Do CD40L and CD40 Contribute to Sickle Cell Anemia?

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ABSTRACT
Sybil A. Yeboah: Do CD40L and CD40 contribute to Sickle Cell Anemia?
(Under the direction of Leslie V. Parise, PhD)

CD40 ligand (CD40L) is an important inflammatory mediator upregulated in inflammatory conditions such as atherosclerosis and sickle cell anemia (SCA). CD40L is expressed on platelets, B cells and epithelial cells, with platelets being its main source. Interaction with its receptor, CD40, has been shown to upregulate adhesion molecule expression, stimulate release of inflammatory molecules and initiate coagulation. These manifestations are also observed in SCA. Previously, our lab reported that the concentration of soluble CD40L ligand in sickle cell patient plasma is 30-fold higher than plasma of normal individuals. Based on these observations, we hypothesize that CD40L and CD40 contribute to SCA by increasing inflammation and blood cell adhesion to the endothelium. To test this hypothesis, we will block the CD40L:CD40 interaction a mouse model of SCA using an anti-CD40L antibody and analyze the effects on lung, liver, spleen and kidney pathology. Furthermore, we will also determine whether this blockade decreases expression of inflammatory mediators such as IL 8 and CD40L. These studies will help to further understand the role of the CD40L:CD40 interaction in SCA, and may eventually help to enhance the overall health of SCA individuals.

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CHAPTER I

HYPOTHESIS AND SPECIFIC AIMS

Sickle cell anemia (SCA) is a genetic disease that mainly affects people of African decent. It is caused by a point mutation in the sixth codon of beta globin, where glutamic acid is replaced with valine. This mutation causes polymerization of hemoglobin in red blood cells, causing them to become sickle shaped. SCA patients suffer from anemia and chronic inflammation. They normally have high leukocyte counts, indicative of inflammation, and mostly have activated platelets that express molecules, which initiate procoagulant activity in the disease. CD40L, a protein found in abundance in the plasma of SCA individuals, is a transmembrane protein expressed in B cells, dendritic cells, monocytes, thymic epithelial cells and platelets. Its receptor, CD40, is an integral membrane protein, and a member of the tumor necrosis factor receptor (TNFR) family. CD40 is expressed as a homotrimer on B cells, monocytes/macrophages, dendritic cells, platelets and epithelial cells. The interaction of CD40L with its receptor CD40 is a major factor in inflammatory conditions in atherosclerosis, acute coronary syndrome (ACS), lymphocytic leukemia and diabetes. Activated platelets are the main source of CD40L. CD40L is either expressed on the platelet surface or released as a soluble form (sCD40L) in plasma. CD40L then interacts with CD40 to initiate immune and inflammatory responses. These responses include an increase in leukocyte proliferation, which correlates with inflammation, and an increase in adhesion molecules, which contributes to endothelial adhesiveness and plaque formation in diseases

such as atherosclerosis. Recent studies have shown that CD40L is ~30 fold higher in plasma concentration of patients with SCA than in unaffected individuals. Correspondingly, CD40L is significantly depleted from sickle cell platelets SCA patients suffer from endothelial and blood cells adhesion to the endothelium, which causes blood vessel blockage and pain crises. They also suffer from chronic inflammation, which is a hallmark of the SCA disease. Since the clinical manifestations of SCA are believed to correspond with the outcome of the CD40L:CD40 interaction in inflammatory diseases like atherosclerosis, **I hypothesize that CD40L and CD40 contribute to the clinical manifestations of SCA.** Preliminary studies from our lab using a mouse model of SCA, demonstrated that depletion of CD40L decreased liver, lung, spleen and kidney pathology in SCA. Since this interesting observation was performed only once, my goal is to confirm these results and to elucidate the pathologic role of CD40L in SCA.

Specific Aim 1

To determine whether blocking CD40L alters the pathologic features of SCA in mice.

In order to generate enough SCA for experimental purposes, I will perform fetal liver transplants from Berkeley SCA mice into lethally irradiated wild type mice. These SCA-generated mice will express endogenous tissue CD40L and exogenous CD40L in their blood cells from the fetal liver transplant. These transplanted SCA mice will be treated with either a control or anti-CD40L antibody. Organ damage (lung, kidney and liver) and hematological parameters (red blood cell, reticulocyte, leukocyte count, etc) will be assessed to determine whether CD40L affects SCA pathology. When mice treated with anti CD40L antibody are compared to mice treated with a control antibody, I expect to see decreased spleen size,

decreased lung, kidney and liver pathology and lower leukocyte count, all of which will be indicative of decreased inflammation in these mice. Furthermore, I expect a decrease in inflammatory molecules such as CD40L, IL-1, and IL-8.

