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Chemokines play a pivotal role in the maturation of the immune system, and in the initiation, and maintenance of an immune response. Because of their key role in the immune response, the aberrant expression of chemokines can have a profound effect on the ability of T cells to respond to antigen. We have found that several breast cancer cell lines produced chemokines capable of recruiting T cells. However, instead of increasing anti-tumor immunity, the tumor-derived chemokines may have prevented an effective immune response by desensitizing T-cell chemokine receptors. Our hypothesis is that disrupting the synthesis of tumor-derived chemokines (using anti-sense technology) will remove tumor-induced immune suppression and enhance the immunogenicity of the tumor. In order to test this hypothesis we have generated stable clones that lack MCP-1 and RANTES production compared to the parental tumor cell line. Using these tumors we will determine whether the T cells are better able to elicit an anti-tumor immune response by comparing the immunogenicity of the tumors that do and do not express chemokines. These tumor cells will be evaluated by immunization/challenge experiments and by the ability to generate tumor-specific T cells in vaccine draining lymph nodes.

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TABLE OF CONTENTS:

Cover.. 1

SF 298... 2

Introduction... 4

Body.. 5

Key Research Accomplishments.. 13

Reportable Outcomes... 13

Conclusions... 14

References... 14

Appendices.. NA

INTRODUCTION

Chemokines play a pivotal role in the maturation of the immune system, and in the initiation, and maintenance of an immune response (1). Because of their key role in the immune response, the aberrant expression of chemokines can have a profound effect on the ability of T cells to respond to antigen. We have found that several breast cancer cell lines produced chemokines [Regulated upon activation, normal T cell expressed and secreted (RANTES) and monocyte chemotactic factor-1 (MCP-1)] capable of recruiting T cells, as well as the chemokine KC (2). Additionally, supernatants derived from the tumor cell line 4T1 could mediate the chemotaxis of T cells. However, instead of increasing anti-tumor immunity, the tumor-derived chemokines may have prevented an effective immune response by desensitizing T-cell chemokine receptors (2). The receptors for RANTES and MCP-1 on T cells were desensitized in tumor-bearing animals. Moreover, there was cross-receptor desensitization of the CC chemokine receptor 7 (CCR7), which impaired the ability of the T cells to respond to secondary lymphoid chemokine, SLC. These data indicate that the aberrant expression of tumor-derived chemokines may help tumors escape immune attack. Our hypothesis is that disrupting the synthesis of tumor-derived chemokines (using anti-sense technology) will remove tumor-induced immune suppression and enhance the immunogenicity of the tumor. We will determine whether the T cells are better able to elicit an anti-tumor immune response by comparing the immunogenicity of the tumors that do and do not express chemokines. These tumor cells will be evaluated by immunization/challenge experiments and by the ability to generate tumor-specific T cells in vaccine draining lymph nodes.

BODY

The first objective of the proposal was to generate sense and anti-sense constructs capable of inhibiting synthesis of the tumor-derived chemokines RANTES, KC and MCP-1. This objective has been completed. We started with isolation of mRNA from the 4t1 tumor cell line. The mRNA was converted to cDNA and used to amplify fragments of the chemokines (Figure 1). These fragments were isolated from the gel using the Qiagen gel extraction kit and cloned into a T vector. The T vectors containing the chemokine fragments (RANTES, MCP-1 and KC) were used to transform competent E. coli (strain JM109). Subsequently, the transformed bacteria were screened by performing 5-10 separate minipreps for each transformation and digesting each set of plasmid DNA with EcoR1 (Figure 2). The figure shows the vector at 3.0 kb and inserts at the correct sizes (KC 302 bp, RANTES 202 bp, MCP-1 345 bp) were present in each of the clones screened. This gave us the plasmid vector, in a bacterial stock (glycerol stocks stored at –80°C), for each of the chemokines that will be used in this study. The next step was to take the chemokine fragments out of the T vector and clone them into a eukaryotic expression vector. For each chemokine this was accomplished by digestion of the T vector, containing the chemokine, with EcoR1 and gel purification of the fragment. The fragments were cloned into the EcoR1 site of the eukaryotic expression vector and used to transform competent E. coli. Ten separate clones were screened for the presence of the inserts (Figure 3). Following identification of vectors with the inserts glycerol stock were made (stored at –80°C) and a sample of the plasmid DNA was sent to Penn State University College of Medicine for sequencing. The sequencing reactions confirmed isolation of the KC, MCP-1 and RANTES gene fragments and identified expression vectors containing them in the sense and anti-sense orientations. For KC clone#5 is the sense stock and clone#10 is the anti-sense stock. For MCP-1 clone #2 is the sense and clone#3 is the anti-sense stock. For RANTES clone #1 is the sense and clone #5 is the anti-sense stock. This work completed most of objective # 1 in the proposal's statement of work for the months 1-9.

