins were increased within an hour in both of these cells. Moreover, the focal adhesion kinase (FAK) was tyrosine phosphorylated in both of these two cell lines. These results suggest that the ECM-mediated early signalling mechanism was normal in IMR-32 cell line. The expression of both NSE and Bcl-2 was increased by ECM treatment in SK-N-SH. However, these components were not changed by ECM in IMR-32 cells to ECM component is likely due to the abnomality of the transcriptional regulation mechanism which is responsible for the neuronal differentiation.

KEY WORDS: Extracellular Matrix (ECM), Neurite Outgrowth, Focal Adhesion Kinase (FAK), Human Neuroblastoma Cell

The extracellular matrix (ECM) affects many aspects of cell behavior, including the migratory properties of cells, their morphology, growth characteristics, and differentiation (McDonald, 1988; McClay and Ettersohn, 1987). There is evidence that many of these actions are mediated through integrins, the family of ECM receptors found on most cells (Buck *et al.*, 1987; Akiyama *et al.*, 1990). Integrins are heterodimers; each subunit has a large extracellular domain, spans the membrane once, and has a short cytoplasmic sequence (Hynes, 1992). The engagement of integrins with ligand (proteins of the ECM) induces the phosphorylation of proteins on tyrosine. It is now firmly established that one of the major substrates for integrin-induced tyrosine phosphorylation is a protein tyrosine kinase (PTK) called focal adhesion kinase (FAK or pp125^{FAK}) (Hynes, 1992; Kornberg *et al.*, 1992; Burridge *et al.*, 1992; Lipfert *et al.*, 1992). The initial event in this signalling pathway is integrin-dependent binding to the ECM and the induction of pp125^{FAK} autophosphorylation. In vitro, pp125^{FAK} autophosphorylation occurs on tyrosine residue 397 (Tyr397), and it perhaps leads to the recruitment and activation of Src-like PTKs, including Src itself, Fyn and Csk. This bipartite pp125^{FAK}-src-like kinase signalling complex may be necessary to catalyze the tyrosine phosphorylation of downstream targets (Cantley

*To whom correspondence should be addressed.

et al., 1991).

When established in culture, human neuroblastoma cell lines typically are comprised of heterogeneous cellular subpopulations, including neuroblastic (N-type), substrate-adherent (S-type), and intermediate (I-type) cells (Ciccarone *et al.*, 1989; Ross *et al.*, 1983).

About one-third of human NB cell lines contain high levels of the 26 kDa protein encoded by bcl-2 proto-oncogene. Bcl-2 protein levels are correlated with other characteristics of the cell lines, including morphology, N-myc gene amplification status, and p75-NGF-receptor expression. A clear association is noted between Bcl-2 and cellular morphology and the expression levels of p26-Bcl-2 is up-regulated as these neoplastic cells undergo further neuronal differentiation (Hanada, M. *et al.*, 1993). High level of Bcl-2 protein appears to contribute to extend cell survival rather than accelerate the rate of cellular proliferation (Vaux *et al.*, 1988). Specificially, p26-Bcl-2 blocks programmed cell death (also termed apoptosis), an active form of cellular demise that typically requires new RNA and proteins synthesis (Wyllie *et al.*, 1980).

The SK-N-SH line was derived from a human metastatic neuroblastoma and contains cells with differing morphological and biochemical characteristics. This cell line lacks N-myc gene amplification and differentiates to neuronal cells by increasing the length of the neuritic processes by the treatment of drugs, such as retinoic acid (RA) or TPA (Pahlaman *et al.*, 1981; Ross *et al.*, 1993; Sidell *et al.*, 1983). However, the IMR-32 line is amplified N-myc gene (Kim *et al.*, 1993).

In this study, to examine the early ECM mediated signalling mechanism, we analysed tyrosine phosphorylated proteins including the focal adhesion kinase when these cells were plated on the laminin/collagen coated plates. Furthermore, we investigated the change of neuron specific enolase (NSE) and Bcl-2 protein that occur in these two cell lines when induced to differentiate in culture.

Materials and Methods

Materials: Laminin and collagen were obtained from Life Technology Inc., while all trans-retinoic acid (RA) was obtained from Sigma. Anti-mouse and anti-rabbit Ig conjugated with horse raddish peroxidase were purchased from Boehringer Mannheim Biochemica, protein A- and G-sepharose from Pharmacia, anti-neuron specific enolase (NSE) and anti-Bcl-2 from DAKO, and enhanced chemiluminescence (ECL) reagent from Amersham.

