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Acute myelogenous leukemia cells with the MLL–ELL translocation convert morphologically and functionally into adherent myofibroblasts

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ABSTRACT

Bone marrow-myofibroblasts, a major component of bone marrow-stroma, are reported to originate from hematopoietic stem cells. We show in this paper that non-adherent leukemia blasts can change into myofibroblasts. When myeloblasts from two cases of acute myelogenous leukemia with a fusion product comprising mixed lineage leukemia and RNA polymerase II elongation factor, were cultured long term, their morphology changed to that of myofibroblasts with similar molecular characteristics to the parental myeloblasts. The original leukemia blasts, when cultured on the leukemia blast-derived myofibroblasts, grew extensively. Leukemia blasts can create their own microenvironment for proliferation.

Introduction

Chromosomal translocations involving the mixed lineage leukemia (MLL) gene on chromosome 11 at q23 are a commonly observed cytogenetic abnormality [1,2]. Approximately 5–10% of adults with acute myelogenous leukemia (AML) and acute lymphocytic leukemia (ALL) have such a translocation [3]. MLL-involving translocations are also observed in cases of therapy-related leukemia, especially in patients previously treated with inhibitors of DNA topoisomerase II [4]. In cases of MLL-related chromosomal aberrations, both AML and ALL are developed; thus, acute leukemia with MLL-abnormality is caused by a malignant transformation of hematopoietic cells of the stem cell level [5].

The MLL gene is significantly homologous with the *Drosophila trithorax* gene, which serves to regulate and/or maintain the homeotic expression of multiple HOX genes during fetal development [6]. MLL adopts an active form through dimerization with the AT-hook of its N-terminal region. Also, the C-terminal half of MLL is processed proteolytically, forming a heterodimer with the N-terminal half [7]. Thus, promotion of the dimerization of MLL triggers activation [8]. Regarding MLL-related chromosomal aberrations, about 30 fusion partners have been identified [9]. ELL, one of these translocation-partners located at 19p13.1, was first identified as a transcriptional elongation factor for RNA polymerase II [10], and also revealed to regulate cell proliferation and survival [11]. When MLL fuses with ELL, molecular dimerization occurs, and subsequently cellular transformation is induced to generate leukemia. In an *in vivo* murine leukemia model, over-expression of MLL–ELL transformed hematopoietic stem cells to generate AML [12].

To elucidate the precise biological characteristics of leukemia blasts with the MLL–ELL translocation, we examined a primary culture of non-adherent leukemia blasts *in vitro*. The blasts were prepared from two AML patients with t(11;19)(q23;p13.1). After culturing for 1 month, leukemia blast-derived adherent myofibroblasts were generated, and then characterized. The implications for these myofibroblasts are discussed with regard to a cancer stem cell.

Materials and methods

Cell culture. Bone marrow cells collected from informed patients and healthy volunteers were centrifuged in Ficoll-Paque (S.G. 1077, Lymphoprep™, Fresenius Kabi Norge AS, Norway) to obtain a mononuclear cell-fraction. A 24-h culture in Dulbecco's Modified Eagle's Medium (DMEM; Nissui, Japan) containing 10% fetal calf serum (FCS; CELLect® GOLD; MP Biomedicals, Germany) in a humidified 5% CO_2 incubator eliminated adherent cell-fractions, and the prepared non-adherent mononuclear cells were further cultured long term. If adherent cells were observed microscopically during the cultures, cells were treated with Trypsin (Sigma, MO), and re-plated every week to eliminate the non-adherent cells, and after 1 month myofibroblasts were prepared. Cells were cultured on a 96-well flat-bottom plate (Corning Incorporated Costar, NY) for the selection of positive clones using reverse transcription-polymerase chain reaction (RT-PCR).

