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VCAM-1 ABLATION IN NON-HEMATOPOIETIC CELLS IN MxCre+ VCAM-1^{f/f} MICE IS VARIABLE AND DICTATES THEIR PHENOTYPE

Tatiana Ulyanova, Gregory V. Priestley, Betty Nakamoto, Yi Jiang, and Thalia Papayannopoulou

University of Washington, Department of Medicine, Division of Hematology, Seattle, WA, USA

Abstract

Objective— The goal of the present study was to assess the extent of VCAM-1 gene deletion in hematopoietic vs. non-hematopoietic cells in the bone marrow (BM) of MxCre⁺VCAM-1^{f/f} mice and its impact on the phenotypic features of these mice.

Methods—VCAM-1 ablation was evaluated at the genomic level by PCR, at the mRNA level by real time PCR and at the protein level by FACS and immunohistochemistry. The homing or mobilization of CFU-Cs was assessed by standard assays.

Results—A previously accepted Interferon-induction scheme yielded efficient VCAM-1 ablation in hematopoietic cells, but variable ablation in BM fibroblasts and endothelial cells. The level of ablation in the latter populations correlated with alterations in the hematopoietic phenotype.

Conclusions—Poly(I:C)-induced MxCre-mediated gene ablation is highly efficient in hematopoietic cells, but variable and partial in non-hematopoietic cells in BM. Ablation of VCAM-1 in hematopoietic cells does not contribute to their mobilization, nor does it impair their homing. The latter is dependent on VCAM-1 ablation in non-hematopoietic cells of BM.

INTRODUCTION

Vascular cell adhesion molecule-1 (VCAM-1) is expressed in endothelial cells, and together with its main counter receptor $\alpha 4 \beta 1$ integrin (VLA-4) is expressed in hematopoietic cells and participates in physiologic and inflammatory processes in many tissues [1–7]. Furthermore, using mice genetically deficient for either VCAM-1 or VLA4, we have previously shown that the VCAM-1/VLA4 pathway is important in mediating retention of hematopoietic progenitor cells (HPC) in bone marrow under homeostatic conditions [8], corroborating earlier studies employing anti-functional antibodies [9–11]. Two mouse models with conditional Cre-mediated VCAM-1 deletion have been described. One employs the Tie2 promoter to control Cre expression in endothelial and hematopoietic cells [12], the other makes use of the interferon inducible MxCre system [13]. VCAM-1, normally expressed in hematopoietic cells of several lineages, in endothelial cells and in a proportion of fibroblasts in BM, is absent from these cell populations in Tie2Cre⁺VCAM-1^{f/f} mice [8]. As a result, life-long changes in the biodistribution of hematopoietic progenitor cells were documented in these mice. However, the specific contribution to the above phenotypic changes of VCAM-1-deficient hematopoietic cells vs. VCAM-1-deficient non-hematopoietic cells was not clear.

Corresponding Author and reprint requests: Dr. Thalia Papayannopoulou, University of Washington, Div. of Hematology, Box 357710, Seattle, WA 98195-7710, USA, phone: 206-543-5756, fax: 206-543-3050, email: thalp@u.washington.edu

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In the MxCre⁺VCAM-1^{f/f} mice, although abnormalities in IgM production and B-cell biodistribution were described [13], no studies addressing progenitor content and biodistribution were done. Furthermore, in this model, although VCAM-1 ablation in BM cells was documented [13], the extent of VCAM-1 deletion, if any, in non-hematopoietic cells in BM was not explored in this study as well as in other studies using the interferon-induced Cre-mediated recombination [14–19]. In the present paper we investigate the extent of MxCre-mediated VCAM-1 ablation in both hematopoietic and non-hematopoietic cells and ascertain whether and to what extent such an ablation influences hemopoietic phenotype and progenitor cell biodistribution. Our present data from MxCre⁺VCAM-1^{f/f} mice and their comparison with data from Tie2Cre⁺VCAM-1^{f/f} mice before or after transplantation with normal cells provide further insight toward the molecular mechanisms of homeostatically regulated retention of progenitor cells in the BM and reveal potential limitations of the MxCre-based conditional gene ablation of non-hematopoietic cells.

