Induction of Programmed Cell Death in Human Hematopoietic Cell Lines by Fibronectin via Its Interaction with Very Late Antigen 5

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Summary

Extracellular matrix (ECM) molecules such as fibronectin (FN), collagens, and laminin have important roles in hematopoiesis. However, little is known about the precise mechanisms by which ECM molecules regulate proliferation of human hematopoietic progenitor cells. In this study, we have investigated the effects of ECM molecules, particularly of FN, on the proliferation of a myeloid leukemia cell line, M07E, which proliferates in response to either human granulocyte/macrophage colony-stimulating factor (GM-CSF) or stem cell factor (SCF). The [^{3}H]thymidine incorporation and cell enumeration assays showed that FN strikingly inhibited GM-CSF– or SCF-induced proliferation of M07E cells in a dose-dependent manner, whereas little or no inhibition was induced by collagen types I and IV. The growth suppression of M07E cells was not due to the inhibitory effect of FN on ligand binding or very early events in the signal transduction pathways from the GM-CSF or SCF receptors. DNA content analysis using flow cytometry after staining with propidium iodide revealed that the treatment of M07E cells with FN did not block the entry of the cells into the cell cycle after stimulation with GM-CSF or SCF, whereas the treatment resulted in the appearance of subdiploid peak. Furthermore, FN was found to induce oligonucleosomal DNA fragmentation and chromatin condensation in the cells even in the presence of GM-CSF or SCF, suggesting the involvement of programmed cell death (apoptosis) in the FN-induced growth suppression. The growth suppression or apoptosis induced by FN was rescued by the addition of either anti-FN antibody, anti–very late antigen 5 monoclonal antibody (anti-VLA5 mAb), or GRGDSP peptide, but not by that of anti-VLA4 mAb or GRGESP peptide, suggesting that the FN effects on M07E cells were mediated through VLA5. In addition, the FN-induced apoptosis was detectable in VLA5-positive human hematopoietic cell lines other than M07E cells, but not in any of the VLA5-negative cell lines. These results suggest that FN is capable of inducing apoptosis via its interaction with VLA5, and also raise the possibility that the FN–VLA5 interaction may contribute, at least in part, to negative regulation of hematopoiesis.

Normal hematopoiesis is regulated by a complex set of events, involving interactions of hematopoietic stem cells with bone marrow stromal cells and/or extracellular matrix (ECM)[1] molecules (1–4). Because of a variety of hematopoietic growth factors being produced by stromal cells, it has been suggested that these growth factors may play a role in stromal cell–mediated hematopoiesis (5–7). In fact, some growth factors such as stem cell factor (SCF) and M-CSF are produced by stromal cells as either membrane bound or soluble forms, and are considered to be indispensable molecules for stromal cell–dependent proliferation and differentiation of hematopoietic cells (8–13). Furthermore, growth factors such as GM-CSF are reported to be capable of binding to ECM, and are proposed to function to promote growth of hematopoietic cells (14, 15).

In addition to hematopoietic growth factors, ECM such as fibronectin (FN), collagens, laminin, thrombospondin, and

[1] *Abbreviations used in this paper:* ECM, extracellular matrix; FN, fibronectin; KitR, *c-kit* receptor; PI, propidium iodide; SCF, stem cell factor; VLA, very late antigen.

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proteoglycans have also been implicated as molecules that promote growth, differentiation, anchorage, spreading, and migration of hematopoietic cells (for reviews see references 16–22). Cell surface receptors for ECM molecules are the integrins that are α/β heterodimeric transmembrane glycoproteins; they are subdivided according to their common β chain subunit into at least eight classes (18–22). A number of studies have demonstrated that integrins of the $\beta 1$ subfamily, also known as very late antigen (VLA), are primarily responsible for the cell–ECM interactions, whereas those of the $\beta 2$ subfamily are the mediators of cell–cell and cell–endothelial interactions (18–22). Among cell–ECM interactions, VLA-4/FN (CS-1 region) or VLA-5/FN (RGDS sequence) interactions have been proposed to have potentially crucial roles in the attachment, migration, or differentiation of hematopoietic progenitor cells (20, 21, 23–29). For example, progenitors of erythroid or lymphoid lineages attach to FN, but differentiated cells of these lineages become unable to adhere to FN because of their loss of FN-receptor expression (26–29). In addition, adhesion of $CD4^-CD8^-$ T cells to thymic stroma cells through FN–FN receptor interaction is considered to be crucial for their differentiation (30). Furthermore, the interaction between FN and VLA4 was demonstrated to play a relevant role in the terminal differentiation of human B cells (31), whereas FN was reported to inhibit suspension-induced terminal differentiation of human keratinocytes via a $\beta 1$ integrin, probably VLA5 (32). However, little is known about the effects of ECM molecules on the proliferation of hematopoietic progenitor cells, especially of human myeloid progenitor cells.

In an effort to investigate the effects of ECM molecules on human hematopoietic progenitor cells, we have primarily used a human myeloid cell line, M07E (33), because this cell line shows about the same proliferative response to GM-CSF or SCF as normal myeloid progenitor cells (34–36), and expresses several $\beta 1$ integrins, including VLA2, VLA4, VLA5, and VLA6 (37). Also, the assay system deprived of signaling between various cell types makes it possible to determine the direct effect of ECM molecules on hematopoietic cells. Of several ECM molecules tested, FN was found to dramatically induce programed cell death (apoptosis), resulting in inhibition of GM-CSF– or SCF-dependent proliferation of M07E cells. The FN-induced apoptosis or growth suppression was rescued by the treatment with either anti-FN serum, anti-VLA5 mAb or GRGDSP peptide, but not with anti-VLA4 mAb or GRGESP peptide, suggesting that VLA5–FN interaction may be involved in the effect of FN. Thus, our data provide unique evidence for the role of VLA5–FN interaction in the growth regulation of hematopoietic cells.