RT-PCR and genomic PCR analysis. RNA was extracted from patient-derived leukemia blasts, normal bone marrow cells, leukemia blast-derived myofibroblasts, and normal bone marrow-derived

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myofibroblasts using the GTC method. The first-strand cDNA was synthesized with an oligo-dT primer using a first-strand cDNA Synthesis Kit (Invitrogen, CA), with which RT-PCR was carried out. The primers used included: MLL-common and ELL reverse [13]; MLL exon-6 forward-1 and ELL exon-2 reverse-1, and MLL exon-6 forward-2 and ELL exon-2 reverse-2 [14]; CD13 forward and reverse [15]; CD33 forward and reverse [16]; CD106 forward and reverse [17]; CD34 forward and reverse [18]; CD133 forward (5′-GATCTGG TGTCCAGCATG-3′) and reverse (5′-ACATGAAAAGA CCTGGGGG-3′) ([19], and GenBank); human fibroblast-specific protein 1 (FSP1) forward-1 and reverse-1, and forward-2 and reverse-2 [20]; and forward and reverse primers for human GAPDH as a control for the reaction [18]. The MLL–ELL PCR product obtained with the MLL common and ELL reverse primers was analyzed with 2.5% agarose gel electrophoresis in Tris–Borate–EDTA buffer, the fusion cDNA fragment was recovered with a Geneclean® II Kit (MD Biomedicals, OH), and the sequence of the fusion product was determined using a BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA) and analyzed with an ABI PRISM 3700 DNA analyzer (Applied Biosystems). Genomic PCR was carried out with a 1st PCR using the MLL common and ELL reverse primers, a 2nd PCR with the MLL exon-6 forward-1 and ELL exon-2 reverse-1 primers, and a 3rd PCR with the MLL exon-6 forward-2 and ELL exon-2 reverse-2 primers.

Southern blot analysis and fluorescent in situ hybridization (FISH) analysis of the genomic DNA. High molecular weight DNA was obtained from the indicated cells (SepaGene Kit®, Sanko-Junnyaku, Japan), and 10 μg was digested with an appropriate restriction enzyme, and electrophoresis was carried out. The procedures for electrophoresis, transferal to the membrane, pre-hybridization, and hybridization have been reported previously [21]. The BamHI-digested fragment of probe X was used as a probe [22]. FISH was carried out as reported [23], with the 5′ portion of genomic MLL labeled with SpectrumGreen (green) and the 3′ part labeled with SpectrumOrange (red) (Abbott, IL). Normal cells appeared yellow because of the fusion of the 5′ and 3′, while cells having the MLL-translocation gave split red and green signals.

Immunocytochemical staining. Cells were cultured on Lab-Tek Chamber Slides (Nunc, NY) for 3–7 days. Cells were fixed with acetone at 4 °C for 5 min, air-dried, and washed with phosphate-buffered saline (PBS). To eliminate an intrinsic peroxidase activity, the slides were treated with 0.6% hydrogen peroxide in PBS for 5 min. After two washes, the slides were reacted with murine monoclonal antibodies at 37 °C for 40 min. The antibodies used included; anti-human CD13 antibody (Ab) (diluted with PBS at 1:100, Novocastra, UK), CD33 Ab (1:100, Immunotech, Beckman Coulter, CA), fibronectin Ab (1:200, Immunotech), smooth muscle actin (SMA) Ab (1:200, DAKO, Denmark), FSP1 (also called S100) Ab (1:200, Becton Dickinson, CA), CD106 Ab (1:200, Becton Dickinson), CD34 Ab (1:50, Becton Dickinson), CD133 Ab (1:50, Miltenyi Biotec, Germany), and myeloperoxidase Ab (1:300, DAKO). After three washes, the slides were incubated with rat anti-murine immunoglobulin G monoclonal Ab at 37 °C for 30 min. They were washed, and stained with 3,3-diaminobenzidine tetrahydrochloride (Histofine; Nichirei, Japan) and hematoxylin [24].

Cytokine assay. The human vascular endothelial growth factor (VEGF) Immunoassay Kit (Quantikine®; R&D systems, MN), human basic fibroblast growth factor (bFGF) Immunoassay Kit (Quantikine®), and human interleukin (IL)-7 Immunoassay Kit (Quantikine®) were purchased commercially. The conditioned media from 48-h-cultures of the myofibroblasts at 1×10^4/ml in 24-well plates (NUNC) were used for the quantification according to the manufacturer's directions.

^{3}H-incorporation assay. The cultured cells were re-suspended to a concentration of 1×10^6/ml for normal bone marrow non-adherent mononuclear cells, and 1×10^4/ml for leukemia blasts. One hundred microliters of the cell suspension was cultured in a flat-bottom 96-well plate (Corning Incorporated Costar). When cells were cultured on the feeder layer, adherent myofibroblasts were prior irradiated at 30 Gray (Gy). Then 0.1 μCi of [methyl-^{3}H]-thymidine (Specific Activity 80.0 Ci/mmol; Amersham Biosciences, NJ) per well was added for 18 h. The cultured cells were trapped and washed on glass-filters (Whatman Laboratory, UK) using a cell harvester (M-24 system; Brandel, MD), and the radioactivity of the filters was counted using an LSC-5100 scintillation counter (Aloka, Japan).