MATERIALS AND METHODS

Mice and treatments

Tie2Cre⁺VCAM-1^{f/f} (T2-V) mice were previously described [8,12]. To generate MxCre⁺/VCAM-1^{f/f} (Mx-V) mice, we interbred VCAM-1^{f/f} mice and mice carrying MxCre transgene. To induce VCAM-1 ablation, Mx mice were treated with interferon inducer poly(I:C) (three intraperitoneal injections of 300 µg of poly(I:C) 48 hours apart) and were used for experiments at least four weeks post ablation. Poly(I:C) from two sources, GE Healthcare Life Sciences (formerly Amersham, Piscataway, NJ, USA) and Sigma Chemical Co., St. Louis, MO, USA, was used. For 5-fluorouracil (5-FU) cytotoxic stress, mice received a single intravenous (i.v.) injection of 5-FU (250 mg/kg of body weight) and were sacrificed 11 days post injection. Mice were bred and maintained under specific pathogen free conditions at the University of Washington. All experimental procedures were done in accordance with IACUC guidelines with approved institutional protocols.

Antibodies

Anti-VCAM-1 (MK-2) and anti-α4 (PS/2) antibodies were from Southern Biotech (Birmingham, AL, USA). CD45-APC and Ter119-PE antibodies were from BD Biosciences (San Diego, CA, USA).

FACS analysis

Cells were stained with an appropriate fluorochrome-conjugated antibody (30 min on ice) washed in PBS containing 0.05% BSA and analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) using CELLQuest software.

Endothelial and fibroblast cell cultures

Primary endothelial and fibroblast cell cultures were established as described [8]. In brief, for endothelial cell cultures, flushed femurs were treated with 0.125% Trypsin/0.2% EDTA for 15 min at 37°C; cells were discarded, bones minced, incubated with 0.1% collagenase, Type I (1–3 hours) and released cells were cultured on collagen IV-coated plates (BD Biosciences; Bedford, MA) using endothelial cell medium (Dulbecco's Modified Eagle Medium (DMEM) +20% Fetal Bovine Serum (FBS) containing 250 ng/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA, USA), 10 ng/ml recombinant murine vascular endothelial cell growth factor, 7.5 ng/ml basic fibroblast growth factor (both from Peprotech, Rocky Hill, NJ, USA), 0.6 U/mL heparin, 5x10⁻³ M 2-mercaptoethanol, Non-essential amino acids and 5x10⁻³ M HEPES. Non-adherent cells were removed 24 hours later and fresh medium was added. Cells were propagated by splitting 1:2, using TrypLE Express solution (Invitrogen,

Carlsbad, CA, USA) for cell dissociation. For fibroblast cultures, bone fragments were plated in DMEM with 20% FBS and cultured for 4–7 weeks on plastic tissue culture dishes. At every passage, cells were incubated with TrypLE Select cell-dissociating solution (Invitrogen) for no longer than two minutes and plated on new plates. Such brief incubation allowed the efficient removal of contaminating macrophages (CD45+) with several successive passages. Before use, fibroblasts and endothelial cell cultures were analyzed by FACS for expression of CD45 and Ter119 and only cells negative for both were used for assays.

DNA/RNA studies

DNA and RNA isolation, RT PCR analysis and primers used were described [8]. Briefly, genomic DNA or RNA was isolated from cultured cells, primary BM cells or mouse tails using appropriate kits (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Isolated RNA (1mg) was reverse transcribed using random nanomer primer (Sigma Chemical) and SuperScript II enzyme (Invitrogen). Quantitative PCR was performed on reverse-transcribed RNA using Power Sybr Green PCR master mix (Applied Biosystems, Foster City, CA, USA); following primer sets were used: VCAM-1 (sense, 5'-TCTCTCAGGAAATGCCACCC, antisense, 5'-CACAGCCAATAGCAGCACAC), actin (sense, 5'-ATCCTCACCCCTGAAGTACCC-3', antisense, 5'-ATTTCCCGCTCGGCCGTGGT-3'). For analysis of genomic DNA to distinguish between VCAM-1 wild type, deleted and floxed alleles following primers were used: (5'-ATCGATCCCTGGATATGTCG3', 5'-AGGCTATCAGTGAGACCCACA-3', 5'-TGGGCTGTCTATCTGGGTTC-3').

