Expression of Vimentin Intermediate Filament Cytoskeleton in Acute Nonlymphoblastic Leukemias

By Koussay Dellagi, Antonio Tabilio, Marie-Madeleine Portier, William Vainchenker, Sylvie Castaigne, Josette Guichard, Jeanine Breton-Gorius, and Jean-Claude Brouet

Since vimentin intermediate filament (IF) expression in hemopoietic cells varies with the cell lineage as well as the state of differentiation of the cells, we studied the vimentin cytoskeleton by direct immunofluorescence and electron microscopy in 50 cases of acute nonlymphocytic leukemias. We found that malignant cells tend to reproduce the vimentin organization characteristic of their normal cellular counterpart. Thus, in M2 and M3 leukemias (French-American-British classification), vimentin was often reduced to a juxtanuclear bundle of filaments contrasting with the rich filamentous network expressed by M4 or M5 leukemias. In erythroblastic leukemias (M6) and megakaryoblastic leukemias, both identified by the expression of lineage-specific antigens, the absence of vimentin IFs could be correlated with the level of differentiation reached by the blasts. M1 leukemias displayed an abnormal pattern of vimentin organization with aggregated filaments giving a ring-like structure. However, no abnormality of the vimentin polypeptide could be detected by two-dimensional electrophoresis. These results show that the expression of the vimentin IF cytoskeleton may be a useful marker of differentiation in the study of leukemic cells.

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THE EUKARYOTIC CELL cytoskeleton comprises three main filamentous networks, namely: microfilaments, microtubules, and intermediate filaments (IFs). IFs differ in cells of different embryologic derivation; thus, glial fibrillary acidic protein is found in glial cells, the neurofilament triplet in neurons, desmin in muscles, keratins in epithelial cells, and vimentin in cells of mesenchymatous origin (reviewed in reference 1). Several recent reports have emphasized the close relationship between the expression of IFs and the process of cell differentiation.1,2 For instance, within a given cell lineage, certain differentiating cells can substitute one IF class polypeptide for another, ie, a transition from vimentin synthesis to neurofilaments occurs in neurons,1 and myoblasts switch from vimentin to desmin as they fuse into myotubes.4 Finally, the expression of IF subunits may decrease dramatically during terminal differentiation.5

Vimentin is the major IF polypeptide of hemopoietic cells, and its quantitative expression and cellular organization is highly variable within the different hemopoietic lineages.6 Thus, vimentin is organized in monocytes into a rich filamentous network spreading through the whole cytoplasm, whereas granulocytes and their precursors express only a single bundle of filaments, usually beneath the nucleus. On the other hand, vimentin disappears completely during the maturation of erythroid cells and megakaryocytes, since platelets and red blood cells are not stained by anti-vimentin antibodies. In the erythroid series, the disappearance of vimentin occurs at random in both large (immature) or small (mature) erythroblasts. In the megakaryocytic series, the switch-off of vimentin synthesis is a very early event.

Due to the tissue specificity of the IF subunits, the characterization of IF polypeptides has recently proved to be a powerful tool for establishing the cellular origin of solid tumors.2,7,8 Indeed, malignant cells usually retain the expression of the IF polypeptides characteristic of their normal cellular counterpart. In view of the special features of vimentin IF expression in hemopoietic cells as outlined above, we decided to study the IF organization in acute nonlymphocytic leukemias (ANLL) and look for correlation with the FAB classification.

MATERIALS AND METHODS

Cells

Mononuclear cells were purified from the blood and bone marrow samples from 50 patients with ANLL on a Ficoll-Hypaque gradient (Flow Laboratories, Scotland) (density, 1.077). In all cases, blast cells represented at least 75% of the preparation. Leukemias were classified according to the French-American-British (FAB) classification.9 In order to more accurately characterize the ANLL, immunofluorescence was performed with a panel of murine monoclonal antibodies (Mab), which included antimyeloid antibodies recognizing either early granulocytic stages of differentiation, and mature granulocytes (80H5 Mab)10 and antimonoocyte antibodies, which recognize both monocytes or their immediate precursors.
Several cells expressed both a perinuclear vimentin bundle and cytoplasmic nonfilamentous staining with anti-vimentin antibody.

This case was a mixed-type leukemia: About 50% of the blasts expressed glycophorin, whereas the remaining blasts expressed platelet lineage-specific markers.

