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ABSTRACT

Friend Virus is a murine retrovirus that causes erythroleukemia in adult mice. Friend Virus has two major forms, Friend Virus – Polycythemia (FV-P) and Friend Virus – Anemia (FV-A). Although both forms of the virus lead to erythroleukemia in mice, FV-P leads to polycythemia, while FV-A leads to anemia. The cells that are infected by the virus are part of the stress erythropoiesis pathway. Friend Virus induces the expansion of stress erythroid progenitors that become the targets for the virus. When early “stem cell like” progenitors are infected they proliferate and form self-renewing leukemia stem cells (LSCs), while infection of late stage progenitors lead to differentiation. Previous analysis of Friend Virus targets characterized mice infected with FV-P. This study examines FV-A and how it affects the stress erythropoietic progenitor cells. My data show that FV-A LSCs have a more limited self-renewal ability than FV-P LSCs.
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Chapter 1

Introduction

In adult mice, Friend Virus causes spleen enlargement and acute erythroleukemia because of how it interacts with the stress erythropoiesis pathway (1). Friend Virus is a complex of two viruses, the spleen focus forming virus (SFFV) and the Friend murine leukemia virus (F-MuLV). SFFV is the pathological component and responsible for the enlarged spleen and the development of erythroleukemia, SFFV is replication defective so F-MuLV provides helper proteins for SFFV replication (2, 3). The stress erythropoiesis pathway is activated in times of hypoxic stress such as anemia (4, 5). It can be split into two stages, an early stage where “stem cell like” progenitors rapidly proliferate and late stage where progenitors differentiate as shown in Figure 1 (5). These progenitor cells are found in the spleen rather than the bone marrow as a way to bypass the traditional erythropoietic pathway.
There are several loci in the mouse genome that affect how Friend Virus infects erythroid progenitor cells (6, 7). *Fv1* inhibits the replication of Friend Virus by interacting with viral capsid proteins leading to an inhibition of infection. *Fv3* encodes cytidine deaminase, which again inhibits viral infection. *Fv4* is an endogenous retroviral envelop protein that blocks the interaction of FV with its receptor. Another set of genes is required for the development of progenitor cells, *W, Sl, f*, and *Fv2* (2). These genes encode essential factors of the BMP4-dependent stress erythropoiesis pathway like the Kit receptor coded by *W*, the Kit receptor’s
ligand SCF coded by $Sl$, and Smad5 coded by $f$ (8). Kit is one of four major markers used to show the maturity of the progenitor cells (Figure 2).

Figure 2 – Erythropoiesis maturity indicators and in vitro culture system: In a culture of HH+BMP4+GDF15+SCF either with, or without, Epo at 1% O$_2$ cell development is shown. Cell membrane indicators of maturity are listed under each stage of development. Early progenitors convert from CD34+CD133+Kit+Sca1+ to CD34-CD133+Kit+Sca1+ “stem cell like” progenitor cells. These cells then grow to CD34-CD133-Kit+Sca1+ to begin differentiation and become mature with a CD34-CD133-Kit+Sca1- indication.

$Fv2$ encodes for the stem cell derived tyrosine kinase receptor (Stk), which also encodes a naturally occurring truncated form referred to as short form Stk (Sf-Stk) (9). Expression of Sf-stk creates susceptibility to Friend Virus. Sf-Stk is activated by gp55, the retroviral envelope glycoprotein of the SFFV component of Friend Virus. Between FV-P and FV-A, gp55 differs at a critical amino acid, L238, which is important because it affects the binding to the erythropoietin receptor (EpoR) (10, 11). As shown in Figure 1 and Figure 2 Epo is a crucial transition signal for red blood cell production and the interaction between gp55 and EpoR is critical in that process. Strong binding activated by gp55-P with EpoR induces differentiation of cells into both BFU-Es and CFU-FVs, while FV-A binding between gp55-A and EpoR leads only to CFU-FV formation. Figure 3 shows how, when infected by Friend Virus, progenitor
cells split their development into either leukemia cells or differentiating erythroid progenitors based on being Sca1+ or Sca1- (12).