Clonogenic Progenitor Assays

Colony forming unit-culture (CFU-C) assays were performed with peripheral blood (PB) or BM samples, using a methylcellulose mixture (Methocult GF; Stem Cell Technologies, Vancouver, BC, Canada) and evaluated as described previously [14].

Immunohistochemistry

Femurs obtained from mice after 5-FU treatment were processed as described [8]. Briefly, bones were fixed in 4% paraformaldehyde followed by decalcification in 10% EDTA for 7–10 days and sucrose treated, then embedded in Tissue-Tek (Sakura Finetechnical, Tokyo, Japan) and frozen in acetone/dry ice. Air dried frozen sections were stained with MK-2 antibody followed by anti-rat Alexa 594 (Invitrogen). Sections were counter-stained with DAPI to visualize cell nuclei.

Anti- α 4 antibody-induced mobilization

Mice were injected i.v. with PS/2 (2.0 mg/kg/day x 3 days, low endotoxin, no azide, a kind gift from Biogen, Cambridge, MA, USA) and 24 hours after the last injection, the number of circulating HPCs (CFU-C) was determined in PB.

Homing experiments

Lethally irradiated (1150 cGy whole body irradiation with a ^{137}Cs source [20]) control, Mx-V or T2-V recipients received transplants of normal BM cells (20×10^6 /recipient) by intravenous route. Mice were sacrificed 20 hours later and samples of peripheral blood or BM were subjected to a clonogenic progenitor assay to determine numbers of CFU-Cs present in these samples (endogenous CFU-C do not survive using this level of irradiation as a single dose). Proportions of homed CFU-C were calculated from the total CFU-C present in the injected inoculum determined by culturing donor cells.

Transplantation experiments

Lethally irradiated control or T2-V recipients were injected i.v. with normal BM cells (5×10^5 cells/recipient). Recipient mice were bled 6 and 10 weeks after transplantation to determine donor cell reconstitution and numbers of circulating progenitor cells (CFU-C).

Soluble VCAM-1

Levels of soluble VCAM-1 (sVCAM-1) were assayed in the Cytokine Analysis Core Facility of Fred Hutchinson Cancer Research Center, as described [8].

Statistical Analysis

All statistical analyses were performed with a two tailed Student's t-Test (Microsoft Excel 2000 software, Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

Efficient ablation of VCAM-1 gene in hematopoietic cells of MxCre⁺ VCAM-1^{ff} mice

MxCre⁺VCAM-1^{ff} mice (Mx-V) were injected with interferon-inducer poly(I:C) and studied from two to several weeks after the last injection. When hematopoietic cells in BM were tested, starting at two weeks after the last poly(I:C) injection, a virtually complete VCAM-1 ablation was documented in these cells (Fig. 1). Both at the protein level (Figure 1 upper panel) and at the mRNA level (Figure 1 middle panel), no VCAM-1 expression was detected in BM cells of Mx-V mice. In addition to total BM cells, CFU-C progeny in vitro (pooled colonies) showed efficient VCAM-1 gene ablation in Mx-V mice at the progenitor cell level (Fig. 1 bottom panel). These findings are in agreement with those previously described for the same mice after direct interferon administration, showing no VCAM-1 mRNA in BM cells [13]. The data with BM cells are also comparable to the ones in Tie2Cre⁺VCAM-1^{ff} mice (T2-V) we have studied previously [8], and consistent with other examples of efficient gene ablation of hematopoietic cells using the MxCre system [14–19].

