Gelsolin Induces Promonocytic Leukemia Differentiation Accompanied by Upregulation of p21CIP1

Reza Shirkoohi1,2*, Hisakazu Fujita2,3, Stephanie Darmanin4, Masato Takimoto2,5

Abstract

Tumor suppressor genes have received much attention for their roles in the development of human malignancies. Gelsolin has been found to be down-regulated in several types of human cancers, including leukemias. It is, however, expressed in macrophages, which are the final differentiation derivatives for the monocytic myeloid lineage, implicating this protein in the differentiation process of such cells. In order to investigate the role of gelsolin in leukemic cell differentiation, stable clones over-expressing ectopic gelsolin, and a control clone were established from U937 leukemia cells. Unlike the control cells, both gelsolin-overexpressing clones displayed retarded growth, improved monocytic morphology, increased NADPH and NSE activities, and enhanced surface expression of the β-integrin receptor, CD11b, when compared with the parental U937 cells. Interestingly, RT-PCR and western blot analysis also revealed that gelsolin enhanced p21CIP1 mRNA and protein expression in the overexpressing clones. Moreover, transient transfection with siRNA silencing P21CIP1, but not the control siRNA, resulted in a reduction in monocytic differentiation, accompanied by an increase in proliferation. In conclusion, our work demonstrates that gelsolin, by itself, is capable of inducing monocytic differentiation in U937 leukemia cells, most probably through p21CIP1 activation.

Keywords: Gelsolin - actin - promonocytic leukemia - differentiation

Introduction

Acute myeloid leukemia (AML) is characterized by blocked or impaired differentiation of haematopoietic stem cells, resulting in abnormal accumulation of immature precursors and suppression of growth and maturation of cells involved in normal haematopoiesis (Craig and Schiller, 2008). Classical treatments are usually toxic and non-specific, and are associated with difficulties (Cervio et al., 2012), such as economic burden (O’Donnell et al., 2012) and poor tolerance to myelo-suppressive chemotherapy, especially in elderly patients (Wiernik, 2011). Recent clinical trials demonstrate the efficacy of differentiation induction in the treatment of AML, either alone (Petrie, 2009), or in combination with intensive chemotherapy, thereby increasing the susceptibility of AML blasts to drug-induced apoptosis (Fredly, 2009).

The process of differentiation is usually accompanied by the activation of various molecules inside the cell, some of which are possibly actin-binding proteins. During dendritic cell maturation, for instance, the actin-regulatory protein, cofillin, is dephosphorylated (activated) and translocated to the cell membrane, suggesting a role for this protein in the rearrangement of the actin cytoskeleton (Verdijk et al., 2004). Also, in megakaryoblastic leukaemic cell lines, the expression of scinderin, another actin-regulatory protein, induces differentiation, maturation and apoptosis (Bhat et al., 2009). The actin cytoskeleton is an essential scaffold for integrating membrane and intracellular function, whose dynamics are regulated by a number of accessory proteins (Sato, 2012). It has been demonstrated that gelsolin, a ubiquitous protein expressed in almost all mammalian tissues (Kuzumaki and Maruta, 2002), also has an important role in actin reorganization (Furnish, 2001).

Gelsolin is a Ca²⁺ and polyphosphoinositide 4,5-bisphosphate (PIP₂)-regulated actin filament-severing and capping protein that is implicated in actin remodeling, both in growing and apoptotic cells (Patel, 2011). It was previously reported that the expression of gelsolin is frequently down-regulated in several types of human cancers, including stomach, bladder, colon and lung cancers (Tanaka et al., 2006). Whereas gelsolin is expressed in macrophages, which are the final differentiation derivatives for the monocytic myeloid lineage, the expression of this protein is strongly down-
regulated in the U937, human myelomonocytic leukaemia, cell line. Interestingly, when this cell line is differentiated using tetradecanoyl-phorbolacetate (TPA) the mRNA and protein levels of gelsolin are elevated (Kwaitkowski, 1988). Additionally, gelsolin mRNA and protein levels are also enhanced during the differentiation of embryonal carcinoma cells (Dieffenbach, 1989).

Taken together these findings suggest the possible correlation of gelsolin levels with cytoskeletal reorganization and morphological changes during the process of differentiation in myeloid cell lines. In this study, we established gelsolin-overexpressing U937 clones, by means of retroviral infection and demonstrated that gelsolin, alone, is capable of inducing differentiation of the monocytic leukemia cell line U937.

