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13. Abstract (Maximum 200 Words) We have proposed that CD34+ vascular endothelial progenitor (VEP) cells in blood can be used to transport therapeutic genes into areas of angiogenesis within growing breast cancer tumors. Our system is tumors (MDA-MB 231) grown in SCID mice. It was difficult to grow these tumors. We learned that NK cells and NKT cells had to be eliminated before tumors could grow. CD34+ cells were isolated from leukopacks and labeled with a lipophilic fluorescent dye, DiI. Leukopacks do not supply us with enough CD34+ cells to further isolate subsets of cells that have phenotypic markers of VEP. CD34+ and CD34- cells were then injected into mice with growing tumors. Mice were sacrificed and tumors removed and sectioned and examined for fluorescent labeled cells and their juxtaposition to vascular elements. We found that there were far more CD34+ cells homing to tumors than CD34- cells. Labeled calls do not accumulate in other tissues except spleen. We were not able to determine the relationship between CD34+ cells and tumor vascular elements. However, labeled cells were still intact one week after injection. We conclude that some CD34+ cells do become incorporated into breast cancer tumors and remain intact long enough to express a transfected gene product.

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Introduction

In our original application we proposed to investigate the possibility that there exists a cell in the blood that would home to sites of angiogeneis within tumors and could be used to deliver genes or radioisotopes to the tumor. Based upon a published study from Dr. Jeffery Issner's lab (1) we speculated that there are CD34+ cells, or subsets of CD34+ cells, in peripheral blood lymphocytes (PBL) that have the property of heamopoeiteic progenitor cells that become incorporated into newly forming blood vessels. Since this study was first proposed there have been studies that confirmed the existence of this cell (2,3) and other showing that this cell can be stably transfected and used to shuttle genes into *in vivo* systems (4,5). Our results show that CD34+ cells, isolated from PBL, will home to human tumors growing in SCID mice. Additionally, the *in vitro* manipulation of these cells, labeling with a lipid intercalating fluorescent dye, does not prevent their homing to tumors. Unfortunately, we have not been able to determine the phenotype of the subset of CD34+ cells from PBL that are involved. This study is now being expanded to incorporate new technologies that will allow us to grow specific subsets of CD34+ vascular endothelial progenitor cells *in vitro*. These studies will give us enough cells of a specific subset to see if transfection with therapeutic genes will permit the homing of the cells to growing tumors.

BODY

We originally proposed to harvest CD34+ cells from a leukophoresis. Access to this source of cells was denied by the IRB at MRMC. We were forced to substitute CD34+ cell from leukopacks purchased from the Red Cross. Unfortunately, the number of CD34+ cells harvested from leukopacks is orders of magnitude less than we could have harvested from a leukophoresis, giving us only 2 to 4 $x10^4$ CD34+ cells/leukopack. After labeling with a fluorescent, lipophilic tracking dye, DiI, we recover only about 1 to 2 $x10^4$ cells from a leukopack. The labeled cells are then injected into SCID mice that have growing breast cancer tumors; grown from the injection of the human breast cancer cell line, MDA-MB 231.

Initial attempts to exploit this model were unsuccessful. We could not find any indications that labeled CD34+ cells had homed to tumors. We found very few labeled cells in tumors of mice that were injected with either CD34+ cells or CD34- cells. These tumors grew very slowly, taking as long as six weeks to become palpable, and we were concerned that there is limited angiogenesis in such slow growing tumors. It was decided to try to increase the rate of tumor growth, and, hopefully, angiogenesis by depleting populations of active lymphocytes. Although SCID mice do not produce significant populations of T-cells and B-cells they do have Natural Killer (NK) cells and NK cells also have anti-tumor activity. NK cells were, therefore, depleted by injecting anti-NK cell antibodies into the mice before implantation of the breast cancer tumors. Unfortunately, the tumors still did not grow very fast.

We then ran a series of experiments to determine if SCID mice might have NKT cells and if these cells might have anti-tumor functions. NKT cell are a primitive lymphocyte that has a T-cell receptor that recognizes CD1 restricted lipid antigens and they have both T-cell and NK cell functions (6). We were surprised to find that there are significant numbers of NKT cells in SCID mice; cells that are $CD3^{low}$, CD4-/CD8-and staining positively for the NK cell marker CD161. We therefore modified our NK cell depletion by switch to an antibody that binds a cell surface protein on both NK cell and NKT cells; anti-IL-2 receptor β-chain. When mice were injected with this antibody before tumor implantation both NNK cells and NKT cells were depleted and the tumors grew significantly faster.

