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The LINC-less granulocyte nucleus

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Abstract

The major blood granulocyte (neutrophil) is rapidly recruited to sites of bacterial and fungal infections. It is a highly malleable cell, allowing it to squeeze out of blood vessels and migrate through tight tissue spaces. The human granulocyte nucleus is lobulated and exhibits a paucity of nuclear lamins, increasing its capability for deformation. The present study examined the existence of protein connections between the nuclear envelope and cytoskeletal elements (the LINC complex) in differentiated cell states (i.e. granulocytic, monocytic and macrophage) of the human leukemic cell line HL-60, as well as in human blood leukocytes. HL-60 granulocytes exhibited a deficiency of several LINC complex proteins (i.e. nesprin 1 giant, nesprin 2 giant, SUN1, plectin and vimentin); whereas, the macrophage state revealed nesprin 1 giant, plectin and vimentin. Both states possessed SUN2 in the nuclear envelope. Parallel differences were observed with some of the LINC complex proteins in isolated human blood leukocytes, including macrophage cells derived from blood monocytes. The present study documenting the paucity of LINC complex proteins in granulocytic forms, in combination with previous data on granulocyte nuclear shape and nuclear envelope composition, suggest the hypothesis that these adaptations evolved to facilitate granulocyte cellular malleability.

Keywords

Neutrophil; Monocyte; Macrophage; Nuclear envelope; Cytoskeleton

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Introduction

The LINC (linker of nucleoskeleton and cytoskeleton) complex of proteins is believed to tether the interphase nuclear envelope (NE) to various cytoskeletal elements and, in turn, to the plasma membrane (Crisp et al., 2006; Stewart et al., 2007). Components of the LINC complex include the nesprins, SUN proteins, actin, intermediate filaments, plectin, lamins A/C and chromatin. Postulated functions of the LINC complex include positioning the nucleus within the cell and transducing external forces and signals to the nucleus.

The principal blood granulocyte (neutrophil) is a key component of innate immunity, rapidly egressing from blood vessels, migrating through tight tissue spaces to the site of infection, phagocytizing and destroying bacteria and fungi (Lee et al., 1999). The neutrophil is a highly deformable cell, enabling the squeezing of this cell (~7 μ m diameter) through ~1 μ m channels created in vascular endothelial walls (Dejana, 2006; Feng et al., 1998). Granulocyte nuclei are lobulated, an important adaptation, facilitating the cellular deformation, likely resulting from increased levels of lamin B receptor, decreased levels of lamins A/C and B1 and the presence of intact microtubules during granulopoiesis in the bone marrow (Hoffmann et al., 2007; Olins and Olins, 2004; Olins et al., 2008).

The human leukemic cell line HL-60 furnishes a convenient (but imperfect) model system for in vitro myelopoiesis: treatment with retinoic acid (RA) induces granulocyte formation; D3 (D3), monocytes; phorbol ester (TPA), macrophage cells (Collins, 1987; Olins et al., 1998, 2001; Rovera et al., 1979; White et al., 2005). The present study compares the cell contents and locations of LINC complex proteins, as well as other NE proteins, in four HL-60 cell states (i.e., undifferentiated, granulocyte, monocyte and macrophage). Most striking, we determined that undifferentiated, granulocytic and monocytic HL-60 cells are deficient in nesprin-1 and -2 giant isoforms, SUN1, vimentin, plectin and lamins A/C; whereas, actin and SUN2 are present. In sharp contrast to these HL-60 cell states, macrophage HL-60 cells reveal increased levels of nesprin-1 giant, vimentin, plectin and lamins A/C, as well as possessing actin and SUN2. Furthermore, undifferentiated, granulocytic and monocytic HL-60 cells grow as suspension cultures; whereas, macrophage forms attach to substrates and exhibit extensive cellular processes. Peripheral blood leukocytes (i.e., granulocytes, monocytes and in vitro induced macrophage) show some similar differences with respect to selected LINC proteins, as those observed with HL-60 cells. These observations suggest an additional adaptation of granulocytes to facilitate cellular deformation; i.e., a paucity of connections between the granulocyte nucleus and the cytoskeleton.

Materials and methods

Cells

Human HL-60/S4 myeloid leukemic cells were cultivated as described earlier (Olins et al., 1998). Differentiation was induced by the addition of: 1 μ M retinoic acid (RA), yielding granulocytes; 100 nM 1,25 vitamin D3 (D3), monocytes; 16 nM phorbol ester (TPA), macrophage. During induced differentiation the flasks were covered with aluminum foil to protect against light. The inducing chemicals were purchased from Sigma-Aldrich Inc., St. Louis, MO. COS1 cells were purchased from ATCC (Manassas VA) and cultivated following the supplier's instructions. Cytocentrifuged preparations of differentiating HL-60/S4 were stained with Hema-3 (Thermo Fisher Corp.) and examined in the light microscope to monitor nuclear shape, percent of mitotic figures and percent of apoptotic cells.