The functional role of VCAM-1 present in hematopoietic cells is yet to be fully explored. We previously showed that VCAM-1 expression among hematopoietic cells of different lineages requires tissue inductive signals not present in circulating cells, and that VCAM-1 could be induced in vitro in proliferating myeloid but not lymphoid cells from PB. This suggests a bias of VCAM-1 expression towards a myeloid lineage [8]. In this context, it is of interest that VCAM-1, present in very early multipotent progenitors, represents a branching point between myeloid and lymphoid lineages, since the latter develop only from VCAM-1-negative cells [21]. Nevertheless, VCAM-1 expression in endothelial cells plays an important role in the trafficking of lymphocytes, for those expressing VLA4 or for the ones with only $\alpha\text{D}\beta 2$, an alternative VCAM-1 counter-receptor with a VLA4 overlapping binding site [22]. For myeloid cells, however, hypothetical cell-cell interactions involving VCAM-1 can be entertained, but no evidence for this has been obtained thus far.

Variability in phenotype correlates with extent of VCAM-1 ablation in non-hematopoietic cells

To test whether Mx-V mice display the phenotypic features that we have previously seen in T2-V mice with VCAM-1 ablation in both endothelial cells and hematopoietic cells, we studied the cellularity and progenitor content in BM, spleen and PB of these mice. After poly(I:C) treatment, BM cellularity ($27.0 \times 10^6 \pm 1.3 \times 10^6$ cells/femur, n=6) and progenitor content in BM ($119.1 \times 10^3 \pm 11.3 \times 10^3$ CFU-C/femur, n=6) was significantly higher than in controls ($17.8 \times 10^6 \pm 1.1 \times 10^6$ cells/femur, p<0.05, n=5, and $59.8 \times 10^3 \pm 4.9 \times 10^3$ CFU-C/femur, p<0.05, n=5). Spleen cellularity and progenitor content in Mx-V mice, was highly variable and not significantly different from controls (data not shown). Further, a highly significant difference

in circulating CFU-C in Mx-V mice was seen compared to controls (Fig. 2A, left panel). Overall however, this increase was much lower than the one seen in T2-V mice and a wide variation in results was seen. For example, CFU-C levels in Mx-V mice ranged from 229 CFU-C/mL PB (similar to the control levels) to 1,298 CFU-C/mL. Such variability was maintained regardless of the source of poly(I:C) used for interferon induction (data not shown). Thus, in Mx-V mice, circulating CFU-C levels ranging from almost normal up to the levels seen in T2-V mice were noted. On the basis of these results, we arbitrarily segregated Mx-V mice into two groups, corresponding to high and low levels of circulating progenitors (Mx-V CP^{hi} and Mx-V CP^{lo}, respectively) using a cut-off level of 700 CFU-C/mL blood (Figure 2A right panel). To test whether the observed variation in CFU-C biodistribution was determined by the variable ablation in BM non-hematopoietic cells of these animals, we correlated levels of PB CFU-C with the extent of VCAM-1 deletion in BM non-hematopoietic cells (endothelial cells or fibroblasts). For this purpose we studied VCAM-1 expression levels in fibroblast or endothelial cell cultures derived from Mx-V CP^{lo} and Mx-V CP^{hi} mice and compared them to those derived from control or T2-V mice. Although the level of surface VCAM-1 expression was significantly lower in fibroblasts derived from both CP^{lo} and CP^{hi} Mx-V than those from control mice, the degree of VCAM-1 ablation was significantly higher in fibroblasts from Mx-V CP^{hi} mice (Fig. 2B, left). A similar trend was observed in cultured endothelial cells from Mx-V CP^{lo} and CP^{hi} mice, both at the protein expression level (Fig. 2B, right) and when a quantitative PCR approach was employed (Fig. 2C). Furthermore, evaluation of VCAM-1 protein in BM using representative samples from Mx-V CP^{lo} or Mx-V CP^{hi} mice was addressed by immunohistochemical means. To increase the sensitivity of results, we subjected the animals to stress conditions known to induce disruption in BM vasculature followed by active remodeling [23] and up-regulation of VCAM-1 [24] in BM endothelial cells: mice were treated with 5-FU. VCAM-1 expression was monitored immunohistochemically in femurs at the recovery phase. Mx-V CP^{lo} mice with no significant difference in circulating CFU-C from control mice were found to display VCAM-1 labeling of bone marrow sections similar to that seen in controls (Fig. 2D). By contrast, virtually no VCAM-1 was seen in the Mx-V CP^{hi} mice (Fig. 2D). These protein expression data were consistent with our data at the mRNA level mentioned earlier.