**Table 1. Vimentin IF Expression in ANLL According to FAB Classification**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>FAB Classification</th>
<th>Vimentin Expression (%)</th>
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<tr>
<td></td>
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<td>Positive Filamentous Staining</td>
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<td>39</td>
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*Several cells expressed both a perinuclear vimentin bundle and cytoplasmic nonfilamentous staining with anti-vimentin antibody.

†This case was a mixed-type leukemia: About 50% of the blasts expressed glycophorin, whereas the remaining blasts expressed platelet lineage-specific markers.
hours with 30 μCi/mL of [35S]-methionine (SJ 204, Amersham, Buckinghamshire, England) in RPMI 1640 medium made 10% FCS and thereafter washed twice with PBS and once with 0.1 mol/L 2-(N-morpholino)-ethanesulfonic acid (pH 6.4), 1.0 mmol/L ethyl-
energylcol-bis (β-aminoethyl ether)N, N’, N”-tetraacetic acid, 0.1
mmol/L EDTA, 1.0 mmol/L mercaptoethanol, 0.5 mmol/L MgCl2,
and 1.0 mmol/L guanosine triphosphate (GTP). They were resus-
pended in the same buffer, which contained, in addition, several
protease inhibitors (1.0 mmol/L phenylmethylsulfonyl fluoride, 5.0
mmol/L N-acetyl-L-lysine chloromethyl ketone, 20 mmol/L p-
amino benzanidine, 40 μg/mL leupeptin, and 20 μg/mL pepstatin).
Cell lysis was performed by adding Triton X-100 (1% final) followed
by gentle agitation through a Pasteur pipette. Lysates were cen-
trifuged at 12,000 g for ten minutes at 4 °C. The supernatant and the
pellet were immediately treated for isoelectric focusing in urea-
containing buffer, and stored at -20 °C until use. The radioactiv-
ity of labeled cell extracts was measured after hot trichloroactic
c acid precipitation.

Two-dimensional gel electrophoresis. The two-dimensional
protein separation method was modified from the O’Farrell proce-
dure.18 Gel were stained with Coomassie brilliant blue (Merck,
Darmstadt, FRG). Industrex AX Kodak films (Kodak, Rochester,
NY) were used for autoradiography.

Identification of proteins. Proteins were identified by their
isoelectric point, their mol wt, and also by a peptide mapping
analysis. The different proteins isolated by two-dimensional gel
electrophoresis were characterized by digestion with Staphylococ-
cus aureus V8 protease (Miles Laboratories, Paris). The peptide
analysis was carried out on 15% acrylamide:0.0875% bisacrylamide
slab gels containing 0.1% sodium dodecyl sulfate (SDS). The general
procedure for peptide analysis was performed according to
Cleveland et al.20

Electron Microscopy (EM)

Cells were fixed with 1.25% glutaraldehyde in Gey’s buffer,21
which preserves granulocytic and platelet peroxidases,22 washed,
and then incubated in the diaminobenzidine medium.23 The cells were
then fixed by osmium tetroxide, dehydrated, and embedded in epon.
In one experiment, C17Mo Mab, which recognizes the platelet
GpIIa,24 was conjugated to colloidal gold25 and applied to unfixed
cells. The pellet was then treated as above.

RESULTS

Expression of Vimentin in ANLL Cells

Vimentin IFs were studied in 50 cases of ANLL by
direct immunofluorescence using a fluorescein-conju-
gated human monoclonal IgM specific for vimentin. The characteristic features of the IF network of mali-
gnant cells are listed in Table 1. Twelve cases of myeloblastic leukemias without maturation (M1 sub-
type of FAB classification) were studied. Nearly all
blasts were vimentin-positive in 11 cases. A striking feature was the unusual organization of the IF net-
work: Filaments accumulated at one pole of the cell leading to a ring- or coil-like appearance of the IF cytoskeleton (Fig 1A and B). This aspect was usually
found in a high percentage of the blasts and observed whether cells were smeared or cytocentrifuged on
slides. The vimentin structure was unchanged when the blasts were previously incubated with colchicine.

A similar aspect was observed in only four of 11
cases of myeloblastic leukemias with partial differen-
tiation (M2 subtype of the FAB classification). In five
of 11 cases of M2 leukemia, the blast expressed a single bundle of filaments usually located along the
nucleus. In addition to the filamentous vimentin, there was a diffuse cytoplasmic staining in five of these
cases. In one case, 83% of the cells appeared vimentin-
negative, even when the blasts were previously incub-
ated with colchicine.