**Figure 3 – Model for the development of Friend Virus induced leukemia:**

(3A) During infection stress erythropoiesis progenitor cells can be differentiated into four groups based on m34, Kit, Sca1 and Spi1 insertion indicators. The red dots show cells without a Spi1 insertion that can differentiate and expand as m34+Kit+Sca1- mature cells/BFU-Es. Blue dots indicate infected m34+Kit+Sca1+ cells that cannot progress and also do not have a Spi1 insertion. Black cells develop into LSCs, which develop late in the infection and are m34+Kit+Sca1+ with a proviral insertion into Spi1 p53+/+. The white cells develop a p53 mutation causing them to not act as LSCs, but as CFU-FV leukemia cells, which are m34+Kit+Sca1+ with a proviral insertion into Spi1 p53-/-.

(3B) The process of infected cells becoming either BFU-E or Leukemia cells is shown as cells rapidly proliferate and produce transformed leukemia cells and Epoind BFU-Es through p53 -/- mutated cells with Spi1 activation. A major indicator that divides the infected stress progenitors into two distinct populations of Sca1-, which leads to Epoind BFU-E or Sca1+, which leads to leukemia cells.
Friend Virus infection with FV-P induces disease in stress erythroid progenitors (13). The primary pathologies associated with infection include the development of polycythemia and the progression to leukemia. These events are not linked in that the development of polycythemia results from the infection of late stage stress erythropoiesis progenitors that differentiate upon infection, while leukemia develops from the infection of early “stem cell like” stress erythropoiesis progenitors (3). These cells have intrinsic self-renewal properties, which allow them to become leukemia stem cells after infection (5, 14). In contrast to FV-P, FV-A induces a different disease. Mice develop anemia following infection and the development of leukemia is much slower (15, 16).

In this study I examined the development of leukemia stem cells in FV-A infected mice. Previous work showed in mice infected with FV-P, early “stem cell like” stress erythroid progenitors form LSCs. These cells are characterized by the expression of CD34, CD133, Kit, and Sca1 (5). We examined FV-A infected cells in culture in order to see how FV-A LSCs survive and self-renew when re-cultured. A similar experiment has already been done with FV-P and the results of this study allow further differentiation in how FV-A and FV-P behave. My data show that FV-A LSCs have more limited self-renewal than FV-P. It is my hypothesis that this difference is due to the difference in gp55 proteins between FV-P and FV-A and as a result, the Sf-Stk signaling pathway. The limited renewal of FV-A infected cells in both Epo and non-Epo environments show a significant difference between the FV strains.
Chapter 2
Materials and Methods

DBA and BALB-c mice were used in this experiment. The mice were infected through retro-orbital injection of FV-A virus. The infection period within the mice was two weeks before the mice were euthanized and the spleen was isolated. A small portion of the spleen was mashed in 1mL of PBS and the liquid was mixed with 5mL of red blood cell lysis buffer for 5 minutes, then with 5mL of PBS to neutralize the RBC lysis buffer. Cells were spun down to a pellet and resuspended in 1mL of PBS and the cells were counted using a hemocytometer and trypan blue.

After counting the cells, 100,000 cells were plated per well of a 6 well plate. Each well of 4 mL of methocult was supplemented with 15ng/mL BMP4, 50ng/mL SCF, 25ng/mL Shh, 30ng/mL GDF15, 50ng/mL IL3 and 1% penicillin-streptomycin were added as well as 3units/mL of Epo when the media needed to be Epo+. These plated cells were then in culture for 10 days before another round of LSC colony counting and re-plating. In order to count the cells the plates were washed with three sets of 3mL of PBS per well, centrifuge to pellet the cells and re-suspended in PBS to count the cells as described above. The cells were then re-plated for another 10 days of culture before re-plating again until the culture no longer self-renewed. Graphs were made using the average of data taken from the mice.

During the re-plating process cells were analyzed by for flow cytometry. Between two mice two separate flow tube were filled with 100μL of cells from each mouse, one with 50μL /50μL from each mouse. The cells were stained with antibodies anti-Kit – Alexa647, anti-Sca1 – PeCy7, anti-CD34 – FITC, and anti-CD133 – PE. The cells were analyzed on the Accuri flow cytometer and the data were analyzed using FloJo software.
Chapter 3

Results

In order to study how FV-A infects the stress erythroid progenitors during the development of erythroleukemia in DBA and BALB-c mice, mice were infected with FV-A and two weeks later spleen cells were harvested. The development of erythroleukemia was observed by the development of LSC colonies in methocult media containing BMP4, SCF, Shh, GDF15, IL3, Epo and antibiotics (12). The first question addressed was how long it takes for LSCs to develop in the culture. The data in Figure 4 show a timeline of colony formation after culturing of cells. After a week, few LSCs had formed, but as time progressed, each day there were more colonies until the ideal number of colonies for this experiment was reached on Day 10.

