Brief Report: Alternative Splicing of Extra Domain A (EIIIA) of Fibronectin Plays a Tissue-Specific Role in Hematopoietic Homeostasis

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ABSTRACT

Fibronectin (FN) is a major extracellular matrix protein implicated in cell adhesion and differentiation in the bone marrow (BM) environment. Alternative splicing of FN gene results in the generation of protein variants containing an additional EIIIA domain that sustains cell proliferation or differentiation during physiological or pathological tissue remodeling. To date its expression and role in adult hematopoiesis has not been explored. Here, we demonstrate that during physiological hematopoiesis a small fraction of BM derived FN contains the EIIIA domain and that mice constitutively including (EIIIA +) or excluding (EIIIA −) the EIIIA exon present comparable levels of hematopoietic stem cells, myeloid and lymphoid progenitors within BM. Moreover, only minor alterations were detected in blood parameters and in hematopoietic frequencies of BM granulocytes/monocytes and B cells. As opposed to other tissues, unique compensatory mechanisms, such as increased FN accumulation and variable expression of the EIIIA receptors, Toll like receptor-4 and alpha9 integrin subunit, characterized the BM of these mice. Our data demonstrate that FN is a fundamental component of the hematopoietic tissue and that the EIIIA exon may play a key role in modulating hematopoiesis in conditions of BM stress or diseases. STEM CELLS 2016;34:2263–2268

SIGNIFICANCE STATEMENT

Fibronectin represents a major extracellular matrix protein implicated in cell adhesion in the bone marrow environment. Alternative splicing of fibronectin gene results in the generation of protein variants containing an additional EIIIA domain that sustains cell proliferation or differentiation during physiological or pathological tissue remodeling. However, its expression and role in adult hematopoiesis has not been explored. In our research, we demonstrate that during physiological hematopoiesis a small fraction of BM derived FN contains the EIIIA domain and that mice constitutively including (EIIIA +) or excluding (EIIIA −) the EIIIA exon present comparable levels of hematopoietic stem cells, myeloid and lymphoid progenitors within BM. Moreover, only minor alterations were detected in blood parameters and in hematopoietic frequencies of BM granulocytes/monocytes and B cells. As opposed to other tissues, unique compensatory mechanisms, such as increased FN accumulation and variable expression of the EIIIA receptors, Toll like receptor-4 and alpha9 integrin subunit, characterized the BM of these mice. Our data demonstrate that FN is a fundamental component of the hematopoietic tissue and that the EIIIA exon may play a key role in modulating hematopoiesis in conditions of BM stress or diseases.

INTRODUCTION

Fibronectin (FN) is an extracellular matrix (ECM) glycoprotein that plays a regulatory role during embryogenesis, wound healing and maintenance of tissue integrity [1]. The FN1 gene encodes 15 type III repeats that are constitutively expressed plus 2 that are alternatively spliced: extra domain A (EIIIA, ED-A) and extra domain B (EIIIB, ED-B) [2]. EIIIA exon is included or excluded from the FN mRNA by exon skipping [3, 4]. Plasma FN lacks both EIIIA and EIIIB segments and is a soluble form secreted by hepatocytes, whereas cellular FN contains variable proportions of EIIIA and EIIIB segments and is found as fibrils in the ECM [5]. Increased expression of the EIIIA + and EIIIB + isoforms of FN are associated with physiological or pathological tissue remodeling, including wound healing [6], tissue fibrosis [7], fibroblast differentiation [8], inflammation [9] and tumor progression [10].