In order to consolidate the findings with Mx-V mice, to test whether several other phenotypic features seen in T2-V mice were present in Mx-V CP^{lo} mice, we tested progenitor cell mobilization in response to anti- $\alpha 4$ antibody. Although anti- $\alpha 4$ treatment elicited no significant mobilization in T2-V mice, it did yield a mobilization response in Mx-V CP^{lo} mice similar to that seen in controls (Fig. 3A). These data indirectly suggested the presence of VCAM-1 in BM of these mice. Next we tested the homing patterns of wild type BM cells using irradiated Mx-V CP^{lo} mice, T2-V mice, or WT controls as recipients. Homing of normal donor cells into Mx-V CP^{lo} irradiated recipients was not significantly different from the one seen using normal recipients, in contrast to the homing in T2-V recipients (Fig. 3B). A concurrent increase of non-homed donor cells in PB of T2-V but not of Mx-V CP^{lo} recipient mice was a corroborating feature (Fig. 3B). BM homing of VCAM-1-deficient cells in normal recipients was tested and found to be similar to that of VCAM-1 +/+ cells (Fig. 3C). In addition transplantation experiments with normal BM cells were carried out in VCAM-1-deficient and normal recipients (Fig. 3D). These experiments provide compelling evidence that the presence of VCAM-1 in the bone marrow environment controls the ability to anchor and retain transplanted hematopoietic progenitor cells, rather than the VCAM-1 on hematopoietic cells.

Finally, circulating levels of soluble VCAM-1, another distinguishing feature of T2-V mice, were measured in Mx-V CP^{lo} mice. sVCAM-1 levels in Mx-V CP^{lo} mice (290.5 ± 12.5 ng/mL blood, $n = 11$), although significantly lower than in controls (630.03 ± 29.0 ng/mL blood, $n = 14$, $p < 0.01$), were significantly higher than sVCAM-1 levels in T2-V mice (147.76 ± 38.0 ng/mL blood, $n = 5$, $p < 0.01$).

In contrast to findings with hematopoietic cells, data on BM non-hematopoietic cells indicate a variable ablation of VCAM-1 in fibroblasts and endothelial cells. Similar studies on MxCre-dependent ablation of other genes in non-hematopoietic cells from BM are not available for comparison. In the one instance that this was tested, partial ablation, consistent with our data, was seen by FACS using a very small number of CD45⁺Ter119⁺ cells [25]. It is possible that different injection schemes (i.e. 4–10 poly(I:C) injections) may have a different outcome. Nevertheless what is emphasized here is that, even using the same injection schedule, variable ablation among animals is to be expected. Such variation gave us the opportunity to explain phenotypic differences among animals and brings forth the awareness of variability in ablation patterns in other animal models using the Mxcre system.

In summary, our data, taken together, suggest that (i) ablation through the MxCre system is efficient in hematopoietic cells, but does not necessarily predict ablation in BM non-hematopoietic cells; (ii) great variability in BM non-hematopoietic cell ablation is to be expected in a given group of mice treated with the same injection schedule using different sources of poly(I:C); (iii) phenotypic alterations in MxCre⁺ VCAM-1^{f/f} mice similar to those found in Tie2Cre⁺VCAM-1^{f/f} mice are attributed to ablation mainly in non-hematopoietic cells with no contribution from VCAM-1 ablation in hematopoietic cells. Therefore, in the Mx-V model, increases in the level of circulating progenitors is a sensitive indicator of partial ablation of VCAM-1 in non-hematopoietic cells and supports the role of VCAM-1 in progenitor BM retention during homeostasis. We believe that our data provide further insight towards the phenotype of MxCre⁺ or Tie2Cre⁺ VCAM-1 mice and the molecular mechanisms of normal hematopoietic progenitor cell retention in BM. Further, our data highlight findings in non-hematopoietic cells in BM that are applicable to inducible ablation of other genes using the MxCre-mediated LoxP-based system.