Materials and Methods

Cell lines and construction of recombinant retrovirus vectors

U937 monocytic leukemia cells were purchased from the Health Science Research Resources Bank (Osaka, Japan) and cultured in 25 mm² flasks using RPMI medium (GIBCO, BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂/air incubator. The gelsolin retrovirus vector pLNCChGSN was constructed from a pLNCX retroviral vector containing the Neo control gene (a kind gift from Dr. AD Miller, Fred Hutchinson Cancer Research Center) as previously described (Banno et al., 1999). Production of VSV-G pseudo-typed retroviruses was achieved by co-transfection of each retroviral vector and pMD.G, the plasmid encoding the VSV envelope protein VSV-G, into the human embryonal kidney packaging cell line 293gp/bsr (kind gifts from Dr. I. Verma, Salk Institute) as previously described (Ory, 1996). After 48 hours, virus-containing supernatants (VCM) of pLNCX and pLNCChGSN were infected into NIH3T3 cells, cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 0.03% L-glutamine, as previously described (Nam, 2011). After 24 hours, the virus-containing supernatant was replaced with VCM containing 10⁶ CFU/ml of the virus and 6 microg/ml polybrene. The cells were then centrifuged at 2000 g for 1 hour at room temperature and incubated in VCM as previously described (Nam, 2011). Cells were treated with G418 (500 microg/ml) for 10 days and cloned by limiting dilution. Four clones were selected for primary analysis (UG3, UG4, UG6, UG11). To establish a gelsolin-overexpressing stable clone, by means of retroviral infection and demonstrated that gelsolin, alone, is capable of inducing differentiation of the monocytic leukemia cell line U937.

Establishment of gelsolin-overexpressing U937 stable clones

To establish a gelsolin-overexpressing stable clone, U937 cells were centrifuged at 300 g for 3 min. The supernatant was replaced with VCM containing 10⁶ CFU/ml of the virus and 6 microg/ml polybrene. The cells were then centrifuged at 2000 g for 1 hour at room temperature and incubated in VCM as previously described (Nam, 2011). Cells were treated with G418 (500 microg/ml) for 10 days and cloned by limiting dilution. Four clones were selected for primary analysis (UG3, UG4, UG6, UG11). To establish a gelsolin-overexpressing stable clone, U937 cells were infected with the Neo control pLNCX retrovirus; one clone (UN5) was selected for further use.

Western blot and biological analysis

Western blot analysis: Whole cell lysates were prepared from parental U937 cells, the Neo clone (UN5), and gelsolin-overexpressing clones (UG3, UG4, UG6 and UG11) in SDS lysis buffer (25 mM Tris–HCl pH 6.8, 0.8% SDS, 4% glycerol, 2% 2-mercaptoethanol, 0.008% bromophenol blue) after washing the cells twice in PBS. For immunoblotting the protein extracts were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Cardiff, UK). Blocking was performed with 5% nonfat dry milk in TBST (50 mmol/L Tris-HCl (pH7.5), 150 mmol/L NaCl, 0.1% Tween-20) for 1 hour. The membranes were rinsed 3 times with TBST and incubated overnight at 4°C with a mouse monoclonal antibody against human gelsolin GS-2C4 (SIGMA) (1:5000), a mouse monoclonal anti-human p21cip1 antibody (Cell Signaling Technology) and a mouse anti-human actin monoclonal antibody (1:5000) (Chemicon, Temecula, CA) in TBST. The next day, the membranes were washed several times with TBST and incubated with the secondary antibody: peroxidase conjugated affinity pure F(ab’)2, fragment goat anti-mouse IgG + IgM (H+L) (Jackson Immunoresearch laboratories Inc., West Grove, PA) in TBST for 1 hour at room temperature. Following another three washes with TBST, the membranes were incubated with ECL Western blotting detection reagents (Amersham Bioscience) for 1 min and the band intensity was analyzed by means of a Fuji film LAS-1000 Cool Scanner (Fuji Film, Kanagawa, Japan).

Growth curve: Parental U937 cells and stable clones (UN5, UG3, UG4, UG6, and UG11) were seeded at a density of 10⁴ per well in 6 well-plates and incubated at 37°C in a 5% CO₂-incubator. Viable cells were counted every 24 hours for 5 days, based on trypan blue exclusion using a haemocytometer.

Apoptosis was investigated by Hoechst 33342 staining (Molecular Probes, Eugene, OR). In brief, 10⁶ cells (U937, UN5, UG3, UG4, UG6, and UG11) were cultured in 6 well-plates and incubated at 37°C in a 5% CO₂-incubator for 24, 48 and 72 hours. Hoechst 33342 (33 ng/ml) was added to the wells 5 min prior to microscopic observation. A minimum of 300 cells were counted for each cell line by means of a haemocytometer, under the UV light source of a Nikon biological microscope Eclipse TE300 (Nikon, Yokohama, Japan).