Materials and Methods

Reagents and Antibodies. Highly purified recombinant human GM-CSF was a gift from Drs. Steve Clark and Gordon Wong (Genetics Institute, Cambridge, MA). Recombinant human SCF was a gift from Dr. Krisztina M. Zsebo (Amgen, Inc., Thousand Oaks, CA) (10). Human plasma FN was purchased from Collaborative Biomedical Products (Bedford, MA). Murine mAbs reactive with CD49d/VLA4-α (SG17 and IgG2a) and CD49e/VLA5-α (KH72 and IgG1) were kind gifts of Dr. Kensuke Miyake (Saga Medical School, Saga, Japan) (38). The anti-phosphotyrosine antibody, a murine mAb generated against phosphotyramine as the immunogen, was generously supplied by Dr. Brian Druker (Dana-Farber Cancer Institute, Boston, MA) (34, 35). Goat $F(ab')_2$ antibodies against human FN, mouse IgG, and purified murine myeloma proteins of IgG1 (MOPC21) and IgG2a (UPC10) isotypes were purchased from Cappel (Durham, NC). Synthetic peptides GRGDSP and GRGESP were purchased from Iwaki Glass (Tokyo, Japan). Chemically defined serum-free medium ASF-102 was purchased from Ajinomoto (Tokyo, Japan). It contains human transferrin, insulin, and BSA.

Source of Cells. A human GM-CSF, IL-3, and SCF-dependent cell line, M07E, obtained from Dr. Steve Clark (Genetics Institute), was originally established by Avanzi et al. (33) from the peripheral blood of an infant with acute megakaryocytic leukemia. M07E cells were cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FCS (Flow Laboratories, North Ryde, Australia) and 10 ng/ml rhGM-CSF. Myeloid leukemia cell lines (HL-60, KG1, THP1, and KU812F), erythroid leukemia cell lines (K562 and HEL), T cell leukemia cell lines (CCRF-CEM and MOLT-3), B cell leukemia cell lines (BALL and RPMI-1788), and a human fetus-derived fibroblast cell line (NTI4) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). These cell lines were adapted to grow and were maintained in RPMI-1640 supplemented with 10% FCS. OTL-1 cell line was established in our laboratory from the peripheral blood of a patient with adult T cell leukemia, and maintained in RPMI-1640 supplemented with 10% FCS.

Peripheral blood was collected from healthy volunteers after obtaining informed consent. PBMC were isolated by Ficoll–Hypaque gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway), and PBL were obtained after depleting monocytes by adherence to plastic dishes for 3 h at 37°C. Neutrophils were obtained from peripheral blood by double Ficoll–Hypaque gradient method as described previously (39). Both lymphocyte and neutrophil preparations were >95% pure as assessed by May-Grunwald-Giemsa staining.

Cell Proliferation Assay. To quantitate the proliferation of cells, we used a [^{3}H]thymidine incorporation assay as previously described (35, 36). In the proliferation assay, both soluble and immobilized ECM were used. For immobilization, flat-bottom, 96-well microtiter wells were incubated first with either 100 μl of FN (0–100 μg in PBS) or 1% BSA for 1 h at 37°C, and the wells were washed with PBS and further incubated with 1% BSA to block nonspecific protein binding sites. In this study, however, we used soluble FN in most experiments because of the following reasons: (*a*) in preliminary studies, it was noted that growth suppression and apoptosis of M07E cells were significantly induced not only by immobilized FN but also by soluble FN; and (*b*) it seemed rather difficult to estimate the amounts of FN immobilized after incubation. Cells were washed twice with ASF-102 medium, and the triplicate aliquots of the cells (3×10^4 cells in 100 μl of ASF-102 medium) were cultured in 96-well, flat bottom microtiter plates with various combinations of GM-CSF, SCF, ECM, and/or antibodies. At various times after the initiation of culture, each well was pulsed for 5 h with 0.5 μCi [^{3}H]thymidine (sp act, 5 Ci/mM; Amersham International, Amersham, Bucks, UK). The cells were then harvested with a semiautomatic cell harvester (model 1295; Pharmacia LKB Biotechnology, Piscataway, NJ) and the incorporation was measured with a liquid scintillation counter.

In some experiments, cell proliferation was assessed by cell enumeration: viable cells were counted either by trypan blue dye exclusion or a phase-contrast microscope with a standard hemocytometer. In addition, the morphological characteristics of the cells were determined by staining the cytospin preparations (Shandon, Pittsburgh, PA) with May-Grunwald-Giemsa.

Flow Cytometry. Nuclear DNA content and cell cycle were analyzed by flow cytometry as described previously (40). Briefly, cells were centrifuged, the cell pellet was gently resuspended in hypotonic fluorochrome solution (50 μg/ml of propidium iodide [PI] in 0.1% sodium citrate containing 0.1% Triton X-100), and the cells were incubated at 4°C overnight in the dark. The fluorescence emitted from the PI–DNA complex was then quantitated after laser excitation of the fluorescence dye by a FACScan® (Becton Dickinson & Co., Mountain View, CA).