Human peripheral blood cell preparations were processed as described earlier to yield granulocytic and mononuclear fractions, employing density gradient centrifugation with HISTOPAQUE 1119 and 1077 (Olins et al., 2008). Reconstituted "buffy coat" was prepared

by mixing (1:1) the PBS-washed preparations of the granulocyte and mononuclear fractions, prior to cyto centrifugation. Blood monocytes were obtained by incubating the mononuclear fractions on LabTec single-chamber slides (Nunc, Naperville IL), based upon published procedures (Keisari, 2005). Differentiation of the adherent monocytes to macrophage was accomplished by making the culture medium 2 nM TPA for ~2 weeks.

Antibodies

A number of the antibodies employed in this study have been used in previous publications from this laboratory, including mouse monoclonal anti-vimentin (3B4), anti- α -tubulin (Sigma T-5168), anti- β -actin (Sigma A-5441), anti-lamins A/C (x67 and x167), anti-lamin B2 (x223) and anti-LAP2 β (6G11) (Olins et al., 1998, 2000, 2001). An additional mouse monoclonal anti-LAP2 (TMPO, clone 6E10) was purchased from Sigma, revealing predominant reactivity with LAP2 β . Mouse monoclonal anti-LBR has been prepared in the Zentgraf laboratory, and described earlier (Olins et al., 2008). Also prepared in the Zentgraf laboratory was a mouse monoclonal anti-lamin A/C (LaA-Z1). Polyclonal guinea pig anti-LBR and guinea pig anti-emerin have been previously described (Dreger et al., 2002; Hoffmann et al., 2002). Polyclonal guinea pig anti-human plectin (P1) has also been described (Stegh et al., 2000). Goat anti-lamin B1 (S20) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), see discussion of the use of this antiserum (Olins et al., 2008). Rabbit polyclonal anti-SUN1 and -SUN2 have been previously described (Hodzic et al., 2004). A rabbit polyclonal anti-N terminus nesprin-1 antibody was detailed earlier (Padmakumar et al., 2004), as was a rabbit polyclonal anti-C-terminus nesprin-2 (pABk1) (Libotte et al., 2005). Secondary antibodies were all made in donkey, with minimal cross-reactivity, and purchased from Jackson ImmunoResearch Laboratory (West Grove, PA).

Immunochemical methods

Immunostaining procedures have been described earlier (Olins et al., 2008). In brief, cyto centrifuged cells or cells grown on chamber slides were fixed either with 4% paraformaldehyde (PFA) in PBS (room temperature, 15 min) or in anhydrous methanol (-20°C, 10 min) depending upon the particular primary antibody (detailed in the figure legends). Confocal images were obtained on a Zeiss 510 Meta microscope, employing the 100 $\times$ objective. Companion conventional DAPI images were obtained on the same microscope equipped with a Zeiss AxioCam MRm CCD camera.

Immunoblotting procedures were also performed exactly as detailed earlier (Olins et al., 2008), employing BioRad 4-20% precast gels for SDS-PAGE experiments. For analysis of HL-60/S4 antigens, total cell extracts from the same number of cells were loaded in each lane (i.e., 3×10^4 cells/lane). Peripheral blood granulocyte extracts were loaded at 6×10^4 cells/lane. Total cell extracts from COS1, A431 and U2OS were included as protein positive controls on the SDS-PAGE gels. Molecular weights of the ECL-positive bands were estimated by comparison with Protein Marker P7702S (New England BioLabs Inc.).

Results

Granulocytic forms of HL-60/S4 are deficient in many LINC components

HL-60/S4 cells were induced into granulocytes with RA, into monocytes with D3 and into macrophages with TPA. Treatments were four days long. The cytopun cells were fixed with PFA or methanol, depending upon the antibody employed, and immunostained with antibodies directed against various components of the LINC complex. Confocal slices of the immunostained HL-60/S4 cells are presented in Figure 1. In order to acquire a semi-quantitative impression of the relative immunostaining of the different cell states by a particular antibody, the gain of the confocal microscope was “optimized” for the strongest staining specimen and

images of other cell states were obtained at the same settings during the same viewing session. For some experiments, parallel immunostaining was performed with COS1 cells (data not shown), which indicated clear positive reactions with anti-SUN1, -SUN2, -nesprin 1 and -nesprin 2. By this procedure, it was clear that anti-vimentin, -plectin, -SUN1, -nesprin 1 and -nesprin 2 exhibit negligible staining of undifferentiated and granulocytic HL-60/S4 cells; whereas, anti-SUN2 stains both of these cell states. By contrast, TPA-treated cells reveal vimentin, plectin and nesprin 1 staining; as well as reaction with anti-SUN2. D3-treated cells appear to have staining properties intermediate between granulocytic and macrophage states of HL-60/S4, exhibiting very weak staining with anti-vimentin and -plectin. Therefore, the immunostaining experiments strongly support the notion that undifferentiated and granulocytic HL-60/S4 cells have an evident deficiency in a number of prominent LINC complex proteins.

