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A Possible Role of Fibronectin on the Differentiation of Monocyte to Macrophage

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Monocyte interaction with fibronectin (FN) mediates specific cell surface receptors and results in cell attachment and differentiation. Several cell-mediated activities for various fragments of FN have been documented. To investigate the regulatory mechanisms of monocyte differentiation by cell binding domains of FN and their receptors, cell attachment-, cell migration-, and its respective inhibition assay were carried out. Monocyte recognizes 38-kDa domain distinctively from its recognition of 85-kDa domain, and the heparin-binding site of the 38-kDa fragment is not involved in monocyte adhesion. Based on these experimental results, it can be suggested that monocyte/macrophage interacts with at least two different sites in FN, which is critical step in cell adhesion and (or) migration.

KEY WORDS: Fibronectin, Monocyte/Macrophage, Differentiation

Adherence to endothelium and then to extracellular matrix (ECM) is a prerequisite for migration of peripheral blood monocytes into injured tissues. Monocytes then undergo final differentiation into specialized tissue macrophages (Bevilacqua *et al.*, 1981; Bianco, 1983). Monocyte attachment to ECM is mediated by specific interaction between a family of cell surface receptors, the integrins, and components of ECM, notably fibronectin (FN) (Yamada, 1989; Hynes, 1990, 1992). The RGDS (Arg-Gly-Asp-Ser) sequence present in the central cell binding domain of FN is the prototype of these sites (Pierschbacher and Ruoslahti, 1984a, 1984b; Pytela *et al.*, 1985a, 1985b, 1986; Ruoslahti and Pierschbacher, 1986, 1987; Buck and Horwitz, 1987; Hynes, 1987). Another cell-adhesion regions are found to be located within the alternatively spliced segment IIICS (or V region) and the HepII domain of FN (Humphries *et al.*, 1987; McCarthy, *et al.*, 1988). Moreover, the integrins bind to a wide variety of ligands including ECM glycoproteins such as laminin, collagen, fibrin, and glycosaminoglycans (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Hynes *et al.*, 1989; Hemler, 1990).

The integrins are membrane-spaning heterodimers, which have been divided into three major subfamilies, each containing a common β subunits associated with multiple α subunits (Hynes, 1987; Hemler *et al.*, 1988; Springer, 1989). Currently, there are at least 20 different integrin subunit combinations, and considerable effort has been directed towards elucidating their functional characteristics (Hemler, 1990).

It is still not known when monocytes begin to express integrins and how these receptors are regulated during the various stages of monocyte differentiation. In order to investigate further these events, FN and its tryptic fragments were purified to homogeneity and the interactions of cellular FN receptors in monocyte and macrophage with tryptic fragments were performed. The digestion fragments used in this study include (1) 85-kDa cell

attachment domain, (2) 58-kDa fragment containing HepII domains, and (3) 38-kDa fragment containing most but all of the IIICS domain. Also the relationship between FN receptors and FN-fragments during the course of differentiation of monocyte to macrophage were characterized based on the functional and structural features of the FN receptors. It was found that cell adhesion and (or) migration of monocyte/macrophage may be related with at least two different sites of FN.

Materials and Methods

Inbred Balb/c mice were obtained from Animal Breeding Center, Seoul National University and Sprague Dawley rats were from Animal Breeding Center in the College of Medicine, Kyungpook National University.

Purification of Fibronectin and Its Tryptic Fragments

FN was purified from citrated rat plasma by following the method of Ruoslahti and Engvall (1978). The 80-kDa fragment containing the RGD sequence and the 38-kDa fragment containing the HepII domain and most of the IIICS region were prepared by TPCK-treated trypsin digestion of FN (1:200, w/w, 90 min, 37°C) and subsequent gelatin-Sepharose and Sephadex G-100 gel filtration chromatography, as previously described (Johansson, 1985). Whereas the 31- and 29-kDa fragment were isolated by mild tryptic digestion of FN (1:1,000, w/w, 15 min, room temperature) as reported (Garcia-Pardo, 1987).

The concentrations of FN was determined by its extinction coefficient $E_{1\%}$=13, and those of FN fragments were by Bradford method (1976). The purity of FN and FN fragments used in this study was verified by SDS-PAGE (Laemmli, 1970).