Statement of Work

Objective 1. Generate anti-sense constructs capable of inhibiting synthesis of tumor-derived chemokines, months 1-9:

a. PCR amplify RANTES, MCP-1 and KC from the murine breast cancer cell line 4T1. (4T1 produces RANTES, MCP-1 and KC)
b. Ligate the chemokines into the T-vector.
c. Transform the vector containing the chemokines into competent E. coli.
d. Screen for clones that contain the correct insert by blue/white screening and a restriction digest.
e. Digest the correct clones and gel purify the chemokine DNA.
f. Separately ligate each chemokine into the expression vector in the reverse orientation.

milestone #1-Single antisense vectors constructed

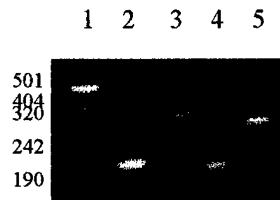

Figure 1. Amplification of tumor-derived chemokines. RT-PCR was used to amplify MCP-1 (lane 3), RANTES (lane 4) and KC (lane 5) from the murine mammary carcinoma 4t1. The PCR fragments were subsequently purified from the gel and cloned into a T vector. GAPDH (lane 2) was used as a positive control. The molecular weight marker (bp) is shown in lane 1.

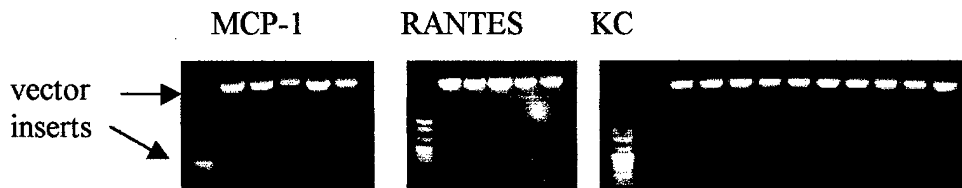

Figure 2. Selection of T vector containing the chemokines. The chemokine fragments were cloned into the PGEM T vector and used to transform competent E. coli. The bacteria were grown overnight on an LB+ampicillin plate and 5-10 colonies were collected and used to start plasmid preparations. The plasmid DNA was isolated and examined for the presence of the chemokine genes by EcoR1 restriction digests. The gels show the 5 MCP-1 and RANTES clones and the 10 KC clones that were screened.

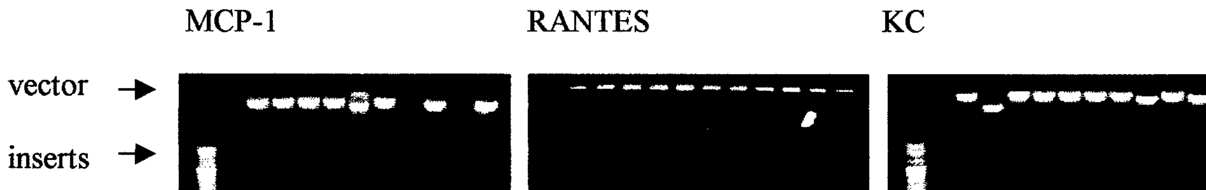

Figure 3. Selection of the expression vector containing the chemokines. The chemokine fragments were cloned out of the PGEM T vector, into the eukaryotic expression vector, and used to transform E. coli. The bacteria were grown overnight on an LB+ampicillin plate and 10 colonies were collected and used to start plasmid preparations. The plasmid DNA was isolated and examined for the presence of the chemokine genes by EcoR1 restriction digests. The figure shows the 10 MCP-1, RANTES, and KC clones that were screened. Sequencing the DNA revealed MCP-1 clone 2 as sense, 3 as anti-sense, RANTES clone 1 as sense, 5 as anti-sense, and KC clone 5 as sense and 10 as anti-sense.