Only one case of promyelocytic leukemia (M3 sub-
type) was studied: 70% of the blasts expressed vimen-
tin as juxtanuclear filaments in a majority of cells.

A characteristic feature of the myelomonocytic and
monoblastic leukemias (M4 and M5 subtypes, respec-
tively) was the density of the vimentin reseau in nearly
all the blasts: the filaments extended through the whole
cytoplasm (Fig 1C and D). Ring-like vimentin
organization was observed in a small percentage of the
blasts in one case of M4 leukemia.

Four cases of erythroleukemias were studied (M6
subtype). In one case, 40% of the blasts expressed a
detectable level of glycophorin and displayed vimentin
IFs as a paranuclear bundle of filaments. In double-
labeling experiments, glycophorin-positive blasts were
either vimentin-positive or -negative. In another case
of M6 leukemias (60% glycophorin-positive cells), 50%
of the blasts had a diffuse cytoplasmic staining for
vimentin, contrasting with a very rich filamentous
network of vimentin IFs in 10% of the cells. Since the
latter cells were glycophorin-negative in double-label-
ing experiments, they likely represent immature eryth-
roid progenitors. In the third case, of M6 leukemia
with the phenotype of a CFU-E (ie, absence of glyco-
phorin and of specific markers of other hemopoietic
lineages and labeling with F4G152 Mab), all cells
exhibited a rich filamentous network. The last case,
which was a mixture of glycophorin-positive blasts and
of promegakaryoblasts, was totally devoid of vimen-
tin.

Eleven cases were classified as megakaryoblastic
leukemias on the basis of expression of platelet peroxi-
dase, detection of specific platelet glycoproteins, and
cytoplasmic factor VIII vWF. Eight cases were blastic
transformation of chronic myeloid leukemia (CML).
The blasts were classified into three types according to
their maturational stage (Table 2). The more immu-
nature cases (type I) exhibited platelet peroxidase in the
near total absence of platelet glycoproteins or cytoplas-
mic factor VIII vWF; the intermediate cases (type II)
both platelet peroxidase and significant amounts of
platelet glycoproteins; the more mature cases (type
III) expressed all the megakaryocytic markers. Vimen-
tin was found in blasts of type I and II leukemias
Vimentin organization as revealed by direct immunofluorescence in (A, B) M1 myeloblastic leukemia, (C, D) M5 monoblastic leukemia, (E) K562 cell line. Note the polar accumulation of vimentin in (A), (B), and (E) and the filamentous organization of IFs in (C) and (D).
Table 2. Vimentin IF Expression in Megakaryoblastic Leukemia

<table>
<thead>
<tr>
<th>Phenotype of Megakaryoblastic Leukemia</th>
<th>Case No.</th>
<th>Vimentin Expression (%)</th>
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<td>Platelet Gps</td>
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<tr>
<td></td>
<td>Factor VIII vWF</td>
<td>—</td>
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<tr>
<td>Type II</td>
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<td>Platelet Gps</td>
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<td></td>
<td>Factor VIII vWF</td>
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<tr>
<td></td>
<td>46</td>
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</tr>
<tr>
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<td></td>
<td>Factor VIII vWF</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
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</tbody>
</table>

PPO, platelet peroxidase detected by electron microscopy; platelet Gps, platelet glycoproteins detected by indirect immunofluorescence using AN51 and 515 Mab; factor VIII vWF, von Willebrand factor VIII detected by indirect immunofluorescence.

presence of protease inhibitors, most of the vimentin migrated to its expected position (Fig 2). The three usual vimentin-derived proteins were also observed. No difference in vimentin migration was detected whether vimentin IFs were organized as a filamentous network or polar aggregate. Furthermore, peptide map analysis was identical to that of human vimentin (data not shown). The highest amount of vimentin was observed in the monoblastic leukemia (Fig 2B) and in one of the four myeloblastic leukemias. In these cases, the vimentin spot represented the main component of the Triton X-100–insoluble material extracted from the cells.