Blood cell formation is controlled by a complex set of events, including interactions between ECM components and hematopoietic cells. Within bone marrow (BM) environment, FN represents a major ECM component supposed to play important roles in various aspects of hematopoiesis through binding to very late antigen 4 (VLA4) and 5 (VLA5) [11]. However, effects of FN variants on hematopoietic stem cell behavior are largely unknown. In particular, the expression and precise localization of FN variants within BM environment could be instrumental for a fine control of the interactions between cells with resulting...
Figure 1. Characterization of BM environment in wild type, EIIIA<sup>+/+</sup> and EIIIA<sup>−/−</sup> mice. (A) BM ECM proteins from wild type mice were analyzed by western blotting and probed with a polyclonal antibody anti fibronectin (FN) and a monoclonal antibody specific for the EIIIA segment. (B) RT-PCR analysis of EIIIA-FN expression in sorted CD45<sup>+</sup>, CD45<sup>−</sup>/CD31<sup>+</sup> endothelial cells and CD45<sup>−</sup>/CD31<sup>−</sup>/CD140a<sup>+</sup> stromal cells. p value *<0.05 and **<0.01, respectively. (n = 3). (C) Analysis of FN1 gene expression and EIIIA inclusion in BM cells of wild type, EIIIA<sup>+/+</sup> and EIIIA<sup>−/−</sup> mice by RT-PCR. (D, E) RT-PCR analysis of alternative exons EIIIB and IIICS region expression, using primers that flank each in BM derived MNCs. (+) mRNA indicates inclusion and (−) mRNA indicates exclusion of the exon. (F) Quantification of BM mononuclear cells. (n = 5 mice in each group). (G) Hematoxylin and eosin (H&E) staining at and immunofluorescence staining of FN (red) of BM sections of mice. Hoechst 33258 (blue) was used to stained nuclei. Original magnification × 20, × 40, and × 10, respectively. Scale Bar = 50, 25, and 100 μm, respectively. Abbreviations: BM, bone marrow; ECM, extracellular matrix; FN, Fibronectin.
changes in cell adhesion, migration, and growth [12, 13]. In the present work we studied the role of EIIIA FN isoform in murine hematopoiesis taking advantage of two mouse strains unable to undergo a regular alternative splicing of the EIIIA exon. One strain contains optimized spliced sites at both splicing junctions of the EIIIA exon and constitutively includes the exon (EIIIA<sup>1/1</sup>), whereas the other strain contains an EIIIA-null allele of the EIIIA exon (EIIIA<sup>2/2</sup>) [6].

**MATERIALS AND METHODS**

**Animals**

C57BL/6 wild type mice were from Charles River Laboratories, Italy. EIIIA<sup>1/1</sup> and EIIIA<sup>2/2</sup> mouse strains were previously generated [6]. Six to eight weeks old mice were used in all experiments. Mice were housed at the animal facility of the Department of Physiology, section of General Physiology, University of Pavia (approval # 3/2013, 19/11/2013). All animals were sacrificed according to the current European legal Animal Practice requirements.

**Antibodies and Reagents**

The following antibodies were used: anti rat FN was from Chemicon (Merck-Millipore, Milan, Italy), anti cellular FN 3E2, anti laminin and anti β-actin antibodies were from Sigma Aldrich. Anti mouse integrin alpha9 and anti type I collagen were from Abcam (Cambridge, U.K.). Anti TLR4 (clone 25) was from Santa Cruz Biotechnology Inc. Human plasma FN was purchased from BD Bioscience (Milan, Italy), cellular FN from human skin fibroblasts was from Sigma-Aldrich (Milan, Italy).

**Statistics**

Values are expressed as mean ± SD. One way ANOVA and two way ANOVA followed by a Bonferroni post-test were used to analyze experiments. p value statistically significant were expressed as *, p < .05, **, p < .01, and ***, p < .001 respectively.

Extended information on experimental procedures are available in Supporting Information.

**RESULTS AND DISCUSSION**

To decipher the role of the FN EIIIA domain in the biology of the hematopoietic system, we first evaluated its expression in the BM matrix of adult mice. As shown in Fig. 1A, deoxycholic acid (DOC) insoluble fibrillar proteins derived from BM were analyzed by western blotting and FN was detected by a polyclonal antibody that recognizes both plasmatic and cellular isoforms (left panel). Importantly, a small fraction of this fibrillar FN stained positive also for a monoclonal antibody directed against the EIIIA domain (right panel). We further performed a detailed analysis of cellular EIIIA-FN mRNA expression. As demonstrated in Fig. 1B higher levels of EIIIA FN expression were detected in sorted CD45<sup>-</sup>/CD31<sup>+</sup> endothelial cells and CD45<sup>-</sup>/CD31<sup>-</sup>/PDGFRα<sup>+</sup>/CD140a<sup>+</sup> stromal cells with respect to hematopoietic mononuclear CD45<sup>+</sup> cells.