Specific Aim 2

To determine whether CD40 expressed in tissue or blood cells contributes to SCA pathology.

The role of the CD40L - CD40 receptor interaction in SCA will be assessed in two main parts:

A. To determine whether blood cell CD40 or tissue CD40 contributes to SCA pathology, SCA fetal liver cells from SCA Berkley mice will be transplanted into lethally irradiated CD40 knock-out mice. These mice will lack endogenous CD40 in their tissues but will express CD40 on their blood cells from the transplantation. As a control, SCA fetal liver cells will be transplanted into wild type mice. Organ damage and hematologic parameters will be assessed for degree of SCA pathology as described in Aim 1. If SCA pathology remains the same in SCA-transplanted CD40 knock-out mice as compared to SCA-transplanted control mice, then I will conclude that CD40 in the tissues does not contribute to SCA pathology. However, if SCA pathology is reduced, I expect to see decreased spleen size, decreased lung, kidney and liver pathology, lower leukocyte and reticulocyte counts in our hematologic parameters and decreased levels of inflammatory molecules. Therefore, I will conclude that expression of CD40 in the tissues contribute to SCA pathology. This possibility will be further tested in the second part of my aim.

B. To determine whether CD40 expressed on the introduced blood cells contribute to SCA pathology, SCA fetal liver cells will be transplanted into CD40 knockout mice, followed by treatment with either an anti-CD40 antibody or a control antibody. These mice will lack endogenous CD40 in their tissues but have CD40 in their blood cells from the transplantation. As described above, organ damage and hematologic parameters will also be assessed. If SCA pathology in SCA-transplanted mice treated with an anti-CD40 antibody is similar to mice treated with a control antibody, then I will conclude that CD40 from the blood cells does not contribute to SCA pathology. However, if the pathology is reduced in the anti-CD40-treated mice, I can conclude that CD40 from the blood cells contributes to SCA pathology.

These two experiments will help me determine which source of CD40 – blood cells or tissues, contributes to SCA pathology.

Background and significance

Sickle cell anemia, the first known genetic disorder to be discovered on a molecular level, was identified in a Ghanaian family in 1670(1, 2). It is caused by a point mutation in codon six of the beta hemoglobin gene of red blood cells, where glutamic acid is replaced with valine (fig.1) (3). Low oxygen tension causes the hemoglobin to polymerize, causing the red cell to become sickle shaped (fig.2), more rigid and more likely to adhere to the blood vessel wall (4, 5). Hemoglobin

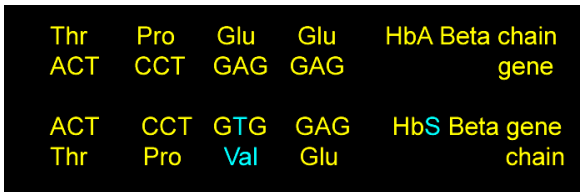

Figure 1 A point mutation at position six of beta globin gene Sickle Cell Anemia is a result of polymerization of the hemoglobin in red cells due to this mutation.

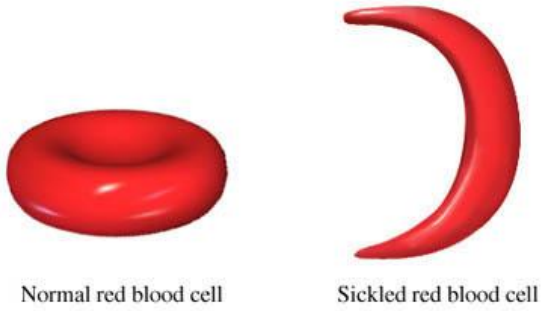

Figure 2 Normal red cell (disc shaped) and sickle shaped red cell Occurs as a result of polymerization of the hemoglobin on red cells (Copyright © 2005 Nucleus Communications, Inc. All rights reserved. www.nucleusinc.com)

is comprised of 2 alpha and 2 beta globin chains. Because sickle cell anemia is a recessive trait, an individual will have to inherit a sickle beta globin gene from each parent to get the disease (31). SCA affects 72,000 Americans (30). One out of every 600 African American is affected by sickle cell anemia (30). Sickle red blood cells are abnormally sticky and adhere to blood vessel walls resulting in the obstruction of blood flow leading to poor perfusion and tissue damage (fig.4), (6). As a result, sickle cell patients suffer from vaso-occlusion; they experience periodic, excruciating pain crises that are caused by obstruction of blood flow to various tissues (6). Other clinical characteristics are commonly found in sickle cell patients are anemia or low red cell count, lung and heart injury, and spleenic sequestration (7).