The second objective of the proposal (months 10-18) is underway as described below. Transfection of the 4T1-9 tumor cell line has been accomplished with the sense and anti-sense RANTES, MCP-1 and KC vectors. The progress with each chemokine is described separately.

RANTES is constitutively expressed by the 4T1 tumor cell line as well as T cells, epithelial cells and platelets following exposure to inflammatory agents or mitogens (1, 2). Mast cells, T cells, natural killer cells, dendritic cells, eosinophils and basophils are capable of responding to RANTES via CCR1, CCR2, CCR3, CCR4 and CCR10 (3). In order to study the impact of tumor-derived RANTES on anti-tumor immunity we attempted to inhibit RANTES production using anti-sense technology. For this purpose the tumor cells were transfected with the sense and anti-sense vectors, cloned and screened for RANTES production by RT-PCR and ELISA (figure 4). Although the RT-PCR was not predictive of protein production, the ELISA revealed a clone that expressed ten-fold less RANTES (clone 5) than the sense transfected and parental tumor cell line. These clones were expanded and supernatants were taken at 1, 4, 8 and 24 hours in order to examine the kinetics of RANTES production (Figure 5A). The data revealed that in three separate experiments RANTES production was consistently normal in the sense clone and consistently low in the anti-sense clone. The stability of the clone expressing low levels of RANTES is critical to the completion of this project because the tumors will be grown in vivo in the absence of G418 selection. In order to examine the stability of the clones RANTES production was examined after the clones were grown in vivo for 4 weeks in the absence of G418 (Figure 5B). The data show that the sense and anti-sense expressing tumor cell clones are stable. Studies with the RANTES clones thus far have focused on an examination of the in vitro and in vivo growth rates. A comparison of the immunogenicity of the two clones will be facilitated if the clones grow at similar rates. The data we have generated so far show that the sense and anti-sense clones grow at similar rates in vitro and in vivo (Figure 6). Thus, these clones designated R1 (sense) and RA5 (anti-sense) will be used for all subsequent studies, which will investigate the role of tumor-derived RANTES on anti-tumor immunity.

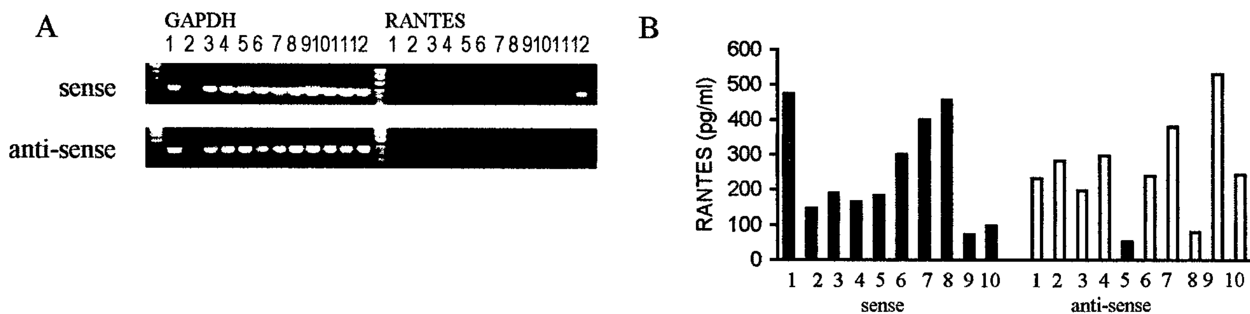

Figure 4. Screening of sense and anti-sense transfected 4T1. A. Ten sense and anti-sense transfected clones were screened by RT-PCR for RANTES mRNA expression. GAPDH was used as a positive control. Lanes 1 and 2 represent the + and – RT-PCR controls. Lanes 3-13 represent the 10 clones that were screened. B. The ten sense and anti-sense clones were plated at $1x10^6$ cells/well in a 24 well plate and 24 hours later the supernatants were harvested and assayed for RANTES by ELISA.