To measure the effects of the EIIIA domain in the hematopoietic system, we analyzed mouse strains unable to undergo alternative splicing of the FN EIIIA exon. As shown in Fig. 1C–1E, EIIIA<sup>1/1</sup> and EIIIA<sup>2/2</sup> displayed opposite levels of spliced EIIIA exon at mRNA level, comparable levels in the splicing rate of IIICS region, while, inclusion of EIIIB domain was not detected in the adult hematopoietic tissue. These results
confirmed the autonomy of the alternatively spliced regions of the FN1 gene in the transgenic EIIIA mice and confirmed the absence of EIIIB domain in the FN of the hematopoietic tissue, as previously described in other tissues during mice development and aging [14].

Both mouse strains presented regular BM cell count (Fig. 1F–1G) and regular FN assembly in the BM extracellular matrix (Fig. 1G). Frequencies and absolute number of KSL stem cells were comparable in all mice strains, as well as myeloid and lymphoid progenitors, suggesting that the splicing of EIIIA exon is dispensable for adult KSL self-renewal and differentiation (Fig. 2A–2G). We then analyzed the frequencies of mature blood cell lineages in the peripheral blood (PB) by differential cell count analysis. Interfering with FN splicing of EIIIA exon did not determine a significant alteration of most of the blood parameters in the PB of EIIIA\(^1/1\) and EIIIA\(^2/2\) mouse models. As reported in Supporting Information Table 1, we only observed a significant difference in the mean platelet volume (MPV) that resulted significantly higher in EIIIA\(^1/1\) mice. In order to better characterized the hematopoietic system of these mice, we then analyzed the immunophenotype of the cell population of the BM, the spleen and the PB by flow cytometry. As shown in Fig. 3A, 3C, EIIIA\(^1/1\) mice displayed a reduction in the percentage of B220\(^+\) cells with respect to wild type and EIIIA\(^2/2\) mice (mean 35.52 vs. 26.43 vs. 32.33%) \((p<.001\) and \(p<.05\), respectively). Differently, BM cells in the EIIIA\(^1/1\) mice were characterized by an increase in granulocyte/monocytic content as revealed by Gr-1/Mac-1 staining of cells by flow cytometry (FACS) (mean EIIIA\(^\text{wt}/\text{wt}\) 34.57 vs. EIIIA\(^1/1\) 39.11 vs. EIIIA\(^2/2\) 45.02\%, \(p<.05\)). No significant alterations in BM T lymphocyte and macrophage content were detected. Importantly, these slight modifications in cell frequencies were specifically found in the BM although no significant differences in structure, FN deposition and cell lineage frequencies were detected in the spleen or PB of both mice models (Fig. 3B, 3D–E; Supporting Information Fig. S1). The reduction in total B-cells within BM in EIIIA\(^1/1\) led us to characterize B cell maturation by analyzing the subsequent stages of B-cell development. As shown in Supporting Information Fig. S1A, we did not identify blocks in B-cell development in EIIIA\(^1/1\) mice, but only a reduction of Pre B and Pro B cell progenitors within BM \((p<.05)\). Furthermore, the frequencies of marginal and transitional T1 B cells in the spleen, mature and transitional T2 cells of spleen and PB in both mouse models were comparable to those of wild type (Supporting Information Fig. S1). Overall, when we calculated

Figure 3. Mature blood cells production in mice with aberrant EIIIA-FN expression. (A, B) Representative FACS analysis of B, T lymphocytes, CD4 and CD8 T lymphocytes and granulo/monocytic cells within bone marrow (BM) and spleen of mice. (C–E) Quantification of B lymphocytes and CD3, CD4, and CD8 T lymphocytes and granulo/monocytic cell frequencies in BM (C) spleen (D) and peripheral blood (E) of wild type, EIIIA\(^1/1\) and EIIIA\(^2/2\) mice. \(p\) value *<.05 and **<.001, respectively. \((n=5\) mice in each group).
the ratio between the frequency of the B cell subpopulation and the absolute number of the BM mononuclear cells, we did not observe significant differences (data not shown). Taken together these data proved that the hematopoietic tissue was comparable, in terms of cell composition, in all the three different genotypes we analyzed.