Immunoblotting experiments with various anti-LINC complex antibodies on total cell extracts of undifferentiated, granulocyte and macrophage states of HL-60/S4 are presented in Figure 2. These experiments examine daily changes (0 to 5 days) in specific protein levels during differentiation of HL-60/S4 into granulocytes with RA (1R to 5R) or into macrophages with TPA (1T to 5T). The HL-60/S4 samples were loaded at the same number of cells/lane (see Fig. 4, for an example of Coomassie Blue staining). Flanking the HL-60/S4 samples are total extracts of A431 and U2OS cells, intended to be positive position markers for the protein of interest. α -Tubulin, β -actin and SUN2 appear to be present in all the HL-60/S4 extracts, with a demonstration that β -actin increases in amount during macrophage differentiation. Whereas undifferentiated and granulocytic HL-60/S4 cells exhibit negligible reactivity with anti-vimentin, -plectin and -nesprin 1 giant, increasing amounts of these proteins appear during TPA-induced macrophage differentiation. None of the HL-60/S4 extracts revealed any significant reactivity with anti-SUN1 or -nesprin 2 giant, even though strong reactions were seen with the A431 extracts.

Nesprin 3 is a recently identified member of the LINC complex, being much smaller than nesprin 1 and 2 (i.e., nesprin 3 is ~100 kDa), lacking the N-terminal actin-binding domain and binding instead to the plakin family member plectin, which is suggested to dimerize and interact with vimentin (Ketema et al., 2007; Wilhelmsen et al., 2005). A polyclonal rabbit antibody directed against mouse nesprin 3, courtesy of A. Sonnenberg (Wilhelmsen et al., 2005) was employed to immunoblot HL-60/S4 extracts of undifferentiated, RA- and TPA-treated cells (data not shown). A band (~100 kDa) with increasing intensity was observed progressively from days 2-5, following TPA treatment. A weak-intensity band of the same molecular weight was observed on day 5 following RA treatment; negligible levels of putative nesprin 3 were seen on other days. Less interpretable, however, there were bands of lower molecular weight (~63 and ~35 kDa) that exhibited unchanged intensities in all the cell extracts. This immunoblot data is consistent with, but does not firmly establish, an increased expression of human nesprin 3 during TPA-induced macrophage differentiation of HL-60/S4 cells. In summary, the immunoblotting experiments are in good agreement with the immunofluorescence studies, indicating that undifferentiated and granulocytic HL-60/S4 cells exhibit a paucity of LINC complex proteins, with indications of their presence in the macrophage cell state.

Granulocyte and macrophage forms of HL-60/S4 cells reveal differences in NE proteins

Differences in the composition of NE proteins, comparing the various cell states of HL-60/S4, have been presented in previous publications from this group (Olins et al., 2001, 2008). A confocal immunofluorescence comparison of the four different cell states, employing "optimized" gain (as in Fig. 1), is shown in Figure 3. Anti-lamins A/C and -B1 are strongest against TPA-induced macrophage; anti-lamin A/C exhibited a weak reaction with undifferentiated cells. Anti-lamin B2 and -LAP2 β reacted comparably well with all four cell states. Anti-LBR reacted most strongly with granulocyte forms, most weakly with macrophage

and moderately with D3-induced monocytes. Thus, the four cell states of HL-60/S4 (undifferentiated, granulocyte, monocyte and macrophage) have distinguishably different immunostaining reactivity with the panel of tested NE antibodies.

Immunoblotting experiments with selected NE antibodies on total cell extracts of undifferentiated, granulocyte and macrophage states of HL-60/S4 are presented in Figure 4. These experiments examine daily changes (0 to 5 days) in specific protein levels during differentiation of HL-60/S4 into granulocytes with RA or into macrophages with TPA. The arrangement of samples on the blot membranes is exactly as shown in Figure 2. Anti-lamins A/C and -B1 give very weak reactions with undifferentiated and granulocytic forms of HL-60/S4, but show increasingly strong reactions with macrophage forms. Anti-lamin B2 and -emerin reacted with undifferentiated, granulocytic and macrophage forms, but exhibited no clear systematic variations in reaction intensity. There is an indication that LAP2 β is decreasing in amount during both RA- and TPA-induced differentiation, compared to undifferentiated HL-60/S4. The significance of this observation remains to be explored, but could represent changing relationships in the NE-heterochromatin interaction. Anti-LBR yielded a complex pattern of reaction bands (i.e., an intact ~58-kDa band, plus aggregates and lower-molecular-weight peptides), with a clear increase during *in vitro* granulopoiesis and an evident decrease during macrophage differentiation.