Preparation of Monocyte and Macrophage

Monocytes were isolated from citrated rat blood (Boyum, 1968). Briefly, whole blood was diluted 1:1 in phosphate buffered saline (PBS) and was centrifuged through Ficoll-Hypaque (Pharmacia) cushion. The buffy coat cells were collected and washed, and then suspended in RPMI 1640/10% fetal calf serum to 5 x 10^6/ml. Cell viability as determined by trypan blue dye exclusion was greater than 90%.

Peritoneal macrophages were isolated from rat spleens according to the method of Mosher (1984). Rats were injected with 10 ml of 7% thioglycolate solution 3 days before obtaining peritoneal lavage. The cell suspension was incubated at 37°C in 5% CO_2/95% O_2 chamber for 2 hr. Nonadherent cells were removed by washing with RPMI 1640, and the adherent monocytes/macrophages were released by incubation with 5 mM ethylenediaminetetraacetic acid. Monocytes and macrophages were assessed by nonspecific esterase staining (Norris *et al.*, 1979).

Cell Attachment Assay

FN-coated plates were prepared by coating each well (Linbro plate) with FN or FN-fragment solutions, and incubated for 2 hr at room temperature. Plates were then rinsed with PBS and incubated at 37°C with 1% BSA to block nonspecific binding sites. The plates were rinsed again with PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and 100 μl of a cell suspension (5 × 10^5/ml) in binding medium was added to each well. After incubation at 37°C, nonadherent cells were removed by gently washing. Attached cells were fixed and stained with toluidine blue, and counted under an inverted microscope (Lavobert, Leitz). The number of attached cells was estimated by determining the absorbance at 600 nm using a Titertek Multiscan Plus (Flow Lab.)

For inhibition experiments, 5 × 10^5 cells in binding medium were preincubated for 1 hr at 4°C with appropriate dilutions of FN or FN fragments.

Acid Phosphatase Assay

Acid phosphatase activity of monocyte and macrophage was measured as described by Akiyama *et al.* (1988). Monocytes (2 × 10^5/ml) cultured in microtiter plates or FN-coated plates were lysed by adding 100 μl of 0.2% Triton-X 100 for 30 min at 37°C. Then one hundred μl of 10 mM *p*-nitrophenyl phosphate in 0.1 M citrate buffer (pH 4.5) was added to each well followed by

incubation at 37°C for 24 hr with gentle shaking. The absorbance was then measured at 405 nm with a Titertek Multiscan.

Results

The two subunits of the FN dimer are held together by a pair of interchain disulfides at the C-terminal end. Figure 1 displays tryptic digestion pattern and specific domains of FN. Trypsin digestion releases two different kinds of fragment containing the HepII domain with molecular mass of 38- and 58-kDa. The difference of molecular weights is due to its absence of IIICS region in B chain. Hence only the 38-kDa fragment comprises the first 67 amino acids of IIICS derived from the A chain. The 58-kDa fragment derived from the B chain does not contain the trypsin-cleavage site, present in the IIICS region.

As a first step to investigate the role of fibronectin and its receptors in differentiation of monocytes to macrophages *in vitro*, FN and its fragments from rat plasma were purified to homogeneity (Fig. 2) using gelatin-Sepharose, heparin-Sepharose, and DEAE-cellulose chromatography as described in 'Materials and Methods'.

The coating efficiency of each fragment was estimated to be 9% (85-kDa), 12% (58-kDa) and 15% (38-kDa). The calculated concentration required for 50% cell attachment was 28 nM (FN), 74 nM (85-kDa), and 98 nM (38-kDa), indicating that the two fragments of 85- and 38-kDa had similar cell attachment activity. Monocytes were attached to plastic wells coated with FN, 85-kDa and 38-kDa fragment in a dose-dependent manner (Fig. 3). In contrast, monocytes did not bind to the wells coated with the 29-, 31-, or 58-kDa fragment.