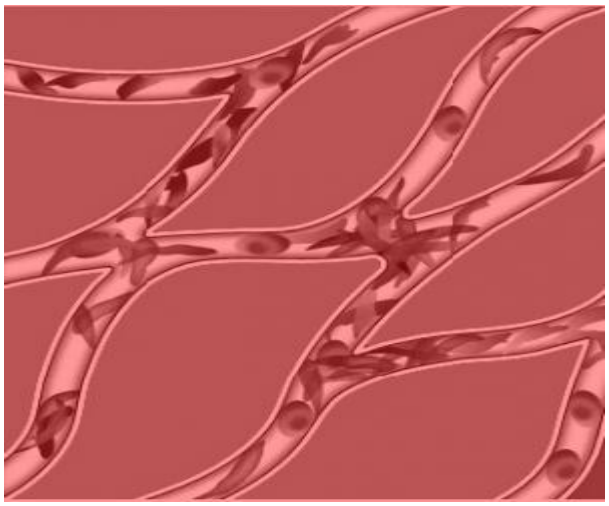

Figure 3 Blood cells adhesion to the vasculature Sickle red cells become more adhesive and stick to blood vessels to obstruct blood flow (Figure from ref 37).

Another major manifestation of SCA is chronic inflammation (8). Previous studies have shown that leukocytes are elevated in SCA and may contribute to the inflammatory state of

this disease (8). Neutrophils, monocytes and platelets also contribute to inflammation; since these cells are activated in SCA, they may also play a role in SCA inflammation (8). In addition to leukocytes, neutrophils, monocytes, platelets and inflammatory mediators such as C-reactive protein, TNF- α, IL-1 and CD40L are also elevated in SCA (9-13). Studies have also shown that CD40L is also elevated in the plasma of individuals suffering from systemic lupus erythematosus, rheumatoid arthritis and sickle cell anemia (17, 34, 35). Previous data published by our lab indicates that the average concentration of soluble CD40L (sCD40L) in SCA plasma is 30-fold higher than in normal plasma, and that platelets, which are the major storage site of CD40L, contain less than half the CD40L found in platelets from normal individuals (Fig. 4) (14). Since CD40L appears to play an important role in many inflammatory-related diseases, my long-term goal is to elucidate the pathologic role of CD40L in SCA.

CD40L

CD40L is a 39kDa type II transmembrane protein, and a member of the tumor necrosis factor (TNF) superfamily (15). CD40L exists as a homotrimer in two forms – membrane bound and soluble. The soluble form is cleaved into plasma as a result of intercellular enzymatic activity (16). Both forms may perform the same functions (16). The membrane bound form is expressed on the surface of activated platelets, leukocyte subsets, smooth muscle cells and epithelial cells (17-20). CD40L initiates many immune and pro-inflammatory responses. It is upregulated in several diseases such as rheumatoid arthritis, lung inflammation, lymphocytic leukemia and atherosclerosis and is believed to contribute to the symptoms and progression of these diseases (14, 21-22). CD40L binds to its receptor CD40 to activate it. Once activated

CD40 gets internalized into cells, which then initiates cascades that release inflammatory and adhesion molecules such as vascular adhesion molecules (VCAM) and intercellular adhesion