D3-induced monocytic HL-60/S4 exhibit similarities and differences compared to both granulocyte and macrophage cell states

Immunostaining of D3-treated HL-60/S4 cells with anti-lamin B2, -LBR, -LAP2 β and -SUN2 (see, Figs. 1 and 3) suggested the presence of nuclear lobulation and NE-limited chromatin sheets (ELCS), much as observed with RA-induced granulocytes (Olins et al., 1998). Staining for the light microscope with Hema-3 and thin-section electron microscopy confirmed the presence of lobulated nuclei with ELCS in D3-treated cells (Fig. 5). The ultrastructural morphology of the ELCS in both the granulocyte and monocyte cell states is virtually identical. ELCS are not observed in undifferentiated or TPA-treated HL-60/S4 cells (data not shown).

D3-induced monocyte HL-60/S4 cells do reveal some similarities to the macrophage cell state. As described earlier, HL-60/S4 monocytes exhibit weak positive immunostaining with anti-vimentin and anti-plectin, characteristics not seen with HL-60/S4 granulocytes (Figs. 1 and 3). However, there is one notable immunostaining difference between HL-60/S4 monocytes and macrophages; i.e., weak-to-negligible staining with anti-lamin A/C of monocyte nuclei, in comparison to macrophage nuclei (Fig. 3).

Immunoblotting comparisons of total cell extracts of D3- and TPA-treated HL-60/S4 cells are presented in Figure 6. Extracts from the same number of cells were loaded in each lane, in agreement with the comparable total protein loads seen in the Coomassie Blue-stained membrane (data not shown). Extracts of D3-treated cells were collected for up to 7 days, since there was little indication of cell death during the period of differentiation (in contrast to RA-treated HL-60/S4 cells; see below). The results demonstrate less lamin A/C, vimentin, plectin and nesprin 1 giant in extracts of D3-treated cells, compared to macrophage extracts. Lamins B1 and B2, LAP2 β , SUN2 and β -actin are present in comparable amounts. LBR increases in amount during the D3-induced differentiation, exceeding the levels seen in macrophage extracts. Overall, the results are consistent with the concept that nuclear lobulation and ELCS formation in HL-60/S4 differentiated myeloid cells correlates with elevation of cellular levels of LBR, in the context of depressed levels of lamin A/C (Hoffmann et al., 2007; Olins et al., 2001).

In view of the similarities (described above) between RA- and D3-treated HL-60/S4 cells, we attempted to document how similar these two cell states are with respect to growth and to

appearance of apoptosis. Therefore, in a separate set of experiments, undifferentiated, RA-treated and D3-treated HL-60/S4 cells were compared by growth curves for up to 8 days, and cytocentrifuged and stained with Hema-3 to calculate the percentages of mitotic and apoptotic cells (data not shown). Simultaneous cultures of these three conditions were started at a concentration of $\sim 1 \times 10^5$ cells/ml. By day 4, no mitotic figures could be found on stained slides from the RA- and D3-treated cultures; some were still observable in the undifferentiated cells after 6 days. The undifferentiated cells achieved “plateau” by day 5, reaching $\sim 2 \times 10^6$ cells/ml. RA- and D3-treated cells stopped growing earlier than the undifferentiated cells, achieving plateau values (by day 4) of $\sim 1 \times 10^6$ cells/ml. These observations on the growth of D3-treated HL-60 cells are entirely consistent with a previous study of D3-treated HL-60 cells, demonstrating 2-3 days of proliferation, followed by growth arrest and maturation (Brown et al., 1999). Clear apoptosis became apparent in RA-treated cells by day 4, steadily increasing from $\sim 3\%$ (day 4) to $\sim 18\%$ (day 7). On the other hand, apoptosis remained minimal ($\sim 1\%$) during the entire period of D3 treatment (up to 8 days). The significant level of apoptosis in the RA-induced granulocytes is well known (Martin et al., 1990) and has been previously discussed (Olins et al., 1998, 2000). The “resistance” to apoptosis in D3-treated HL-60 cells has also been noted before, with evidence presented that viability is preserved by an increased sensitivity of the induced monocytes to insulin in the growth medium (Marcinkowska et al., 2001). Clearly, whatever the morphological and biochemical similarities between RA- and D3-treated HL-60/S4 cells, they exhibit a very different propensity for apoptosis.