Statistical analysis. The data are represented as the means + standard error of the mean (SEM). The significance of differences among the groups was determined using Student's *t*-test, and is indicated ($P < 0.01$).

Results

Molecular analysis of leukemia blasts and blast-derived myofibroblasts

Mononuclear cells were prepared from two informed AML patients (Fig. 1B and C) using density centrifugation, in which the

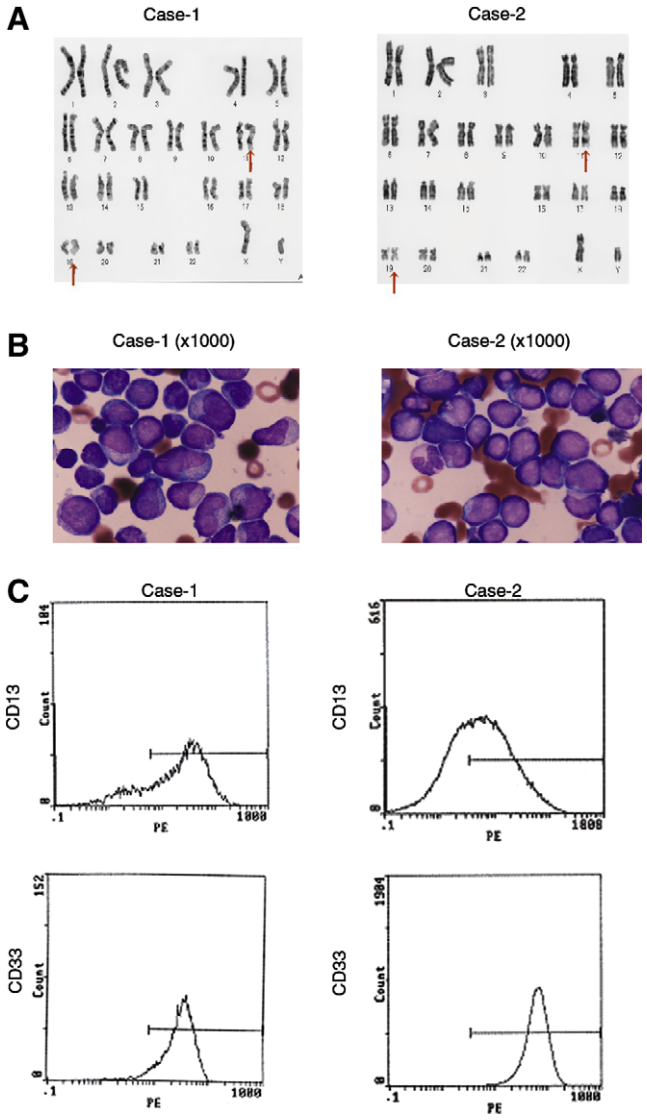

Fig. 1. Characteristics of the patients' leukemia blasts. (A) A karyotypic analysis of bone marrow cells from the patients using the G-banding method. Red arrows indicate t(11;19)(q23;p13.1). (B) May-Giemsa staining of bone marrow cells. (C) Analysis of the cell-surface molecules expressed in the patients' blasts (COULTER EPICS XL-MCL, Beckman Coulter, CA).

chromosomal translocation t(11;19)(q23;p13.1) was identified (Fig. 1A, red arrows). The cells were cultured short term to eliminate adherent fractions. The primary non-adherent mononuclear cell-culture was continued, and the morphology of the cultured cells was observed. When adherent cells were observed in the cultures, the cells were treated with Trypsin, and re-plated every week to eliminate the non-adherent fractions. A prominent expansion of myofibroblasts was observed after 1 month (Fig. 2A), and further analysis was performed.

