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Rapid Communication

HIV-1 determinants of thrombocytopenia at the stage of CD34+ progenitor cell differentiation in vivo lie in the viral envelope gp120 V3 loop region

Menghua Zhang [a], Stella Evans [a], Jinyun Yuan [a], Lee Ratner [b,c,d], Prasad S. Koka [a,*]

[a] Laboratory of Stem Cell Biology, Divisions of Immunology, Cell and Molecular Biology, Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, California 92121, USA
[b] Department of Medicine, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, Missouri 63110, USA
[c] Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, Missouri 63110, USA
[d] Department of Pathology and Immunology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, Missouri 63110, USA

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ABSTRACT

HIV-1 V3 loop clones of virus isolates derived from patients suffering from thrombocytopenia were used for infection of the human thymus/liver conjoint hematopoietic organ that developed in the severe combined immunodeficient mouse (SCID-hu Thy/Liv). The V3 loop clones showed a significantly greater degree of inhibition of megakaryopoiesis than myelopoiesis and erythropoiesis of the human CD34+ progenitor cells, in vivo. Inhibition of megakaryopoiesis occurs through reduction in c-Mpl expression and consequent decrease in STAT5 activation. Therefore HIV-1 V3 loop sequences of thrombocytopenic patients exhibit preferential inhibition of megakaryocyte lineage-specific differentiation of CD34+ progenitor cells, thus reflecting the patients' clinical condition.

HIV infected patients often suffer from multiple hematopoietic abnormalities which include anemia, thrombocytopenia, lymphocytopenia, monocytopenia, neutropenia, and myelodysplastic/hyperplastic alterations of the bone marrow microenvironment (Mir et al., 1989; Ratner 1989; Sun et al., 1989). Cytopenias are also induced by continued AZT treatment or HAART of HIV infected patients (Miles et al., 1991a,b), with thrombocytopenia being more persistent than the other cytopenias (Mir et al., 1989). Thrombocytopenia can present a greater risk for human immunodeficiency virus (HIV) infected individuals receiving HAART and who also require cardiovascular surgery (Utley 1990).

Thrombocytopenia is a major risk factor for morbidity and mortality due to severe bleeding and it is a common occurrence among patients with HIV infection. The factors that contribute to insufficient platelet production include the circulating thrombopoietin (Tpo) levels and Tpo receptor (c-Mpl) expression (Espanol et al., 1999; Li et al., 2000; Moliterno et al., 2004). At the early stages of megakaryocyte development, megakaryopoiesis of CD34+ progenitor stem cells does occur (Koka et al., 2004). At the advanced stages of platelet development, the major causes of thrombocytopenia include accelerated peripheral platelet destruction by anti-platelet antibodies and insufficient production of platelets from the mature megakaryocytes (Espanol et al., 1999; Li et al., 2000; Moliterno et al., 2004). Interactions between the Tpo receptor, c-Mpl, and its ligand Tpo play a major role in megakaryocyte development (Kirito and Kaushansky, 2006). It has been reported by us and others that there are functional defects in human multi-lineage hematopoiesis including megakaryopoiesis, in HIV-1 infection in vivo in the SCID-hu model system (Koka et al., 2004; Koka and Reddy, 2004), and also in the infected humans (Cole et al., 1998; Zauli et al., 1996). It was reported that in HIV infected patients suffering from thrombocytopenia, the bone marrow fails to increase megakaryopoiesis when the platelet count drops (Zauli et al., 1991). This is in concurrence with our previous results that HIV-1 infection inhibits multi-lineage hematopoiesis including megakaryopoiesis (Koka et al., 1998, 2004), since de novo megakaryocyte production fails to occur.

Results and discussion

The HIV-1 V3 loop is a variable region that is contained in the gp120 envelope protein of HIV-1. The V3 loop sequences of thrombocytopenic patient HIV-1 isolates have been cloned into the corresponding region of the wild type NL4-3 strain of the virus. We have investigated the potential presence of determinants in the V3 loop sequences of these thrombocytopenic patients that will correlate with a preferential megakaryocytic lineage-specific inhibition. Such inhibition was assessed by a decreased megakaryopoiesis (colony formation) of the

* Corresponding author. Laboratory of Stem Cell Biology, Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, CA 92121, USA. Fax: +1 858 597 3804.
E-mail address: pkoka@tpims.org (P.S. Koka).

