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# Monocyte macrophage differentiation *in vitro*: Fibronectin-dependent upregulation of certain macrophage-specific activities

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**Abstract** Transendothelial migration of monocytes followed by their differentiation into macrophages involves interaction of monocytes with subendothelial matrix. The influence of extracellular matrix on monocyte–macrophage differentiation was studied using an *in vitro* model system with human PBMC maintained on different matrix protein substrata. Upregulation of macrophage specific marker activities such as endocytosis of modified proteins, changes in expression of cell surface antigen, and production of matrix metalloproteinases was studied. Cells maintained on Fibronectin (Fn) showed significantly higher rate of endocytosis and production of MMP2 and MMP9 when compared to other matrix protein substrata. Immunoblot analysis, ELISA, and zymography showed that Fn-dependent upregulation of MMPs was blocked by antibodies to $\alpha_5\beta_1$ integrin indicating that the Fn effect was mediated by integrins. The Fn effect on mo–mΦ was blocked by genistein and herbimycin. As monocytes differentiate to macrophages there was an increase in the rate of production of Fn. These results indicate the influence of the microenvironment of the cell, particularly Fn, on mo–mΦ differentiation and integrin-mediated downstream signaling through focal adhesion kinase and Src type tyrosine kinase is involved in this.

**Keywords** Monocyte–macrophage · MMPs · Fibronectin · Integrin signaling

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## Introduction

Adhesion of circulating monocytes (mo) to the endothelium and their migration into the subendothelial matrix form the crucial early event in the formation of macrophages (mφ) [1]. Macrophages are versatile cells found in every tissue where their chief function is as antigen presenting cells to T cells. Mφs also play a crucial role in normal as well as in many pathophysiological conditions particularly in the development of early lesions of atherosclerosis [2]. Another important feature involved in host defense is the scavenger function where they mediate the uptake of unphysiologic modified protein. Apart from the endocytotic potential, mφs are also involved in the secretion of growth factors, inflammatory mediators and cytokines, which induce or modulate the proliferation of cells [3, 4].

Monocytes and macrophages are the basic cells of the reticuloendothelial system originating from the bone marrow and undergoing differentiation from stem cells to blood monocytes and finally to tissue-specific macrophages. Macrophages are seen in almost all tissues where they have their own tissue-specific functions which include the histiocytes of the connective tissue, Kupffer cells of liver, alveolar mφs of the lungs, free and fixed mφs of the spleen and the lymph, pleural, and peritoneal mφs of the serous fluids, histiocytes and Langerhans cells of the skin, etc. [5]. Monocytes can be considered as immature mφs because upon migration into tissues, they undergo differentiation to multifunctional tissue mφs. Monocytes also represent the circulating mφ population which are fully functional for their location when they change their phenotype in response to factors encountered in the microenvironment of specific tissue, particularly the nature of extracellular matrix (ECM) components leading to mφ heterogeneity.

Mo–mφ differentiation is favored *in vivo* and *in vitro* by various diffusible and non-diffusible factors. Several endogenous ligands are found to be responsible for monocyte activation and mo–mφ differentiation [6], which includes modified unphysiologic proteins, certain growth factors, and cytokines which in turn mediate their effect through the activation of certain transcription factors. PPARs, which belong to the nuclear hormone receptor superfamily, are reported to play an important role in inflammatory response. PPARγ is induced in human monocyte-derived macrophages when exposed to unphysiologic proteins. Ligand activation of PPARγ:RXRα heterodimer in myelomonocytic cell lines induce changes characteristic of macrophage differentiation such as the transcriptional induction of scavenger receptors for the uptake of modified proteins [7]. The differentiation of monocytes to macrophages prepares the cell for active participation in inflammatory and immune responses which are mediated through the activation of transcription factor, NFκB [8].