Genetic manipulation of FN1 gene in the EIIIA+/+ mice was demonstrated to induce a striking decrease in FN levels in most tissues of the body [6]. Interestingly, the hematopoietic tissue was not affected by a reduction in FN amount, highlighting the importance of FN in the regulation of hematopoietic tissue homeostasis. As shown in Fig. 4A, 4B, FN protein level was increased in BM-derived MNCs from EIIIA+/+ mice, whereas comparable levels of FN were present in spleen-derived MNCs among all mice genotypes.

The increase in BM content of FN in the EIIIA+/+ mice did not seem to be a consequence of increased gene expression, as a comparable expression of the FN1 gene was detected in BM cells derived from all mice (Fig. 4C). Therefore this increase in FN protein in the EIIIA+/+ mice might derive from mechanisms of extravasation or uptake of plasma-derived FN that was previously shown to constitute an additional source of tissue FN in several organs [5]. In support of this statement, deposition of other ECMs, such as type I collagen or laminin, in the BM was unaffected by FN gene manipulation (Supporting Information Fig. S2).

The EIIIA segment of FN has been showed to contain binding sites for leukocyte integrins α9β1, α4β1, and α4β7, providing a novel mechanism for regulating cell adhesion by alternative splicing [15]. Additionally, EIIIA domain was recently demonstrated to activate the Toll Like Receptor 4 (TLR4), involved in the defense in the innate immune response following recognition of pathogens associated molecular patterns [16]. Interestingly, the signaling associated with these receptors has been implicated in the direction of granulopoiesis and stem cell differentiation during the immune response [17], [18]. Analysis of EIIIA receptor expression in our mice, revealed that the higher amount of cellular EIIIA FN in the BM of EIIIA+/+ mice was accompanied by an increased expression of both TLR4 and alpha9 integrin subunit (Fig. 4D–4F) in BM-derived MNCs, but not in spleen-derived MNCs which were exposed to regular amount of tissue FN (Fig. 4D–4F). Similarly, only adhesion to cellular FN (cFN, containing the EIIA domain) differently modulate TLR4, alpha9 and beta1 integrin expression in BM-derived MNCs. (N = 3). *, p < .05. All data are expressed as mean ± SD.

Figure 4. Compensatory mechanisms in Fibronectin (FN) accumulation and receptors expression characterized bone marrow (BM) environment of mice with aberrant FN splicing. (A) Western blot analysis of FN content in BM- and spleen-derived mononuclear cells from wild type, EIIIA+/+ and EIIIA−/− mice (two mice per genotype are shown). (B) Quantification of FN protein content in BM and spleen-derived mononuclear cells lysate. Band intensities were normalized for beta-actin content. *, p < .05, ***, p < .001. All data are expressed as mean ± SD of three independent experiments. (C) Analysis of FN1 gene expression in BM-derived mononuclear cells from wild type, EIIIA+/+ and EIIIA−/− mice. Expression of ribosomal 18S gene was used for comparative analysis. (D) Western blot analysis of TLR4 and alpha9 integrin expression in BM and spleen-derived mononuclear cells of wild type, EIIIA+/+ and EIIIA−/− mice. (E, F) Densitometric analysis of TLR4 and alpha9 expression in BM (E) and spleen-derived mononuclear cells lysate (F). Band intensities were normalized for beta-actin content. *, p < .05, ***, p < .001. All data are expressed as mean ± SD. (G) Adhesion to plasmatic FN (pFN, lacking the EIIIA domain) or cellular FN (cFN, containing the EIIA domain) differently modulate TLR4, alpha9 and beta1 integrin expression in BM-derived MNCs. (N = 3). *, p < .05. All data are expressed as mean ± SD.
suggest that alternative splicing of FN may play an important role in maintaining the steady state of the hematopoietic tissue. Alternative spliced EIIIA and EIIIB isoforms of FN have been demonstrated to play a key role in physiological tissue repair [6, 19, 20], thus further experiments are required to clarify the role of spliced FNs in conditions of hematopoietic stress or hematological malignancies.

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Author Contributions

A.M., C.G., G.C., B.R. performed the research; A.M. and A.B. designed research, A.F.M. provided reagents, interpreted and analyzed the data; L.D.M. and L.L. analyzed data; A.M. and A.B. wrote the paper.

Disclosure of Potential Conflicts of Interest

The authors indicate they have no conflicts of interest.

References


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