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Antigen-Independent Methods to Improve Radioimmunotherapy of Prostate Cancer

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Radioimmunolocalization of prostate cancer with radiolabeled antibodies is widely used in a clinic but radioimmunotherapy (RIT) fails to produce objective responses. Physiological barriers to the delivery of macromolecules to solid tumors are usually blamed for these failures. These studies are designed to improve the outcome of RIT in prostate adenocarcinoma by the inclusion of the antigen-independent peptides in the RIT protocol. To date two peptides able to modify vascular permeability were tested. Cytotoxicity studies indicate dose-dependent changes in cell metabolic activities after treatment with the C5aAP peptide; whereas peptide able to interact with a formyl peptide receptor-like 1 (FPRL1) does not seem to have any effect on the growth of these cells in vitro. In vivo results indicate that both peptides significantly augment RIT with 131I-CC49. Three xenografts were tested to date: LNCaP, PC3 and DU145. These xenografts do not show differences in the growth pattern between the untreated tumors and peptide only-treated tumors, but there is a considerable delay in the tumor growth when peptides are combined with 131I-CC49. The mechanism of this effect is more complicated than observed for LS174T tumors evaluated in the pilot studies and appears to vary depending on the tumor model and the peptide. The pattern of dependence on key two factors emerged from these studies: (1) the improved vascular permeability and (2) the generation of reactive oxygen species.

Prostate cancer, radioimmunotherapy, antibodies, vascular permeability, C5a agonists

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Introduction

The efficacy of radioimmunotherapy (RIT) of solid tumors depends on a number of factors related to the characteristics of antibody, radionuclide, and the tumor physiology such as the tumor heterogeneity, its size, the antigen distribution, radiosensitivity, microvessel perfusion, and vascular permeability. It is apparent that a success of radioimmunotherapy in solid tumors will require a combination of therapeutic strategies.

One widely studied approach involves the use of systematically administered vasoactive agents, such as angiotensin II (Kinuya et al., 2000), interleukin-2 (Hornick et al., 1999), alpha v beta 3 integrin receptor antagonist (Burke et al., 2000), and the C5a agonist peptides (Kurizaki et al., 2002). The complement derived inflammatory mediator C5a evokes broad anaphylactic and chemotactic responses, including increased vascular permeability, changes in adhesiveness, smooth muscle contraction, and chemotactic migration of a number of cells. These biological activities are mediated through its binding to the C5a receptor (C5aR; CD88). C5a-derived small agonist peptides (C5aAP) from C-terminal region behave as full agonist, but with reduced potency. One analog, YSFKPMPLaR, expressed between 2 and 10% of full C5a activity in increasing vascular permeability and it was stable in the presence of mouse and human serum carboxypeptidases. Our studies showed that combination RIT with C5aAP (YSFKPMPLaR) resulted in two- to five-fold better LS174T xenografts responses than RIT alone. This therapeutic improvement in colorectal adenocarcinoma model was primarily attributed to the agonist-induced increase in the tumor vascular permeability (Kurizaki et al., 2002). In the current study, the effects of C5aAP and other peptides able to change vascular permeability of tumor blood vessels of radioimmunotherapy of prostate cancer are evaluated.

Summary of the Statement of Work Progress

These studies were designed to evaluate the effect of antigen-independent peptides on the outcome of RIT in experimental models of the human prostate adenocarcinoma. In Objective 1: (determination of the uptake of \(^{125}\)I-labeled B72.3 monoclonal antibody in three human prostate adenocarcinoma models: PC-3, DU-145 and LNCaP in athymic mice) the following specific tasks are included:

1. In vitro culture of PC-3, DU-145, LNCaP for implantation into mice: \(\text{months 1 – 36}\)
   \(\text{In progress}\)
   This is an ongoing task. Because TAG-72 antigen is only expressed in tumors grown in vivo, a constant supply of large number of cultured cells for implantation into mice is needed.

2. Radiolabeling of B72.3 with iodine-131 and iodine-125 \(\text{months 1 – 36}\)
   \(\text{In progress}\)
   This is also an ongoing task. All biodistribution studies are done with iodine-125-labeled antibodies. For therapy studies we are using iodine-131 as the radioisotope.

3. Immunohistochemistry to determine TAG-72 expression \(\text{months 1 – 6}\)
   \(\text{Completed: reported in the progress for the first year of funding.}\)
   In addition to biodistribution studies, we have also evaluated the expression of the antigen in frozen tumors collected during the necropsy.
For Objective 2 (to measure the effect of C5aAP on the uptake of 125I-labeled B72.3 monoclonal antibody in a prostate cancer model selected in Aim 1 for the expression of TAG-72 antigen) the following specific tasks were planned:

1. **Synthesis of peptides**  
   *In progress*  
   This is an ongoing task in years 2 and 3 of this project. Initial studies were done with the previously used C5aAP analog. Currently a new peptide is evaluated in search of a more response-selective derivatives.