From the data in Figure 4, 10 days was determined to be the consistent period of time to culture the cells for the rest of the experiment. This time of culture ensured that significant colonies would form allowing for the analysis of LSC self-renewal.
The next question addressed was the ability for FV-A infected cultures to self renew. With a culture time of 10 days, LSC colonies were counted during primary, secondary and tertiary platings in media containing BMP4, SCF, Shh, GDF15, IL3, Epo and antibiotics growth factors. The data shown in Figure 5 show that after the first 10 days there were too many colonies to count. On subsequent platings, there was a significant drop in number of LSCs occurs upon the secondary culture and finally the cells were unable to self-renew in the tertiary culture.

Figure 5 – Serial re-plating of FV-A infected spleen cells: Spleen cells from FV-A infected DBA mice harvested 2 weeks after infection were plated in methylcellulose media containing BMP4, SCF, Shh, GDF15, IL3, Epo and antibiotics growth factors for 10 days. Colonies were counted, and then cells were re-suspended and re-plated in same media. This procedure was replicated until no colonies were generated.

*TMTC – Too Many To Count

This observation was significantly different than that observed with LSCs cultured from mice infected with FV-P. In that case the cells self-renewed for over 6 weeks. The FV-A infected cell’s inability to self-renew in the tertiary culture led to questions on how FV-A LSCs differed from FV-P. For this reason, the culture was changed to further investigate Epo’s role in cell renewal and growth because of the difference in gp55 between FV-A and FV-P and how it interacts with EpoR.
To determine the role that gp55 plays, the culture was changed so that one was with Epo and the other was without Epo. The comparison of LSC colonies formed in presence or absence of Epo is shown in Figure 6. Two replicate experiments were done. In the data shown in Figure 6A Epo appears to increase LSC colony formation, but in subsequent experiments that affect was not observed. This result is not surprising in that Epo drives differentiation, which would inhibit self-renewal.

**Figure 6 - Cultures with Epo compared against cultures without Epo:**

(6A) After the first round of culturing cells from BALB-c mice an average of 29.835 LSC colonies resulted in an Epo+ environment and 12 from an Epo- environment. No colonies were present with or without Epo when re-cultured for another 10 days. (6B) Another round of BALB-c mice infected with FV-A were cultured to test for self-renewal. After the initial 10 days of culture 42.835 colonies were seen in an Epo+ environment, while 44.665 were seen in an Epo- environment. These numbers went down to 20.665 and 20 respectively upon self-renewal and then were unable to renew upon the third round of re-culturing.
We next examined whether FV-A induced LSCs expressed the same cell surface markers as FV-P LSCs. These experiments addressed the question of whether the difference in self-renewal was caused by a different target population. Flow cytometry shows the maturity of the cells within the cultures and the abundance of different cell populations present. Maturity is measured based on the presence of CD34, CD133, Kit, and Sca1 on the surface of the cells. The data in Figure 7 shows what cells are present in vivo from the spleen of the mouse after infected with FV-A for 14 days.

One difference between FV-P and FV-A is the percentage of Kit^+Sca1^+. In contrast, FV-A infection leads to only 3-4% Kit^+Sca1^+ cells. This observation is consistent with our in vitro
data. We next examine the cell populations generated by in vitro culture for 10 days. The percentage of Kit+Sca1+ cells increases significantly (3-4% to 15-23% Epo- and 17% Epo+). These data show that Kit+Sca1+ LSCs do expand in culture, but they do so less than FV-P infected cells (12). We next used CD34 and CD133 to further characterize the cells. Kit+Sca1+ cells that are CD34+CD133+ are more immature while CD34-CD133- are most mature and do not represent LSCs. At day 10, the difference between a population of cells in its first culture is different than during its secondary culture at Day 20 and these flow diagrams show this difference.
Figure 8 – Flow cytometry diagrams of primary and secondary cultures with and without Epo: Two BALB-c mice infected with FV-A were put into HH media with and without Epo to determine the difference in cell population during cell growth and self-renewal. (8A-C) Shows the cell population after 10 days of culture and is measured against (8A) Kit and Sca1, (8B) CD34 and CD133 on a Kit+Sca1+ Gate, and (8C) CD34 and CD133 on a Kit+Sca1- Gate. (8D-F) The same cultures were renewed to create the Day 20 cultures and once again measured using flow cytometry with the same parameters.