The ECM, which is an intricate assembly of proteins such as collagens, laminin, fibronectin, etc., influences various cellular activities and differentiation [9–11]. Monocytes during their egress from circulation encounter with the subendothelial matrix components of a particular tissue and differentiate into tissue specific macrophages. Studies have shown that mo–mφ differentiation is modulated by the chemical nature as well as the physical state of the matrix protein substrata. Interaction of monocytes with non-enzymatically glycated matrix protein results in a faster rate of induction of mo–mφ differentiation leading to foam cell formation, which is a critical event occurring in diabetic conditions eventually leading to the initiation and development of atherosclerosis [12]. The degree of expression of mφ specific function is also influenced by the nature of the matrix protein. Mφ specific activities such as endocytosis and production of MMPs were significantly higher in monocytes maintained on Fn and Col I than on Col IV indicating that cells which contact with stromal components differentiate at a faster rate [13]. Another factor crucial in regulating the cell behavior and differentiation is the shape of the cell as provided by the matrix which was evident from the rapid rate of differentiation of monocytes to macrophages when they were maintained *in vitro* on three dimensional Col I lattice [14]. Morphological analysis revealed that cells adhering to different matrix proteins acquire different shapes showing that mechanical properties of ECM are critical in regulating actin cytoskeletal organization and signaling [15].

Individual components of the matrix have been shown to interact with cells through specific matrix receptors causing the generation of signals to modulate various intracellular activities in a specific tissue-dependent manner [16]. Results have indicated that integrin-mediated downstream signaling is required to induce intracellular events associated with mo–mφ differentiation [17].

Although there are limitations in studying the specific marker activities of tissue specific macrophages, a general approach can be made to assess the extent of differentiation of PBMC to macrophages by monitoring the upregulation of macrophage-specific activities such as production of MMPs, changes in the expression of cell surface antigens, endocytotic potential as well as the down regulation of monocyte-specific functions such as myeloperoxidase activity and changes in the cell surface antigen expression. The studies carried out using human PBMC maintained *in vitro* on different matrix protein substrata indicate that interaction of monocytes with vessel-wall matrix leading to the differentiation to macrophages occurs in a matrix-dependent manner.

## Materials and methods

### Isolation and culture of PBMC

PBMC were prepared from peripheral blood of healthy donors using Histopaque 1077 as described by Huh *et al.* [18]. Cells were collected, washed, and resuspended in RPMI-1640 medium and seeded into matrix protein-coated culture dishes. Non-adherent cells were removed and the adherent cells were maintained in RPMI-1640 medium supplemented with penicillin (100 U/l), streptomycin (100 mg/l), gentamycin (100 mg/l) as described before and maintained in culture under 5% $CO_2$ atmosphere at 37°C in a forma $CO_2$ incubator. Cells were dislodged with PBS containing 2 mM EDTA at 4°C and the phenotypic and functional characteristics of monocyte-derived macrophages were analyzed.

### Immunofluorescence microscopy

PBMC were maintained in culture on matrix and non-matrix protein-coated coverglass slips and were blocked with 1% goat serum for 2 h followed by paraformaldehyde (1%) fixing for 10 min at 4°C. Cells were treated with primary antibody to CD14 or CD71 overnight at 4°C followed by FITC-conjugated secondary antibody for 1 h at RT in dark. Cells fixed on coverslips were thoroughly washed with PBS and viewed under a fluorescence microscope. Flow cytometric analysis was done using a FACS Vantage (B.D., CA, USA) and a 488 nm argon laser as detailed before [12].

### Zymography

Activities of MMPs in the medium secreted by cells were determined by gelatin zymography [19]. Zymogram gels