Cell culture: SK-N-SH and IMR-32 cells were cultured in RPMI 1640 media containing 10% fetal calf serum under 5% CO_2 at 37°C on either uncoated or laminin/collagen coated plates. Every 3 days fresh media was supplied. In some experiments, cells cultured on uncoated plates for 24 hrs were treated with1 mM all trans-retinoic acid.

Immunoprecipitation: Cells were plated on either uncoated or laminin/collagen coated culture dishes (75 cm^2) for an hour, washed with ice cold PBS, and then lysed with immunoprecipitation buffer (1 ml/dish) containing 10 mM Tris-Cl pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1% Triton-X100, and 0.5% NP-40 for 30 min. on ice with occasional agitation. Lysed cells were collected and centrifused at 13,000 $\times$g for 15 min. Supernatant was incubated with appropriate antibody (5 μg/ml) and protein A-sepharose (50 μl of 10%/ml) for 1 hr, then washed three times with immunoprecipitation buffer by brief centrifugation. Pellet was dissolved in Laemmli sample buffer, and then analysed on 10% SDS-polyacrylamide gel eletrophoresis (Laemmli, 1970).

Western blot: Proteins that had been electrophoretically fractionated on the10% SDS-gel were blotted to a nitrocellulose paper as described previously (Towbin *et al.*, 1979). Antibody labeling of protein bands was detected with ECL reagents according to the supplier's protocol.

Results

The effect of ECM proteins on the neuronal differentiation of SK-N-SH and IMR-32 human neuroblastoma cell lines was examined. Fig. 1 shows examples of the morphological changes that occurred in IMR-32 and SK-N-SH cells by treatment with RA or laminin/collagen. The IMR-32 cell line is neuronal in appearance with neuritic processes and round or stellate cell bodies that are