To assess the specificity of monocyte attachment to the surfaces coated with FN or 38-kDa fragment, monocytes were preincubated with the 85-kDa or with the synthetic peptide GRGDSPC prior to the addition to FN- or 38-kDa-coated wells. The preincubation of the cells with 85-kDa or with the synthetic peptide GRGDSPC inhibited the cell's attachment to FN-coated wells, but showed no effect on the cell's attachment to 38-kDa-coated wells (Fig. 4). Preincubation of the cells with soluble FN had not inhibited their subsequent adhesion to either FN- or 38-kDa-coated wells. Preincubation of monocytes with the soluble 38-kDa fragment slightly inhibited cell's attachment to FN-coated well, but abolished attachment to 38-kDa coated wells (Fig. 4A and B). This result indicates that monocyte recognizes 38-kDa domain distinctively

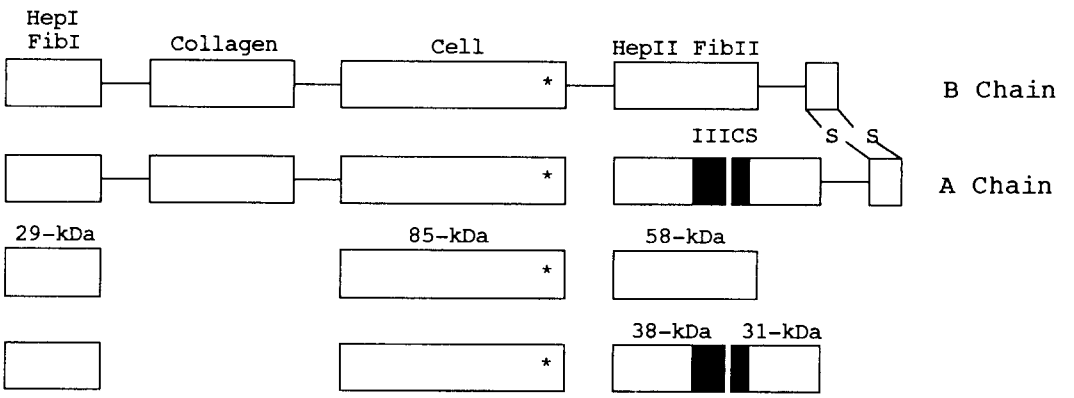

Fig. 1. Domain structure of rat plasma fibronectin. The 85-kDa fragment contains the cell binding domain (Cell) and the RGDS sequence of fibronectin (*). The 58- and 38-kDa fragment contains heparin binding domain II (HepII). The 38-kDa fragment comprises the first 67 amino acid residues of the alternatively spliced connecting segment of fibronectin (IIICS) indicating that is derived from the A chain. The 58-kDa fragment derived from B chain lacks the IIICS region (Garcia-Pardo *et al.*, 1990) and HepII and FibII domain.

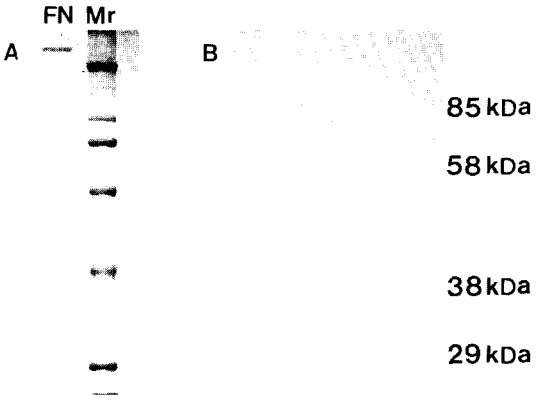

Fig. 2. Purified FN (A) and its tryptic fragments (B) from rat plasma. A polypeptide of the FN and each fragments were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Coomassie brillant blue R stained.

from its recognition of 85-kDa domain.

To examine whether the degree of monocyte differentiation to macrophages was influenced by the addition of FN, acid phosphatase activity of monocyte was measured along the culture period. The activity of lysosomal acid phosphatase tightly correlated with the morphological maturation of monocyte to macrophage *in vitro*. The enzyme activity increased linearly from day 1 to day 9 of the cell culture (Fig. 5A). The coated well with FN promoted effectively the macrophage maturation. In contrast, monocytes cultured in serum-free media did not bring the cells to the state of fully differentiated macrophage within 9 days. In addition, increase in the acid phosphatase activity of these cells was not significant during the culture periods (data not shown). Upon preincubation with 85-kDa, GRGDSPC peptide or 38-kDa fragment, the acid phosphatase activity of monocytes was decreased as compared to that of