Morphological and flow cytometric analysis, and enzymatic staining

Giemsa staining: Cells, at a density of 10⁶, were smeared onto glass slides, fixed with methanol for 1 min and air-dried. Slides were then stained with Giemsa solution (MERCK, Darmstadt, Germany) for 10 min and examined under a light microscope (Olympus BX50, Tokyo, Japan) to ascertain their morphology. At least three different fields were observed and more than 100 cells were counted per field.

Nitroblue tetrazolium (NBT) assay: Superoxide-generating activity of neutrophils was examined by the nitroblue tetrazolium (NBT) assay. NBT reduction
Gelsolin Induces Promonocytic Leukemia Differentiation Accompanied by Upregulation of p21CIP1

Non-specific Esterase staining: Parental U937 cells and stable clones (UN5, UG6, and UG11), at a density of \(10^6\), were smeared on glass slides, fixed for 1 min with methanol and air-dried. The slides were then stained for alpha-naphthyl esterase activity with 0.4% 1-naphthyl acetate (Sigma) in acetone mixed with pararosaniline HCl/sodium nitrite solution (Sigma) for 1 min. The slides were then rinsed in tap water, counter-stained with Mayer’s Haematoxylin, rinsed again and air-dried (Kim and Feldman, 2002). Cells were then viewed under a light microscope using 40X, 60X and 100X magnification. At least three different fields were observed and more than 100 cells were counted per field. Cells that contained black-blue formazan precipitates were considered positive.

Flow cytometric analysis: The surface marker antigen (β-integrin receptor) CD11b was monitored in U937 parental cells and UN5, UG6, and UG11 clones by flow cytometry (Yamamoto et al., 2009). Cells, at a density of \(10^6\), were washed 3 times in PBS containing 0.1% bovine serum albumin (BSA) (Wako, Osaka, Japan) and 0.05% sodium azide (FACS buffer). The cells were re-suspended in 90 microl FACS buffer and incubated with 10 microl fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-human CD11b (Sigma), or mouse IgG1/FITC as a negative control (Dako, Gotsstrup, Denmark), at 4°C for one hour in the dark. Cells were again washed three times in FACS buffer, passed through a mesh to remove debris and re-suspended at a concentration of 10^5/ml in FACS buffer. Fluorescence intensity was detected by means of a BECTON DICKINSON FACS Calibur (San Jose, CA).

PCR, RNAi, statistics

RT-PCR analysis: In an effort to delineate the molecules affected by gelsolin over-expression, flanking primers were designed for the gelsolin, PU1, and p21CIP1 genes, including GAPDH as an internal control. The forward and reverse PCR primers used were gelsolin: 5’-TAT GAA TTC GAC ACC GCC AAG GAG GAT-3’ and 5’-TAT CTC GAG TTA GGA AAG GTA GGA CAA GCC-3’ and PU1: 5’-CCA TCA GAA GAC CTG GTG C-3’ and 5’-GAT GCT GTC CTT CAT CTC G-3’; respectively. For p21CIP1 and GAPDH previously reported primers (Matsuzaki et al., 2003; Zeng and Davis, 2003) were used. Total RNA was isolated from parental U937 cells, the UN5 control clone and gelsolin-overexpressing clones (UG6 and UG11) using TRIZol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Two micrograms of RNA were reverse-transcribed using SuperScript II and random primers (Invitrogen). Two dilutions for cDNA were prepared (10X and 100X) and PCR reactions were performed using a Gene Amp PCR 9700 (PE Applied Biosystem, Foster City, CA). The PCR reaction cycle consisted of an initial denaturation step at 94°C for 5 min, followed by 33 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min (for gelsolin and PU1), 30 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec (for p21CIP1) or as previously described (for GAPDH) (Matsuzaki et al., 2003), and then followed by a final 7 min extension at 72°C. PCR reactions were run in 2% agarose gel electrophoresis and band images were detected as previously described (Tanaka et al., 2006).

RNA interference: To elucidate the role of p21CIP1 in the differentiation process induced by gelsolin overexpression, UG6 and UG11 clones were transfected by means of a siRNA system for p21CIP1 (Cell Signalling Technology, Beverly, MA). Fluorescein conjugated-control siRNA (SignalSilence: Cell Signaling) was used as a negative control. Experiments were performed using Nucleofector technology (Amaza Biosystems, Gaithersburg, MD), and the electroporation was performed according to the manufacturer’s instructions. Briefly, for each reaction, 10^6 UG6 or UG11 cells were mixed with a total of 100 nM of each siRNA for knockdown of expression in 100 µl of V solution (Amaza). Following electroporation, samples were transferred into 12-well plates and incubated at 37°C in 5% CO2 condition. Cell counting, Giemsa staining, and Western blot analyses for p21CIP1, were performed using an anti-p21CIP1 mouse monoclonal antibody (Cell signaling technology) at 24, 48, and 72 hour intervals, as mentioned earlier in this section.