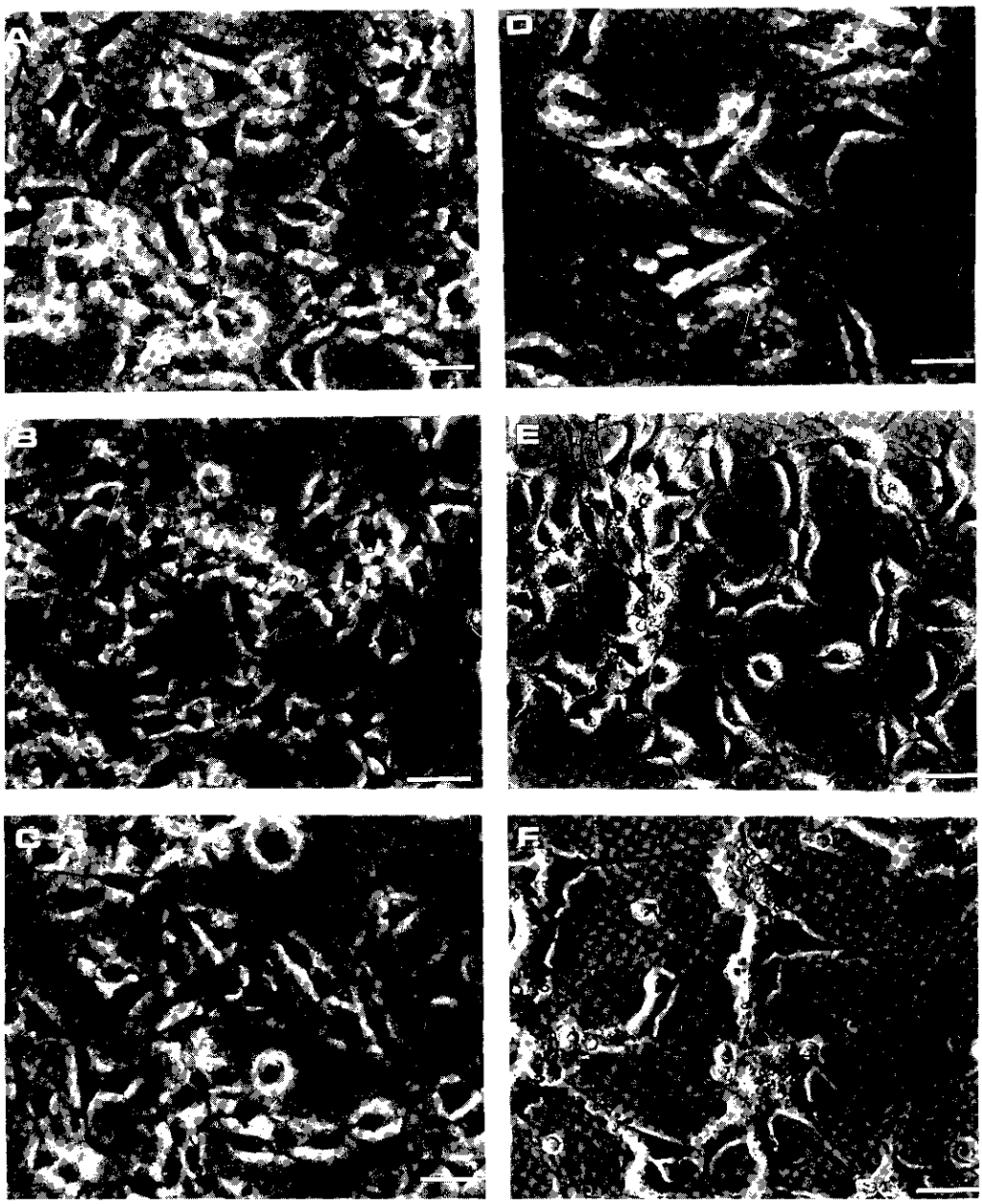

Fig. 1. Phase-contrast micrographys of IMR-32 (A, B, C) and SK-N-SH (D, E, F) cells, on the uncoated plate (A, D), collagen/laminin coated plate (B, E) and in the presence of RA (C, F) for 7 days.

loosely adherent to the culture substratum (Fig. 1A). When cells were cultured on laminin/collagen coated plate for 7 days, no distinct morphological change was detected in this cell line (Fig. 1B). And no significant morphological change was detected in most cells in RA-treated cultures (Fig. 1C). The SK-N-SH cell line composes of heterogeneous phenotypic characteristics, but most of the cells growing as monolayer on culture plates showed flattened epithelial cell-like morphology (Fig. 1D), while cells cultured on either laminin/collagen coated plates (Fig. 1E) or treated with retinoic acid (Fig. 1F) started to form neurite within 5 days, and cell bodies rounded up and lots of neurites were formed after 7 days (Fig. 1E). However, the average length of formed neurites was longer in retinoic acid treated cells than that in the cells cultured on laminin/collagen coated plate. These results indicate that SK-N-SH cells can be differentiated to neuronal cells by both retinoic acid and extacellular matrix proteins such as laminin and collagen, however, IMR-32 cells did not respond to these reagents.

To address the reason why IMR-32 cell line did not respond to ECM proteins, the early ECM mediated signalling mechanisms were analysed in both SK-N-SH and IMR-32 cells. Having demonstrated that the increase of tyrosine phosphorylation in several proteins by ECM components (Hynes, 1992), we first examined tyrosine phosphorylated proteins in the cells which plated on laminin/collagen coated plates by analysing the anti-phosphotyrosine immunoprecipitates. Several species of proteins interacting with anti-phosphotyrosine were observed in the immunoprecipitates from both IMR-32 (Fig. 2, lane 1) and SK-N-SH (Fig. 2 lane 3)cells plated on uncoated dishes. However, the number of tyrosine phosphorylated proteins as well as the intensity of tyrosine phosphorylation were increased in the immunoprecipitates from both IMR-32 (Fig. 2, lane 2) and SK-N-SH (Fig. 2, lane 4) cells plated on laminin/collagen coated dishes. This increased tyrosine phosphorylation observed in the cells plated on ECM coated dishes is likely due to the activation of FAK by laminin and collagen.