The data on chromosomal translocation indicated the MLL–ELL fusion gene to be present in these two AML cases; thus, leukemia blasts were analyzed with RT-PCR and the genomic PCR as well as Southern blotting. The RT-PCR analysis revealed the transcript of MLL–ELL to be present in leukemia blasts (Fig. 2B and C). At DNA level, an abnormal band was detected by Southern blot analysis in leukemia blasts using probe X, which can recognize the breakpoint region of MLL [22] (data not shown). The genomic PCR analysis demonstrated that the MLL gene and ELL gene were fused in two cases of AML (Fig. 2D). The cultured myofibroblasts were divided into sub-clones, further cultured, and then analyzed using RT-PCR. As shown in Fig. 2B, the fusion transcript was identified in 13 of 96 clones in case-1, and 8 of 96 clones in case-2. The genomic PCR analysis demonstrated a specific band of approximately 3.5-kbp in the leukemia blast-derived myofibroblast clones (Fig. 2D). Also, in the generated myofibroblasts, the 5′ and 3′ parts of the MLL gene were observed to have split in the FISH analysis

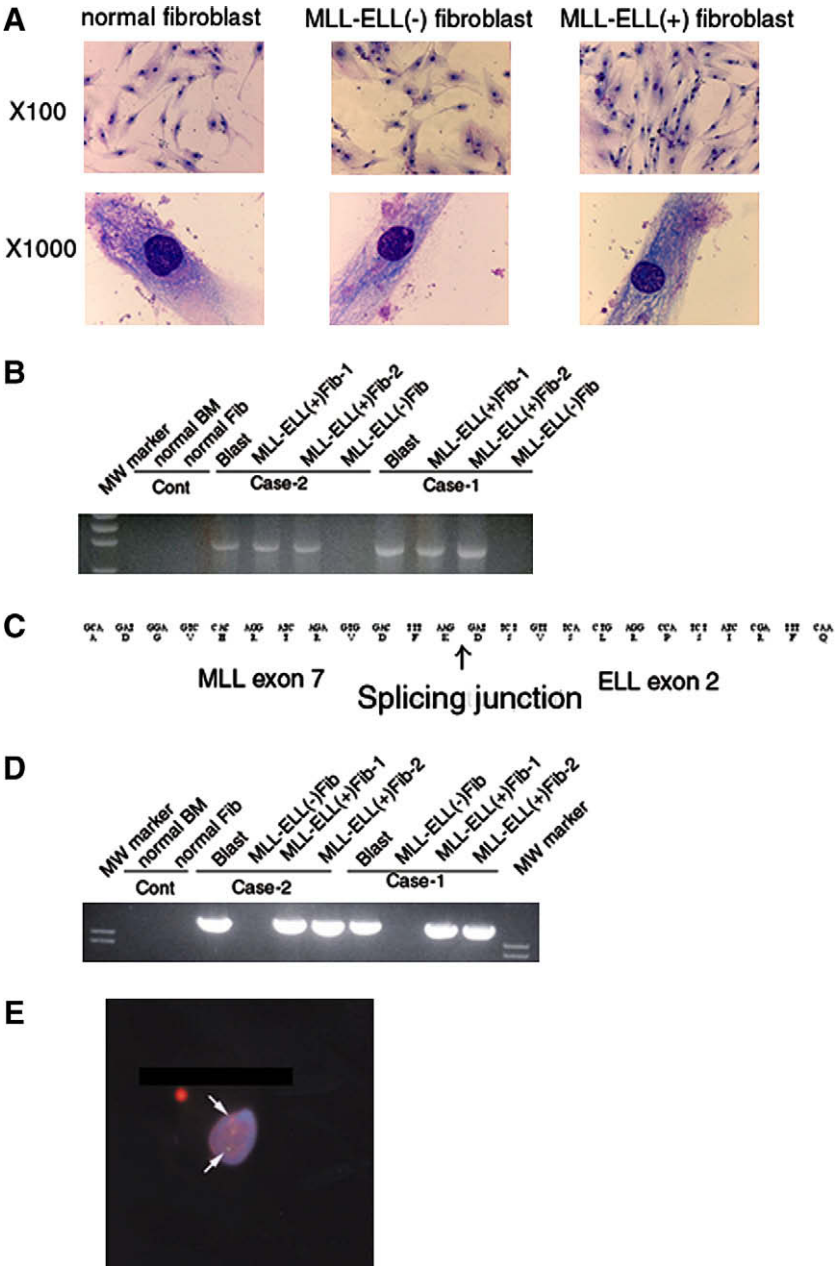

Fig. 2. Morphology and characteristics of leukemia blast-derived myofibroblasts. (A) The morphology of the cultured myofibroblasts (May-Giemsa staining). (B) RT-PCR analysis. Both the patients' blasts and the cloned myofibroblasts expressed the MLL–ELL fusion transcript. (C) cDNA sequence of the fusion product obtained from RT-PCR analysis. The splicing junction of MLL and ELL is indicated with an arrow. (D) Genomic PCR analysis using three different primer pairs indicated the fusion of MLL and ELL. (E) FISH analysis. The 5′ and 3′ MLL genomic locus was labeled. Arrows indicate split signals of the 5′ and 3′ MLL genomic locus. In (B), and (D), MW indicates molecular weight; BM, bone marrow non-adherent mononuclear cells; and fibroblast and Fib, myofibroblasts.