Statistical analyses

Statistical analyses for data were done using two-tailed, unpaired Student’s t-tests. Differences were considered to be statistically significant at P<0.01.

Results

Gelsolin overexpression induces changes in morphology and growth patterns

Gelsolin was not detected in both parental U937 cells and the Neo control clone UN5, while gelsolin-overexpressing U937 clones UG3, UG4, UG6 and UG11, displayed expression of gelsolin (90 kDa) (Figure 1A). The level of actin, as an internal control, remained unchanged in all cell lines. UN5 cells exhibited exponential growth and continued to proliferate in a fashion similar to the parental U937 cells. Growth pattern of the lowest gelsolin expressing clone, UG3 was the same as parental cells and UN5. Overexpression of gelsolin in both UG6 and UG11 clones, however, resulted in clear inhibition of cellular proliferation between days 3 and 6. Growth rate of UG4 clones (UN5, UG6, and UG11) using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.
Figure 2. Induction of Monocytic Differentiation in Gelsolin-overexpressing U937 Clones. A) Giemsa staining of parental U937 cells, and UN5, UG6 and UG11 clones. Gelsolin-overexpressing UG6 and UG11 cells show greater monocytic morphology than UG3, UG4, UN5 and UG11. Actin was used as an internal control. B) The percentage of monocytic differentiation in parental U937 ( ), UN5 ( ), UG3 ( ), UG4 ( ), UG6 ( ) and UG11 ( ).

Figure 1. Isolation and Growth Suppression. A) Western blot analysis in parental U937 cells, the Neo control clone UN5, and gelsolin-overexpressed stable clones UG3, UG4, UG6 and UG11. Actin was used as an internal control. B) Growth curve of parental U937 ( ), UN5 ( ), UG3 ( ), UG4 ( ), UG6 ( ) and UG11 ( ).

Figure 1. Isolation and Growth Suppression. A) Western blot analysis in parental U937 cells, the Neo control clone UN5, and gelsolin-overexpressed stable clones UG3, UG4, UG6 and UG11. Actin was used as an internal control. B) Growth curve of parental U937 ( ), UN5 ( ), UG3 ( ), UG4 ( ), UG6 ( ) and UG11 ( ).

Figure 2. Induction of Monocytic Differentiation in Gelsolin-overexpressing U937 Clones. A) Giemsa staining of parental U937 cells, and UN5, UG6 and UG11 clones. Gelsolin-overexpressing UG6 and UG11 cells show greater monocytic morphology than UG3, UG4, the parental U937 and UN5 control cells (100x). B) The percentage of monocytic differentiation in the parental U937 cells and UN5 control cells, UG3, UG4, UG6 and UG11 clones. The error bars indicate standard deviations from three different microscopic fields that were randomly chosen and contained more than 100 cells per field having monoblast or monocyte characteristics.

Induction of monocytic differentiation markers in gelsolin-overexpressing U937 clones

Functional differentiation of monoblasts into monocytes was histologically quantified by the cell’s ability to produce a superoxide, generated via NADPH oxidase, and to reduce the tetrazolium salt NBT to insoluble formazan, which forms a black-blue precipitate in these cells (Jiang et al., 2008). As shown in Figure 3, NBT-reducing ability was enhanced in UG6 (70%) and UG11 (68%) cells, when compared with UN5 (2%) and parental (2%) cells (Figure 3B).

Since the NBT-reducing ability has also been used to observe differentiation into the granulocyte lineage, the measurement of non-specific esterases activity was used to distinguish differentiation into the monocyte lineage. NSE is a heterogeneous and ubiquitous group of carboxyl esterases which are detected cytochemically with alpha-naphthyl ester substrates and diazotized couplers (Miranda, 2002). In monocytes NSE can react with alpha-naphthyl ester substrates that lead to the appearance of red-brown granules (Miranda et al., 2002). As shown in Figure 4A and 4B, 55-45% of UG6 and UG11 cells were positive for NSE staining, when compared with UN5 (5%) and parental U937 (3%) cells.

The expression of CD11b (a beta-integrin receptor) is enhanced in myelomonocytic progenitors following differentiation to more mature cell types (Yamamoto et al., 2009). Flow cytometric analysis revealed that the mean percentages of CD11b-positive cells in parental U937 and UN5 cells were 12%–10%, whereas those in UG6 and UG11 cells increased to 40%–36% (Figure 5A).