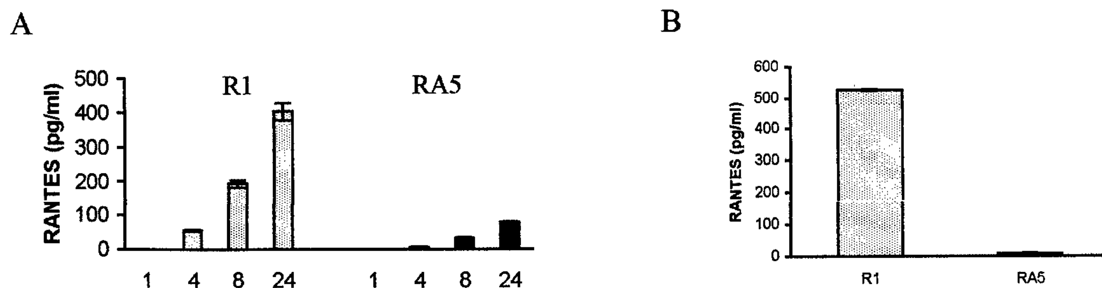

Figure 5. Kinetics and stability of RANTES production. A. The sense transfected clone (R1) and anti-sense clone (RA5) were plated at 1×10^6 cells/well in a 24 well plate and supernatants were harvested at 1, 4, 8 and 24 hours and analyzed for RANTES by ELISA. The experiment was repeated three times with similar results. Error bars represent standard deviation of duplicate samples. B. The sense and anti-sense clones were injected into Balb/c mice and recovered after 30 days of tumor growth. The tumors were digested in collagenase cocktail and plated in cRPMI. After 3 days the tumor cells were harvested and plated at 1×10^6 cells/ well in a 24 well plate. Twenty-four hours later the supernatants were harvested and analyzed for RANTES by ELISA. The results are representative of three separate experiments with the standard deviation of duplicate samples shown.

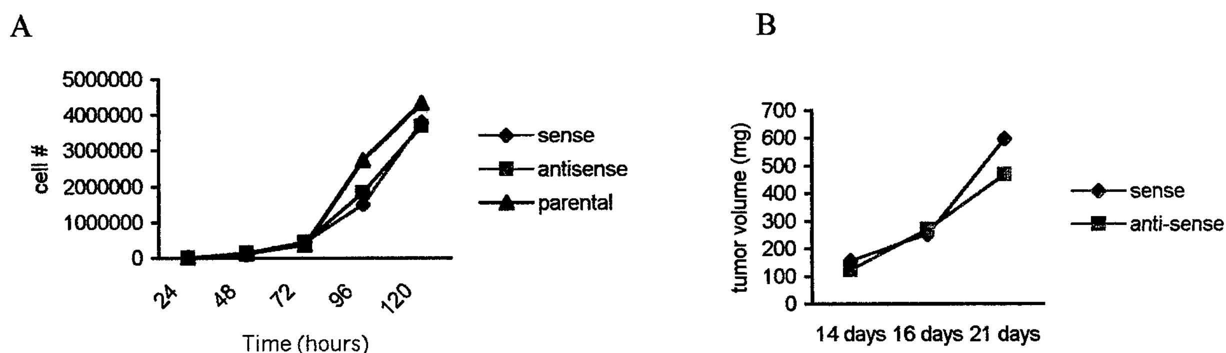

Figure 6. Growth kinetics of the RANTES clones. A. In order to examine the in vitro growth rate of the RANTES sense and anti-sense clones 2×10^4 cells were plated in tissue culture flasks, collected at 24-120 hours and counted. The experiment was repeated three times with similar results. B. In order to examine the in vivo growth rates of the sense and anti-sense clones 5×10^4 cells were delivered subcutaneously (sc) to each mouse and the tumors were measured for 21 days. The average of 5 mice is shown. The experiment was performed three times with similar results. Parental tumor cells (4T1) were used as controls.