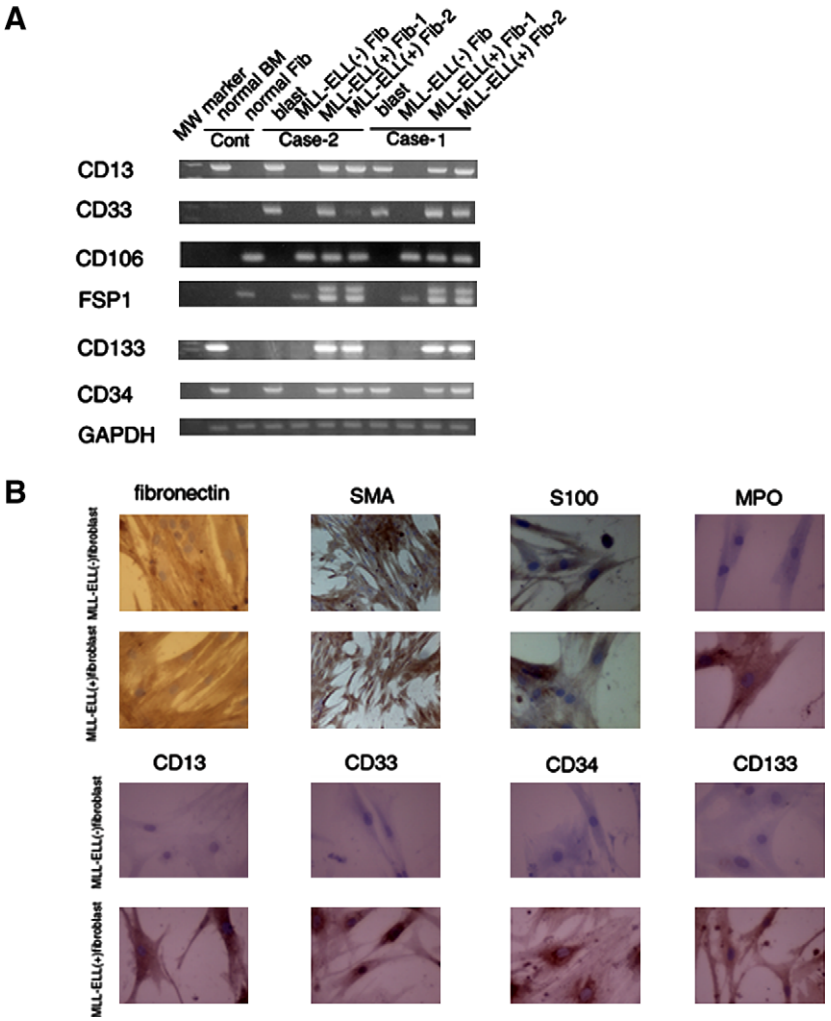

Fig. 3. Expression of specific molecules in leukemia blast-derived myofibroblasts. RT-PCR analysis (A), and immunocytochemical staining (B).

(Fig. 2E). The RT-PCR products were sequenced, with case-1 and case-2 found to have a similar fusion product with a similar splicing junction of MLL and ELL (Fig. 2C).