The LINC complex of peripheral blood leukocytes

Previous studies have compared the NE and heterochromatin composition of peripheral blood granulocytes with granulocytic HL-60/S4 cells, employing confocal immunofluorescence and immunoblotting techniques (Olins et al., 2008). These studies indicated a surprising paucity of lamins A/C, B1 and B2, of LBR, LAP2 β and emerin in blood granulocytes, compared to HL-60/S4 granulocytes. Also described was the dramatic fragility of blood granulocyte nuclei to methanol fixation, confounding immunostaining experiments with antibodies whose epitopes are destroyed by PFA fixation. In the present study, peripheral blood granulocytes and mononuclear cells were obtained from HISTOPAQUE step density gradient centrifugation as previously described (Olins et al., 2008). In some experiments, a reconstituted “buffy coat” was created by mixing the granulocyte and mononuclear fractions back together (1:1), eliminating the massive presence of erythrocytes. In other experiments, blood monocytes were enriched from the mononuclear fraction by adherence to glass chamber tissue culture slides (Keisari, 2005), washing away lymphocytes and other non-adherent blood cells. Some of the monocyte chamber slides were made 2 nM TPA in RPMI 1640 plus 10% heated fetal calf serum and incubated for ~ 2 weeks to induce macrophage differentiation (Keisari, 2005; Markovich et al., 1994). Examples of confocal immunostaining results of the reconstituted “buffy coat” are presented in Figure 7. Especially as viewed with DAPI staining, the results indicated that blood monocytes have deeply indented nuclei; but could be distinguished from the lobulated granulocyte nuclei. Blood monocytes yielded positive staining by anti-vimentin, -plectin and -SUN2 antibodies. Granulocytes revealed no plectin staining; but did react with anti-SUN2 and - vimentin. Indeed, one clear difference between peripheral blood granulocytes and HL-60/S4 granulocytes is the presence of vimentin in the peripheral blood cells. The presence of vimentin in blood granulocytes is well documented in the literature (Moisan et al., 2007; Pryzwansky and Merricks, 1998), although its functional role is not clear. In addition, anti-SUN1 yielded no immunostaining reaction with peripheral blood granulocytes (data not shown). Immunoblotting experiments (Fig. 8) comparing HL-60/S4 and blood granulocyte total cell extracts, prepared as previously described (Olins et al., 2008), revealed the absence of nesprin 1 giant and the presence of SUN2 in blood granulocytes. A comparison of TPA-induced macrophages from enriched blood monocytes and from HL-60/S4 cells is presented in Figure 9. There is considerable morphologic resemblance between the TPA-induced

macrophage cells, whether derived from blood monocytes or from HL-60/S4 cells. Furthermore, both types of macrophage, as well as blood monocytes, were positive for immunostaining with anti-lamin B1, - vimentin and -plectin. It seems very likely that many components of the LINC complex are present in macrophages, as well as in blood monocytes.

Discussion

This study demonstrated that many of the LINC complex proteins (Crisp et al., 2006; Stewart et al., 2007) are depleted in amount within the granulocytic forms of HL-60/S4 cells; whereas, these same proteins are clearly present within the macrophage cell state of HL-60/S4 (see Table 1, for a summary of observations on HL-60/S4 cells). Thus, these two alternative differentiation states of a myeloid leukemic cell line, which mimic their normal myeloid counterparts, reveal major differences in the structural coupling between the NE and cytoskeletal elements. These studies also demonstrated that undifferentiated HL-60/S4 exhibited a similar paucity of LINC complex proteins; but, D3-induced monocytic forms revealed low levels of several LINC proteins.

The major cytoskeletal proteins (i.e. α -tubulin, β -actin and vimentin) are clearly present in undifferentiated HL-60/S4 cells, with a reduction in the amount of vimentin during RA-induced granulocytic differentiation (Olins et al., 2000). In the present study, TPA-induced macrophage differentiation of HL-60/S4 cells revealed increased amounts of β -actin and vimentin, compared to undifferentiated and granulocytic forms. Thus, the cellular levels of the major cytoskeletal proteins change, as do the LINC complex proteins, during in vitro differentiation of HL-60/S4. The cellular levels of vimentin have been examined during in vitro RA- and TPA-induced differentiation in the promyelocytic leukemic cell line NB4 (Bruehl et al., 2001), with very similar results (i.e., RA down-regulated vimentin; TPA increased vimentin content). Normal peripheral blood granulocytes possess α -tubulin, β -actin and vimentin. In both HL-60 and peripheral blood granulocytes microtubules are involved in cell polarity, and actin microfilaments are involved in cell migration (Fenteany and Glogauer, 2004; Olins et al., 2000). The role of vimentin in blood granulocyte structure and function is not yet clearly established (Moisan et al., 2007).