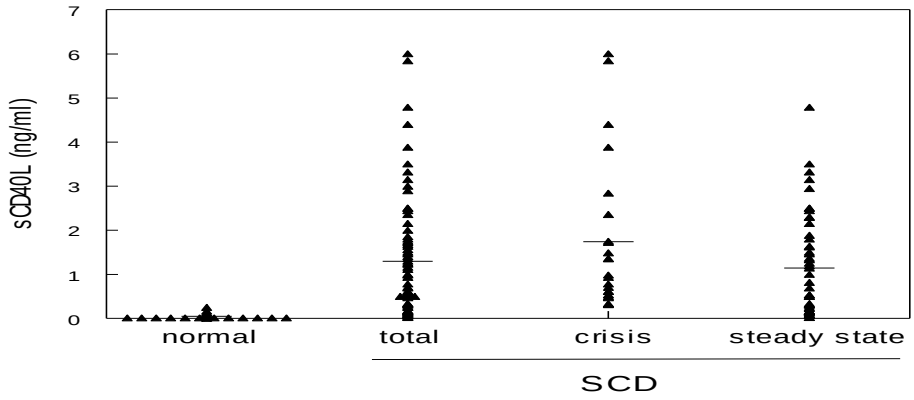

Figure 4 Soluble CD40L is elevated in SCD plasma. Quantitative ELISA results comparing sCD40L in HbAA (n=16) plasma to HbSS anemia (n=10; P=0.0002), and to total HbSS anemia (n=45; P 0.0001). HbSS crisis plasma sCD40L content (n=10) was compared with HbSS steady state (n=37; P=0.065) (Figure from ref 14).

molecules (ICAM) (fig.5) (32). Platelets are the main storage sites for CD40L (23) and release molecules CD40L upon activation. As a result of the role of CD40L in inflammatory conditions, we hypothesize that CD40L may perform the following functions in SCA:

- **<u>CD40L may elevate leukocyte counts in SCA patients (6):</u>** CD40L is known to be involved in B cell proliferation and differentiation (fig. 6, 7) (21, 23). This activity increases leukocyte counts (18). Elevated leukocytes are indicative of inflammation.