To examine the surface expression of VLAs, cells were incubated with the indicated antibodies at 4°C for 30 min, rinsed, and developed with FITC-conjugated goat anti-mouse IgG (Becton Dickinson & Co.) at 4°C for 30 min. The cells were then rinsed and analyzed on a FACScan®.

Analysis of DNA Fragmentation. Fragmentation of DNA was analyzed as described previously (41). Cells were incubated for the indicated time periods under various culture conditions. After incubation, the cells were washed twice in PBS and immediately used for DNA extraction. Equal numbers of the cells were lysed in a lysis solution containing 200 mM Tris-HCl, pH 8.0, 100 mM EDTA, 1% SDS, and 50 μg/ml proteinase K (Nacalai Tesque). After a 4-h incubation at 37°C with constant rotation, DNA was extracted with an equal volume of Tris buffer–saturated phenol and chloroform/isoamylalcohol (24:1). The aqueous phase was mixed with sodium chloride to give a final concentration of 150 mM of sodium chloride, followed by addition of 2 vol absolute ethanol. The mixture was incubated at −20°C overnight, and the ethanol-precipitated DNA was dried and resuspended in TE (10 mM Tris-HCl and 1 mM EDTA) buffer. The suspension was treated with 50 μg/ml of DNase-free RNase for 5 h at 37°C, and then with 300 μg/ml proteinase K for 5 h at 37°C. DNA was precipitated again by ethanol and resuspended in TE buffer. Subsequently, 5 μg DNA per lane was separated by electrophoresis on a 1% agarose gel in the presence of ethidium bromide. The electrophoresis was carried out in TBE buffer (90 mM Tris-HCl, 90 mM boric acid, and 2 mM EDTA).

Stimulation with Factors and Cell Lysis. Exponentially growing M07E cells were washed free of serum and growth factors and incubated in serum-free ASF-102 medium for 12 h at 37°C to factor deprive the cells. The cells (10^7 cells suspended in 1 ml of ASF-102 medium) were treated with FN (50 μg/ml) at 37°C for 20 min, and then exposed to GM-CSF (10 ng/ml) or SCF (100 ng/ml) at 37°C for 15 min. After stimulation with factors, cells were washed with cold PBS and lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40) containing protease and phosphatase inhibitors as described previously (34, 42). Insoluble material was removed by centrifugation at 10,000 *g* for 15 min at 4°C.

Gel Electrophoresis and Immunoblotting. The procedures of gel electrophoresis and immunoblotting were performed according to the methods described previously (34, 42, 43). Briefly, cell lysates (150 μg for 13× 12-cm gels) were mixed 2:1 with 3× SDS-sample buffer with 2-ME, heated at 100°C for 5 min before one-dimensional SDS-PAGE with 7.5% polyacrylamide. After electrophoresis, proteins were electrophoretically transferred from the gel onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol at 0.4 A for 4 h at 4°C. Residual binding sites on the filter were blocked by incubating the membrane in TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing 1% gelatin (Bio-Rad Laboratories, Richmond, CA) for 1 h at 25°C. Immunoblotting was then performed with anti-phosphotyrosine mAb (1.5 μg/ml) and alkaline phosphatase–conjugated anti-mouse IgG (Promega Corp., Madison, WI).

Results

FN Is Inhibitory to the Proliferation of M07E Cells Stimulated with GM-CSF or SCF. We first examined the effects of various ECM molecules on GM-CSF–induced proliferation of M07E cells. M07E cells were cultured in a serum-free medium containing GM-CSF (10 ng/ml) in the presence or absence of collagen type I, collagen type IV, laminin, or FN (each at a concentration of 25 μg/ml) for 3 d, followed by measurement of cell proliferation with a [^{3}H]thymidine incorporation assay. Despite little effect by collagen types I and IV, GM-CSF–induced proliferation of M07E cells was significantly suppressed by laminin and particularly by FN (Fig. 1). These suppressive effects were also observed using immobilized forms of laminin and FN, albeit to a lesser degree than soluble forms (data not shown).

Since the most striking inhibition was observed by FN, we examined the dose–response effect of FN and GM-CSF and SCF-induced proliferation of M07E cells. The [^{3}H]thymidine incorporation assay showed that FN inhibited GM-CSF or SCF-induced proliferation of M07E cells in a dose-dependent manner (Fig. 2 *A*). In addition, the time course study showed that FN (50 μg/ml) inhibited the GM-CSF– or SCF-induced proliferation of M07E cells throughout the culture period (Fig. 2 *B*). In addition to the [^{3}H]thymidine incorporation

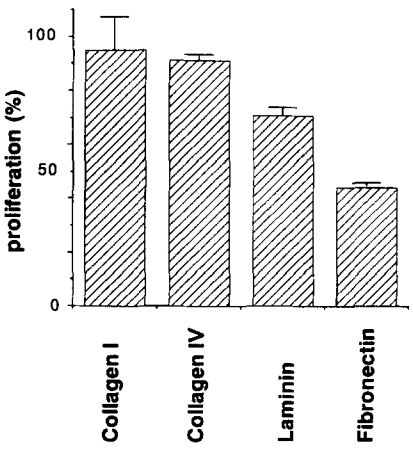

Figure 1. Effect of ECM molecules on proliferation of M073 cells. Triplicate aliquots of M073 cells (3×10^4) were incubated for 72 h in a serum-free AFS102 medium containing 10 ng/ml of GM-CSF in the presence of either collagen type I, collagen type IV, laminin, or fibronectin (all 25 μg/ml). The cells were pulsed for the final 5 h of 72-h culture period with 0.5 μCi [^{3}H]thymidine; [^{3}H]thymidine incorporation was measured with a liquid scintillation counter; and percent control was measured. The results are shown as mean ± SD of three independent experiments.