MCP-1 is constitutively expressed by the 4T1 tumor cell line as well as macrophages, neutrophils, mast cells and fibroblasts during an inflammatory response (1, 2). Macrophages, dendritic cells and T cells are capable of responding to MCP-1 via CCR2 and CCR4 (3). In order to study the impact of tumor-derived MCP-1 we attempted to inhibit MCP-1 production

using anti-sense technology. For this purpose the tumor cells were transfected with the sense and anti-sense vectors, cloned and screened for MCP-1 production by RT-PCR and ELISA (figure 7). Although the RT-PCR was not predictive of protein production the ELISA revealed a clone that did not produce detectable levels (<15pg/ml) of MCP-1. The sense and anti-sense expressing clones were expanded and supernatants were taken at 1, 4, 8 and 24 hours in order to examine the kinetics of MCP-1 production (Figure 8A). The data revealed that in three separate experiments MCP-1 production was consistently normal in the sense clone and consistently below detection in the anti-sense clone. The stability of the clone expressing undetectable levels of MCP-1 is critical to the completion of this project because the tumors will be grown in vivo without G418 selection. In order to examine the stability of MCP-1 production we examined MCP-1 production after the clones were grown in vivo for 21 days in the absence of G418 (Figure 8B). The data show that the production of MCP-1 (sense) and lack of production (anti-sense) was stable. Studies with the MCP-1 clones thus far have focused on an examination of the in vitro and in vivo growth rates. A comparison of the two clones will be facilitated if the clones grow at similar rates. The data we have generated so far show that the sense and anti-sense clones grow at similar rates in vitro and in vivo (Figure 9). Therefore, these clones designated A4 (sense) and G7 (anti-sense) will be used for all subsequent studies, which will investigate the role of tumor-derived MCP-1 on anti-tumor immunity.

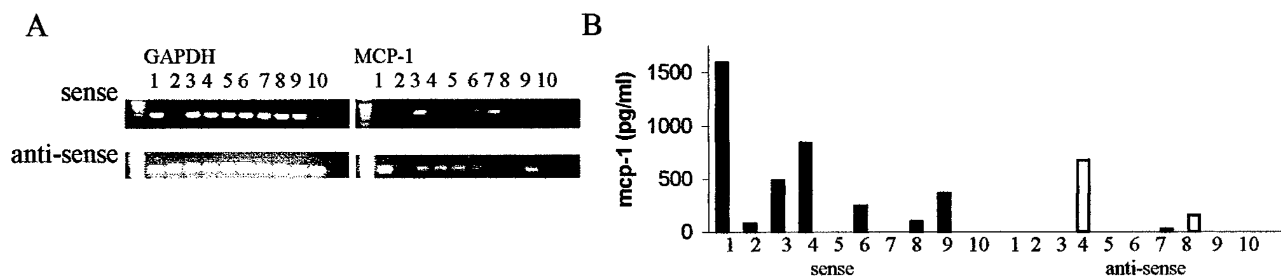

Figure 7. Screening of sense and anti-sense transfected 4T1. A. Ten sense and anti-sense transfected clones were screened by RT-PCR for MCP-1 mRNA expression. GAPDH was used as a positive control. Lanes 1-10 represents the 10 clones that were screened. B. The ten sense and anti-sense clones were plated at $1x10^6$ cells/well in a 24 well plate and 24 hours later the supernatants were harvested and assayed for MCP-1 by ELISA.