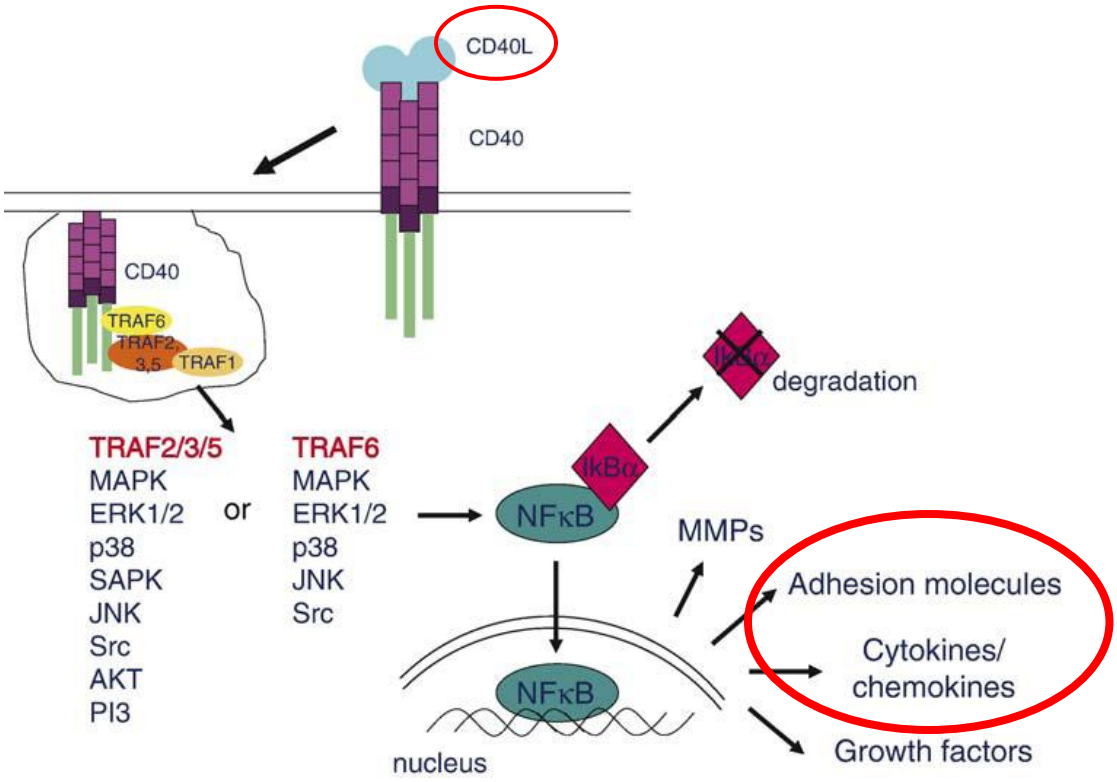

Figure 5. **Downstream signaling of CD40L:CD40 interaction** CD40L binds to CD40. Activated CD40 recruits adaptor molecules, the TRAFs. CD40 has 2 TRAF binding domains: 1 for TRAF2, 3, and 5 (a binding domain that can also bind TRAF1 after TRAF2/3/5 have bound), and 1 for TRAF6. After binding of TRAF1, 2, 3, 5, or 6, further downstream signaling is elicited. Signaling translocates nuclear factor kappa B (NFκB) into the nucleus and this results in the expression of pathways of inflammation, thrombosis proteolysis, etc (figure adapted from ref 32).

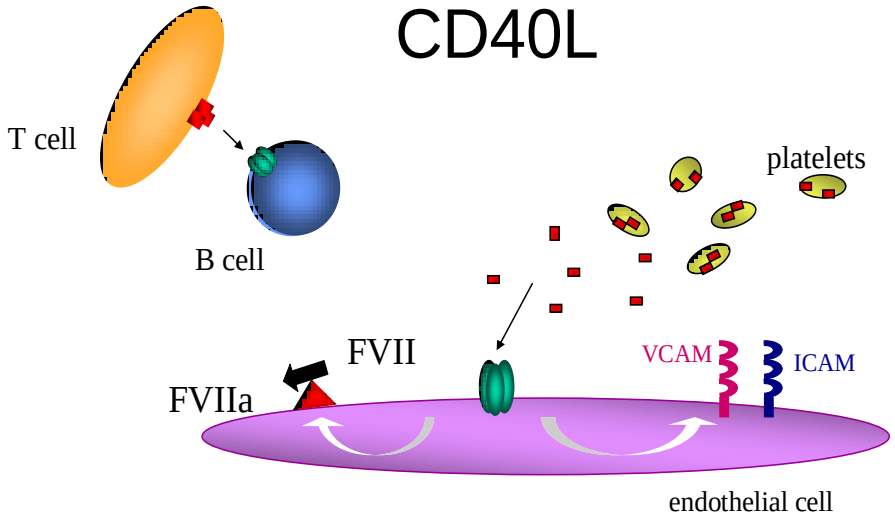

Figure 6 Functions of CD40L.CD40L induces B cell proliferation and activation, increases endothelial cell adhesion molecule expression and promotes coagulation (Lee, S. 2006).

CD40Lmay promote cell adhesion and procoagulant activity: CD40L activity increases secretion of P-selectin, E-selectin VCAM and ICAM, which are all adhesion molecules (fig 6) (14). Increased secretion of these adhesion molecules may facilitate sickle cells adhesion to the vasculature, which may increase the possibility of vaso-occlusion in SCA patients. CD40L increases the production of tissue factor (TF) which is involved in procoagulant activity, and downregulates thrombomodulin, a protein that mediates anticoagulation expression (27-29). This process may increase thromboembolic events in SCA (fig. 6).

- **CD40L may promote inflammation in SCA:** The CD40L:CD40 interaction is known to initiate cascades that increase plasma levels of cytokines - IL-1, IL-6, IL-8,

and TNF (24 – 28, 32). These cytokines that are known to increase adhesion in the vasculature therefore may contribute the pathology of SCA (24-28).