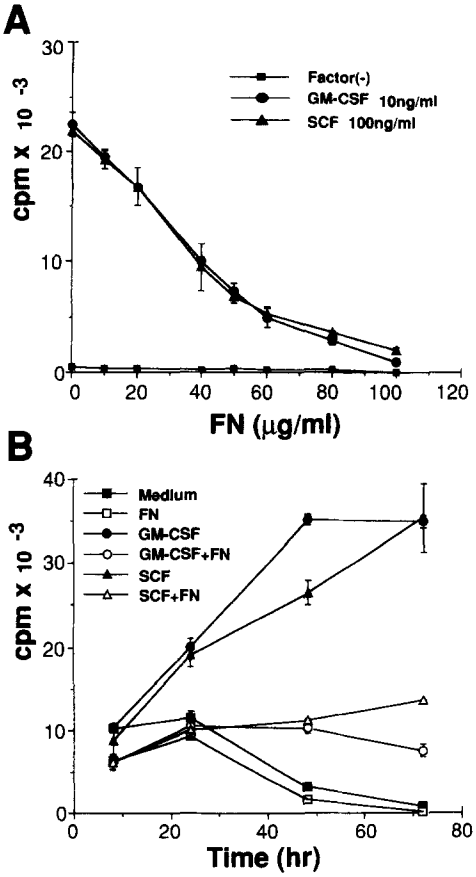

Figure 2. Effects of FN on proliferation of M073 cells. (*A*) Triplicate aliquots of M073 cells (3×10^4) were incubated for 72 h in a serum-free medium containing control media, GM-CSF (10 ng/ml) or SCF (100 ng/ml) with the indicated concentrations of FN. (*B*) Triplicate aliquots of M073 cells were incubated with or without 50 μg/ml of FN in the presence or absence of GM-CSF (10 ng/ml) or SCF (100 ng/ml) for the indicated times. Cell proliferation was measured using a [^{3}H]thymidine incorporation assay. The results are shown as mean ± SD of triplicate cultures.

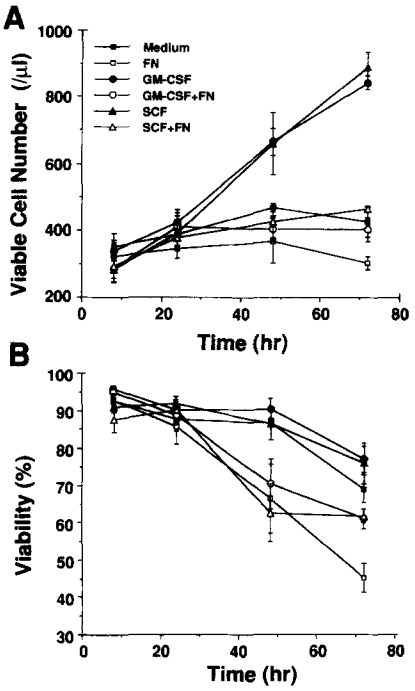

Figure 3. Effects of FN on viable cell number and viability of M073 cells. Triplicate aliquots of M073 cells were incubated in a serum-free medium containing control media, GM-CSF (10 ng/ml), or SCF (100 ng/ml) with or without 50 μg/ml of FN for the indicated times. At various times after initiation of the culture, viable cell number (*A*) and viability (*B*) of M073 cells were determined by trypan blue dye exclusion, a phase-contrast microscope, and May-Grunwald-Giemsa staining. The results are shown as mean ± SD of triplicate cultures.

assay, the inhibitory effect of FN on M07E proliferation was assessed by cell enumeration method. Inconsistent with the data on [^{3}H]thymidine incorporation, FN almost completely inhibited the GM-CSF– or SCF-induced proliferation of M07E cells (Fig. 3 *A*). Moreover, it was revealed that FN treatment led to a decrease in the proportion of viable cells in the presence or absence of GM-CSF or SCF (Fig. 3 *B*).

Effect of FN on the Initial Signaling Mediated by GM-CSF or SCF. One of the possible mechanisms that might explain the suppressive effect of FN was that FN inhibited the binding of GM-CSF or SCF to the corresponding receptor, thereby blocking the initial signaling of GM-CSF or SCF. By using M07E cells, we have previously demonstrated that GM-CSF and SCF induce rapid, dose-dependent tyrosine phosphorylation of a 93-kD cytoplasmic protein (p93) (34, 35) and the 145-kD c-*kit* receptor (KitR) (36, 43), respectively, and also that the tyrosine phosphorylation of p93 or KitR is correlated with the rate of cell proliferation (34–36). We therefore determined whether FN inhibited tyrosine phosphorylation of p93 or KitR in M07E cells. M07E cells were deprived of factor for 12 h, pretreated with 50 μg/ml of FN or control media for 20 min, and then further cultured with control media, GM-CSF, or SCF for an additional 15 min. The cells were then lysed and phosphotyrosine-containing proteins detected by immunoblotting. The pretreatment of M07E cells with FN did not affect tyrosine phosphorylation of p93 and KitR (c-*kit*) whose phosphorylation was induced by GM-CSF and SCF, respectively (Fig. 4). Further, tyrosine phosphorylation of p93 and KitR was not inhibited even after long-term exposure to FN (up to 12 h). These results suggest that FN is inhibitory to neither ligand binding nor very early steps in the signal transduction pathways from the GM-CSF or SCF receptors.