To investigate whether FAK can be activated in IMR-32 cells by extracellular matrix proteins, we

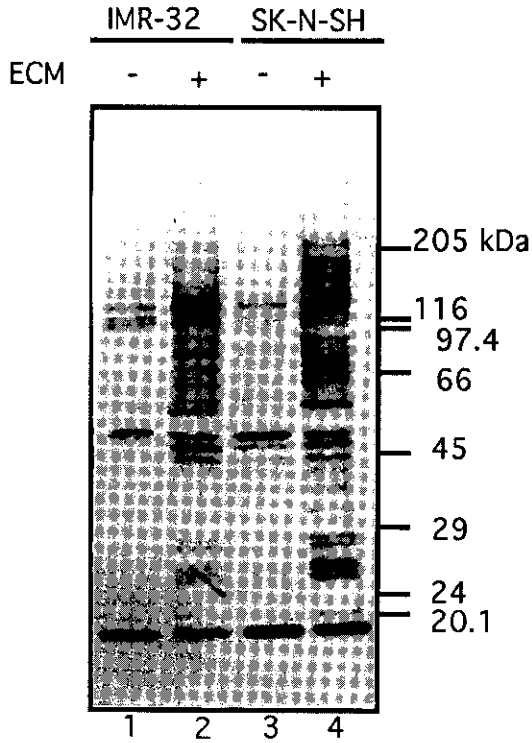

Fig. 2. Anti-phosphotyrosine immunoblot of the anti-phosphotyrosine immunoprecipitates from IMR-32 cells (A) and SK-N-SH cells (B) incubated in the absence of ECM protein (lane 1,3) or in the presence of collagen/laminin (lane 2, 4) for 1hr.

examined the existence and activation of FAK by analyzing the anti-FAK immunoprecipitates from these two cell lines. The immunoblotting with anti-FAK (Fig. 3B) of anti-FAK immunoprecipitates from IMR-32 (lanes 1 and 2) and SK-N-SH (lanes 3 and 4) revealed almost identical amount of a 125 kDa component either cells plated on the uncoated dishes (lanes 1 and 3) or laminin/colagen coated dishes (lanes 2 and 4). A slightly lower molecular weight component (about 116 kDa) also appeared to interact with anti-FAK. To examine whether FAK was activated by laminin/collgen in the anti-FAK immunoprecipitates from these two cell lines, the anti-FAK immunoprecipitates used in figure 3B was reblotted with anti-phosphotyrosine (Fig. 3A). Anti-phosphotyrosine binding components identified in the FAK immunoprecipitates appeared almost identical with those of the anti-

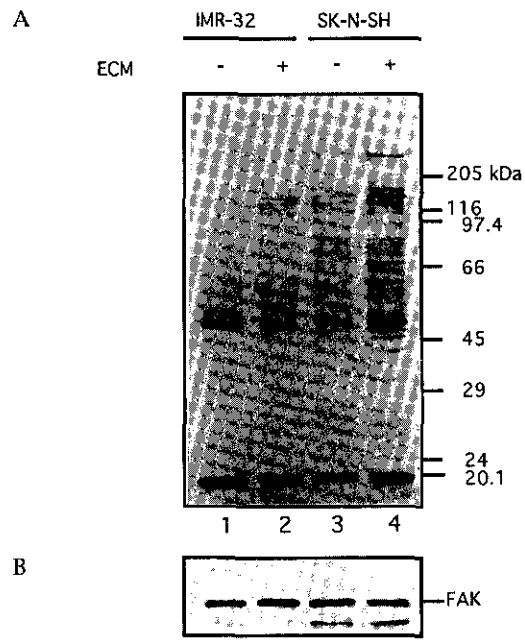

Fig. 3. Anti-phosphotyrosine (A) and anti-FAK immunoblots of the anti-FAK immunoprecipitates from IMR-32 and SK-N-SH cells. A. Anti-phosphotyrosine immunoblot of anti-FAK precipitates from IMR-32 (lane1, 2) and SK-N-SH (lane 3, 4)cells incubated in the absence of ECM protein (lane 1, 3) or in the presence of collagen/laminin (lane 2, 4) for 1hr. B. Anti-FAK immunoblot of anti-FAK immunoprecipitates from IMR-32 (lane 1, 2) and SK-N-SH (lane 3, 4) cells incubated in the absence of ECM protein (lane 1, 3) or in the presence of collagen/laminin (lane 2, 4) for 1hr.

phosphotyrosine immunoprecipitates (compare Fig. 3B with Fig. 2). The anti-FAK immunoprecipitates from IMR-32 (lane 1) and SK-N-SH (lane 3) cells plated on uncoated dishes have several tyrosine phosphorylated components. However, the number of tyrosine phosphorylated proteins and intensity of phosphorylation to these components were increased both IMR-32 (lane 3) and SK-N-SH (lane 4) by ECM components (compare lane 1 with 2 and lane 3 with 4 in arrow area) These results suggest that the ECM-mediated early signalling mechanism is normal in IMR-32 cell line.