Nesprins 1 and 2 are spectrin-repeat integral membrane proteins coded by separate genes and expressed in many tissues with numerous alternate splice products (Padmakumar et al., 2004; Zhang et al., 2001; Zhen et al., 2002). Both nesprins exist in “giant” forms (nesprin 1 giant, >700 kDa; nesprin 2 giant, >1 MDa), believed to be important “cytolinkers” connecting the NE with the cytoskeletal elements. Both “giant” forms possess N-terminal actin-binding domains and C-terminal transmembrane domains, and appear to be embedded in the outer nuclear membrane, binding to actin-rich cytoplasmic structures. There is evidence that the presence of lamins A/C in the NE is necessary for nesprin 2 localization, and that nesprin 2 is necessary for proper localization of emerin within the NE (Libotte et al., 2005; Zhang et al., 2005). In addition, experiments indicate that SUN1 expression within the NE is a requirement for proper localization of nesprin 2 into the NE, mediated by the C-terminus of SUN1 present within the perinuclear space (Haque et al., 2006; Padmakumar et al., 2005). These SUN-domain-dependent “bridges” apparently interact directly with the KASH domain of nesprin 1 and 2, as well as other KASH domain proteins (Tzur et al., 2006). Indeed, there is evidence that SUN1 and SUN2, and nesprin 1 and nesprin 2 have overlapping functions, and “interact promiscuously” (Ding et al., 2007; Stewart-Hutchinson et al., 2008; Zhang X. et al., 2007). Therefore, functional LINC complexes might still be formed in the absence of SUN1 or nesprin 2, with the “weak link in the chain” being the absence of lamin A/C and the mislocalization of emerin. The data presented in this study fit very well with recent literature indicating that nesprin 2 is involved in nuclear architecture. Its absence in lamin A/C mutant fibroblasts affects nuclear morphology (Kandert et al., 2007). Similarly, when nesprins are mutated NE

architecture is compromised (Zhang Q. et al., 2007). These results were further corroborated by siRNA nesprin 2 experiments. When nesprin 2 giant was absent, there resulted an increase in the nuclear area and in the number of heavily deformed nuclei (Luke et al., 2008). In addition, recent studies indicate that LINC complexes play a role in cellular mechanical stiffness (Stewart-Hutchinson et al., 2008).

What might be the functional significance of the reduced levels of LINC complex proteins within granulocytic cells, in comparison to their presence in macrophage cells? The neutrophil is a highly deformable cell. Serial thin-section electron microscopy of activated blood neutrophils indicates that these cells (~7 μ m diameter) can crawl through ~1 μ m diameter intracellular channels (i.e. transcellular route) within the endothelia of post-capillary venules (Feng et al., 1998). Apparently, human neutrophils primarily cross endothelia at cell junctions (paracellular), but a significant percentage employ the transcellular route (Dejana, 2006). In contrast to this “active deformation” (i.e. directed migration towards a source of chemotactic agents, arising from infection and tissue breakdown), neutrophils also experience “passive deformation” during passage through the pulmonary microcirculation, which consists of ~50-100 sequential capillary segments with ~2-15 μ m diameter (Yap and Kamm, 2005). A number of cellular adaptations have evolved that may facilitate the deformability of peripheral blood granulocytes. Most prominent of the adaptations, the differentiated granulocyte nucleus is lobulated in humans (ring-shaped in rodents), involving the required expression of LBR during granulopoiesis (Hoffmann et al., 2002, 2007; Shultz et al., 2003). In the absence of sufficient levels of LBR, the granulocyte nucleus is ovoid and neutrophils migrate more slowly through narrow pores, than do normal neutrophils (Gaines et al., 2008; Park et al., 1977). Since the interphase cell nucleus is regarded as being significantly stiffer than the surrounding cytoplasm (Dahl et al., 2008; Rowat et al., 2008), a malleable nuclear shape offers advantages for cells migrating through tight tissue spaces. As a second adaptation, mature blood granulocyte NE and HL-60/S4 granulocyte NE appear to have a paucity of lamins A/C, compared to more typical cells (Olins et al., 1998, 2001; Rober et al., 1990). From a study of embryo fibroblasts of lamin A/C-deficient mice, it is clear that such NE are more readily deformable to applied mechanical strain, than are NE of wild-type embryo fibroblast cells (Lammerding et al., 2004). Furthermore, recent data from our laboratory indicate that mature human blood granulocytes have low-to-negligible levels of LBR, lamins B1 and B2, LAP2 β and emerin, consistent with enhanced NE flexibility (Olins et al., 2008). In summary, we hypothesize that the blood granulocyte nucleus has a number of adaptations (i.e. malleable nuclear shape, weak and flexible NE and negligible connections between the nucleus and cytoskeleton) which collectively generates a highly deformable cell. The mature neutrophil appears highly specialized to squeeze through tight tissue spaces, migrate rapidly to the site of infection and destroy the invading bacteria by phagocytosis or by suicidal explosion (Brinkmann et al., 2004; Fuchs et al., 2007; Olins et al., 2008).