FN Induces Apoptosis. To further understand the mechanism by which FN inhibited the factor-dependent proliferation of M07E cells, we investigated the effect of FN on the state of cell cycle in M07E cells. In the presence or absence of FN (50 μg/ml), M07E cells were cultured in a serum-free media containing medium alone, GM-CSF, or SCF for 36 h, and the cells were stained with PI and analyzed by flow cytometry. As shown in Fig. 5 *A*, FN treatment did not block the entry of M07E cells into the cell cycle when stimulated with GM-CSF or SCF. However, the treatment of M07E cells with FN resulted in the appearance of subdiploid peak in

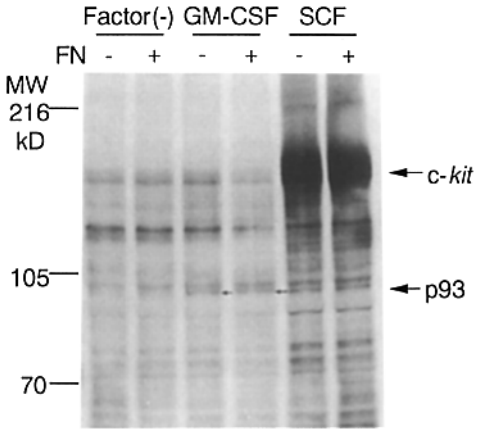

Figure 4. Effects of FN on factor-induced protein tyrosine phosphorylation. M07E cells were factor deprived for 12 h, exposed to FN (50 μg/ml) or control media for 20 min, and then further cultured with control media, GM-CSF (10 ng/ml), and SCF (100 ng/ml) for an additional 15 min. The cells were then lysed and phosphotyrosine-containing proteins were detected by immunoblotting with an anti-phosphotyrosine mAb. (*Right*) The mobilities of p93 and KitR (c-*kit*).

the presence or absence of stimulation with GM-CSF or SCF (Fig. 5 *A*). Moreover, the DNA content analysis revealed that the proportions of subdiploid peak were 17.8, 55.3, and 52.6% after the treatment with FN (50 μg/ml) and GM-CSF (10 ng/ml) for 24, 48, and 72 h, respectively, raising the possibility that the FN-induced cell death observed in Fig. 3 *B* was due to apoptosis.

To test this possibility, M07E cells were treated with or without FN, and subjected to DNA fragmentation and morphological analyses. In the absence of FN, DNA fragmentation was barely detected in M07E cells when cultured with GM-CSF or SCF, whereas factor deprivation induced DNA fragmentation to a modest degree (Fig. 5 *B*). By contrast, DNA fragmentation into oligonucleosomal-sized pieces was readily visible after the treatment with FN even in the presence of GM-CSF or SCF (Fig. 5 *B*). FN treatment was also found to lead to distinctive condensation of chromatin and fragmented nuclei (Fig. 5 *C*). These results indicated that FN induced apoptosis of M07E cells, thereby leading to growth suppression of the cells.

Involvement of VLA5 in the Effect of FN. Interaction of hematopoietic cells with FN is known to be mediated by at least two integrins, VLA4 and VLA5, which recognize the CS-1 region and RGDS sequence of FN, respectively (18, 20–24). The expression of VLA4 and VLA5 on M07E cells was examined by flow cytometry using anti-VLA4-α (SG17) and anti-VLA5-α (KH72) mAbs. This analysis showed that VLA4 and VLA5 were abundantly expressed on the cell surface of M07E cells at an almost equal level (Fig. 6).

To determine whether each integrin expressed on M07E cells was involved in the FN effects, M07E cells were cultured with GM-CSF (10 ng/ml) and FN (15 μg/ml) in the presence of anti-VLA4 mAb, anti-VLA5 mAb, anti-FN Ab, or RGD-containing peptide. The thymidine incorporation