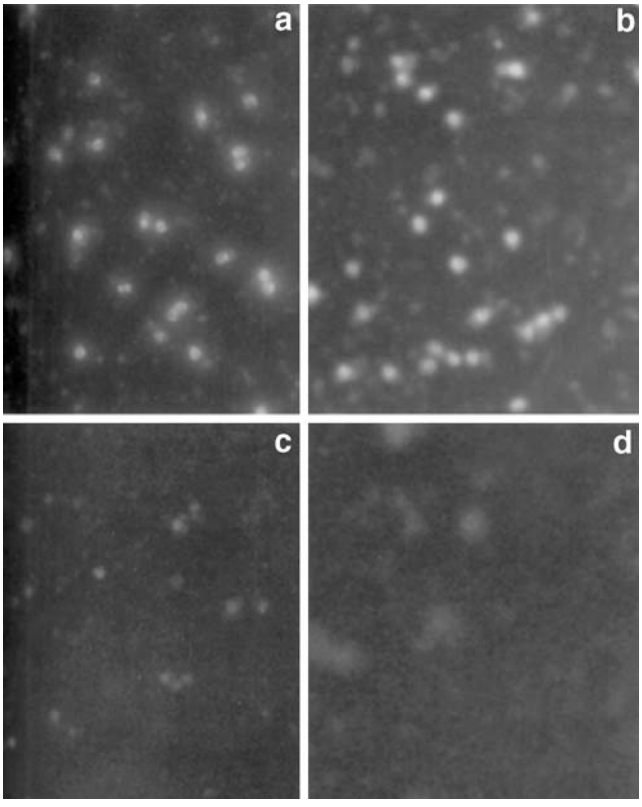


**Fig. 1** Immunofluorescence staining of surface antigens: Mononuclear cells were maintained on Col I-coated coverslips for 4 and 96 h. Cells were fixed and treated with primary antibody for CD14 and CD71 followed by FITC-labeled secondary antibody and viewed under fluorescence microscope. (a) $CD14^+$ 4 h 20×, (b) $CD71^+$ 96 h 20×, (c) $CD14^-$ 96 h 20×, (d) $CD71^-$ 4 h 20×

consisted of 7.5% polyacrylamide gel polymerized together with gelatin (1 mg/ml). After electrophoresis the gels were washed with Triton X-100 and incubated with substrate buffer (50 mM Tris–HCL, 5 mM $CaCl_2$, 0.02% $NaN_3$, pH 7.5) at 37°C for 24 h and stained with Coomassie brilliant blue R-250, and destained with water.

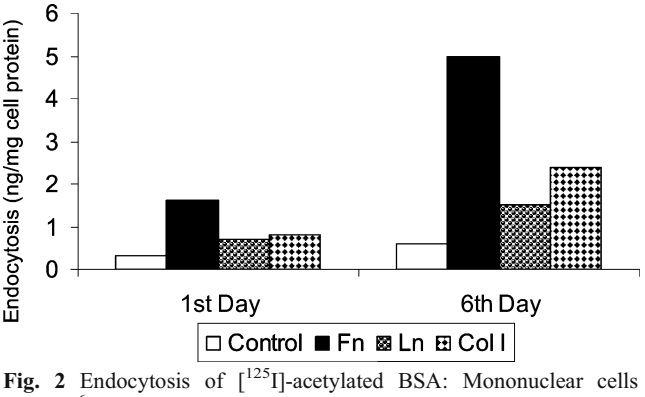


**Fig. 2** Endocytosis of [$^{125}$I]-acetylated BSA: Mononuclear cells ($1.5\times10^6$ cells/ml) were maintained in culture on Fn, Col I, Ln, or Poly lysine-coated multiwell plates for different time intervals. Cultures were supplemented with [$^{125}$I]-acetylated BSA (3.1 μg/ml) on the first day and the sixth day of culture. The amount of protein endocytosed after 6 h incubation at 37°C was measured. Values given are averages of triplicate cultures

Immunoblot analysis

Cells were lysed in lysis buffer and subjected to SDS-PAGE on 10% acrylamide gels. Proteins were transferred from mini gels to nitrocellulose membrane using a semi-dry blotting system and were probed with specific antibody and developed using HRP-conjugated secondary antibody [20].

ELISA for MMPs

MMPs in culture medium were quantified by ELISA. Cell culture medium pre-coated onto ELISA plates served as the antigen. Using specific antibodies to MMP2 and MMP9, the production of MMPs was quantitatively estimated using *o*-dianisidine as substrate for HRP [21].

Endocytosis

Endocytosis of modified protein was studied by supplementing the culture medium with radioiodinated acetyl BSA for 6 h at 37°C. Acetylation and radioiodination of BSA were done as previously described [22, 23]. The cellular uptake (acid precipitable) and degradation (acid soluble) of [$^{125}$I]-acetyl BSA were determined by precipitation with 10% TCA and radioactivity was measured in a LKB minigamma counter. Protein was estimated by the method of Lowry *et al*. [24].