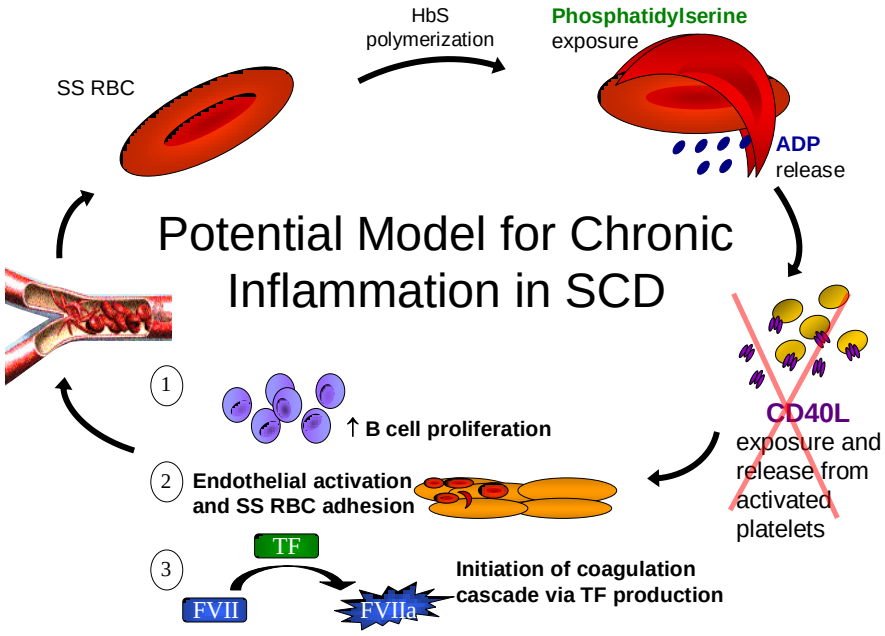

Figure 7 Model of Chronic. Chronic inflammation in SCA may be reduced by usinf an antibody to block CD40L (lee, S. 2006).

Murine studies using an anti-CD40L antibody to block CD40L activity in low density lipoprotein receptors (LDLR) knockout mice prevented progression of antheroslerotic plaques (33), suggesting that CD40L plays a significant role in the progression of cardiovascular disease. The vaso-occlusive crises SCA patients suffer from is due to sickle red cells adhering to the vascular endothelium (36). CD40L binding to its receptor increases the expression of VCAM, ICAM, P-selectin and E-selectin which are adhesion molecules that contribute the vaso-occlusion crises in SCA. Since CD40L is elevated in the plasma of SCA individuals, using an anti CD40L antibody to block its interaction with its receptor

CD40 may reduce release of VCAM, ICAM, P-selectin and E-selectin, thereby decreasing the occurrence of vaso-occlusion (fig. 7). In addition, proinflammatory cytokines - TNF α, IL-1 and IL-4 are upregulated in SCA (fig. 7). Since CD40L also increases the release of these cytokines, blocking CD40L may decrease cytokine levels in the plasma, thereby decreasing the chronic inflammation suffered by these individuals (fig. 7).

Based on these findings, I hypothesize that **CD40L and CD40 may contribute to the manifestations of SCA**. This hypothesis in will be addressed in the following specific aims: specific aim 1: **To determine whether blocking of CD40L alters the pathologic features of SCA in mice** and specific aim 2**: To determine whether CD40 expressed in tissues or blood cells contributes to SCA pathology.**

To test Aim 1, I will use Berkeley mice. These are genetically engineered mice that have human alpha and beta globin genes, with a mutation in the beta globin gene (39). Their sickle red blood cells are irreversible (39). They exhibit anemia and multi-organ pathologies, which are similar to the clinical manifestations of SCA individuals (38, 39). I will transplant Berkeley SCA fetal liver cells into normal irradiated mice.

For Aim 2, I will use CD40 knockout mice (40). These mice do not express CD40 and therefore will allow me to test the hypothesis of whether tissue or blood cell CD40 contributes to SCA pathology.

CHAPTER II

PRELIMINARY STUDIES AND RESEARCH DESIGN AND METHODS

A preliminary study using a monoclonal antibody (MR1) to block CD40L was performed by a former graduate student in our lab. Her results suggested that treating sickle cell mice with an anti-CD40L antibody improved the overall health of the SCA mice. Interestingly, liver and kidney pathology was significantly reduced, as well as spleen size (14). Additional preliminary data showed that SCA bone marrow cells transplanted into CD40 knockout mice, followed by treatment with an anti-CD40 antibody, decreased lung pathology when compared to mice treated with a control antibody. These results suggest that CD40 expressed on blood cells may contribute to SCA pathology. Since these experiments were performed once, my goal is to ensure these results are reproducible, and to further determine the contributing source of CD40 to SCA pathology. In this experiment, I will examine the pathology of the lung, liver, spleen and kidney, specifically.

To prepare for this project, I have learned mouse handling, breeding and transplantation technologies. In addition, I have performed fetal liver transplants to produce sickle mice, have treated mice with an anti platelet aggregation drug, and performed surgery on the mice to obtain their organs for viewing. Practicing these experiments has given me hands-on experience on how to handle the mice for my study. Currently I am in the process of purifying the (MR1) anti CD40L antibody which will be needed for my experiments.

Research Design and Methods

Specific Aim 1: To determine whether blocking of CD40L alters the pathologic features of SCA in mice.