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CD34+ progenitor cells derived from the virus infected SCID-hu Thy/Liv implants in Megacult-C. The colonies formed were compared to those formed by CD34+ cells derived from mock infected implants. The numbers of megakaryocytoid colonies formed in Megacult-C were also compared with the myeloid and erythroid colonies of the CD34+ progenitor cells that were formed in methylcellulose. The SCID-hu model enables the evaluation of the consequences of HIV-1 infection and the viral V3 loop determinants on megakaryopoiesis, in the absence of other confounding factors potentially present in humans. Such megakaryopoiesis of the CD34+ hematopoietic progenitor stem cells is in the early stages of megakaryocyte development when c-Mpl but not the lineage phenotypic marker CD41 (Zauli et al., 1996) is expressed. Expression of the late stage megakaryocyte marker CD41 can make the differentiated CD34+ cells, susceptible to HIV-1 infection (Voulgaropoulou et al., 2000). In humans, the late stages of megakaryocyte development can cause platelet formation by the anucleation of mature megakaryocytes. Also, since the CD34+ cells are known to be resistant to HIV-1 infection, in vitro studies are not possible as they do not reproduce the cell–cell contact or interactions between CD34+ and CD3+ cells that occur in vivo. Thus the SCID-hu model is ideally suited to determine the role of HIV-1 V3 loop sequences (Table 1) in the inhibition of lineage-specific hematopoiesis of the immature CD34+ progenitor stem cells that are relevant to a particular cytopenia.

Previously we found that HIV-1 isolates (PT3MO and PT8MO) from pediatric patients suffering from severe hematopoietic abnormalities inhibited myeloid and erythroid colony formation. This happened despite the fact that thymocytes were not depleted, even at 6 weeks post-infection of SCID-hu animals (Koka et al., 1998). Hence it was inferred that these isolates have a preferential "tropism" towards non-T lineage cells, the precursors of myeloid and erythroid lineages. We did not at that time perform studies on megakaryocytoid colony formation. Herein we have used the HIV-1 V3 loop clones constructed from virus isolates derived from bone marrow of thrombocytopenic patients to infect the SCID-hu animals (Koka et al., 1998, 2004; Voulgaropoulou et al., 1999), to determine the role of V3 loop in lineage-specific inhibition of hematopoiesis including megakaryopoiesis.

CD34+ cells were isolated from the infected implants 3 weeks post-infection since we have previously reported that inhibition of colony forming units (CFU) occurs by 3 weeks post-infection and precedes thymocyte depletion which occurs beyond 4.5 weeks post-infection (Koka et al., 1998, 2004). This also preserves the infected implants from shrinking in size as the infection advances which makes it difficult to isolate sufficient numbers of CD34+ cells for analysis. Further, differentiation of the immature CD34+ progenitors begins shortly after 3 weeks post-infection (Koka et al., 2004). We have herein used the HIV-1 V3 loop clones derived from patients with the clinical condition of thrombocytopenia, to determine the role of these V3 loops in vivo, using the SCID-hu Thy/Liv model.

These V3 loop sequences are generally considered to be of subtype B HIV-1 isolates derived from patients in the United States (Table 1) (Jensen et al., 2006). For the V3 loop sequences, genotype predictors have been developed based on position-specific scoring matrices (PSSM) (Jensen et al., 2003). This methodology has also been applied to subtype C V3 loop sequences (C-PSSM) (Jensen et al., 2006). The peptide sequences of the V3 loops of HIV-1 isolates derived from patients suffering from different cytopenic conditions were determined (20). We have used the PSSM methodologies to confirm the co-receptor (CCR5 or CXCR4) usage of these V3 loop sequences (Goodenow and Collman, 2006; Jensen et al., 2006; Lathey et al., 2000). We have determined if the co-receptor usage has relevance to thrombocytopenia, for example, whether R5 viruses preferentially cause or are more severe on inhibition of megakaryocytoid colony formation. Our results have suggested that inhibition of lineage-specific megakaryopoiesis or multi-lineage hematopoiesis is not necessarily dependent on the co-receptor usage (or viral tropism) but more so, on the patient's clinical cytopenic condition (Table 3 and Figs. 1A and B). We report here that the HIV-1 V3 loop clones derived from patients suffering from thrombocytopenia exhibit a preferential megakaryocytic lineage-specific inhibition (Table 1 and Fig. 1B). This has enabled correlation of the role of viral envelope gene V3 loop sequences to the cytopenic condition of the patients. Viruses utilizing CXCR4 are generally considered to be more aggressive in replicative capacity than those using CCR5. We have ensured that the viral loads or replicative capacity of these different HIV-1 strains and clones are similar (Table 2) with comparable infectivity of the SCID-hu implants. We have maintained the replicative capacity to be similar between the different HIV-1 strains and clones by injecting the same amount of infectious units (100 IU) or p24 titer (50 ng) into the SCID-hu Thy/Liv implants (Table 2). The V3 loop clones of CXCR4 co-receptor usage caused inhibition of multi-lineage CFU (Table 3 and Fig. 1A), whereas those of CCR5 usage were more severe on inhibition of megakaryopoiesis (Table 3 and Fig. 1B).