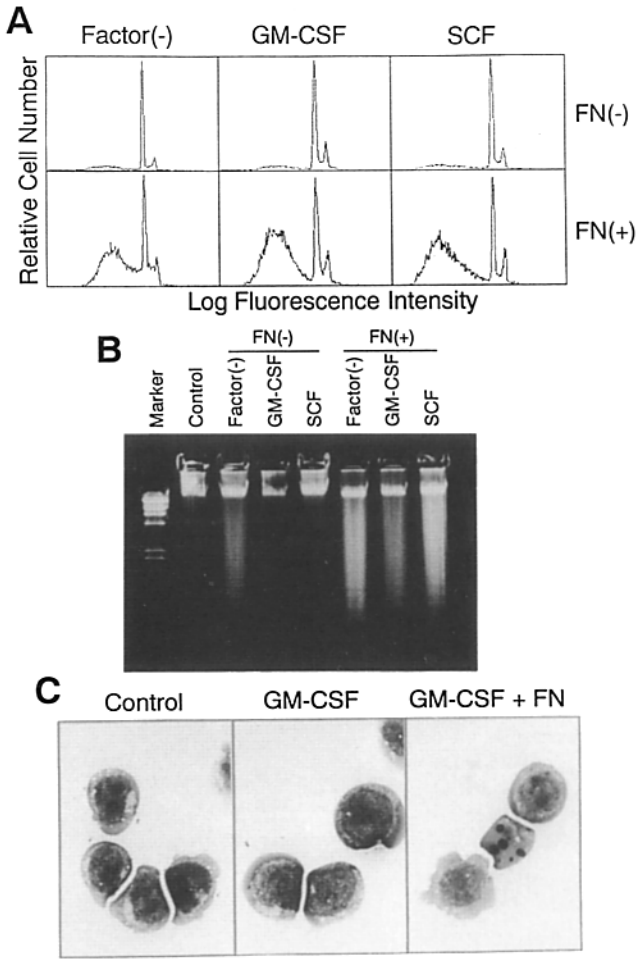

Figure 5. Induction of oligonucleosomal DNA fragmentation and chromatin condensation by FN. M073 cells were incubated in a serum-free medium containing control media, GM-CSF (10 ng/ml), or SCF (100 ng/ml) with or without 50 μg/ml of FN for 36 h, and then subjected to nuclear DNA content (*A*), DNA fragmentation (*B*), and morphological (*C*) analyses as described in Materials and Methods. Exponentially growing M07E cells that were cultured with GM-CSF (10 ng/ml) were used as Control (*B* and (*C*). Each figure shows one of three similar experiments.

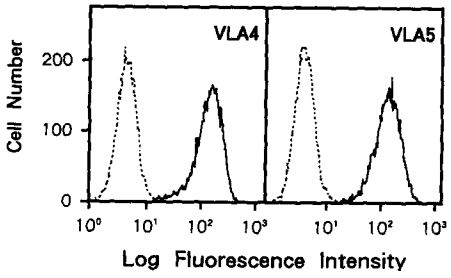

Figure 6. Expression of VLA4 and VLA5 on M07E cells. The cells were incubated in either negative control antibody (- - -) or the indicated anti-VLA-α mAb (—), washed, incubated with fluorescein-conjugated goat $F(ab')_2$ anti-mouse IgG antibody, and analyzed on a FACScan®. Anti-VLA4-α (SG17 and IgG2a) and anti-VLA5α (KH72 and IgG1) were used in this study.

assay showed that, when compared with controls, the suppressive effect of FN on GM-CSF–induced proliferation of M07E cells was significantly rescued by the addition of either anti-FN-antibody or anti-VLA5-α mAb, but not anti-VLA4-α mAb (Fig. 7 *A*). Also, the growth suppression induced by FN was considerably abrogated by the incubation with GRGDSP peptide but not with GRGESP peptide (Fig. 7 *A*). In accord with data on the proliferation assay, further, FN-induced apoptosis of M07E cells was canceled by incubation with either anti-FN-antibody, anti-VLA5-α mAb, or GRGDSP peptide, but not with anti-VLA4-α mAb or GRGESP peptide (Fig. 7 *B*). Similar results were also obtained with SCF (data not shown). These results suggested that FN exhibited the growth suppression or apoptosis, at least in part, via its interaction with VLA5 expressed on M07E cells.

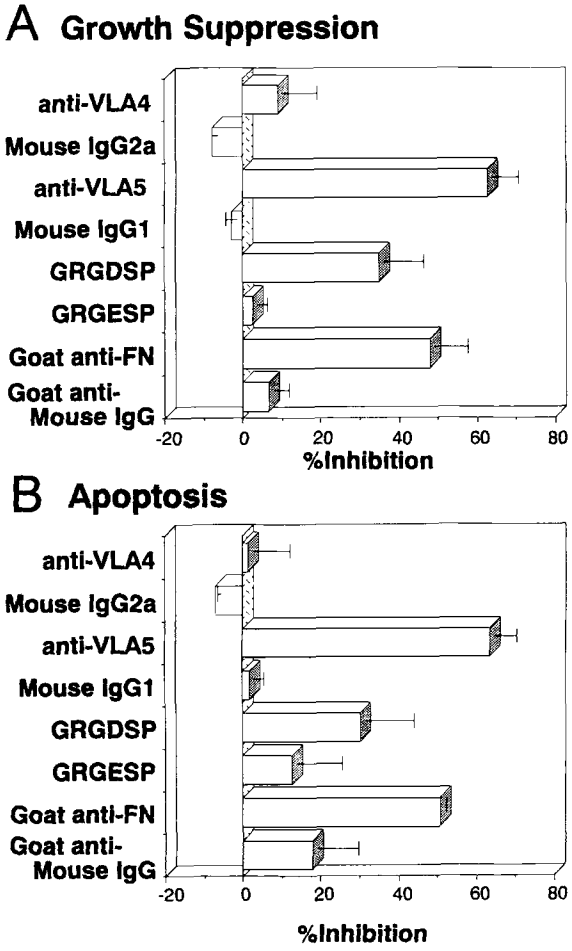

Figure 7. Involvement of VLA5 in the growth suppression (*A*) and apoptosis (*B*) of M07E cells by FN. (*A*) Triplicate aliquots of M073 cells (3×10^4) were cultured in a serum-free medium containing GM-CSF (10 ng/ml) with or without FN (15 μg/ml) for 72 h in the presence or absence of the indicated antibodies or peptides, and cell proliferation was measured using a [^{3}H]thymidine incorporation assay. Shown are percent inhibition by the indicated antibodies or peptides of FN-induced growth suppression. (*B*) Triplicate aliquots of M073 cells (2×10^5) were cultured with FN (50 μg/ml) for 48 h as described above, and percent inhibition by the indicated antibodies or peptides of FN-induced apoptosis was measured. In this study, the cells in apoptosis were determined by DNA content analysis (36). The concentrations of antibodies and peptides used in these studies were as follows: anti-VLA4 mAb, anti-VLA5 mAb, and their control mAbs of mouse IgG1 and IgG2a isotypes were used at 50 μg/ml; goat anti-FN and anti-mouse IgG antibodies at 0.5 mg/ml; and GRGDSP and GRGESP peptides at 0.5 mg/ml. All of the results are shown as mean ± SD of triplicate cultures.