Ultrastructural Results

IFs measuring 8 to 10 nm in diameter were particularly developed in the two cases of acute megakaryoblastic leukemia, in which promegakaryoblasts exhibited platelet peroxidase (PPO) as the only marker of the megakaryocytic differentiation; ie, these cells lacked platelet-specific proteins and platelet organelles. IFs were organized in one or several large bundles of parallel filaments (Fig 3) that occupied a vast zone of the cytoplasm. Mitochondria and ribosomes were excluded from these zones; the Golgi apparatus was pushed to the periphery of the cytoplasm, close to the cell membrane. Some microtubules were arranged in a parallel direction at the external border of the filaments. Since these zones containing the IFs were totally devoid of ribosomes, they could be identified on Giemsa smears as white areas contrasting with the intense basophilia of the remaining cytoplasm. In such cases, some blasts without any differentiation markers also exhibited the same large extension of the IFs. In one patient, in whom we simultaneously detected expression of the platelet GpIIa by C17 Mab coupled to colloidal gold and PPO, large bundles of IFs were only present in the PPO-positive blasts, which were devoid of GpIIa. Such expression of IF was not detected in the acute megakaryocytic leukemia with a more differentiated phenotype.

In M1 acute leukemias, IFs were well developed, with a crescent shape frequently applied against the nucleus. Similarly, in some acute leukemias in which

![Fig 2. Autoradiograms of two-dimensional electrophoresis of Triton X-100–insoluble crude cytoskeletal material extracted from [35S]-methionine–labeled cells (only a part of the gel is shown): (A) M1 myeloblastic leukemia; (B) M6 monoblastic leukemia; (C) K562 cell line. Vi, vimentin; αT, actin; αT, βT, tubulin subunits; bracket, vimentin-derived proteins.](image)
VIMENTIN CYTOSKELETON IN ANLL

Fig 3. Leukemic promegakaryoblast: The nature of the blast could be determined only by detection of platelet peroxidase localized in the nuclear envelope and in the endoplasmic reticulum, since there are no granules or demarcation membranes. A large bundle of IFs (700 nm wide), cut in longitudinal sections and arranged in a ring, excludes ribosomes and mitochondria (original magnification x 15,800; current magnification x 11,613).

no myeloperoxidase was detected at the light microscopy level but with small myeloperoxidase-positive granules only detected by ultrastructural cytochemistry, large bundles of IFs were present (Fig 4). In M3 leukemias, only short parallel IFs were located near the nucleus (Fig 5).

DISCUSSION

Recently, many studies have been devoted to the characterization of IFs in normal or malignant cells.

This cytoskeleton network of eukaryotic cells possesses two unique features: First, its constitutive polypeptides vary according to the embryonic origin of the cells, and second, its expression may be altered during the process of cell differentiation. The former observation stimulated studies aiming to define the origin of solid tumors according to the expression of a given type of IF polypeptide. Such studies are of limited interest in human leukemias, since these malignancies originate from a common pluripotential hemopoietic stem cell, and accordingly, cells from the various subgroups of ANLL all express vimentin, the constitutive polypeptide of mesenchymal cell IFs. Likewise, cells from lymphoid leukemias, either of B or T cell lineage, express vimentin IFs (K.D. and J.-C.B., unpublished data, December 1982). However, it should be noted that the absence of detectable vimentin IFs does not exclude the diagnosis of ANLL, as discussed below.

In the present report, we studied the organization of the IF network in ANLL, because previous work from our laboratory on human normal myeloid cells demonstrated that the organization of vimentin cytoskeleton is highly variable within cells of the human hemopoietic system, depending on the cell lineage considered and the maturation level reached by the cell in a given lineage. On the whole, we found that malignant cells tend to reproduce the vimentin organization characteristic of their normal cellular counterpart, and that such studies may help delineate the stage of differentiation reached by the malignant cells. However, one striking exception was offered by the pattern of IF organization in M1 leukemias (FAB classification): IFs accumulated in a dense aggregate with a barely visible filamentous network at one pole of the cell. This aspect may be reminiscent of the juxtanuclear coil of...
vimentin IFs induced in normal cells by in vitro treatment with colchicine.\(^{28,29}\) In fact, this abnormal pattern of vimentin organization in myeloblasts was not modified after treatment of the cells with colchicine. Although this pattern was most prominent in M1 leukemias, it may be observed in a smaller percentage of cells from other subtypes of ANLL except monoblastic leukemias. A very similar structure had been previously described in acute myeloid leukemias as “cytoplasmic fibrillar body” at the EM level\(^{30,31}\) or by optical polarizing microscopy\(^{32}\); this structure, however, was until now felt to be related to microfilaments.