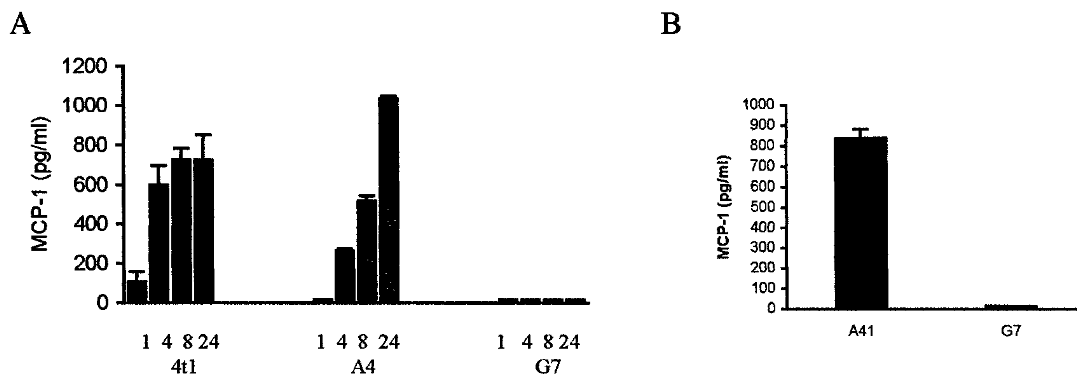

Figure 8. Kinetics and stability of MCP-1 production. A. The parental tumor (4T1), sense (A4) and anti-sense clone (G7) were plated at $1x10^6$ cells/well in a 24 well plate and supernatants were harvested at 1, 4, 8 and 24 hours and analyzed for MCP-1 by ELISA. The experiment was repeated three times with similar results. Error bars represent standard deviation of duplicate samples. B. The sense and anti-sense clones were injected into Balb/c mice and recovered after 21 days of tumor growth. The tumors were digested in collagenase cocktail and plated in cRPMI. After 7 days the tumor cells were harvested and plated at $1x10^6$ cells/ well in a 24 well plate. Twenty-four hours later the supernatants were harvested and analyzed for MCP-1 by ELISA. The results are representative of three separate experiments with the standard deviation of duplicate samples shown.

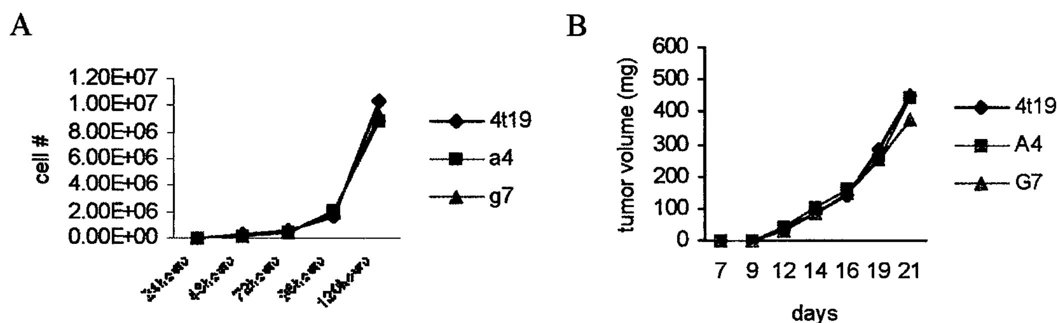

Figure 9. Growth kinetics of the MCP-1 clones. A. In order to examine the in vitro growth rate of the MCP-1 sense and anti-sense clones $2x10^4$ cells were plated in tissue culture flasks, collected at 24-120 hours and counted. The experiment was repeated three times with similar results. B. In order to examine the in vivo growth rates of the sense and anti-sense clones $5x10^4$ cells were delivered sc to each mouse and the tumors were measured for 21 days. The average of 5 mice/group is shown. The experiment was performed three times with similar results. Parental tumor cells (4T1) were used as controls.

KC is constitutively expressed by the 4T1 tumor cell line as well as neutrophils, epithelial cells and platelets following exposure to inflammatory agents or mitogens (1, 2). Mast cells, neutrophils, eosinophils and basophils are capable of responding to KC via CXCR2 (3). In order to study the impact of tumor-derived KC we attempted to inhibit KC production using anti-sense technology. For this purpose the tumor cells were transfected with the sense and anti-sense vectors, cloned and screened for KC production by RT-PCR and ELISA (figure 10). Of the ten anti-sense clones screened there were none with greater than 5-fold decrease in KC expression. In order to generate a clone that expressed lower levels of KC, the clone with the lowest level of KC expression (clone 7) was re-transfected, cloned and screened again (Figure 11A). The data revealed a clone that produced no detectable KC, and two clones that produced ten-fold lower levels of KC compared to the parental tumor cell line. These clone were expanded and the kinetics of KC expression was examined. The data revealed that the clones now expressed high levels of KC (Figure 11B). Additionally, in order to identify a clone with low KC expression 20 more clones were screened (Figure 12). We failed to identify a KC – tumor cell line. Overall, we found 1 negative and 2 clones with low levels of KC expression, however these clones were not stable. These data suggest that KC expression is more difficult to control than MCP-1 and

RANTES. We are in the process of placing the anti-sense KC gene fragment under a different promoter in order to generate a KC- tumor cell line. The lack of a reliable method to inhibit KC gene expression has also prevented us from generating the triple anti-sense tumor cell line, which will lack MCP-1, RANTES and KC. Once we have the parameters to stably block KC expression we will precede with that portion of the project. Collectively with the RANTES, MCP-1 and KC progress objective #2 is near completion (months 10-18).