Effect of FN on Various Types of Cells. Using flow cytometry analysis, the expression of VLA4 and VLA5 was examined on a variety of human hematopoietic cell lines including myeloid cell lines (HL-60, KG1, THP-1, and KU812F), erythroid cell lines (K562 and HEL), T cell lines (CCRF-CEM, MOLT-3, and OTL-1) and B cell lines (BALL and RPMI1788), and also on a human fibroblast cell line (NTI4) as a control for non-hematopoietic cells. In addition, we analyzed the expression of VLA4 and VLA5 on PBL and neutrophils. In hematopoietic cell lines, VLA4 and VLA5 were easily detectable on all but three cell lines tested. OTL-1, BALL, and RPMI1788, expressed only VLA4 on their surface (Table 1). In NTI4 cells, VLA5 was readily detectable on their surface, whereas VLA4 was expressed on only a minor population of the cells (Table 1). Furthermore, both VLA4 and VLA5 were expressed on PBL, but neither of them was expressed on neutrophils (Table 1).

To examine the effects of FN on these cells, cells were cultured in a serum-free medium containing FN (50 μg/ml), and subjected to proliferation and apoptosis assays. In the hematopoietic cell lines, the proliferation of VLA5-positive cell lines was inhibited by incubation with FN, whereas proliferation of VLA5-negative cell lines was rather stimulated. Moreover, although the proportion of apoptosis was not necessarily correlated with the rate of the growth suppression, apoptosis was found to be induced by the treatment with FN in most, if not all, of the VLA5-positive cell lines (Table 1). In addition, the FN-induced growth suppression and apoptosis were also observed in PBL, although FN had no effects on neutrophils that were used as a negative control. By contrast, growth suppression or apoptosis by FN was only minimal or absent in NTI4 cells (Table 1). These results showed that the growth inhibition or apoptosis induced by FN was not limited to M07E cells but was detectable in at least some of the other VLA5-positive hematopoietic cells, whereas VLA5-negative hematopoietic cells showed neither growth suppression nor apoptosis in response to FN.

Discussion

FN, a ubiquitous glycoprotein that can be produced by many types of cells such as fibroblasts, endothelial cells, and macrophages, has been implicated as an important molecule for the attachment of hematopoietic stem or progenitor cells (for reviews see references 17, 21, 22, 26–29). However, the role of FN in growth regulation of hematopoietic stem or progenitor cells is largely unknown. In this study, we found that FN inhibited GM-CSF– or SCF-dependent proliferation of M07E cells in both thymidine incorporation and cell enumeration assays, whereas little or no effect was observed with collagen types I and IV. The inhibitory effect of FN on GM-CSF– or SCF-induced proliferation of M07E cells

Table 1. *The Expression of VLA4 and VLA5 Integrins, and the Effects of FN on the Proliferation and Apoptosis of Human Cell Lines and Leukocytes*

Cells	Expression		Effects of FN*	
	VLA4	VLA5	Proliferation (SI)	Apoptosis
	%			%
Hematopoietic cell lines				
Myeloid				
M07E	99.5	99.7	0.20‡	40.1§
HL60	99.7	95.4	0.40	33.7
KG1	98.2	99.5	0.21	19.3
THP1	99.7	99.9	0.23	23.7
KU812F	94.5	97.6	0.19	33.5
Erythroid				
K562	91.8	99.6	0.51	15.7
HEL	99.6	99.6	0.29	11.3
T cell				
CCRF-CEM	99.3	84.1	0.46	29.3
MOLT-3	99.7	74.1	0.43	21.3
OTL-1	98.8	0.4	1.25	2.9
B cell				
BALL	99.9	9.9	1.10	3.6
RPMI-1788	92.8	1.1	1.39	2.1
Fibroblast cell line				
NT14	16.1	90.8	0.85	<0.1
Normal leukocytes				
PBL	97.3	80.5	0.23	20.1
Neutrophil	1.6	1.5	ND‖	<0.1

The data are shown as mean of two independent experiments.

* Triplicated aliquots of cells were cultured in serum-free media for 72 h with or without the addition of FN (50 μg/ml), and subjected to proliferation and apoptosis assays. For culture of M07E cells, GM-CSF (10 ng/ml) was added to the media.

‡ Cell proliferation was measured using a [^{3}H-thymidine incorporation assay. The effect of FN on cell proliferation was expressed as stimulation index (SI); SI = (thymidine incorporation [cpm] with FN)/(thymidine incorporation [cpm] without FN).

§ Cells were stained with PI, and their nuclear DNA content was analyzed by a FACScan® (40). The cells whose DNA content was < 2 N were in apoptosis. The results show the increase in the proportion of apoptotic cells after treatment with FN.

‖ Cell enumeration assay did show, however, that viable cell numbers of neutrophils were not affected by 72-h treatment with FN.

was considered to result from the induction of apoptosis, because the treatment of M07E cells with FN induced oligonucleosomal DNA fragmentation and chromatin condensation even in the presence of GM-CSF or SCF.