2. **Biodistribution of 125IB72.3 in tumor-bearing mice**  
   *In progress*  
   The biodistribution of the antibody is established in the selected tumor models. We are still also evaluating the effects of peptides dose and dosing schedule on the fate of radiolabeled antibodies. This is be done either via a terminal procedures, i.e., necropsy of tumor-bearing animals as well as via an MRI studies.

3. **MRI of vascular permeability in tumor-bearing mice**  
   *In progress*  
   The initial results on tumor perfusion and water content with and without contrast were reported in the progress report for year 1. Current studies are centered on the effect of peptides on tumor vessel permeability, blood flow and the transport of macromolecules. These are evaluated using contrast agents with different molecular weights such as DTPA conjugated to IgG or albumin and labeled with a mixture of 153Gd and non-radioactive Gd isotope. The development of data analyses of tumor vessel permeability is in progress.

The Objective 3 is primarily concerned with the therapeutic studies (To conduct experimental therapy studies with 131I-B72.3 RIT and in combination 131I-B72.3 with peptide in human prostate cancer model) and the following specific tasks are associated with this Objective:

1. **RIT of prostate cancer in tumor-bearing mice**  
   *In progress*  
   This is an ongoing task in years 2 and 3 of this project and overlaps with the next task 2 in this Objective.

2. **Biodistributions and termination of therapy protocols**  
   *In progress*  
   All therapy protocols are terminated when the tumor size in control groups reaches about 1g. We continue to evaluate antibody retention and tumor vascular changes in necropsied tumor samples. When possible, i.e., sufficient recovery of radiolabeled material in blood, a determination of the immunoreactivity of recovered radioactive species and levels of TAG-72 in treated and non-treated tumors is also performed.

In the final Objective 4 a comparison of the efficacy of 131I-labeled versus 90Y-antibodies in a peptidetic-augmented RIT protocol will be evaluated. These studies will begin in the next few months. The experimental evidence from carcinoma xenografts indicate that 90Y may be a superior choice as a therapeutic radioisotope. To date, there are no studies to confirm this in prostate cancer xenografts. There are also no studies on the effect of biological response modifiers, e.g., our C5aAP on the outcome of 90Y-RIT. We will investigate this question in the last three months.
of these studies. The end points will be the same as for $^{131}$I-RIT, i.e., tumor growth delay, VP changes and the general health of the treated animal.

**Results**

**In Vitro Studies**

Several reports indicate that the C5a complement may play an important role in apoptosis (Riedemann et al., 2002; Perianayagam et al., 2002). The likelihood that C5aAP has a direct effect on apoptosis of prostate carcinoma cells was investigated in vitro. Because C5AP binds to C5aR the expression of C5aR on the PC-3, DU-145 and LNCaP cells was evaluated by flow cytometry (the results of these experiments were detailed in the Progress Report for year 1). To measure the cytotoxicity, if any, of the C5aAP peptides on these cells, different concentrations of C5aAP were added to the growth medium and proliferative or cytotoxic effect were measured using either a clonogenic assay or the CellTiter 96® cell proliferation assay. The effects of C5aAP on the radiosensitivity of the prostate adenocarcinoma cells were also measured. Only at high concentrations of the C5aAP peptides, the fraction of metabolically active cells was diminished. For example, after a 48-h continuous exposure to C5aAP, approximately 30% of PC3 cells did not survive the treatment. However, after 120 hours the cells recovered and there were no detectable statistical differences between irradiated cells treated with C5aAP and cells “sham” treated with PBS. Combination of irradiation and C5aAP treatment does not seem to produce any synergistic or additive effects. To establish if C5aAP influence the cell cycle and apoptosis cells were seeded into 25 cm$^2$ flasks (5 x 10$^5$ cells/ flask) with different concentration of C5aAP. Then, 24 hrs after seeding, some flasks were irradiated at 1 Gy or 6 Gy. After 48 hrs to allow for repair/death, the cells were fixed with 70 % ethanol and stained with the Telford reagent overnight at 4°C to allow maximal intercalation of propidium iodide. The cells were analyzed by flow cytometry. There were statistical differences between cells that were irradiated and the ones that were not, however these changes were not dependent on the presence or absence of C5aAP in the growth medium. Also, there were not statistically differences in apoptotic fractions after C5aAP treatment.