Majority of cultured cells were CD34-CD133- suggesting that the LSC were losing potential (Figure 8B). Kit+Sca1- cells are Epo independent BFU-E in FV-P infections, these cells are also present in these cultures.

When we look at Day 20 culture, we observe that the Kit+Sca1+ population further decreased (Figure 8D). When we gated on the Kit+Sca1+ population we observed that most cells were CD34+CD133-, which represents an aberrant cell population of unknown function.
Similarly, when we examined Kit+Sca1- cells the CD34+CD133- population predominated (Figure 8D, E, and F).
Chapter 4
Discussion

This study found that FV-A developed LSCs at a slower rate than FV-P and has limited self-renewal abilities compared to FV-P infections. The data from Figure 4 show the pace of colony formation and that it takes 10 days for the LSCs to self-renew, while the data from Figure 4, Figure 5, and Figure 6 show that colonies do not renew past a secondary culture. The way Epo affects the infection is highlighted by the data in Figure 6, which showed the Epo signaling was not the difference between FV-A and FV-P LSCs. Epo presence does not increase differentiation in cells. These results are backed up by flow cytometry shown in Figure 7 and Figure 8, which show the same LSC markers.

I conclude from this information that the difference between the Gp55-A viral protein in FV-A and Gp55-P in FV-P is most likely the primary cause of the slow LSC growth and limited self-renewal (10, 11). Gp55-P activates Sf-stk, which is necessary for FV pathogenesis, but Gp55-A does not. Without strong activation, slower LSC expansion like what is seen in this experiment would be expected. This is because FV-A is unable to expand early “stem cell like” progenitors. Another way the difference in Gp55 proteins affects the development of the cell population is its interaction with EpoR. Analysis of FV-A infected cells in both an Epo+ and Epo- media as I did in the experiment displayed by Figure 6 shows the strength of reaction between Gp55-A and EpoR. A strong interaction with EpoR drives differentiation of progenitor cells, not LSCs. The data in Figure 6 show that an Epo+ or an Epo- environment have similar LSC counts. This similarity indicates a weak Gp55-A interaction with EpoR rather than what would be a strong Gp55-P interaction. Gp55-A interacting with EpoR in this way supports the idea that Sf-stk was not strongly activated by Gp55-A either.
By looking at the flow cytometry diagrams in Figure 8 the development of stress erythropoiesis cells can be determined. In vivo, before culture (Figure 7) shows the spleen cell population within the BALB-c mice infected with FV-A. These mice exhibited fewer Kit+Sca1+ cells than FV-P infected mice suggesting that these differences between FV-A and FV-P are already apparent at Day 14 post infection (12). Figure 8 offers comparison between the cell populations with Epo, without Epo, and how each of these media affect renewal in the secondary culture. We observed that the longer cells are cultured FV-A LSCs lose markers associated with LSCs. They develop an aberrant population that is CD34+CD133-. These data support the idea that FV-A induced signals are different than FV-P induced signals, which leads to slower leukemogenesis.

In conclusion, this study shows that FV-A infection slows LSC expansion compared to FV-P and that FV-A induced LSCs have limited self-renewal ability compared to FV-P, which can self-renew indefinitely. It is my belief that these differences in FV-A infection are caused by the difference in the FV-A Gp55 protein. Gp55-A does not activate Sf-stk as robustly as FV-P, which is required for FV pathogenesis and weakly binds to EpoR, an interaction that does not lead to differentiation. The next step in studying FV-A and how it interacts with the stress erythropoiesis pathway is to show the specific differences between the FV-A Gp55 pathway and the FV-P Gp55 pathway.
BIBLIOGRAPHY

Education
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