All these data demonstrate that gelsolin plays an important role in the monocytic differentiation of U937 cells, also in terms of functional activity.

Gelsolin overexpression upregulates p21CIP1

Availability of PU1 is crucial for normal myeloid...
Gelsolin Induces Promonocytic Leukemia Differentiation Accompanied by Upregulation of p21CIP1

Figure 3. Nitroblue Tetrazolium (NBT)-reducing activity in Gelsolin-overexpressing U937 Clones. A) Safranin counterstaining in parental U937 cells, control UN5 cells and gelsolin-overexpressing UG6 and UG11 cells. Parental U937 cells and UN5 cells contained few NBT positive cells whereas gelsolin-overexpressing UG6 and UG11 cells displayed a lot of NBT-positive cells (100X). B) Cells that contained dark precipitation were counted as positive for NBT-reducing activity. The error bars indicate standard deviations from three different microscopic fields that were randomly chosen and contained more than 100 cells per field (*P<0.01).

Figure 4. Nonspecific Esterase Activity in Gelsolin-overexpressing U937 Clones. A) 1-naphtyl acetate staining with haematoxylin counterstaining in parental U937 cells, Neo control UN5 cells, and gelsolin-overexpressing UG6 and UG11 cells. Low esterase activity was observed in parental U937 cells and UN5 cells whereas high esterase activity was observed in the gelsolin-overexpressed UG6 and UG11 cells (100X). Right bottom corner insert shows higher magnification (200X). B) Cells that contained red-brown granules were counted as positive for NSE staining. The error bars indicate standard deviations from three different microscopic fields that were randomly chosen and contained more than 100 cells per field (*P<0.01).

Figure 5. CD11b Expression and RT-PCR Analysis. A) Flow cytometry, using a FITC-conjugated monocyte/macrophage terminal differentiation cell surface marker CD11b antibody in parental U937 cells, Neo control UN5 cells, and gelsolin-overexpressing UG6 and UG11 cells. The error bars indicate standard deviations from triplicate experiments (*P<0.01). B) RT-PCR analysis of gelsolin, PU1 and p21CIP1 mRNAs in parental U937 cells, control UN5 cells, and gelsolin-overexpressing UG6 and UG11 cells. The level of p21CIP1 mRNA increased 13-25 fold in gelsolin-overexpressing clones while the level of PU1 mRNA remained unchanged in all cells. GAPDH mRNA was used as an internal control. C) Western blot analysis for p21CIP1 expression also shows up-regulation of p21CIP1 at the protein level.

P21CIP1 silencing reverses the differentiation and growth suppression induced by gelsolin

To understand the role of the p21CIP1 gene in gelsolin overexpression-induced monocytic differentiation, gene knockdown experiments were performed using siRNAs. Western blot analysis for UG6 and UG11 cells, transfected...
A) Western Blot Analysis for U937 Cells Transfected with siRNA for p21CIP1. Control basal p21CIP1 mRNA levels before transfection and proliferation of p21 siRNA-transfected UG6 and UG11 cells, compared to control siRNA transfected cells (siRNA proliferation: 72h after transfection).

B) Growth Curve for U937 Gelsolin-overexpressing Clones Transfected with siRNA for p21CIP1. Control time points were selected according to the maximal effect of siRNA on proliferation (The optimal time point for siRNA was 48h). UG6 Si-Cont (○), UN11 Si-Cont (△), UG6 Si-p21CIP1 ( ■), and UG11 Si-p21CIP1 ( ● ). The error bars indicate standard deviation.

C) The Percentage of Monocytic Differentiation in U937 Gelsolin–overexpressing Clones Transfected with siRNA for p21CIP1 Compared to the Same Clones Transfected with Control siRNA. Data points were selected according to the maximal effect of siRNA on proliferation which was determined in separate experiments. (The optimal time points were 48h). Error bars; Standard deviation from three different microscopic fields that were randomly chosen and contained more than 100 cells per field that had characteristics of monoblasts or monocytes.

with siRNA for p21CIP1, demonstrated 90.6% and 95.2% knockdown respectively, after 48 hours, which was the best point for silencing (Figure 6A). The growth ratio of the p21CIP1-silenced UG6 and UG11 cells demonstrated a slight increase, when compared with that of the control siRNA-transfected cells (Figure 6B). Morphological analysis, 48 hours after nucleofection, revealed that the monocyte ratio of the p21CIP1-silenced UG6 and UG11 cells was reduced to 28% and 34%, respectively, while control siRNA-transfected UG6 and UG11 cells showed the same monocytic ratio (47% and 68%) as that of untreated UG6 and UG11 clones (50% and 70%) (Figure 6C). All these results strongly suggest that p21CIP1 plays an important role in the growth inhibition and monocytic morphological differentiation observed in gelsolin overexpression-induced monocytic differentiation of U937 cells.