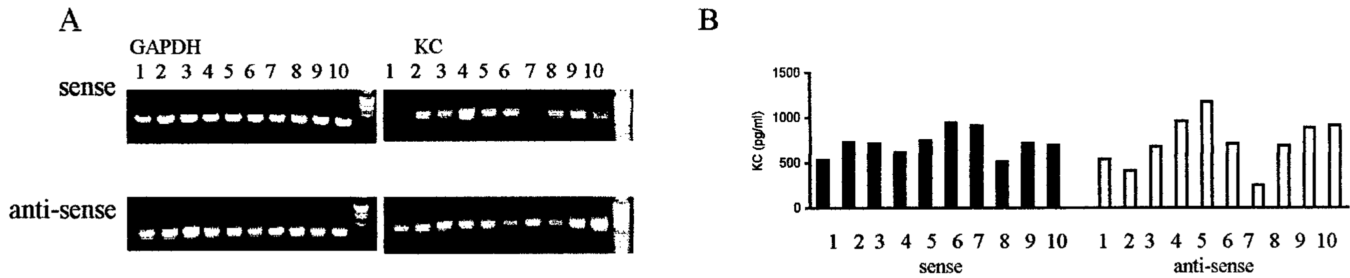

Figure 10. Screening of sense and anti-sense transfected 4T1. A. Ten sense and anti-sense transfected clones were screened by RT-PCR for KC mRNA expression. GAPDH was used as a positive control. Lanes 1-10 represent the 10 clones that were screened. B. The ten sense and anti-sense clones were plated at $1x10^6$ cells/well in a 24 well plate and 24 hours later the supernatants were harvested and assayed for KC by ELISA.

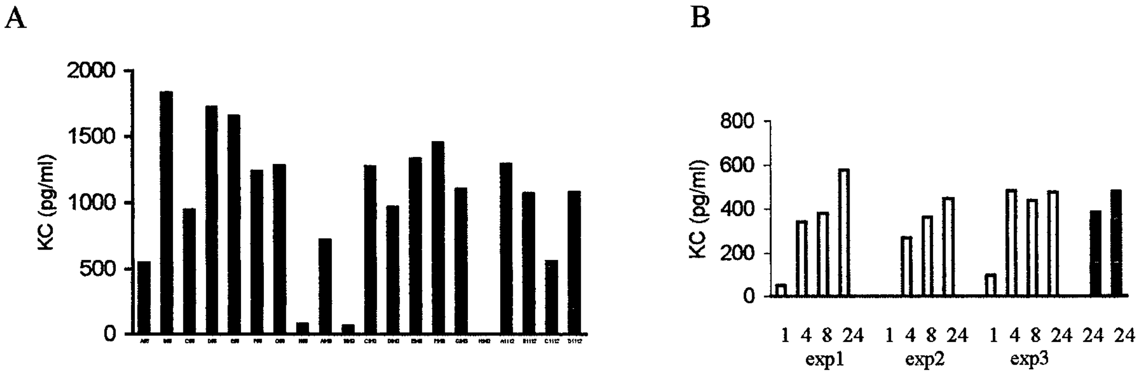

Figure 11. Retransfection of anti-sense clone 7. A. The tumor cell clone (7) that expressed the lowest levels of KC was retransfected, recloned and 20 new clones were screened for KC expression by ELISA. B. The anti-sense clone (with undetectable KC) was plated at $1x10^6$ cells/well in a 24 well plate and supernatants were harvested at 1, 4, 8 and 24 hours and analyzed for KC by ELISA. The three separate experiments are shown. Twenty-four hour supernatants of the low KC expressing (■ and ■) clones were also repeated and found to express high levels of KC.