Because previous report demonstrated that the Bcl-2 gene expression is up-regulated as the neoplastic cells undergo further neuronal differentiation (Ross *et al.*, 1991), we examined

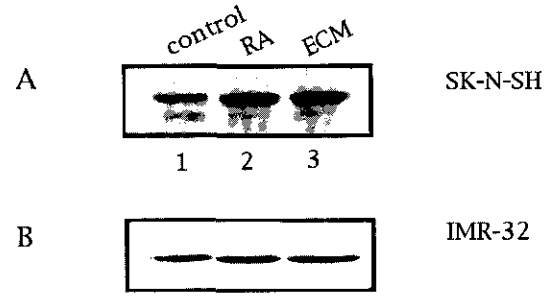

Fig. 4. Anti-Bcl-2 immunoblot of total protein from SK-N-SH (A) and IMR-32 (B) cells in the absence of ECM or RA (lane 1) or in the presence of RA (lane 2) and ECM (lane 3).

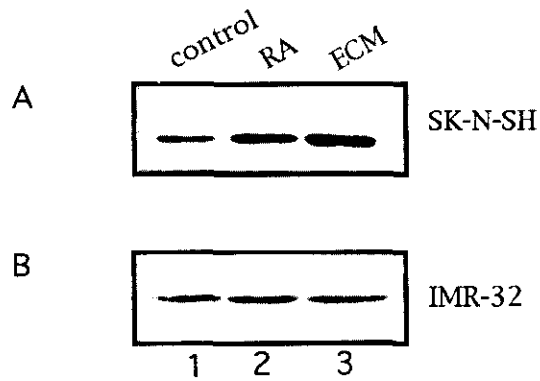

Fig. 5. Anti-NSE immunoblot of total protein from SK-N-SH (A) and IMR-32 (B) cells in the absence RA or ECM (lane 1) or in the presence of RA (lane 2) or ECM (lane 3).

the level of Bcl-2 protein and the neuronal marker, NSE. The expression of both Bcl-2 (Fig. 4) and NSE (Fig. 5) was increased by either retinoic acid (lane 2) or ECM (lane 3) treatment in SK-N-SH (compare lane 1 with 2 and 3 in Fig. 4A and 5A). However, these components were not changed by neither retinoic acid (lane 2) and ECM (lane 3) in IMR-32 (compare lane 1 with 2 and 3 in Fig. 4B and 5B). These results indicate that the early ECM mediated signalling mechanism is normal in these two cell lines, but the failure of differentiation in IMR-32 is likely due to the defect of late mechanism such as transcriptional process which is responsible for the neuronal differentiation.

Discussion

In this report, we studied the effect of ECM proteins on neurite outgrowth in human neuroblastoma cell lines and whether ECM mediated signalling is normal to examine the reason why IMR-32 cell line does not respond to ECM proteins.

Morphological data demonstrated that SK-N-SH cells formed extensive neurites by ECM proteins, but IMR-32 cells did not respond to them. To address the reason why IMR-32 cells did not respond to ECM proteins, the early ECM mediated signalling mechanism was analysed. The results in this report demonstrate that ECM proteins activate FAK and the activated FAK induces tyrosine phosphorylation of several other proteins in both SK-N-SH and IMR-32 cells. These results suggest that the initial signalling event of ECM which mediated through integrins and the resulting phosphorylation of proteins on tyrosine is normal in these two cell lines.