Discussion

Tumor suppressor genes have received much attention for their roles in the development of human malignancies. Their gene products, however, function as negative growth regulators and are highly expressed in many tissues during embryonic development, suggesting that they might also act as critical proteins in differentiation and development (Lai, 2012).

Previously, it was reported that the expression of gelsolin is frequently silenced in various cancers, both in cell lines as well as in human tissue tumors (Tanaka et al., 2006). Since overexpression of wild-type gelsolin inhibited the growth of colon, bladder and lung cancer cell lines in vitro and in vivo, it was proposed that gelsolin can function as a tumor suppressor (Sazawa et al., 2002; Sagawa et al., 2003). It has been shown that one mechanism accounting for the inhibition of cell proliferation and tumorigenicity was related to gelsolin suppressing the activation of protein kinase Cs (PKCs) involved in phospholipid signaling pathways (Sagawa et al., 2003). Furthermore, it has also been demonstrated that transfectants of a highly metastatic murine melanoma cell line, B16-BL6, expressing ectopic gelsolin, exhibited retardation of cell spreading, reduced chemotactic migration to fibronectin and suppressed lung colonization in a spontaneous metastasis assay (Fujita et al., 2001).

There is evidence implicating other tumor suppressors, such as, the retinoblastoma gene (Goodrich, 2006), p53 (Spike and Wahl, 2011), DCC gene (Zhao, 2012), and Differentiation-Related-Gene-1 (Drg-1) (Dong et al., 2005) in cellular differentiation. Results of the molecular control of changes in the normal developmental program in myeloid leukaemia have shown that genetic abnormalities which give rise to malignancy can be bypassed, and their effects nullified, by inducing differentiation, which stops cells from multiplying (Lai et al., 2012). Specific haematopoietic transcription factors are crucial for differentiation to particular lineages during normal differentiation, and are controlled by specific patterns of expression and protein interactions (Petrie, 2009). PU1, an Ets family member, is one of the master transcription factors identified to regulate development of both granulocytes and monocytes/macrophages (Shima and Kitabayashi, 2011). However, in our gelsolin-induced monocytic differentiation, the mRNA expression of PU1 was unchanged.

Myeloid maturation of CD34+ precursor cells is associated with a marked increase in the tumor-suppressor p21CIP1 expression, both at RNA and protein levels (Ullmannová, 2003). In our study, p21CIP1 was also upregulated in gelsolin-overexpressing U937 cells, however the level of the tumor suppressor p53 (activated form) remained unchanged (data not shown). These data suggest that expressions of gelsolin and p21CIP1 are functionally associated with the regulation of cell differentiation. To confirm whether the up-regulation of the p21CIP1 gene was associated with the progression
of monocytic differentiation of the U937 cell line, we investigated the effect of a siRNA, which inhibits the expression of p21CIP1. SiRNA caused reduction in p21CIP1 but a control siRNA did not. The knockdown of p21CIP1, however, showed reduced growth rate and a morphological monocytic differentiation phenotype. Therefore, taken together, our results strongly suggest that p21CIP1 acts as an effector of cytoplasmic gelsolin and plays an important role in gelsolin-induced monocytic differentiation of U937 cells.

A myeloid leukaemia suppressor gene encoding sequence-specific single-stranded DNA binding protein 2 (SSBP2) which may function by direct interaction with cytoskeletal elements, and thus altering signal transduction, was reported to be a potential novel regulator of haematopoietic growth and differentiation, whose loss confers a block in differentiation advantage to myeloid leukaemic cells (Liang, 2005). Our work confirms that the cytoskeletal tumor suppressor, gelsolin, can induce monocytic myeloid differentiation in addition to growth retardation in the human monocytic cell line U937, thus providing further evidence that several tumor suppressor genes have differentiation-inducing function.

