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A Functional Role of Fibronectin Receptors in the Monocyte Differentiation

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The interaction between fibronectin (FN) and its receptors controls cell attachment and migration, two crucial events during monocyte development and differentiation. To investigate the functional role of FN and its receptor, we have studied adhesion of monocyte to two different regions of FN (38- and 85-kDa domain), as well as the expression of the integrin during monocyte differentiation. Anti-integrin β1 subunit antibody completely blocked the attachment of FN-coated latex beads to macrophage, but the effect of anti-integrin α4 antibody was much less significant. Rat monocyte expressed integrin α4β1 predominantly, while macrophage expressed α5β1 as analyzed by flow cytometer and western blot. From these results, it can be suggested that these two integrins play different roles during monocyte differentiation.

KEY WORDS: Fibronectin Receptor, Monocyte/Macrophage, Differentiation

Fibronectin (FN) has a remarkably wide variety of functional activities. The capacity of FN to interact with different molecules indicates its involvement in a variety of biological processes such as embryonic development, cell adhesion and migration, wound healing, and tumor cell metastasis (Carsons *et al.*, 1989; Colvin, 1989; Ginsberg and Plow, 1989; Thiery *et al.*, 1989). FN contains distinct functional domains linked by protease sensitive regions. A central domain containing the Arg-Gly-Asp sequence, known as the cell binding domain, can promote adhesion by interaction with a cell surface receptor (Ruoslahti and Pierschbacher, 1986, 1987; Hynes, 1987; Yamada, 1989).

FN has been found to play a role in host defence and immunoregulation that is independent of its function as an adhesion molecule. Soluble FN and FN fragments induces the differentiation of hematopoietic progenitor cells, displays chemotactic activity for monocytes, and stimulate tumor necrosis factor secretion (Clark *et al.*, 1988; Weistein *et al.*, 1989; Beezhold and Personius, 1992).

Migration across the vascular endothelium to gain access to the sites of inflammation and tumor growth requires monocyte recognition of extracellular matrix (ECM) molecules (Pommier *et al.*, 1983; Ruoslahti, 1988). The cellular receptors that recognize these sites belong to the integrin family, which are heterodimeric integral membrane glycoproteins composed of noncovalently associated α- and β-subunits (Helmer *et al.*, 1988; Hynes, 1990, 1992). Thus, the FN/integrin-mediated signal seems to be of fundamental importance in immune differentiation.

The various events involved in monocyte interactions with FN have not been examined extensively. In our previous studies, rat monocyte

interacts with at least two different sites in FN, that is, monocyte recognizes 38-kDa domain distinctively from its adhesion of 85-kDa domain (Bang *et al.*, 1993). To investigate further events, attachment inhibition assay of monocyte which is pretreated with anti-integrin subunit was performed. Also the expression and regulation of integrin during the course of differentiation of monocyte to macrophage were characterized.

Materials and Methods

Materials

Sprague Dawley rats were from Animal Breeding Center in the College of Medicine, Kyungpook National University. Hank's balanced salt solution, fetal calf serum, RPMI 1640, and other culture media and equipments were purchased from Flow Lab (Worth Lyde, Australia) or GIBCO (Grand Island, USA). Mouse anti-human β1 integrin and anti-human α4 monoclonal antibodies were purchased from Chemicon (Temecula, USA).

Preparation of monocytes and macrophages

Monocytes were isolated from citrated rat blood through Ficoll-Hypaque cushion (Boyum, 1968) and macrophages were isolated from rat spleens according to the method of Mosher (1984). Monocytes and macrophages were assessed by nonspecific esterase staining (Norris *et al.*, 1979).

Bead binding and phagocytosis assay

Polystyrene latex beads were incubated with 340 μg/ml of FN for 10 min at 22°C and blocked with 2% BSA. The coated beads were collected by centrifugation and resuspended in RPMI 1640 with 2% BSA. Monocytes (1×10^6 cells/ml) and FN-coated beads (1.3×10^6) were incubated for 2 hr and half of the samples were treated with 0. 25% trypsin for 5 min to remove surface-bound beads. The number of binding or ingested beads was measured by counting the cell-associated beads under dark field microscopy. Quantitation of the data was based on the counts obtained from 100-200 cells.

Immunofluorescence assay

Cells were incubated with anti-integrin β1 or $-\alpha$4 antibody for 30 min. Cells were washed twice with cold PBS containing 1% BSA and reacted with fluorescein-conjugated F(ab')2 fragments of goat antibodies to mouse IgG for 30 min. Fluorescence measurements were obtained by using fluorescence activated cell sorter (FACS; Beckton-Dickinson, USA). A primary 200-mW argon laser tuned to 488 nm was used to excite FITC and the fluorescence emission peak at 530 nm was monitored.