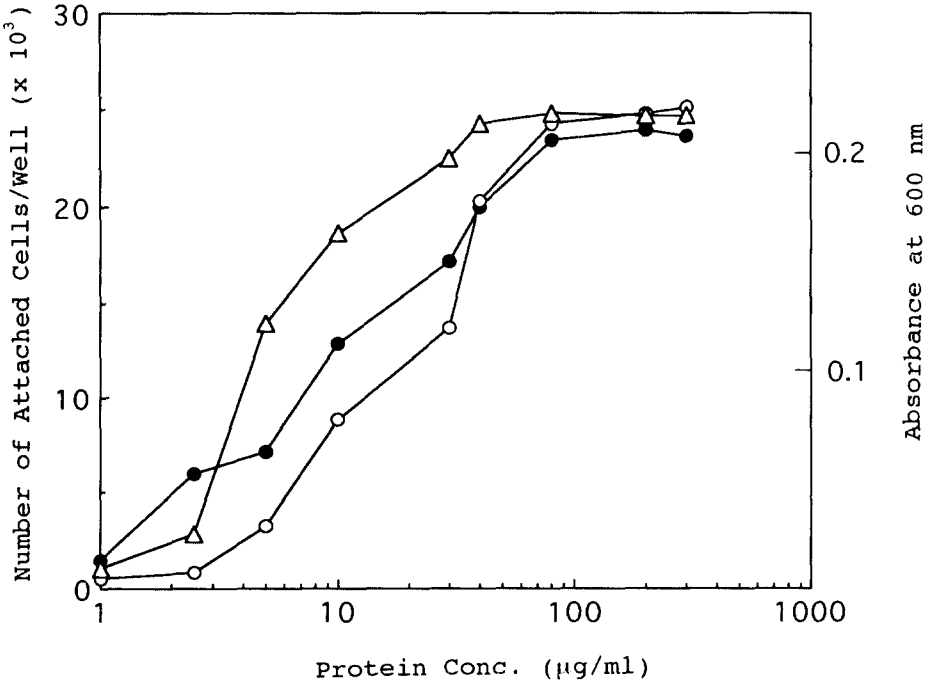

Fig. 3. Attachment of rat monocyte to FN (●) and its fragments, 38-kDa (△), and 85-kDa (○). Microtiter wells were coated with FN or its fragments followed by addition of monocytes (5×10^4/well) and incubation at 37°C for 30 min. The degree of cell attachment was quantified by counting the cells after toluidine blue staining and(or) measuring absorbance at 600 nm. Values are from three independent experiments.