As the tumors grew faster we hoped for more blood vessel formation and better incorporation of labels CD34+ cells into tumors. When tumors were about 4x4mm we injected DiI labeled cells. Approximately 2 to $4x10^4$ cells were injected IV into mice. Mice were injected with either CD34+ cells or CD34- cells. One week after injection of human lymphocytes tumors were removed and sections were fixed or frozen for histological analysis. In tumors from mice injected with either CD34+ or CD34- cells we find labeled cells. Tumor from mice injected with CD34+ cell shows about three times more cells residing in the tumor, Figure 1.

It is of significant interest that labeled cells remain intact one week after injection. Remembering that one of our long range objectives is to deliver genes coding for therapeutic agents to tumors after transfection into CD34+ cells, it is imperative that the CD34+ not only home to tumors but that they remain intact for some period of time. Future experiments will investigate the longevity of CD34+ injected cells within tumors.

Attempts to sort phenotypic subsets of CD34+ cells were abandoned when we were unable to recover a significant number of cells for *in vivo* assay. Phenotypes that are associated with endothelial progenitors have been described. CD34+ VEP in blood have been found to also

express VEGFR-2 and AC133 as well as VE-cadherin, E-selectin and the chemokine receptor, CXCR-4 (7). They also share the endothelial TK receptor Flk-1 and VwF (8). When we tries to separate CD34+ subsets based upon these markers we ended up with far to few cells to try to label and inject.

We have not been able to correlate the position of labeled CD34+ cells within growing tumors with vascular elements. Attempt to counter-stain tissue sections with fluorescent tagged antibodies, like CD31, have shown vascular elements but have failed to clearly locate the injected CD34+ cells as components of blood vessels. In future experiments we need to inject only the subsets of CD34+ cells that are known to incorporate into newly forming vascular elements. Unfortunately isolating these rare cells from PBL is probably not going to provide enough cells for *in vivo* experiments.

Perhaps, more important is the presence of labeled CD34+ cells to other tissues. If we propose to use CD34+ cells to deliver therapeutic genes to tumors we do not want them to go to every other tissue as well. We failed to find significant numbers of labeled CD34+ cells in liver, lung intestine, skeletal muscle and skin. We did find CD34+ and CD34- cells in spleen. The numbers of labeled CD34+ and CD34- cells in the spleen was approximate equal. Therefore, we conclude that this is a non-specific event, that the spleen is acting as a filter to accumulate the injected cells. The total number of injected cell in the spleen was far less than was seen in the tumor of mice injected with CD34+ cells. This suggests some tissue specificity for homing to tumors.

The hypothesis that there are CD34+ cells in blood that can go to tumors seems to be valid. Several other studies have recently defines vascular endothelial progenitor (VEP) cells within the peripheral blood CD34+ cells (2,3). Unfortunately, VEP cells make up a very small percentage of the CD34+ cells found in peripheral blood, only about 0.4% to 0.2% of PBL having VEP phenotype. It seems advisable that future work be done exclusively with these cells to determine their potential for delivering genes to blood vessels within tumors. However, the small numbers of cells found in blood would make these experiments extremely difficult, even if we could start with a leukophoresis. To obviate this problem we are currently growing VEP cells *in vitro*. Techniques for expanding these cells in the presence of VEGF and bFGF have been reported (3,8,9) and we are currently repeating these experiments with the intention of testing the tumor homing capacity of these cells.

We are encouraged to continue this work and are presently preparing a grant for support of future studies. The problems of the model have been solved and we can now grow human breast cancer tumors in SCID mice. Our experiments have also confirmed our hypothesis that there are CD34+ cells in blood that will home to tumors. Future work will first define whether the *in vitro* expanded VEP cells home to tumors. Perhaps most encouraging was the observation that CD34+ cell that home to tumors were intact one week after injection. This bodes well for using these cells to deliver genes to tumors. Our next series of experiments will be to raise CD34+ VEP cells *in vitro* and to transfect them with a reporter gene; probably GFP. These cells will then be injected into SCID mice with growing human breast cancer tumors to see if they will home to the tumor and continue to express the gene. If they do we will repeat the experiment transfecting the cells with a genes for the IL-12; a cytokine that we know has anti-tumor effects when injected into tumors.