Our experimental results indicate that the macromolecular extravasation induced by the C5aAP is neutrophil-dependent (see discussion below in the section on vivo studies). Other possible mechanisms attribute the macromolecular efflux to extracellular liberation of neutrophils-release products such as oxygen radicals that can undermine vascular integrity by direct or indirect actions on endothelial cells or other components of vascular walls, glycocalyx, basement membrane, etc. The production of reactive oxygen species by C5aAP interaction with neutrophils is of particular interest to RIT. The generation of superoxides of hydrogen peroxide may be responsible for the enhanced responses of tumors to RIT. Superoxide and its derived active oxygen species are believed to be responsible for the polymorphonuclear leukocyte (PMN)-mediated tumoricidal activity of the intraperitoneal OK-432, a biological response modifier (Yoshikawa et al., 1995). The role of C5aAP in the production of the hydrogen peroxide and other reactive species was studied in vitro in cancer cells and in vivo in blood cells.

The production of hydrogen peroxide was measured in the DCF assay by the oxidation of the nonfluorescent compound 2',7'-dichlorofluorescein (DCFH) to the fluorescent compound 2',7'-dichlorofluorescein (DCF). The nonpolar and nonfluorescent form of DCFH is 2',7'-
dichlorofluorescein diacetate (DCFH-DA). DCFH-DA readily diffuses across the cytoplasmic membrane of PMNs and is trapped inside the cell by the hydrolysis of acetyl groups by cytoplasmic carboxylases to the nonfluorescent and charged DCFH. Stimulation of the oxidative burst of DCFH-labeled phagocytes can be readily detected by flow cytometry. The advantage of this assay is the ability to evaluate PMNs in whole blood samples, thus avoiding the stimulatory effects often encountered in many PMN purification procedures.

EDTA-anticoagulated whole blood (0.5 ml) was obtained from mice treated with C5aAP 3 h before blood collection and from normal control mice injected with PBS also 3 h before blood collection. Each blood sample was analyzed separately and also blood collected from 3 mice in each group was pooled and the result compared to individual samples. Phorbol 12-myristate, 13-acetate (PMA) was used for ex vivo stimulation (positive controls). Red blood cell lysing buffer was added to each sample. White blood cells were pelleted by centrifugation at 1,000xg for 1 min and the cell pellet was washed with PBS and resuspended in 0.1 ml of PBS. An aliquot of 0.1 ml 10 μM DCFH-A was added to each tube, vortexed, and incubated for 15 min at 37°C at which time 0.7 ml of catalase solution was added. PMA working solution (0.1 mL of 1 μg/ml in PBS) was added to each positive control samples (PMA-stimulated). The “resting” samples received 0.1 mL PBS in place of PMA. The experimental samples were stimulated with the C5aAP peptide at 100 nM. All samples were incubated for 15 min at 37°C and evaluate samples for fluorescence by flow cytometry. A standard protocol for whole blood samples and one-parameter fluorescence histogram acquisition was used accumulating 5,000 – 10,000 events on either gated neutrophils or monocytes or both. Gating on lymphocytes also allowed the monitoring of any possible extracellular cross-feeding of cells with hydrogen peroxide produced by stimulated phagocytes that were not degraded by catalase. The control resting sample were evaluated first and the fluorescence cursor was set to less than 2%
positive cells. This cursor setting was used for the evaluation of all remaining samples. The mean channel fluorescence of the fluorescence histogram should be recorded for all samples (Figure 1). The in vivo effects of C5aAP on the production of hydrogen peroxide were apparently lost during the cell preparation (compare Fig. 1A and 1B). However, the PMA-stimulated PMNs obtained from normal (Fig. 1E) and C5aAP-treated mice (Fig. 1F) yielded at least 95% positive cells with high mean channel fluorescence values indicating high levels of hydrogen peroxide production. The ex vivo C5aAP-stimulation also produced H2O2 but at much lower levels than PMA (Fig. 1C and 1D). The data is summarized in Figure 2. The cytotoxic potential of neutrophil-release oxygen products prompts further studies to clarify the precise nature of the interactions between these products, cell injury and radiation. The evaluation of superoxide levels measurements in PMNs and tumor cells in vivo and in tumor cells grown in vitro in the presence of neutrophils is in progress.

In Vivo Studies
Mechanism: The studies on the mechanism of the vascular permeability changes caused by the C5aAP peptides are completed. The interactions of the C5aAP peptides with its receptors expressed on polymorphonuclear leukocytes and the role of these interactions on the expression of the inducible nitric oxide synthase as well as the role of cyclooxygenases in vascular permeability changes induced by C5a agonist peptides are discussed in detail in the manuscript included in the Appendix A of this Progress Report (Appendix A: Role of polymorphonuclear leukocytes, nitric oxide synthase, and cyclooxygenase in vascular permeability changes induced by C5a agonist peptides. Takashi Kurizaki, Michio Abe, Sam D. Sanderson, Charles A. Enke, Janina Baranowska-Kortylewicz. Mol. Cancer Ther. 2004; 3(1):1-7.).