An unexpected observation of the present study was the identification of ELCS affiliated with the NE of D3-induced monocytes of HL-60/S4 cells, resembling those previously described in the granulocytic state of HL-60/S4 (Olins et al., 1998). In parallel with this observation, immunostaining and immunoblotting data were consistent with a paucity of lamin A/C and the elevation of LBR during the in vitro monocyte differentiation process, a pattern also observed during ELCS formation in RA-induced granulocytes of HL-60 cells (Olins et al., 2001). Rudimentary ELCS are only rarely observed by thin-section electron microscopy in normal blood granulocytes (Olins et al., 1998). Only about 1% of normal blood neutrophils, monocytes or lymphocytes exhibit ELCS (also called “nuclear pockets”), cited in (Ghadially, 1997). We have suggested that LBR may be responsible for membrane growth in myeloid NE (Zwenger et al., 2008). The reason why HL-60 cells and certain other leukemia and lymphoma cells exhibit ELCS, whereas normal myeloid cells seldom do, may be attributable to an over-expression of LBR (in the context of low levels of lamin A/C) in these specific malignant states.

A comprehensive survey of LBR and lamin A/C expression in various hematological malignancies would be important for testing this supposition (Prokocimer et al., 2006).

This study also presents data consistent with a paucity of LINC complex proteins in normal blood granulocytes, and their presence in normal blood monocytes and in vitro derived macrophages. The resemblance between monocytes and macrophages by this criterion is consistent with the prevalent view that blood monocytes are considered to be circulating “immature macrophages”. Blood monocytes migrate out of the circulation into various tissues, where they differentiate into macrophages and, possibly, dendritic cells and osteoclasts (Lehtonen et al., 2007; Pham et al., 2007). However, it is also clear that there are subpopulations of blood monocytes (Strauss-Ayali et al., 2007; Ziegler-Heitbrock, 2007) with distinct phenotypes and functions (including variations in nuclear morphology). This realization justifies a more in-depth examination of the blood monocytes in terms of nuclear morphology, NE composition and cellular level of LINC complex proteins.

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Fig. 1.

Confocal immunofluorescent images (single slices) of various states of HL-60/S4 stained for LINC complex proteins. Cell states: O, undifferentiated; RA, 1 μ M retinoic acid for 4 days; D3, 100 nM D3 for 4 days; TPA, 16 nM phorbol ester for 4 days. Fixation conditions: anti-vimentin, -plectin, -SUN1 and -SUN2, PFA fixation; anti-nesprin 1 and -nesprin 2, methanol fixation. Microscope gain was “optimized” as follows: vimentin and plectin, optimized for TPA cells; SUN1, SUN2, nesprin 1 and nesprin 2, optimized for COS1 cells. The DAPI images are from the anti-nesprin 2 slides, revealing nuclear shape changes. Bar: 20 μ m.

Fig. 2.

Immunoblotting of extracts from granulocyte and macrophage differentiating HL-60/S4 cells with antibodies to LINC complex proteins. Lane designations: 0, undifferentiated; 1R-5R, days 1 to 5 treatment with 1 μ M RA; 1T-5T, days 1-5 treatment with 16 nM TPA; A, A431; U, U2OS; M, molecular weight markers; BI, empty lane. Antibodies shown: mouse anti- α -tubulin (T-5168); mouse anti- β -actin (A-5441); mouse anti-vimentin (3B4); guinea pig anti-plectin (P1); rabbit anti-SUN1 and anti-SUN2; rabbit anti-nesprin 1 and nesprin 2 (pABk1). The "tics" at the right margins of the plectin and nesprin 1 blots indicate the approximate positions of molecular mass markers (212 and 158 kDa). Intact plectin should have a molecular mass of

~500 kDa, but frequently shows degradation during sample preparation. The principal band corresponding to intact SUN2 (~85 kDa) is indicated by an asterisk.

Fig. 3.

Confocal immunofluorescence images (single slices) of various states of HL-60/S4 stained for NE proteins. Cell states: O, undifferentiated; RA, 1 μM retinoic acid for 4 days; D3, 100 nM D3 for 4 days; TPA, 16 nM phorbol ester for 4 days. Antibodies using PFA fixation: LA/C, anti-lamin A/C (LaA-Z1); LB1, anti-lamin B1 (S20); LBR, guinea pig anti-LBR; anti-LAP2β (TMPO). Using methanol fixation: LB2, anti-lamin B2 (x223). Microscope gain was “optimized” as follows: lamins A/C, B1, B2 and LAP2β, optimized for TPA cells; LBR, optimized for RA cells. Bar: 20 μm.

Fig. 4.