Rationale: The interaction of CD40L with CD40 contributes to atherosclerosis, chronic lymphocytic leukemia and other inflammatory diseases (21, 22). It also promotes prothrombotic and procoagulant activity. Since our preliminary data demonstrated that sCD40L is increased in SCA plasma, it may contribute to these characteristics in SCA. Therefore, our goal is to determine if CD40L contributes to the pathology of the lung, kidney and liver of SCA individuals.

Experimental design: The Berkeley mouse model of SCA will be used in my study. Because of the decreased survival of these mice, I will generate enough mice for the experiments by irradiating wild type (WT) mice, then transplanting fetal liver cells with hematopoietic potential from SCA mice into WT mice. The resulting mice with acquired SCA will be treated with control or anti-CD40L antibody (MR1), and analyzed as described below. Fetal liver transplanted mice will be treated with an anti-CD40L antibody or an isotype-matched control IgG, 8-10 weeks after the FLT. Histologic preparations of perfusion-fixed lung tissue will be prepared with Hematoxylin & Eosin (H&E), which will help to detect inflammation by examining the morphology of the tissues. Gomori iron and the fibrin-indicator phosphotungstic acid-hematoxylin (PTAH) stains will help detect iron and fibrin deposits respectively in the organ tissues. I will compare the degree of inflammation in lungs, liver and kidney of the anti-CD40L treated mice with the control IgG-treated mice to assess if SCA pathology is improved.

Fetal liver transplantation: Eight-week old wild-type C57BL/6 mice will be exposed to two doses of total body irradiation, 700 Rad, and after a 4 hour period, exposed to 500 Rad, in a cesium-137 irradiator. Two – three million fetal liver cells from SCA mice will be retro-orbitally injected into these mice. We will then perform hemoglobin electrophoresis and PCR analysis 8 - 10 weeks following the transplant, to confirm if these mice engrafted the SCA cells.

Anti-CD40L treatment: Transplanted mice with acquired SCA after 8 – 10 weeks will be treated with monoclonal antibody MR1, an Armenian hamster antibody known to inhibit CD40L in vivo (41). The mice will be weighed, and injected intraperitoneally with 250 μg of MR1 twice per week for 6 weeks (28). A non-specific, isotype-matched control antibody will also be administered to some of the SCA mice. After treatment, we will weigh the mice again, to determine if they gained any weight during the treatment process, then we will draw blood by cardiac puncture, and perform CD40L ELISA to analyze CD40L levels (14).

Organ histology: Mice will be sacrificed by CO_2 inhalation and organs preserved by infusing 4% paraformaldehyde followed by 70% ethanol. Lung, liver, spleen and kidney will be embedded, sectioned, and stained with hematoxylin & eosin (H&E), gomori iron and the fibrin-indicator phosphotungstic acid-hematoxylin (PTAH) stains. SCA treated and control organ pathology will be compared.

Measurement of inflammatory markers:

We will use Luminex technology to measure plasma levels of inflammatory cytokines including CD40L (42). Any changes measured by Luminex technology will be verified by other methods such as ELISA (14).

Blood cell count: We will compare red blood cell and leukocytes of sickle cell mice treated with anti-CD40L versus control antibody to determine whether the anti CD40L antibody treatment reduced the anemia and improved the inflammatory state.

Expected results:

If treatment with the anti-CD40L antibody works as proposed, then I will expect to see a tremendous decrease in the lung, kidney and liver damage when compared to the control IgG treated mice. Spleen size for the CD40L treated mice is expected to be smaller than the control because the spleen in the CD40L antibody-treated mice will not work as hard in clearing sickle red cells, as compared to the IgG treated mice. I will also expect to see a decrease in inflammation markers such as CD40L in the plasma. These results should correlate with a decrease in leukocyte counts, since reduced leukocyte counts indicates reduced inflammation. Red blood cells are sickled and continuously being depleted in SCA, so new red cells are made at a higher rate than normal in SCA individuals. As a result, reticulocyte counts are normally high in SCA individuals. I therefore expect to see decreased reticulocyte counts in the CD40L antibody-treated mice, when compared to the untreated since, the treated mice will have less red cells being cleared from their system.