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Figure 1. Ablation of VCAM-1 in hematopoietic cells

Bone marrow cells from control VCAM^{f/f} (f/f-V), Mx-V, and T2-V mice were assayed for VCAM-1 protein expression by FACS (upper panel: f/f-V, gray area; Mx-V, thin black line; T2-V, thick black line; *isotype matched control, gray line) and for mRNA expression by RT PCR (middle panel). PCR with actin primer set served as internal control. Lower panel: Progeny of cultured BM hematopoietic progenitors (pooled colonies) from poly(I:C) treated mice was assessed for the presence of deleted (Δ) VCAM allele by genomic PCR. Results from 3 mice are presented. A sample from BM cells (BM) before poly(I:C) treatment is shown for comparison.

Figure 2. Variability in circulating hematopoietic progenitors and in VCAM-1 expression by BM endothelial and stromal cells

(A) Levels of circulating progenitor cells (CFU-C) in PB of control (f/f), T2-V, and Mx-V mice (left panel) and in sub-groups of Mx-V mice corresponding to low (Mx-V CP^{lo}) and high (Mx-V CP^{hi}) levels of circulating progenitors (right panel). *significant difference over control mice, $p < 0.05$, ‡, significant difference over T2-V mice, $p < 0.05$, and #significant difference between Mx-V CP^{lo} and Mx-V CP^{hi} mice, $p < 0.05$. (B) Mean Fluorescence Intensity (MFI) of FACS analyzed VCAM-1 surface expression in fibroblast (left) and endothelial cell cultures (right) derived from control, T2-V, Mx-V CP^{lo}, and Mx-V CP^{hi} mice. Cells were stained with anti-CD45 and MK-2, an anti-VCAM-1 antibody, and mean fluorescence intensity for MK2

was analyzed in the CD45-negative gate. *significant difference over control, $p < 0.05$, ‡significant difference over T2-V. (C) VCAM-1 mRNA expression in endothelial cell cultures derived from control, T2-V, Mx-V CP^{lo} and Mx-V CP^{hi} mice analyzed by relative quantitative PCR. (D) Immunohistochemical evaluation of VCAM-1 expression in the BM in representative Mx-V CP^{lo} and Mx-V CP^{hi} mice recovering from 5-FU-induced hemopoietic stress (see Materials and Methods). Staining with the secondary antibody only, serving as negative control, showed no reaction (data not shown). Arrows point to the blood vessels. Scale Bar: 10 μ m.

The number of mice in each group shown in Fig. 2 is indicated within the corresponding bar.

Figure 3. Progenitor cell behavior is dictated by the VCAM-1 expression in bone marrow non-hematopoietic rather than hematopoietic cells

(A) Mobilization of progenitor cells in response to anti-α4 antibody. Control (f/f), Mx-V CP^{lo}, and T2-V mice were injected i.v. with P/S2, an anti-α4 antibody (3 daily injections) and circulating progenitor levels were assessed before and 24 hours after the last injection.

*significant difference over control, $p < 0.05$ (B) Homing of wild type BM cells into irradiated control (f/f), Mx-V CP^{lo}, and T2-V recipients. CFU-C levels were assessed in BM (left panel) and PB (right panel) 20 hours after injection of the BM cells (20×10^6 cells/recipient).

*significant difference over control, $p < 0.05$, ‡ significant difference between Mx-V CP^{lo} and T2-V mice, $p < 0.05$. (C) Homing of the VCAM-1 control (f/f) or VCAM-1 deficient (T2-V)

bone marrow cells into normal recipients. CFU-C levels in the BM and PB were assessed 20 hours after injection of the BM cells. (D) Transplantation of normal BM cells (5×10^5 cells/recipient) into control and T2-V recipients. Levels of circulating progenitor cells were assessed 6 and 10 weeks after transplantation. *significant difference over control, $p < 0.05$. Number of mice in each group is indicated within the corresponding bar.