## Results

To study the influence of extra cellular matrix on monocyte macrophage differentiation, an *in vitro* model system,

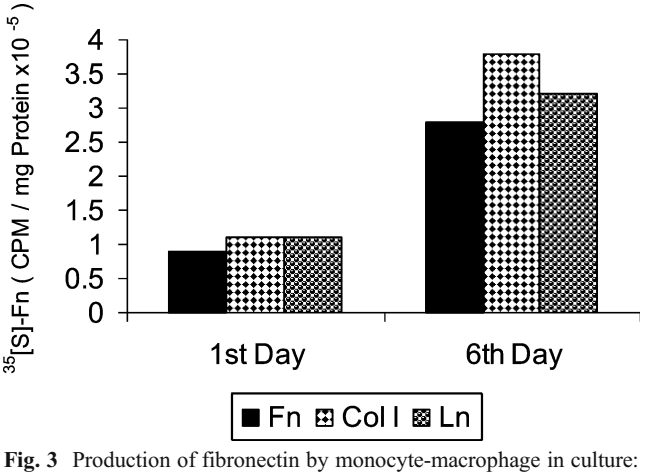


**Fig. 3** Production of fibronectin by monocyte-macrophage in culture: Mononuclear cells were maintained in culture on Fn-, Col I-, Ln-coated multiwell plates for different time intervals. Cultures were metabolically labeled with [$^{35}$S]-methionine (25 μCi/ml) for 20 h on the first day and the sixth day. Medium was collected; Fn was immunoprecipitated and electrophoresed. Values given are averages of triplicate cultures

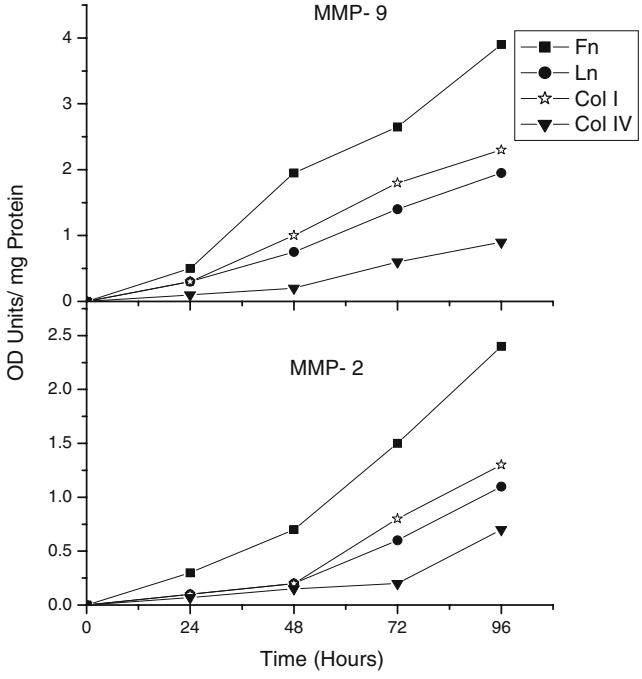


**Fig. 4** Production of MMPs by monocyte-macrophage in culture: Mononuclear cells were maintained in culture on plastic petri dishes coated passively with different matrix proteins, Ln, Fn and Col I and poly lysine-coated multiwell plates served as control. Medium was collected at different time intervals and MMP2 and MMP9 protein levels were determined by ELISA. Values given as OD units are the average of triplicate cultures done in duplicates

which was developed in our laboratory, was used. Isolated peripheral blood mononuclear cells were maintained in culture on specific matrix protein substrata and the rate of loss of monocyte-specific markers and rate of appearance of macrophage-specific activities were analyzed as a measure of monocyte differentiation to macrophages. Expression of monocyte specific surface antigen CD14, the LPS receptor and the macrophage specific surface antigen CD71 were studied by immunofluorescence analysis (Fig. 1). During the initial stages of culture, while CD14 was present, no CD71 staining could be observed. During later stages, after 96 h, the staining corresponding to CD14 decreased while significant fluorescence staining for CD71 could be observed, indicating the expression of macrophage-specific surface antigen. The rate of loss of CD14 was significantly more in cells maintained on Fn (< 20% of initial level) when compared to those maintained on Col IV or non-matrix protein. The rate of loss of monocyte-specific myeloperoxidase was significantly more in cells maintained on Fn substrata than that on cells maintained on non-matrix proteins; after four days in culture, only less than 10% of the initial level of myeloperoxidase was detected. Freshly isolated monocytes have not endocytosed any significant amount of modified protein such as acetylated BSA. As the cells were maintained in culture, the ability to endocytose and degrade the radioiodinated modified protein increased, and this was greater in cells maintained in culture on Fn substrata than in either laminin or Col I (Fig. 2).