Recently, it has been demonstrated that the induction of $pp125^{FAK}$ autophosphorylation may lead to binding of src-like kinase (Cantly *et al.*, 1991). These PTKs may phosphorylate each other to regulate activity or complex formation with other signalling molecules, e.g. phosphatidylinositol 3′ kinase (PI-3 K). In addition, the recruitment/ activation of molecules such as phosphatidylinositol-3 kinase or C3G may trigger the activation of other signalling pathway, e.g. the $p21^{ras}$ pathway (Tanaka *et al.*, 1994; Birge *et al.*, 1993). Recent evidence suggest that subsequent phosphorylation of FAK by src-related PTKs creates binding sites for other SH2 proteins such as Grb2, Nck and PI-3 kinase (Chen *et al.*, 1994; Skolnik *et al.*,1993; Songyang *et al.*, 1993; David *et al.*, 1994). In particular, Grb2 binding to FAK at Tyr 925 may lead to the formation of multiprotein signalling complex which promotes activation of the Ras pathway (David *et al.*, 1994). When IMR-32 and SK-N-SH cells plated on the laminin/collagen coated plate, tyrosine phosphorylation in the FAK from these two cell lines as well as in the anti-Ras immunoprecipitates were increased (data not shown). This increase of tyrosine phosphorylation suggests that ECM mediated signal transduction system from binding of ECM proteins to their receptors to Ras activation in both of these two cell lines looks like normal.

The up-regulation of Bcl-2 and NSE protein level is induced by ECM proteins in SK-N-SH cells. In contrast, these components are not changed by ECM in IMR-32 cells. These data indicate that the unresponsiveness of IMR-32 cells to ECM proteins is likely due to the abnomality of transcriptional regulation mechanism which is responsible for neuronal differentiation. This possibility was also supported by the result of RA effect in IMR-32 cells; RA, which is known to differentiate cells by controlling transcription of a certain set of genes, did not change the quantity of both Bcl-2 and NSE in IMR-32 cells like ECM proteins. Which transcriptional controling mechanism is responsible for the unresponsiveness to ECM proteins in IMR-32 cells is a matter of future study.

Acknowledgements

This work was partially supported by grants from the Basic Science Research Fund, Ministry of Education, The Republic of Korea and from The Korean Research Institute for Better Living, Ewha Womans University.

References

Akiyama, S.K., K. Nagata, and K. Yamada, 1990. Cell surface receptor for extracellular matrix components. *Biochim. Biophys. Acta* **1031:** 91-110.

Birge, R.B., J.E. Fajardo, C. Reichman, S.E. Shoelson, Z. Songyang, L.C. Cantley, and H. Hanafusa,1993. Identification and characterization of high affinity interaction between v-Crk and tyrosine-phosphorylated Paxillin in CT10-transformed fibroblast. *Mol. Cell. Biol.* **13:** 4648-4656.

Buck, C.A., and A.F. Hrwitz, 1987. Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* **4:** 487-525.

Burridge, K., C.E. Turner, and L.H. Romer, 1992. Tyrosine phosphorylation of paxillin and $pp125^{FAK}$ accompanies cell adhesion to extracellular matrix: a role in the cytoskeletal assembly. *J. Cell Biol.* **119:**

893-903.

Cantley, L.C., K.R. Anger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff, 1991. Oncogenes and signal transduction. *Cell* **64:** 281-302.

Chen, R.H., C. Sarnecki, and J. Blenis, 1992. Nuclear localization and regulation of erk- and rsk- encoded protein kinases. *Mol. Cell. Biol.* **12:** 915-927.

Ciccarone, V., B.A. Spengler, M.B. Meyers, J.L. Biedler, and R.A. Ross, 1989. Phenotypic divercification in human neuroblastoma cells. *Cancer Res.* **49:** 219-225.

David, D.S., K.H. Steven, H. Kony, and G. Peter, 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372:** 786-791.

Hanada, M., S. Krajewski, S. Tanaka, D.C. Haten, B.A. Spengler, R.A. Ross, J.L. Biedler, and J.C. Reed, 1993. *Cancer Res.* **53:** 4978-4986.

Hynes, 1992. Integrin: versatility, modulation, and signalling in cell adhesion. *Cell* **69:** 11-25.

Kim, C.J., Y.J. Choe, and C.W. Kim, 1993. Changes of intracellular adhesion molecule expression and cytogenic abnormalities during the differentiation process in human neuroblastoma cell lines. *J. Korean Cancer Associ.* **25:** 563-571.

Kornberg, l., H. Earp, J.T. Parsons, M. Schaller, and R. L. Juiano, 1992. Cell adhesion or integrin clustering increase phosphorylation of focal adhesion associated tyrosine kinase. *J. Biol. Chem.* **267:** 23439-23442.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.