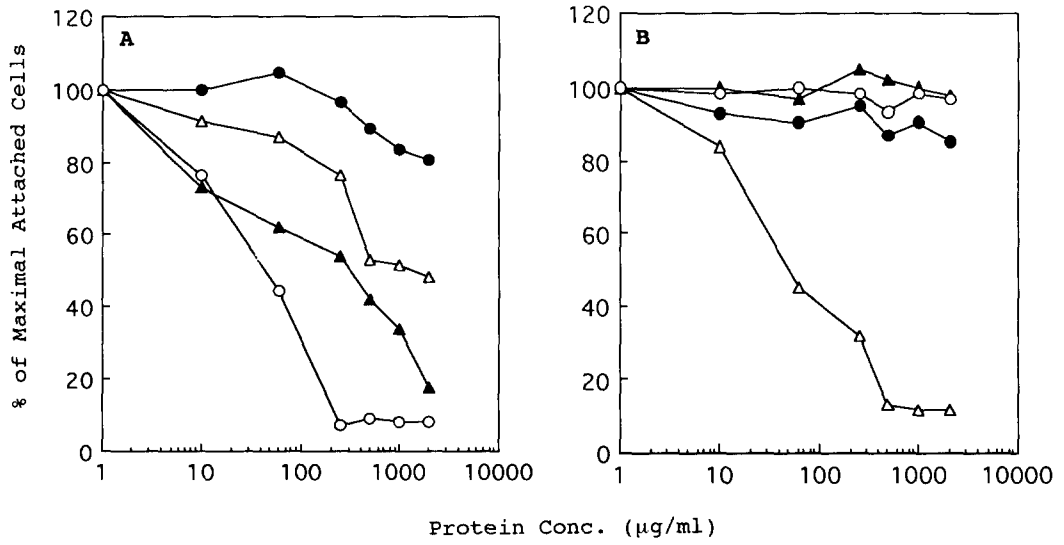

Fig. 4. Dose dependent inhibition of rat monocyte attachment by soluble FN or its fragments against to FN (A) and 38-kDa fragments (B) coated wells. The synthetic peptide GRGDSPC (▲), soluble FN (●), 85-kDa (○), or 38-kDa (△) were used as inhibitors. Cells were preincubatede for 30 min with inhibitors and added to FN (25 μg/ml) or 38-kDa (50 μg/ml)-coated wells. Values represent the average number of attached cells in control wells from three separate experiments.

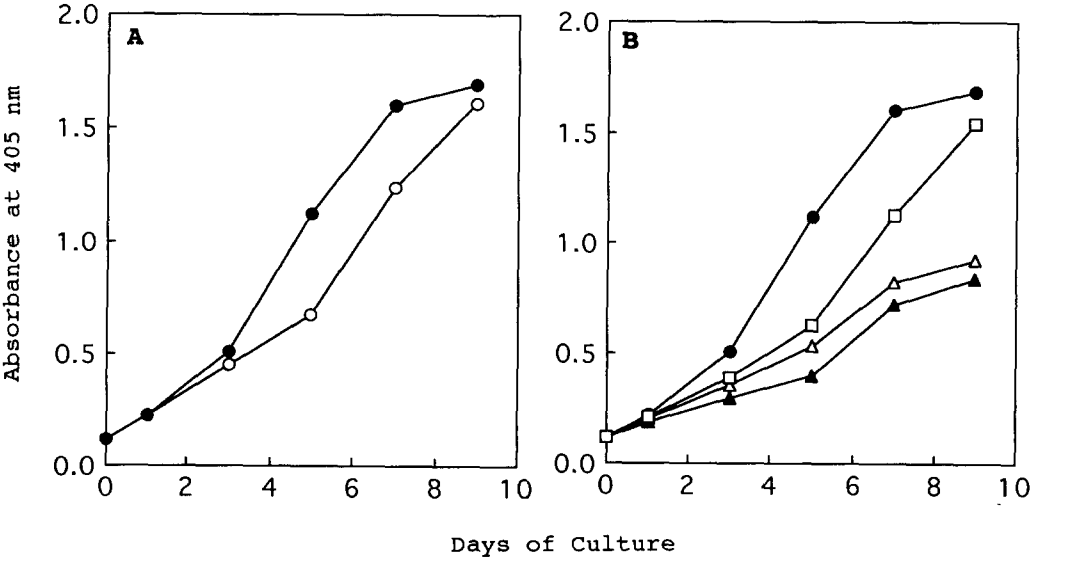

Fig. 5. Acid phosphatase activity of monocytes. (A) Monocyte (5×10^5/ml) were cultured in microtiter wells (○) or in FN-gelatin coated wells (●) with RPMI containing 20% rat serum. (B) Monocyte (5×10^5/ml) were preincubated with synthetic peptide GRGDSPC (▲), 85-kDa (△) or 38-kDa (□) fragments and cultured in FN-gelatin coated wells. The cells were lysed at indicated culture day and reacted with 10 mM *p*-nitrophenylphosphate/0.1 M citrate buffer (pH 4.5) for 12 hr, and the absorbance was measured at 405 nm.

control (Fig. 5B). Intact FN and 85-kDa fragment showed strong inhibition.

Discussion

Many of the monocyte functions are regulated by specific interactions between plasma membrane receptors and components of the ECM. The attachment of monocytes to surfaces containing FN, one of important components of the ECM, not only serves to localize the cells in the matrix but also leads to expression of macrophage-associated activities such as changes in the expression of membrane receptors, enhanced phagocytosis, appearance of oxidative metabolites and secretion of growth factors (Bevilacqua *et al.*, 1981; Bianco, 1983; Pommier *et al.*, 1983; Wright *et al.*, 1983; Brown, 1986). Several investigators have reported cell-mediated functions for the fragments of FN. These functions have been described on numerous cell types including normal and transformed fibroblasts, endothelial cells, and human macrophages.