KEY RESEARCH ACCOMPLISHMENTS

1. Development of the tumor model and the definition of NK cells and NKT cells as having anti-tumor function in SCID mice. By depleting the IL-2 receptor β-chain+ NK cells and NKT cells we are now able to grow human tumors faster and, as a result, increase angiogenesis. This has allowed increased incorporation of vascular endothelial progenitors into the tumor, making evaluation of our experiments easier.
2. Although we have not been able to define the phenotype of the CD34+ cell in blood that home to tumors, our study supports the presence of such a cell.
3. CD34+ cells that home to tumors remain in the tumor, apparently intact, for at least a week. This encourages future studies that these cells can be used to deliver genes to tumors.

REPORTABLE OUTCOMES

1. We are preparing an abstract for submission to the 2003 AACR meeting.
2. The presence of NKT cell in SCID mice that influence tumor growth has not previously been reported.

CONCLUSIONS

We can demonstrate that there is a CD34+ cell in blood that appears to home to breast cancer tumors. These cells reside intact within the tumor environment for at least a week. These results are consistent with our model for delivery of genes or imaging enhancing agents to tumors. Our future work, currently underway, will grow large numbers of a defined vascular endothelial progenitor cell *in vitro* for use in this model. These cells will be transfected with a reporter gene or, in later studies, with a cytokine gene before injection. We hope to show that genes can be delivered to tumors.

"So what"; in clinical trials unrelated to this study we have recently shown that the injection of GM-CSF into tumors, including breast cancer tumors, will result in tumor regression (10). This is clinical proof that the maintenance of immune activation cytokines within tumor has therapeutic effects. Our clinical trial showed that tumors need to be injected daily for several weeks, however, while encouraging, not all tumors can be injected. Delivery of genes to tumors via a CD34+ cell that homes to tumors is an alternative to direct tumor injection and might someday be a viable clinical option for treatment of currently untreatable disease.

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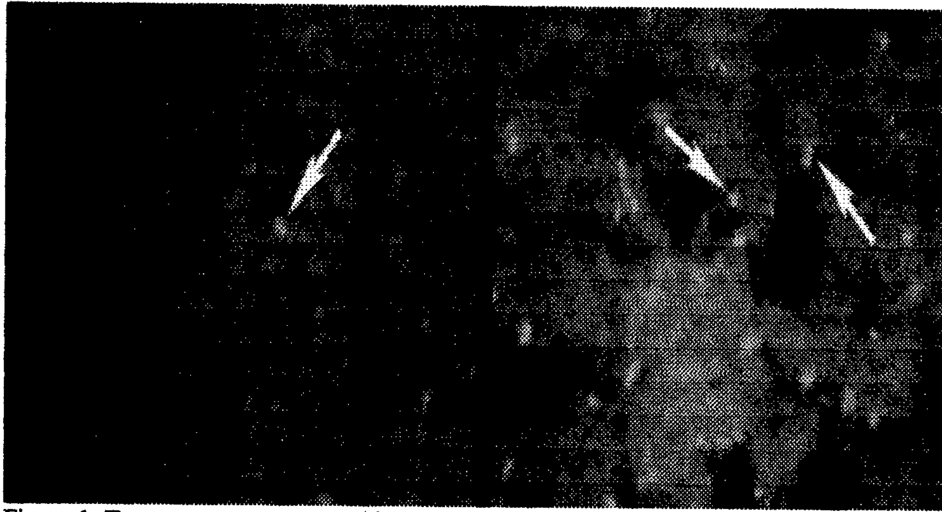

Figure 1. Two computer generated images taken under a fluorescent microscope of MDA-MB 231 tumors one week after injection of CD34- cells, on the left, or CD34+ cells, on the right, that had been labeled with DiI. The tumor injected with CD34+ cells has many more identifiable DiI labeled cells and the high background fluorescence suggests that there are more labeled cells below the plane of focus.