The functions of FN on a variety of hematopoietic cells are known to be mediated through binding of FN to two VLA integrins, VLA4 and VLA5 (18, 20–24). Recently, a large number of investigators have examined the expression and function of VLA4 and VLA5 in murine and human hematopoietic stem or progenitor cells (25, 37, 44–50). For example, Williams et al. (47) have found that day 12 CFU spleen (CSF-S_{12}) expresses VLA4, and that the adhesion of murine hematopoietic stem cells with stromal cell ECM is partly mediated through interaction between VLA4 and the CS-1 portion of FN (47). In addition to murine hematopoietic stem cells, human primitive hematopoietic progenitor cells have also been demonstrated to adhere to the RGDS-independent heparin-binding fragment of FN (48). Miyake et al. (49) have

reported that VLA4 plays a role in the adhesion of B cell progenitor cells to marrow stromal cells, whereas its interaction is not mediated by FN but by vascular cell adhesion molecule 1 that is expressed on stromal cells. Also, they have demonstrated that lymphopoiesis in a murine long-term culture is abrogated by the addition of anti-VLA4 mAb, suggesting a functional requirement for this adhesion interaction (50). Thus, although VLA4-mediated adhesion and growth of hematopoietic stem or progenitor cells has been extensively studied, the functional significance of VLA5 in the growth control of hematopoietic stem or progenitor cells remains elusive.

Our data suggest that VLA5 may be involved in the growth control of hematopoietic cells through interaction with FN. Both growth inhibition and apoptosis induced by FN in M07E cells were substantially abolished by the treatment with either anti-FN Ab or anti-VLA5-α mAb, but not with anti-VLA4-α mAb. Also, the addition of a GRGDSP peptide was found to result in significant abrogation of the effects of FN on M07E cells, whereas little or no effect was obtained by the incubation with a GRGESP peptide that was used as a control. In our preliminary study, it has been noted that growth inhibition or apoptosis of M07E cells can be generated by a RGD sequence–containing 120-kD fragment of FN at a level almost similar to that of native FN, but not by either a 45-kD FN fragment of the gelatin binding domain or a 40-kD FN fragment of the heparin binding domain containing CS-1 region. In addition, despite little effect of FN–VLA5 interaction on a fibroblast cell line (NTI4), FN could induce growth suppression or apoptosis in VLA5-positive PBL and hematopoietic cell lines other than M07E cells. By contrast, FN had no activity in inducing growth suppression or apoptosis in any of the VLA5-negative hematopoietic cell lines or in neutrophils that were lacking in VLA5 expression. These results suggest that the interaction between FN and VLA5 may be important for FN-induced growth inhibition or apoptosis in hematopoietic cells such as M07E cells. Furthermore, these results raise the possibility that the FN–VLA5 interaction may contribute, at least in part, to negative regulation of hematopoiesis in vivo, although it remains to be determined whether the FN–VLA5 interaction has similar effects on normal hematopoietic stem or progenitor cells.

The expression level of some integrins, including VLA5, is known to be diminished in oncogenic transformed cells (51). Moreover, there is evidence suggesting that neoplasmic transformation results in reduced binding of FN to integrins even when integrin expression is not affected (52). Giancotti and Ruoslahti overexpressed human VLA5 in transformed CHO cells, and examined the proliferative potential and tumorigenicity of the overexpressor cells (53). They found that the overexpressor cells deposited more FN in their ECM than control CHO cells, reduced the ability to grow in both liquid and semisolid cultures, and lost the ability to form tumors when injected into nude mouse (53). Although the mechanism for the growth suppression in the overexpressor cells is not characterized, these results provide an additional support for the idea that VLA5 may play a role in the control of cell growth. Also, it is possible that the defective interaction between FN and VLA5 may result in failure to provide negative signal to neoplasmic cells, thereby participating in the abnormal growth of the cells.

Although this study provides unique evidence for the involvement of the FN–VLA5 interaction in the induction of apoptosis, the mechanisms responsible for apoptosis in M07E cells remain to be elucidated. Apoptosis is a normal physiological process that occurs in most animal tissues at some stage of development, and is thought to play a role in the maintenance of cell numbers at homeostatic levels by competing for proliferation or survival signals (for reviews see references 54 and 55). In the case of factor-dependent hematopoietic cell lines, it is well known that apoptosis can be prevented by hematopoietic growth factors that are essential for proliferation or survival of the cells (56). In this study, the pretreatment of M07E cells with FN did not have any effect on tyrosine phosphorylation of p93 and KitR that have shown to be phosphorylated on tyrosine by GM-CSF and SCF in a dose-dependent manner (34, 35). This suggests that the FN-induced apoptosis was not attributable to the inhibitory effect of FN on very early steps, including ligand binding, in signal transduction from the GM-CSF and SCF receptors.

It has recently been demonstrated that the *bcl*-2 and *c-myc* oncoproteins and the tumor suppressor p53 regulate apoptosis (57). In some target cells, furthermore, the stimulation of TNF receptor or the Fas/APO-1 antigen can lead to cell death by apoptosis (57, 58). In addition, some signaling molecules, such as intracellular Ca^{2+}, cAMP, and protein kinase C, have been suggested to be involved in the regulation of apoptosis (57). Also, recent studies on integrins indicate that they are capable of transducing a variety of signals into the cell, including protein tyrosine phosphorylation, increases in cytoplasmic pH, changes in intracellular Ca^{2+} or cAMP levels, activation of the Na/H antiportor, and induction of gene expressions (59–64). It is therefore possible that the signaling molecule(s) described above may be responsible for the induction of apoptosis in M07E cells. Experiments are currently in progress to identify the VLA5-mediated signal transduction molecule(s) that is involved in induction of apoptosis. Clear and decisive information from such studies should not only lead to a more precise understanding of mechanisms underlying regulation of normal and abnormal hematopoiesis, but also should suggest novel therapeutic procedures for patients with hematological disorders.

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