Pitfalls

FLT mice might not fully acquire SCA, but the hemoglobin electrophoresis might show that these mice acquired SCA. If this happens, the organ histology will appear normal, showing that the organs of these mice recovered from SCA as a result from treatment, although this might not be the case. To avoid this, we will have to wait more than 8 -10 weeks after FLT, just to make sure that these mice become fully engrafted with SCA cells. Furthermore, there

might be no rescue with the anti-CD40L antibody, because there might be other ligands besides CD40L that interact with CD40.

Summary

These experiments will tell us whether CD40L contributes to the pathology of these organs in SCA. As a result, we will help determine if CD40L mediates inflammation and organ damage in SCA patients. This knowledge may result in the development of drugs that could decrease the painful crises and symptoms of SCA.

CHAPTER III

SPECIFIC AIM 2: TO DETERMINE WHETHER CD40 EXPRESSED IN TISSUES OR BLOOD CELLS CONTRIBUTES TO SCA PATHOLOGY.

Rationale: CD40 is a major receptor for CD40L. It is expressed as a homotrimer on platelets and epithelial cells. My objective is to inject SCA fetal liver cells into CD40 knockout mice to determine whether CD40 from tissues or blood cells promotes SCA pathology.

Experimental design: For the first part of this experiment we will use the procedure in Aim 1 to generate SCA mice by transplanting SCA fetal liver cells into irradiated CD40 knock-out or wild type mice. These mice will lack endogenous CD40 in their tissues but will express CD40 on their blood cells from the transplantation. Eight weeks following transplant, we will measure inflammatory and hematologic parameters as described in Aim 1, and compare organ histology. For the second part, we will use the same procedure to generate SCA mice by transplanting SCA fetal liver cells into CD40 knockout mice, followed by treating the mice with an anti-CD40 antibody or control antibody. Note that these mice will have CD40 from only the sickle blood cells that were transplanted. Both experiments will help determine whether CD40 from the tissues or the blood cells contribute to SCA pathology.

Fetal Liver Transplantation: We will generate SCA mice as in Aim 1.

CD40 antibody treatment: SCA transplanted mice will undergo treatment with an anti-CD40 function blocking antibody, HM40-3, or its control IgG. Mice will be treated with 250 μg of

HM40-3 or control antibody twice a week for 6 weeks. SCA pathology will be analyzed as in the CD40L experiment.

Organ histology: Mice will be sacrificed by CO_2 inhalation and organs removed for perfusion with 4% paraformaldehyde followed by 70% ethanol. Lung, liver, and kidney will be embedded, sectioned, and stained with hematoxylin & eosin (H&E), gomori iron and the fibrin-indicator phosphotungstic acid-hematoxylin (PTAH) stains to examine degree of inflammation, and iron and fibrin deposits respectively.

Expected results

In the first experiment, I will transplant SCA fetal liver cells into the CD40 knockout mice, and my control will be SCA fetal liver cells transplanted into wildtype mice. If SCA pathology in SCA-transplanted CD40 knockout mice is similar to control SCA-transplanted mice, then I will conclude that CD40 in the tissues does not contribute to SCA pathology. However, if SCA pathology is reduced, I expect to see decreased spleen size, decreased lung, kidney and liver pathology, lower leukocyte and reticulocyte counts and a decrease in inflammatory molecules. My conclusion will be that CD40 in the tissues contribute to SCA pathology.

In the second experiment, SCA fetal liver cells were transplanted into CD40 knockout mice. After engraftment, these animals will be treated with an anti-CD40 antibody or control IgG. If SCA pathology is the same as compared to my control, then I will conclude that CD40 from the blood cells does not contribute to SCA pathology. If the pathology is reduced, then CD40 from the blood cells contributes to SCA pathology. To further investigate the major source of CD40 in SCA pathology, we will perform the same experiment as in Aim 2. We will treat SCA transplanted mice on a wild type background with HM40-3 or a control

antibody. Organs from this experiment will be compared to organs from treatment with anti CD40 antibody on CD40knockout background SCA mice. The goal is to verify which source of CD40 – tissues or blood cells – contributes most to SCA pathology.

Pitfalls

Mice might not fully engraft SCA from the fetal liver transplantation. As a result, we may have to wait for a longer period than 8 - 10 weeks after transplantation (i.e. 12-14 weeks), to make sure we have full SCA engraftment.

Conclusion

These experiments will help determine whether CD40 increases SCA pathology. We will also be able to identify which source of CD40 contributes most to the SCA disease. These results will take us closer to finding drugs that can improve the health of SCA individuals.

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