Analysis of the molecular expression in leukemia blast-derived myofibroblasts

Cells were stained with murine monoclonal antibodies, used included anti-human fibronectin Ab [25], and CD106 (VCAM-1) Ab [26] for the detection of adherent cells, CD13 Ab [15], CD33 Ab [16], and myeloperoxidase Ab [27] for myeloid leukemia, which was also expressed in their leukemia blasts (Fig. 1C), and CD34 Ab and CD133 Ab for stem cell-markers. For the myofibroblast marker, FSP1 Ab, which is expressed in myofibroblasts but not in monocytes nor macrophages [20], and SMA Ab were also examined. Fig. 3B shows the results, in which myofibroblasts generated from the leukemia blasts originating from case-2 are demonstrated. Results of immunocytochemical staining of normal bone marrow-derived myofibroblasts (n-Fib) were similar to those for leukemia blast-derived myofibroblasts that did not express the MLL–ELL fusion transcript ((−)-Fib), and in the figure the results from (−)-Fib and leukemia blast-derived myofibroblasts that constitutively expressed MLL–ELL ((+)-Fib) are demonstrated. Fibronectin was strongly stained in n-Fib (data not shown), (−)-Fib, and (+)-Fib. The anti-CD106 Ab was stained very weakly (data not shown). RT-PCR analysis revealed that CD106 was expressed in these three kinds of myofibroblasts, but not in bone marrow mononuclear cells, or in leukemia blasts (Fig. 3A). SMA was also positively stained in (−)- and (+)-Fib. FSP1 showed positive staining in the (−)- and (+)-Fib as well, which indicated that the newly generated cells are not macrophage that had differentiated from the leukemia blasts, but have the characteristics of myofibroblasts. CD13 and CD33 were expressed in (+)-Fib, but not in n-Fib (data not shown), or in (−)-Fib. Myeloperoxidase was not expressed in n-Fib (data not shown) or (−)-Fib, but was detected in (+)-Fib. RT-PCR analysis showed similar results for CD13 and CD33 staining (Fig. 3A). The expression of CD133 was also demonstrated in (+)-Fib, as well as in (−)-Fib at very low levels (Fig. 3B). Leukemia blasts were not stained by the anti-CD133 Ab (data not shown). RT-PCR analysis revealed that CD133 was expressed in (+)-Fib as well as (−)-Fib and n-Fib (Fig. 3A, and data not shown). (+)-Fib was also stained by the anti-CD34 Ab, and at the RT-PCR level, the expression of CD34 was demonstrated in leukemia blasts and (+)-Fib (Fig. 3A and B).

Biological characterization of MLL–ELL (+) myofibroblasts

On the growth of the myofibroblasts, almost the same capacity to proliferate was observed between (−)- and (+)-Fib; however, after 5 months of culture, the growth of the myofibroblasts diminished (data not shown). A functional analysis was also carried out. The supernatants of the cultured cells were analyzed for the pro-

duction of VEGF and IL-7, both of which are produced by n-Fib, and (+)-Fib produced sufficient amounts of these cytokines (data not shown).

Normal bone marrow-derived non-adherent cells and leukemia blasts from case-2 were cultured on the irradiated myofibroblasts (Fig. 4). When the normal cells were cultured, similar proliferation was observed between n-Fib and (+)-Fib (clone-1 of case-2) (Fig. 4B and C). When the original leukemia blasts were cultured on (+)-Fib, an approximately 20-fold increase in the incorporation of ^{3}H-thymidine was observed that of when leukemia blasts were cultured on n-Fib (Fig. 4B, $P < 0.01$). Microscopically, leukemia blasts expanded in number and foci of the proliferating cells formed on (+)-Fib, but not on n-Fib (Fig. 4A).

Discussion

In hematological malignancies, the abnormal proliferation of fibroblasts has been reported in primary myelofibrosis and chronic myelogenous leukemia (CML) in the accelerated/blast phase. In such pathological conditions, bFGF is related to the expansion of fibroblasts [28], and a reactive fibrosis is observed [29]. Also, regarding CML it was reported that abnormally differentiated macrophages derived from CML cells formed bone marrow-stroma [30]; however, to our knowledge, blast cells from AML patients have not been reported to change into myofibroblasts in a culture system. In sera from the patients and the conditioned media obtained from the culturing of non-adherent leukemia blasts, bFGF levels were not increased in our cases (below the lower limit of the detectable range, data not shown). Thus, it is possible that the leukemia blasts abnormally differentiated into myofibroblasts.

The regulation of self-renewal and abnormal differentiation are important issues for clarifying the malignant transformation of stem cells [31]. This implies that when stem cells succumb to malignant transformation, even they maintain the ability to differentiate, allowing the production of heterogeneous malignant clones [32]. Leukemia stem cells are reported to express myeloid markers on their surfaces, such as CD13, 33, and 123 [33], which are expressed in normal hematopoietic cells. Among such molecular markers, CD133 antigen plays an important role for maintaining stem cells, and is thought to be one of the candidate antigens for cancer stem cells [34]. In (+)-Fib, CD133 molecule was detected with the immunocytochemical staining and RT-PCR analysis. The CD34 transcript was also identified with RT-PCR. Mesenchymal stem cells are reported to express CD106 strongly; however, they do not express CD34. These observations indicate that (+)-Fib analyzed in our two cases have no characteristics of mesenchymal stem cells [35].