Interestingly, cells from the K562 cell line which originates from CML blast crisis and exhibits both erythroid and megakaryocytic phenotype,\(^{33,34}\) also have a large vimentin-positive paranuclear aggregate. Although the study of normal bone marrow precursors is obviously difficult in humans, it should be stressed that we have never observed this pattern of vimentin organization in such cells by immunofluorescence or EM. The significance of this unusual IF organization is still undetermined; the possibility that it may be due to a structural abnormality of vimentin polypeptide is unlikely, since bidimensional electrophoresis of Triton X-100-insoluble cytoskeleton extracted from the K562 cell line and four M1 leukemias having redistributed vimentin IFs yielded a normal vimentin spot. In view of the close interrelationship between IF and microtubule networks,\(^{35}\) we studied by fluorescence the organization of microtubules, which turned out to be quite normal in such cells (data not shown). The possibility exists that an IF- or a microtubule-associated protein is nonfunctional in these cells. The relationship of this peculiar IF organization with the malignant nature of the cells is at present only speculative; however, it is worth noting that a similar pattern has been observed in fibroblasts transformed by the Rous virus.\(^{36}\) Finally, in other models, cytoskeleton proteins such as vinculin may be the target of oncogenic events.\(^{37,38}\)

Cells from monoblastic leukemias always express a very rich filamentous IF network extending all over the cytoplasm as found in normal cells from these series. In contrast, in M2 and M3 subtypes of myeloblastic leukemias, vimentin IF often appeared reduced to a few juxtanuclear bundles of filaments as seen in normal granulocytic precursors and polymorphonuclears.\(^{6,21}\) In addition to the filamentous structures, a cytoplasmic staining was observed in some M2 cases, the significance of which is unknown. As reported elsewhere,\(^{26}\) the contrasting organization between the IFs of cells engaged in the monocytic or granulocytic differentiation pathway is confirmed by the study of the HL-60 promyelocytic cell line: only 10% to 30% of the cells spontaneously express vimentin IFs. However, the vimentin content increases considerably as they are induced to differentiate into macrophages under phorbol-ester treatment. In contrast, under dimethyl sulfoxide (DMSO), cells differentiate into mature polymorphonuclears and retain a single paranuclear bundle of filaments.

Cells from megakaryoblastic leukemias also express vimentin. Interestingly, this expression of vimentin correlates with the degree of maturity of the leukemic cells. Blasts with a very immature megakaryocytic phenotype (low expression of specific platelet markers) synthesize a high level of vimentin, while leukemic cells that have turned on the synthesis of all megakaryocytic markers were devoid of IFs. In many ways, this expression of vimentin is identical to that found during the normal megakaryocytic differentiation. Indeed, we showed elsewhere\(^{9}\) that only rare, normal, small megakaryocytic precursors, identified by the presence of platelet glycoproteins present in the bone marrow or in the megakaryocytic colonies grown in vitro, exhibit a vimentin IF network. In contrast, cultured or bone marrow megakaryocytes lacked vimentin by our immunofluorescence assay.

In erythroleukemia, a variable percentage of blasts express a rich filamentous network; its presence correlates with the degree of differentiation of the leukemic erythroid cells. In one case with a CFU-E phenotype, the blasts express a filamentous vimentin network. In contrast, in another case where erythroid blasts reach the stage of differentiation of normoblasts, vimentin was not detected. In the other two cases where a mixture of erythroid cells at different stages of differentiation was present, double-staining experiments showed that cells expressing a filamentous vimentin network were often glycophorin-negative, indicating that expression of vimentin correlates with the imma-
turity of the blasts. It is of interest to note that some of these erythrocyte- or megakaryocytic leukemias would have been classified as undifferentiated leukemias in the absence of studies with rather sophisticated markers; in such cases, a well-developed vimentin network may indicate the proliferation of very early precursors of these series.

In conclusion, the study of the expression of IFs provides a new tool for the study of stage of differentiation of ANLL cells from a given lineage and FAB subtype and outlines the frequent heterogeneity of such ANLL subsets. The abnormality of IF organization in some type of leukemias may give new insights into the biology of the leukemic cells.

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Expression of vimentin intermediate filament cytoskeleton in acute nonlymphoblastic leukemias

K Dellagi, A Tabilio, MM Portier, W Vainchenker, S Castaigne, J Guichard, J Breton-Gorius and JC Brouet