Studies with myeloid leukaemic cells have shown that some leukaemic cells can be induced to differentiate by cytokines that control normal haematopoiesis, and that myeloid leukaemic cells resistant to normal cytokines can be induced to differentiate by compounds that use alternative differentiation pathways (Humeniuk, 2009). The maturation arrest of acute promyelocytic leukaemia (APL), for instance, can be reversed with all-trans retinoic acid (Germain, 2006). More recent trials involving differentiation induction demonstrate that this can be applied even in combination with intensive chemotherapy to increase the susceptibility of AML blasts to drug-induced apoptosis (Fredly et al., 2009). Besides the standard therapy for AML, the use of site-specific ligands, receptors and cytokines, disruption of dominant fusion leukaemogenic proteins, chromatin remodeling and the combination of the above with cytotoxic chemotherapy have been shown to be synergistic in inducing myeloid differentiation and apoptosis in several AML cell lines and in patients with APL (Koeffler, 2010). In this report, we demonstrate that an actin regulatory-protein, gelsolin, by itself, has induced growth retardation and monocytic differentiation in U937 cells. Recent evidence suggests that several members of the gelsolin family might possess unexpected nuclear functions, including roles in regulating transcription (Li, 2012). For future studies, an in vivo system is necessary to develop a model for gene therapy. This could provide promising new strategies for the use of gelsolin as a differentiation-inducing agent in the therapy of promonocytic leukaemia cells.

Acknowledgements

We thank Dr. Masanabu Kobayashi for his helpful suggestions about this work. This research was supported in parts by a grant-in-aid from the Ministry of Education, Science, Sports and Culture, Japan.

References

Banno Y, Fujita H, Ono Y, et al (1999). Differential phospholipase D activation by bradykinin and sphingosine 1-phosphate in NIH 3T3 fibroblasts overexpressing gelsolin. J Biol Chem, 274, 27385-91.

Bhat R, Malinge S, Gamis A, et al (2009). Mutational analysis of candidate tumor-associated genes in acute megakaryoblastic leukemia. Leukemia, 23, 2159-60.

Cervio C, Barotti D, Ibanez J, et al (2012). Early mortality in children with advanced mature b-cell malignancies in a middle-income country. J Pediatr Hematol Oncol, 34, 266-70.

Craig C, Schiller G (2008). Acute myeloid leukemia in the elderly: conventional and novel treatment approaches. Blood Rev, 22, 221-34.

Dieffenbach C, SenGupta D, Krause D, Swazak D, Silverman, RH (1989). Cloning of murine gelsolin and its regulation during differentiation of embryonal carcinoma cells. J Biol Chem, 264, 13281-8.

Dong Z, Arnold R, Yang Y, et al (2005). Modulation of differentiation-related gene 1 expression by cell cycle blocker mimosine, revealed by proteomic analysis. Mol Cell Proteomics, 4, 993-1001.

Fredly H, Ersvær E, Stapanes C, Gjertsen B, Bruserd Q (2009). The combination of conventional chemotherapy with new targeted therapy in hematologic malignancies: The safety and efficiency of low-dose cytarabine supports its combination with new therapeutic agents in early clinical trials. Current Cancer Therapy Rev, 5, 243-55.

Fujita H, Okada F, Hamada J, et al (2001). Gelsolin functions as a metastasis suppressor in B16-BL6 mouse melanoma cells and requirement of the carboxyl-terminus for its effect. Int J Cancer, 93, 773-80.

Furnish E, Zhou W, Cunningham C, Kas J, Schmidt CE (2001). Gelsolin overexpression enhances neutrite outgrowth in PC12 cells. FEBS Letters, 508, 282-6.

Germain P CP, Eichele G, Evans RM, et al (2006). International union of pharmacology. LX. Retinoic acid receptors. Pharmacol Rev, 58, 712-25.

Goodrich D (2006). The retinoblastoma tumor-suppressor gene, the exception that proves the rule. Oncogene, 25, 5233-43.

Humeniuk R, Mishra P, Bertino J, Banerjee D (2009). Molecular targets for epigenetic therapy of cancer. Curr Pharm Biotechnol, 10, 161-5.

Jiang F, Guo N, Dusting G (2008 ). Modulation of nicotinamide adenine dinucleotide phosphate oxidase expression and function by 3',4'-dihydroxyflavonol in phagocytic and vascular cells. J Pharmacol Exp Ther, 324, 261-9.

Kim J, Feldman R (2002). Activated Fes protein tyrosine kinase induces terminal macrophage differentiation of myeloid progenitors (U937 cells) and activation of the transcription factor PU.1. Mol and Cell Biochem, 22, 1903-18.

Koeffler H (2010). Is there a role for differentiating therapy in non-APL AML? Best Pract Res Clin Haematol, 23, 503-8.

Koschmieder S, Agrawal S, Radomska H, et al (2007). Decitabine and vitamin D3 differentially affect hematopoietic transcription factors to induce monocytic differentiation. Int J Oncol, 30, 349-55.

Kuzumaki N, Maruta H (2002). Cytoskeletal tumor suppressor Genes, tumor-suppressing viruses, genes and drugs innovative cancer therapy approach, 177-197.