Importantly, when cultured on (+)-Fib, the leukemia proliferated extensively. One possibility is that (+)-Fib produce significant

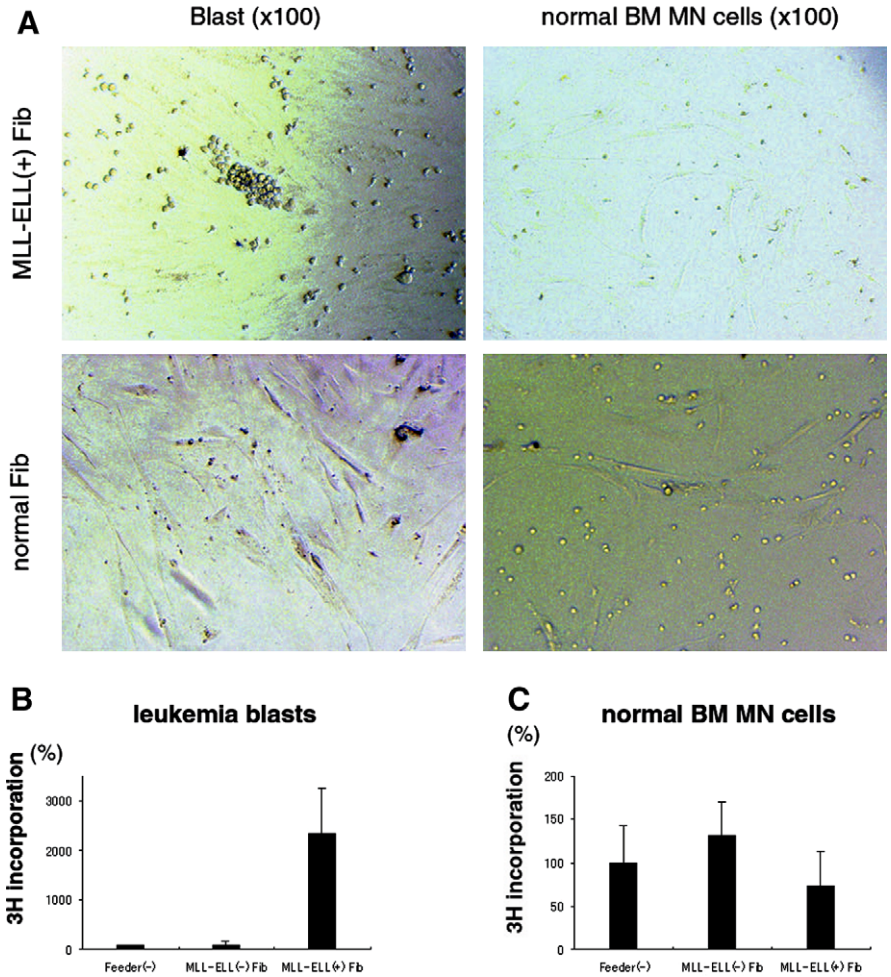

Fig. 4. Effects of myofibroblasts on the expansion of non-adherent normal bone marrow-derived mononuclear cells, and patients' leukemia blasts. (A) (+)-Fib and n-Fib were cultured with leukemia blasts and normal bone marrow-derived non-adherent mononuclear cells. After 72 h, cells were photographed. Leukemia blasts (B), and normal bone marrow-derived non-adherent mononuclear cells (C) were cultured on the irradiated myofibroblasts, and ^{3}H-incorporation was assayed. In (B), and (C), the mean value is taken as 100% when the indicated cells were cultured without feeder layers, and values represent the mean% ($n = 3$) ± SD. BM indicates bone marrow non-adherent mononuclear cells; and Fib, myofibroblasts.

amounts of cytokines that induces to grow the leukemia blasts. These observations indicate that leukemia blasts can differentiate and create an adequate microenvironment for their own growth. Further investigation, including the observation of other types of AML, will reveal the real characteristics of leukemia stem cells and their myofibroblastic conversion.

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