## Production of fibronectin by monocyte–macrophage in culture

The ability of mo–mφ system to produce fibronectin was studied by metabolic labeling. Freshly isolated cells in culture did not produce significant amounts of Fn. With the progression of culture, there was a progressive increase in the amount of Fn produced by mo–mφ in culture and on the fourth day there was about five fold increase in the level of Fn produced. The amount of Fn produced by the cells as they differentiate to macrophages appeared to depend on the nature of matrix protein substrata. Cells maintained on

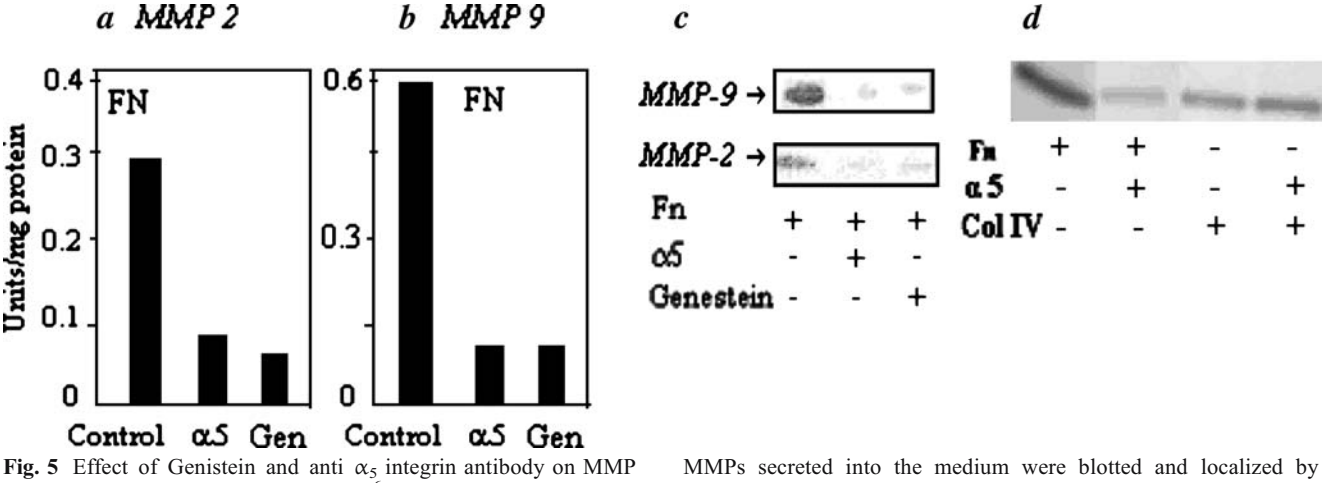


**Fig. 5** Effect of Genistein and anti $\alpha_5$ integrin antibody on MMP production: Mononuclear cells ($1.5\times10^6$ cells) were pretreated with 10 μl of $\alpha_5$ integrin antibody and genistein (50 μM) for 30 min and maintained on Fn- or collagen-coated substrata for 24 h. Cell-associated MMP2 (a) and MMP9 (b) were determined by ELISA. MMPs secreted into the medium were blotted and localized by antibody against MMP2 and MMP9 (c). MMP9 produced by cells pretreated with anti $\alpha_5$ integrin antibody and maintained on Fn and Col IV-coated substrata were immunoblotted (d)