To characterize further the interactions between FN receptors in monocyte and macrophage and cell-binding domains of FN, FN and its tryptic fragments were purified (Fig. 2). Trypsin digestion releases two different kinds of fragment containing the HepII domain with molecular mass of 38- and 58-kDa. The difference of molecular masses is due to its absence of IIICS region in B chain. Hence, only the 38-kDa fragment comprises the first 67 amino acids of IIICS derived from the A chain. The 58-kDa fragment derived from the B chain does not contain the trypsin-cleavage site, present in the IIICS region. And yet the 38-kDa and 58-kDa fragments have identical NH_2-terminal sequences (Fig. 1).

From the cell attachment and attachment-inhibition assay (Fig. 3 and 4), it was found that monocyte recognizes 38-kDa domain distinctively from its recognition of 85-kDa domain, and that monocyte interaction with the 38-kDa domain does not involve the RGDS sequence and the cell surface receptors for 85-kDa fragment. The experimental results also showed that the heparin-binding site of the 38-kDa fragment is not involved in monocyte adhesion, because two other fragments, the 29-kDa fragment containing the HepI domain and the 58-kDa fragment containing the HepII domain, failed in monocyte attachment (Fig. 3). Monocyte attached effectively to FN-coated well, whereas soluble FN did not inhibit monocyte attachment to FN-coated well (Fig. 4B). Therefore, it can be suggested that conformational changes in FN are functionally important as in hepatocyte-FN interaction reported by Johansson and Hook (1984).

The activity of lysosomal acid phosphatase tightly correlated with the morphological maturation of monocyte to macrophage *in vitro*. The enzyme activity increased linearly from day 1 to day 9 of the cell culture (Fig. 5A). The coated well with FN promoted effectively the macrophage maturation. Upon preincubation with 85-kDa, GRGDSPC peptide or 38-kDa fragment, the acid phosphatase activity of monocytes was decreased as compared to that of control (Fig. 5B). Intact FN and 85-kDa fragment showed strong inhibition. This indicates that FN influences the differentiation of monocytes to macrophage, especially through by 85-kDa.

The presence of different cell-binding sites on FN may indicate distinct functional significance. For example, cell interactions with a second FN site may regulate the delivery of intracellular signals. Fibroblasts, the major producers of cellular FN, can synthesize FN molecules with and without IIICS region. Therefore, fibroblast could play a role in the regulation of monocyte interactions with extracellular matrices by varying the type of FN deposited. Plasma FN contains both cell-binding sites, the RGDS and the IIICS region as showed above. Thus, extravasation of plasma FN during inflammatory processes generates a uniform matrix with high attachment capacity for monocytes accumulation and differentiation. In this report we present the isolation and characterization of FN and its fragments from rat plasma. At least two domains may mediate the binding activity of FN and surface receptor and is critical responsible for the adhesion and migration of monocyte and macrophage.

Acknowlegement

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단핵구 분화에 대한 Fibronectin 및 그 단편의 역할

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혈구세포의 한 종류인 단핵구가 혈액을 따라 순환하다가 세포외 기질이나 혈관 내피세포에 결합하면, 혈관을 빠져나가 간, 폐, 복강, 및 비장 등의 조직 특이 대식세포로 분화하게 된다. 이러한 일련의 분화과정에서 fibronectin (FN)과 세포표면에 존재하는 FN-수용체의 역할을 규명하기 위하여 쥐의 혈액으로부터 FN을 순수분리한 다음, trypsin으로 절단한 후 각종 chromatography방법으로 여러 단편들은 분리하였다. 분리간 각 단편으로 단핵구의 세포부착능 분석과 부착능 저해 실험을 실시하여 세포부착에 관여하는 FN-단편을 검정하였다. 단핵구에서 대식세포로의 분화시에 fibronectin분자내 적어도 두개의 85-kDa domain과 38-kDa domain이 세포의 부착 및 이주에 관여하며, 이들 domain의 각각에 대한 세포표면 수용체의 발현 양상이 변화함에 따라 분화의 조절기능이 이루어질 것으로 추정됨을 알았다.