Kwaitkowski D (1988). Predominant induction of gelsolin and actin binding protein during myeloid differentiation. J Biol Chem, 263, 13857-62.

Lai D, Visser-Grieve S, Yang X (2012). Tumour suppressor genes in chemotherapeutic drug response. Biosci Rep, 32, 361-74.
Li G, Arora P, Chen Y, McCulloch C, Liu P (2012). Multifunctional roles of gelsolin in health and diseases. Med Res Rev, 32, 999-1025.

Liang H, Samanta S, Nagarajan L (2005). SSBP2, a candidate tumor suppressor gene, induces growth arrest and differentiation of myeloid leukemia cells. Oncogene, 24, 2625-34.

Mak K, Funnell A, Pearson R, Crossley M (2011). PU.1 and Haematopoietic Cell Fate: Dosage Matters. Int J Cell Biol, VOL?, PAGE?-?

Matsuzaki K, Minami T, Tojo M, et al (2003). PML-nuclear bodies are involved in cellular serum response. Genes Cells, 8, 275-86.

Miranda M, McGuire T, Johnson D (2002). Importance of MEK-1/-2 signaling in monocytic and granulocytic differentiation of myeloid cell lines. Leukemia, 16, 683-92.

Moosavi M, Yazdanparast R (2008). Distinct MAPK signaling pathways, p21 up-regulation and caspase-mediated p21 cleavage establishes the fate of U937 cells exposed to 3-hydrogenkwadaphnin: differentiation versus apoptosis. Toxicol Appl Pharmacol, 230, 86-96.

Nam K, Choi M, Yoo E, Paeng D (2011). Influence of cell packing by centrifugation on 40-MHz ultrasound backscatter. Ultrasonics, 51, 197-201.

O’Donnell M, Abboud C, Altman J, et al (2012). Acute myeloid leukemia. J Natl Compr Canc Netw, 10, 984-1021.

Ory D, Neugeboren B, Mulligan R (1996). A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci USA, 93, 11400-6.

Patel D, Ahmad S, Weiss D, Gerke V, Kuznetsov S (2011). Annexin A1 is a new functional linker between actin filaments and phagosomes during phagocytosis. J Cell Sci, 124, 578-88.

Petrie K, Zelant A, Waxman S (2009). Differentiation therapy of acute myeloid leukemia: past, present and future. Current Opinion in Hematology, 16, 84-91.

Sagawa N, Fujita H, Banno Y, et al (2003). Gelsolin suppresses tumorigenicity through inhibiting PKC activation in a human lung cancer cell line, PC10. Br J Cancer, 88, 606-12.

Sato M, Maruoka M, Takeya T (2012). Functional mechanisms and roles of adaptor proteins in Abl-regulated cytoskeletal actin dynamics. JOURNAL?, VOL?, PAGE ?-?:

Sawada A, Watanabe T, Tanaka M, et al (2002). Adenovirus mediated gelsolin gene therapy for orthotopic human bladder cancer in nude mice. J Urol, 168, 1182-7.

Shima Y, Kitabayashi I (2011). Deregulated transcription factors in leukemia. Int J Hematol, 94, 134-41.

Spive B, Wahl G (2011). p53, stem cells, and reprogramming: tumor suppression beyond guarding the genome. Genes Cancer, 2, 404-19.

Tanaka H, Shirkoohi R, Nakagawa K, et al (2006). siRNA gelsolin knockdown induces epithelial-mesenchymal transition with a cadherin switch in human mammary epithelial cells. Int J Cancer, 118, 1680-91.

Ullmannová V, Stockhauer P, Hradcová M, Soucek J, Haskovec C (2003). Relationship between cyclin D1 and p21(Waf1/Cip1) during differentiation of human myeloid leukemia cell lines. Leuk Res, 27, 1115-23.

Verdijk P, van Veelen P, de Ru A, et al (2004). Morphological changes during dendritic cell maturation correlate with coflin activation and translocation to the cell membrane. Eur J Immunol, 34, 156-64.

Wiertik P (2011). Acute lymphoblastic leukemia of adulthood: progress or not? Curr Treat Options Oncol, 12, 303-11.

Yamamoto T, N Sakaguchi, Hachiyama M, et al (2009). Role of catalase in monocytic differentiation of U937 cells by TPA: hydrogen peroxide as a second messengerRole of catalase in monocytic differentiation. Leukemia, 23, 761-9.

Zeng H, Davis C (2003). Down-regulation of proliferating cell nuclear antigen gene expression occurs during cell cycle arrest induced by human fecal water in colonic HT-29 cells. J Nutr, 133, 2682-7.