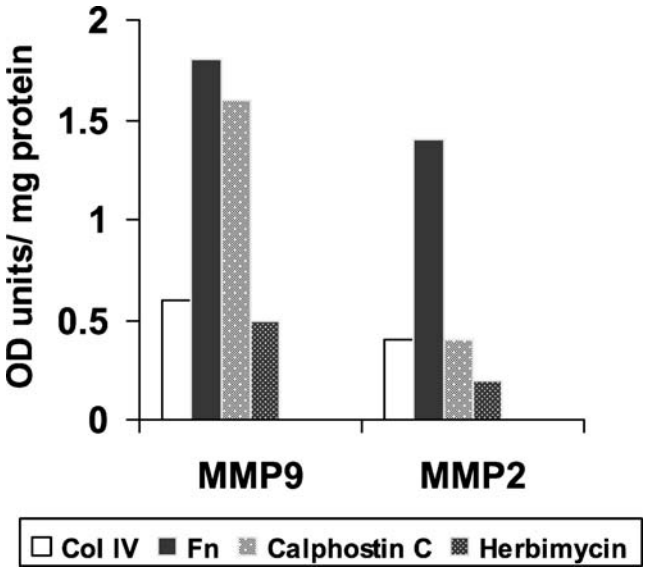


**Fig. 6** Effects of inhibitors of integrin-mediated signaling pathway on MMP production: Mononuclear cells ($1.5\times10^6$ cells) were pretreated with herbimycin A or calphostin C (1 μM) for 30 min and maintained in culture on Fn-coated substrata for 24 h. Cells maintained on Col IV also served as control. Medium was collected and the amount of MMP2 and MMP9 protein was determined by ELISA. Values given as OD units are averages of triplicate cultures

Col IV produced a significantly higher amount of Fn than those maintained on Fn or non-matrix proteins (Fig. 3).

Fn-dependent upregulation of MMPs

To study how the matrix protein influences the mo–mφ differentiation, modulation of macrophage-specific activity, such as the production of MMPs by PBMCs maintained in culture on fibronectin substrata, was studied in detail. Results of the present experiments and those reported earlier showed that freshly isolated inactivated monocytes do not produce significant amounts of MMPs. As they were maintained in culture, they produced significant amounts of MMPs, the relative level of MMP9 being more than MMP2. Analysis of the kinetics of production of MMPs by monocytes maintained in culture for different periods on Fn substrata showed about five fold to six fold increase in the rate of production of MMP9 and about three fold increase in the level of MMP2 when compared to that in cells maintained on non-matrix protein substrata (Fig. 4).

Cell–matrix interactions are mediated by cell surface receptors particularly heterodimeric transmembrane integrin type receptors; $\alpha_5\beta_1$ integrin is the integrin receptor for fibronectin. In order to study the mechanism of Fn-dependent upregulation of MMPs, mononuclear cells were treated with antibodies against $\alpha_5\beta_1$ integrin and were studied for the production of MMPs. Zymographic analysis and immunoblotting showed that treatment with blocking antibodies caused a reduction in the activity of both MMP2 and MMP9 and a reduction in the amount of the enzyme protein produced by the cells (Fig. 5). This appeared to be specific for Fn as the $\alpha_5$ blocking antibody did not produce any significant inhibition of MMP9 production by cells maintained on Col IV (Fig. 5). Our previous studies have indicated the matrix-dependent production of MMPs involved phosphorylation-dependent pathways [17]. This was further investigated by using the phosphorylation-inhibitor genistein. Pretreatment with genistein caused inhibition of MMP2 and MMP9 production by cells maintained on Fn. Zymographic analysis and ELISA of MMPs in cells maintained on Fn pretreated with genistein and immunoblotting of the secreted MMPs in the media showed that pretreatment with genistein caused inhibition of production of both MMP2 and MMP9. These results indicated that the Fn-dependent upregulation of MMP9 and MMP2 involves integrin-dependent phosphorylation signaling pathways.

Fibronectin activated down stream signaling process

Interaction of ligands with integrins triggers complex downstream signaling pathways. Integrin-mediated cell adhesion leads to PKC activation. As anti-integrin antibodies reversed the Fn effect, the integrin-mediated signaling pathway was studied. To examine whether PKC activation was involved in this, the effect of calphostin, an inhibitor of PKC on the Fn-dependent upregulation of MMPs, was studied. Treatment with calphostin caused only less than 10% inhibition of the MMP9 production indicating that Fn-dependent upregulation of MMP9 may not involve PKC-dependent signaling pathways (Fig. 6). Treatment with herbimycin, an inhibitor of Src type tyrosine kinase caused significant inhibition of both MMP9 and MMP2 (Fig. 6).