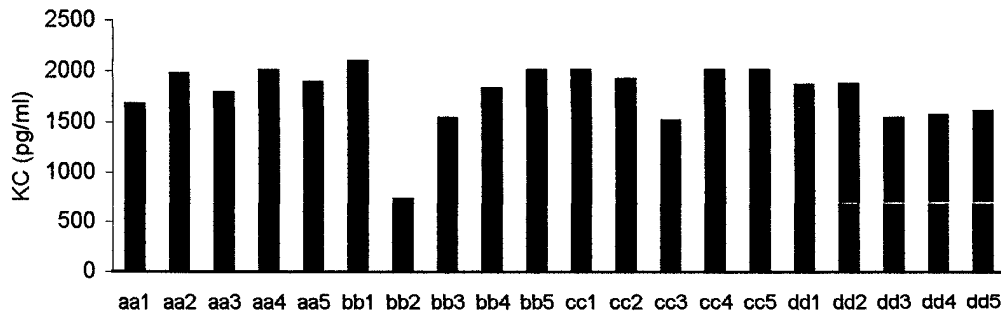

Figure 12. Kc expression in additional tumor clones. Twenty additional anti-sense KC clones were screened for KC expression. The clones were plated at $1x10^6$ cells/well in a 24 well plate and 24 hours later the supernatants were harvested and assayed for KC by ELISA.

Objective 2. Transduce and clone tumor cells that lack production of chemokines, months 10-18:

a. Package the retroviral construct by transfecting the PA317 packing cell line.
b. Collect, concentrate and titre the virions.
c. Transduce 4T1 (clones from Dr. Fox; collaborator) with each of the retroviral constructs.
d. Drug selection and reclone the transduced tumor cells.
e. Screen the transduced clones (4T1αRANTES, 4T1αMCP-1, 4T1αKC, 4T1αRANTES/MCP-1/KC) by RT-PCR for the presence of the antisense construct, and chemokine mRNA synthesis.
f. Screen the transduced clones by ELISA for chemokine protein synthesis.
g. Screen the supernatants from the transduced clones for T cell chemotactic ability. The chemotaxis assays will be performed three times with each clone that shows the lowest levels of chemokines by RT-PCR and ELISA. With three mice/experiment, including the controls, we will use 36 mice for this portion of the study. milestone #3-Tumor cells cloned with antisense transgenes.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning of KC, MCP-1 and RANTES gene fragments into a T vector.
- Construction of sense and anti-sense expression vectors for KC, MCP-1 and RANTES.
- Generation of stable sense and anti-sense RANTES expressing tumor cells, with similar in vitro and in vivo growth rates.
- Generation of stable sense and anti-sense MCP-1 expressing tumor cells, with similar in vitro and in vivo growth rates.

REPORTABLE OUTCOMES

1. Data generated from this project were presented at the 78th annual Pennsylvania Academy of Science (PAS) meeting. title: Blockade of the Tumor-Derived RANTES and the Impact on T-Cell Migration, Evan Adler and Robert A. Kurt, April 5-7, 2002.

2. Data generated from this project will be presented at the 2002 Department of Defense Era of Hope meeting. title: Inhibition of tumor-derived MCP-1 and anti-tumor immunity, Robert A. Kurt, Erin Allison, Mara Shainheit, and Peter Vitiello, Sept 25-28, 2002.

3. Data generated from this project will be used as preliminary data for an American Cancer Society grant that will be submitted October 2002 by Robert A. Kurt.

Conclusions

We have hypothesized that the constitutive expression of chemokines can impair anti-tumor immunity. In order to test this hypothesis a tumor cell line that does and does not express chemokines was necessary. Using anti-sense technology we were able block RANTES and MCP-1 expression from tumor cells that normally constitutively produce these chemokines. These data indicate that MCP-1 and RANTES are not required for tumor growth. Additionally, the tumor cell clones that were generated were stable and exhibited similar growth rates compared to the sense transfected clones and parental tumor cell line. Therefore, in vivo studies can be accomplished with these clones. A comparison of the immunogenicity of the clones is currently underway. The generation of a tumor cell clone that lacks KC expression has proven more difficult. Although we have generated clones with a ten-fold decrease in KC expression and a clone with undetectable KC expression, these clones were not stable and subsequent analysis revealed high levels of KC production. Studies are under way to examine the ability of a different promoter to drive the anti-sense KC expression and generate a stable tumor cell clone that lacks KC production. These tumor cells therefore give us the tools from which to evaluate whether blockade of tumor-derived chemokines will be of value in treating patients with cancer.

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