Immunoblotting of extracts from granulocyte and macrophage differentiating HL-60/S4 cells with antibodies to NE proteins. Lane designations: 0, undifferentiated; 1R-5R, days 1 to 5 treatment with 1 μ M RA; 1T-5T, days 1-5 with treatment with 1 16 μ M nM TPA; A, A431; U, U2OS; M, molecular weight markers; BI, empty lane. Antibodies employed: LA/C, mouse anti-lamin A/C (LaA-Z1); LB1, goat anti-lamin B1 (S20); LB2, mouse anti-lamin B2 (x223); guinea pig anti-LBR; mouse anti-LAP2 β (6G11); guinea pig anti-emerin. The arrow designates the position of intact LBR (~58 kDa). CB: membrane stained with Coomassie Blue.

Fig. 5. (A-C) Hema-3-stained cytocentrifuged preparations of undifferentiated (A), RA-treated (B) and D3-treated (C) HL-60/S4 cells. (D, E) Thin-section electron micrographs (same field) of an HL-60/S4 cell treated with 100 nM D3 for 4 days. Bars: 1 μ m (D); 0.1 μ m (E).

Fig. 6.

Immunoblotting of extracts from monocyte and macrophage differentiating HL-60/S4 cells with antibodies to LINC complex and NE proteins. Lane designations: 0, undifferentiated; 1D-7D, days 1 to 7 treatment with 100 nM D3; 4T, 5T, days 4 and 5 treatment with 16 nM TPA. Antibodies employed: mouse anti- β -actin (A-5441); mouse anti-vimentin (3B4); guinea pig anti-pectin (P1); rabbit anti-SUN2; rabbit anti-nesprin 1; LA/C, mouse anti-lamin A/C (LaA-Z1); LB1, goat anti-lamin B1 (S20); LB2, mouse anti-lamin B2 (x223); guinea pig anti-LBR; mouse anti-LAP2 (TMPO). The “tics” at the right margins of the plectin and nesprin 1 blots indicate the approximate positions of molecular mass markers (212 and 158 kDa). Intact

plectin should have a molecular mass of ~500 kDa, but frequently shows degradation during sample preparation. The arrow designates the position of intact LAP2 β (~53 kDa).

Fig. 7.

Confocal immunofluorescence images (single slices) of reconstituted “buffy coat” stained for LINC complex proteins. Fixation conditions: PFA. Panels (a-c) and (d and e) show the same fields, respectively. Antibodies employed: mouse anti-vimentin (3B4); guinea pig anti-plectin (P1); rabbit anti-SUN2. The arrows in (a and c) point to blood granulocytes; the other two cells in this field are blood monocytes. Bar: 10 μ m.

Fig. 8.

Immunoblotting of extracts from COS1, HL-60/S4 and isolated blood granulocytes with rabbit anti-nesprin 1 and rabbit anti-SUN2. Lane designations: C, COS1; 0, undifferentiated; R, RA-induced granulocytes (day 4); D, D3-induced monocytes (day 4); T, TPA-induced macrophage (day 4); G, blood granulocytes. The principal band corresponding to intact SUN2 (~85 kDa) is indicated by an asterisk.

Fig. 9.

Confocal immunofluorescence images (single slices) of monocytes and macrophages adhering to chamber slides and stained for LINC complex proteins. Fixation conditions: PFA. Rows: top, freshly isolated blood monocytes after nonadherent cells were washed off the chamber slide; middle, similar field of cells after 2-week incubation with 2 nM TPA; bottom, HL-60/S4 cells after 4-day incubation with 16 nM TPA. Columns: left, goat anti-lamin B1 (S20); middle, mouse anti-vimentin (3B4); right, guinea pig anti-plectin (P1). Bar: 20 μ m.

Table 1

Immunocytochemistry* of HL-60/S4 cell states

	0	RA	D3	TPA
α -Tubulin ^a	+	+	+	+
β -Actin ^a	+	+	+	++
Vimentin ^a	+/-	0	+/-	++
Plectin	0	0	+/-	+
Nesprin 1 giant	0	0	+/-	+
Nesprin 2 giant	0	0	0	0
Nesprin 3 ^b	(0)	(+/-)	ND	(+)
SUN 1	0	0	0	0
SUN 2	+	+	+	+
Lamin A/C	+/-	0	+/-	++
Lamin B1	+/-	+/-	+/-	+
Lamin B2	+	+	+/-	+
LBR	+/-	++	++	0
LAP 2 β	+	+	+	+
Emerin	+ ^c	+ ^c	ND	+

* Combining data from present immunofluorescence and immunoblotting experiments and from earlier publications. Scale: 0, negligible; +/-, trace level; +, moderate level; ++, high level; ND, not determined.

^a Quantitative comparisons between 0 and RA, see (Olins et al., 2000).

^b Appearance of a ~100 kDa band (data not shown).

^c Primarily cytoplasmic, see (Olins et al., 2001).