We have thus established a correlation between the nature of the V3 loop of the virus and lineage-specific inhibition of CFU of the CD34+ cells derived from Thy/Liv implants. This enables our understanding the virus's role in causing a particular type of cytopenia. The V3 loop clone of an anemic and thrombocytopenic patient (A/BM9) with CXCR4 co-receptor usage inhibited multi-lineage differentiation of CD34+ cells (Fig. 1A). All the other clones that were used (B/BM4, B/BM8, C/BM6 and C/BM11) were exclusively of CCR5 usage as deduced from the PSSM (Table 3) and were derived from patients suffering from thrombocytopenia.

It has been suggested that HIV co-receptor usage and the viral target cell tropism differ from each other in understanding the mechanisms of pathogenesis (Goodenow and Collman, 2006). Further it was proposed that co-receptor usage was more predictive than NSI/SI phenotype and the latter might be insufficient to predict HIV replication in macrophages (Lathey et al., 2000). Hence the phenotype predictor based on envelope V3 loop sequences that was developed (Jensen et al., 2006), was used in our studies. We were thus able to show that CCR5 usage was primarily but not exclusively associated with the V3 loops of HIV-1 isolates derived from patients suffering from thrombocytopenia. Even though all the three different types of colonies that were studied had decreased due to the CXCR4 and CCR5 tropic V3 loops, the megakaryoid colonies showed an even greater decrease due to the CCR5 tropic V3 loop clones. The V3 loop clone, A/BM9, is constructed from the sequences of an isolate from a

Table 1
Alignment of the amino acid sequences of the V3 loops of the clones and control strains (JR-CSF and NL4-3) of HIV-1 (Chavda et al., 1994; Voulgaropoulou et al., 1999). The asterisks are used to maintain the alignment of the presence of additional or the absence of amino acids.

JR-CSF	C	T	R	P	S	N	N	T	R	K	S	I	H	I	*	*	*	G	P	G	R	A	F	Y	T	T	G	E	I	I	G	D	I	R	Q	A	H	C
NL4-3	C	T	R	P	N	N	N	T	R	K	S	I	R	I	Q	R	*	G	P	G	R	A	F	V	T	I	G	K	I	G	N	M	*	R	Q	A	H	C
A/BM9	C	T	R	P	N	N	N	T	S	K	R	I	S	I	*	*	*	G	P	G	R	A	F	Y	A	S	R	R	I	T	G	D	I	R	Q	A	H	C
B/BM1	C	T	R	P	N	N	N	T	R	K	S	I	S	T	*	*	*	G	P	G	R	A	L	Y	A	T	G	E	I	I	G	D	I	R	Q	A	H	C
B/BM4	C	T	R	P	N	N	N	T	R	K	S	I	H	T	*	*	*	G	P	G	R	A	L	Y	A	T	G	E	I	I	G	D	I	R	Q	A	H	C
B/BM8	C	T	R	P	N	N	N	T	R	K	S	I	R	T	G	H	I	G	P	G	R	T	L	Y	A	T	G	G	I	I	G	D	I	R	Q	A	H	C
C/BM6	C	T	R	P	N	N	N	T	R	K	S	I	S	I	*	*	*	G	P	G	R	A	F	Y	A	T	G	E	I	I	G	D	I	R	Q	A	H	C
C/BM11	C	T	R	P	N	N	N	T	R	K	S	I	S	I	*	*	*	G	P	G	R	A	F	Y	A	T	G	D	I	I	G	D	I	R	Q	A	H	C