## Discussion

Extravasation of blood mononuclear cells involves initial tethering, rolling, and adhesion to the vessel wall followed by trans-endothelial migration into the subendothelial space. On trans-endothelial migration, monocytes interact with the extracellular matrix components and differentiate into macrophages. Results of *in vitro* studies presented earlier and those reported here relating to the appearance of macrophage-specific activity indicate that the rate of differentiation to macrophages depends on, among other factors, the nature of matrix also. Further evidence in support of the role of extracellular matrix in modulating the mo–mϕ differentiation is presented here by analyzing in detail the expression of MMPs, by mo–mϕ *in vitro* in a

matrix protein-dependent manner. Earlier studies have shown that monocytes maintained in culture on different matrix proteins showed significant difference in the activity of MMP2 and MMP9 and the maximum effect was shown by Fn [13]. Results presented above also indicate that the protein level expression of production of MMP9 increased as the monocyte differentiate into macrophages; and further that, when cells were maintained on different matrix protein, maximum upregulation of MMPs, particularly MMP9, was observed in cells maintained on Fn substrata. These results indicate Fn-dependent upregulation of MMPs in monocytes. The down-regulation of monocyte-specific activities such as loss of CD14 and MPO and upregulation of certain macrophage-specific activities such as endocytosis of modified proteins were significantly high in cells maintained on Fn indicating that Fn is a major extracellular matrix component contributing to differentiation of monocyte to macrophages.

The mo–mφ system in culture produce fibronectin, the amount of which increases with the progression of culture, suggesting this is also a macrophage-dependent activity. Mφs have been reported to produce matrix components like Fn and Col VIII [25]. A comparison of the relative ability of the monocyte maintained on different matrix protein substrata to produce the matrix protein like Fn showed that cells maintained on Col IV produced significantly higher amounts of Fn than those maintained on Fn, indicating a probable feedback-type inhibition by Fn in the extracellular space on Fn production by mo–mφs. Similar effects have been reported for Fn synthesis by isolated hepatocytes in culture [26].

Cell surface matrix receptors specific for various components of extracellular matrix are critical in mediating cell matrix interaction and the effect of matrix on cellular function. Integrins have been shown to mediate a variety of cell functions such as cell adhesion, migration, growth, and gene expression [27]. The results presented above indicating the matrix modulates mo–mφ differentiation and that the blocking of specific marker activity such as Fn-dependent upregulation of MMPs by $\alpha_5$ integrin, indicate that the Fn effect is mediated through its integrin receptors. Interaction of matrix protein ligands with integrins triggers a complex signaling pathway. Suppression of Fn-dependent upregulation of MMPs by inhibition of phosphorylation by genistein suggested that phosphorylation cascades are involved in signaling process. Integrin mediated signaling leads to tyrosine phosphorylation of FAK, which in turn may create sites for interaction with proteins having $SH_2$ domain [28]. Src-type tyrosine kinase is one such protein, and inhibition of Src-type tyrosine kinase by herbimycin A significantly reduced MMP9 and MMP2 expression suggesting the involvement of Src type tyrosine kinase in integrin-mediated signaling leading to upregulation of MMPs.

MMP expression by human monocytes is regulated by cytokines and growth factors. Normal inactivated monocytes do not express any significant amounts of MMP2 or MMP9. Following stimulation with agents like LPS or Con A, there is upregulation of MMPs apparently through a $PGE_2$-cAMP-dependent pathway [29–31]. But stimulation of monocytes by TNFα causes upregulation of MMP9 in a $PGE_2$ independent pathway [32] suggesting that multiple pathways operate in the regulation of MMP production in monocytes. It has been suggested that such activation-dependent MMP production in monocytes involve the transcription factor NFκB activation [33]. But no such activation of NFκB and translocation into the nucleus during the early stages of culture were observed in our system (data not shown). It therefore appears that in Fn-dependent upregulation of MMPs in monocytes, contribution of NFκB during early stages may not be significant. Our findings suggest that monocyte differentiation to macrophages is influenced by extracellular matrix protein and the expression of mφ specific activities such as MMP production in a matrix-dependent manner promote their migration.

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