Western blot analysis

For western blotting, lysates of monocyte and macrophage were prepared as described (Pytela *et al.*, 1985) and separated by SDS-PAGE. Proteins were transferred to nitrocellulose sheets by standard procedure (Towbin *et al.*, 1979). The membrane was washed with Tris-buffered saline containing 0.1% Tween 20, and incubated with anti-integrin β1 or $-\alpha$4 antibody. After incubation with secondary antibody, blots were detected using ECL kit (Amersham) and exposed to X-ray films.

Other methods

FN and its tryptic fragments were purified from rat plasma according to the method of Bang *et al.*, (1993). For inhibition of cell attachment assay, cells were preincubated with diluted antibody and plated to FN fragment coated well.

Results and Discussion

Interactions between fibronectin and its receptors on monocyte and macrophage are involved in cell adhesion and differentiation. The presence of different cell-binding sites on FN may indicate distinct functional significance. Therefore, cell attachment to FN fragment and phagocytic activity of rat macrophage were analyzed using anti-FN-receptors. These antibodies were not toxic to monocyte and macrophage upon incubation for 1 hr at 37°C as assessed by trypan blue exclusion (data not shown). Anti-integrin β1 subunit antibody completely blocked the attachment of FN-coated

latex beads to macrophage, but the effect of anti-integrin $\alpha 4$ antibody was much less significant (Table 1).

Moreover, preincubation of monocytes with the anti-integrin $\beta 1$ strongly inhibited the cell attachment to both 85-kDa and 38-kDa fragment coated wells at 1/100 antibody dilution (Fig. 1). In contrast, anti-integrin $\alpha 4$ showed little or no inhibition in monocyte adhesion to 85-kDa-coated wells at all antibody dilution tested (Fig. 1A), and yet were capable of complete inhibition of the cell's attachment to 38-kDa-coated wells at 1/100 antibody dilution tested (Fig. 1B). Therefore, it can be said that anti-integrin $\beta 1$ interact with all kind of FN receptors that recognize intact FN, 85-kDa and 38-kDa domain, while anti-integrin $\alpha 4$ interact with receptor recognizing only 38-kDa domain.

Expression patterns of integrin in monocyte and macrophage were analyzed by FACS. A majority of cells in the monocytes and macrophages population, defined by its light-scattering properties, were intensely stained with both anti-integrin $\beta 1$ and anti-integrin $\alpha 4$. From FACS analysis, the expressions of integrin on monocyte and macrophage were compared by mean fluorescence intensity (Table 2). As shown in Table 2, monocyte expressed $\alpha 4$ integrin predominantly, while the macrophage expressed $\alpha 5$. Western blot analysis also showed that $\beta 1$

Table1. Comparison of phagocytic actiity of macrophages from rat spleen.

Parameter Measured	Macrophages	Cell Treatment*	
		Anti-$\beta 1$	Anti-$\alpha 4$
Number of bound beads/cell	5.1 ± 0.2	0.7 ± 0.6	3.7 ± 0.5
Percent of cells with bound beads	45.7	3	32.0
Number of ingested beads per cell	4.4 ± 0.3	0.6 ± 0.2	3.1 ± 0.6
Percent of cells with ingested beads	41.7	4	29.6

* Cells were preincubated with anti-$\beta 1$ or anti-$\alpha 4$ for 2 hr and then incubated with FN-coated beads and half of the samples were treated with trypsin toremove surface-bound beads in bead ingestion assays.

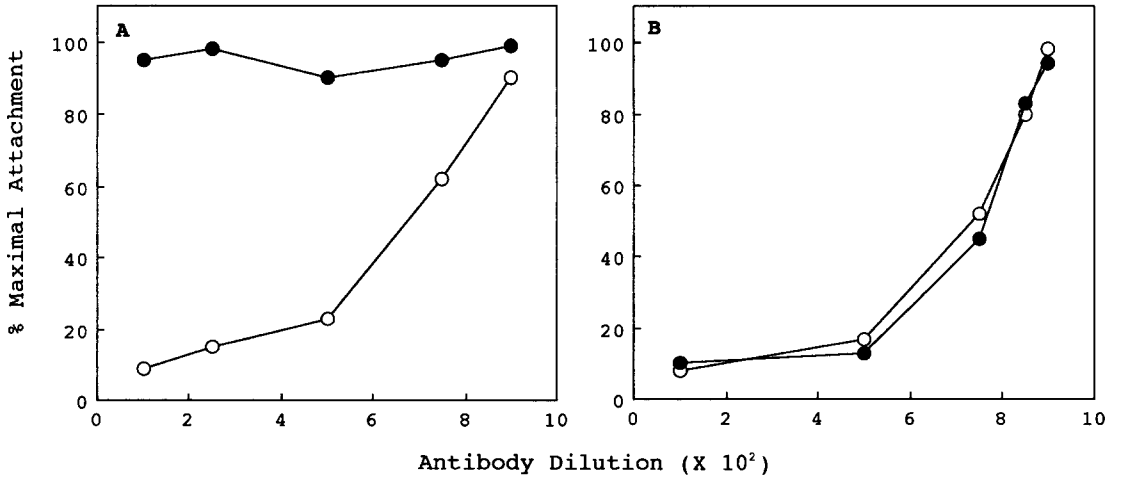

Fig. 1. Inhibition of monocyte cell attachment to 85-kDa (A) and 38-kDa (B) fragments by anti-$\beta 1$ (○) or anti- 4 (●). Cells were preincubated for 2 hr with the indicated dilutions of antibodies and plated to 85-kDa (10 μg/ml) or 38-kDa (5 μg/ml) coated wells. Cells were incubated at 37°C for 30 min and attachment was quantitated. Points shown are the means of triplicate experiments.