Lipfert, L., B. Haimovich, M.D. Schaller, B.S. Cobb, J. T. Parsons, and J.S. Brugge, 1992. Integrin-dependent phosphorylation and activation of the protein kinase pp125^{FAK} in platelets. *J. Cell Biol.* **119:** 905-912.

MaClay, D.R. and C.A. Ettersohn, 1987. Cell adhesion in morphogenesis. *Annu. Rev. Cell Biol.* **3:** 319-345.

McDonald, J.A., 1988. Extracellular matrix assembly. *Annu. Rev. Cell Biol.* **4:** 183-207.

Pahlman, S., L. Odelstad, E. Larsson, G. Grotte, and K. Nilson, 1981. Phenotypic changes of human neuroblastoma cells in culture induced by 12-tetradecanoyl phobol-13-acetate. *Int. J. Cancer* **28:** 583-589.

Ross, R.A., B.A. Spengler, J.L. Biedler, 1983. Coordinate morphological and biochemical interconversion of human neuroblastoma cells. *J. Natl. Cancer Inst.* **71:** 741-749.

Ross, R.A., B.A. Spengler, W.R. Rettig, and J.L. Biedler, 1993. Permanent phenotypic conversion of human neuroblastoma I-type cells. *Proc. Am. Assoc. Cancer Res.* **34:** 44.

Sidell, 1987. Retinoic acid induced differentiation of human neuroblastoma: a cell variant system showing two distinct responses. *Exp. Cell Biol.* **54:** 287-300.

Skolnik, E.Y., 1993. The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine phosphorylated IRS1 and Shc. *EMBO J.* **12:** 1929-1936.

Tanaka, S., T. Morishita, Y. Hashimoto, S. Hattori, S. Nakamura, M. Shibuya, K. Matuoka, T. Takenawa, T. Kurata, K. Nakashima, and M. Matsuda, 1994. C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src Homology 3 Domains of CRK and GRB2/ASH proteins. *Proc. Natl. Acad. Sci. USA* **91:** 3443-3447.

Towbin, H., T. Staehelin, and J. Gordon, 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76:** 4350-4354.

Vaux, D., S. Cory, and J. Adams, 1988. Bcl-2 gene promotes hematopoietic cell survival and coorperates with c-myc to immotalize pre B-cells. *Nature* **335:** 440-442.

Wyllie, A., J.F.R. Kerr, and A.R. Currie, 1980. Cell death, the significance of apoptosis. *Int. Rev. Cytol.* **68:** 251-306.

(Accepted October 2, 1995)

ECM 단백질이 IMR-32 및 SK-N-SH 세포주 신경축색생장에 미치는 영향

최윤정 · 김철우[1] · 허규정(이화여자대학교 생물과학과, [1]서울대학교 의과대학 병리학과)

Extracellular matrix(ECM) 단백질이 SK-N-SH 및 IMR-32 세포주가 신경계 세포로 분화되는 데 미치는 영향을 조사하였다. Laminin과 collagen으로 도말한 배양기에서 7일간 배양했을 때 SK-N-SH세포는 잘 발달된 신경축색생장을 보였으나 IMR-32세포는 뚜렷한 형태변화를 나타내지 않았다. 왜 IMR-32세포가 ECM 단백질에 반응을 하지 않는가를 규명하기 위하여 ECM단백질에 의한 초기 신호전달기작을 두 세포주에서 분석하였다. ECM 단백질을 도말한 배양기에 세포를 깔았을 때 한시간 만에 tyrosine 인산화된 단백질이 두 세포 모두 증가함을 볼 수 있었다. 아울러 focal adhesion kinase(FAK)의 tyrosine 인산화도 두 세포주 모두에서 증가하였다. 이러한 결과는 두 세포주가 ECM 단백질에 의한 초기 신호전달체계가 정상임을 의미한다. 신경세포 분화과정에 증가한다고 알려진 Bcl-2 및 NSE의 량을 ECM 단백질 처리후 조사하였을 때 SK-N-SH 세포주는 두 단백질이 증가 했지만 IMR-32 세포주는 변화가 없었다. 이러한 결과는 IMR-32 세포주가 ECM 단백질에 반응하지 않는 것이 ECM 단백질에 의한 신호전달체계에 문제가 있다기 보다 신경계세포로 분화되는 데 필요한 유전인자의 발현조절에 문제가 있음을 시사한다.