integrin subunit was increased and $\alpha 4$ integrin was decreased during differentiation of monocyte to macrophage (Fig. 2). These results indicate that $\alpha 5\beta 1$ and $\alpha 4\beta 1$ involved in cell adhesive interaction are distinctly regulated during cell differentiation. Increased adhesion of cultured monocyte to FN appears to be primarily due to the up regulation of $\alpha 5\beta 1$ receptors at the cell surface. Up regulation of $\alpha 5\beta 1$ expression was previously observed in THP-1 monocytic cells after treatment with transforming growth factor-β_1

Table 2. Expression of integrin $\beta 1$ and $\alpha 4$ fibronectin receptors on monocyte and macrophage

Antibody	Mean Fluorescence Intensity[a]	
	Monocyte	Macrophage
$\beta 1$	113	161
$(\alpha 5)^b$	(31)	(99)
$\alpha 4$	82	62

a: Values converted from a logarithmic scale to linear units.

b: Values calculated from the values of $\beta 1$ and $\alpha 4$

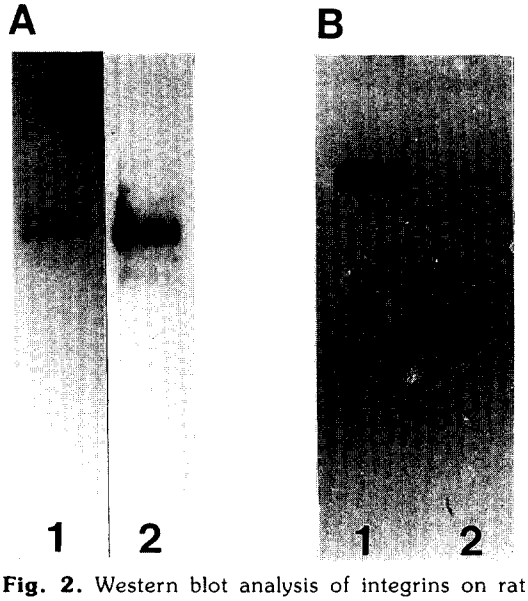

Fig. 2. Western blot analysis of integrins on rat monocyte and macrophage. One hundred μg of monocyte (lane 1) and macrophage (lane 2) lysate was electrophoresed by SDS-PAGE. After transfer to membrane, blots were incubatde with anti-integrin $\beta 1$ (A) or anti-integrin $\alpha 4$ antibody (B), and exposed to x-ray films by using ECL detection kit.

and in murine peritoneal macrophages after inflammatory stimuli (Ignotz and Massague, 1987; Holers *et al.*, 1989). Thus, up-regulation of the $\alpha 5\beta 1$ integrin appears to be concomitant with cell activation and differentiation events. The signal tranduction events involved in monocyte interactions with FN have not not been examined. Recent evidence has suggested that 120-KDa fragment of FN induced early signal tranduction pathways result in the activation of protein kinase C (Chang *et al.*, 1993). Therefore, although both integrins of monocyte are able to bind to FN, the intracellular signals that they deliver are likely to be different. This fact may be crucial for monocyte differentiation and may explain why monocyte would bear two different receptors for the same ligand, the detailed mechanism of which remains to be elucidated.

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단핵구 분화에 있어서 fibronectin 수용체의 역할

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단핵구의 발생 및 분화에 있어서 중요한 과정인 세포 부착과 이주는 세포외기질 성분의 하나인 fibronectin(FN)과 그 수용체의 상호작용에 의해 일어난다. 이러한 FN과 그 수용체의 역할을 규명하기 위하여, FN의 38- 와 85-kDa domain에 대한 단핵구의 부착능 저해실험과 단핵구 분화에 따른 FN 수용체의 발현양상을 알아 보았다. FN 수용체인 $\beta 1$ integrin의 항체를 전처리한 대식세포의 경우 세포부착이 억제되었으나, $\alpha 4$ 수용체의 항체에 대해서는 억제효과가 나타나지 않았다. 단핵구 세포에서는 $\alpha 4\beta 1$ 수용체의 발현이 증가한 반면, 대식세포로의 분화시에는 $\alpha 5\beta 1$ 수용체의 발현이 증가하는 것으로 나타났다. 이러한 결과를 보아, 단핵구 세포에서 대식세포로의 분화시 두가지 FN 수용체가 서로 다른 역할을 담당할 것으로 추정되었다.