Therapy: The effects of C5aAP on radioimmunotherapy of PC3 and DU145 xenographs was evaluated in a subcutaneous tumor model in athymic mice. In all radioimmunotherapy experiments, male athymic mice (nu/nu) obtained from either NIH or Charles River (Wilmington, MA) at 4-6 weeks of age, were injected subcutaneously on the back with 1 x 10^7 tumor cells (DU145) or 2 mm x 2 mm PC-3 tumor sections. Tumor growth was determined using caliper measurements of the long and short axis of each tumor and the tumor volume was calculated as follows: Volume = \( \pi \times (\text{mm, short axis})^2 \times (\text{mm, long axis})/6 \). Three to four weeks after the tumor implantation the mice were assigned into groups (n=10) in such a way as to give a similar tumor size distribution in all treatment groups. The average tumor volume was maintained in all groups at 400 mm^3 on the day of therapy. Control mice were injected IV with PBS; the treatment groups included mice treated with a single dose of 250 \( \mu\)Ci \(^{131}\)IICCC49; mice treated with 200 \( \mu\)g C5aAP peptide; and finally mice treated with a combination of 250 \( \mu\)Ci \(^{131}\)IICCC49 and 200 \( \mu\)g C5aAP peptide. The peptide was

![Figure 2. Mean fluorescence of 2',7'-dichlorodihydrofluorescein in polymorphonuclear cells isolated from blood either control mice (green bars) or C5aAP-treated mice (red bars) either resting or stimulated ex vivo with PMA or C5aAP.](image-url)
injected 3 hours before the $^{131}$I-ICC49 injection. Body weights and tumor sizes were monitored twice a week. Mice were observed until tumors exceeded 10% of the total body weight or until the tumors began to ulcerate through the skin or if mice lost 20% or more of their original weight, at that time the animals were removed from the group and euthanized. The survival fraction of each treatment group was evaluated according to the method of Kaplan and Meier. The survival curves were compared and p values were generated using the logrank test. The GraphPad Prism software (GraphPad Software Inc., San Diego, CA) was used for these analyses. The survival curves for PC3 tumors built based on the quadrupling of tumor volume are shown in Fig. 3 and in Table 1.

A similar treatment was applied to mice bearing DU145 xenografts. The data is summarized in Figure 4 below. Statistical analyses of the therapy data after treatment of DU145-bearing mice with either $^{131}$I-ICC49 alone or in combination with the C5aAP peptide is shown in Table 2. Statistically significant differences between various treatment and control groups emerge as soon as 14 days after the treatment and persist until the termination of the therapy experiments due to the uncontrolled growth of the untreated tumors. The comparison was made using an unpaired t-test with Welch correction for comparison of treatment groups with controls. The Kaplan-Meier analyses of these data gives results similar to these reported for PC3 tumor with $P < 0.02$ (analyses not shown). Of
Table 2. Summary of the Kaplan-Meier analyses of the tumor quadrupling times survival curves.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>131I-CC49</th>
<th>C5aAP</th>
<th>131I-CC49 + C5aAP</th>
<th>No treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaluated sample size</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Median survival</td>
<td>37 days</td>
<td>23 days</td>
<td>Not determined</td>
<td>23 days</td>
</tr>
</tbody>
</table>

Chi-square = 8.9448; Significance P = 0.0300

Table 1. Statistical analyses of the therapy data after treatment of DU145-bearing mice with either 131I-CC49 alone or in combination with the C5aAP peptide (P values). The comparison was made using an unpaired t-test with Welch correction for comparison of treatment groups with controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>day 0</th>
<th>day 5</th>
<th>day 7</th>
<th>day 9</th>
<th>day 12</th>
<th>day 14</th>
<th>day 16</th>
<th>day 19</th>
<th>day 21</th>
<th>day 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5aAP vs PBS</td>
<td>0.261</td>
<td>0.384</td>
<td>0.524</td>
<td>0.464</td>
<td>0.689</td>
<td>0.683</td>
<td>0.683</td>
<td>0.783</td>
<td>0.930</td>
<td>0.741</td>
</tr>
<tr>
<td>131I-CC49 vs PBS</td>
<td>0.695</td>
<td>0.595</td>
<td>0.980</td>
<td>0.668</td>
<td>0.471</td>
<td>0.192</td>
<td>0.137</td>
<td>0.347</td>
<td>0.427</td>
<td>0.475</td>
</tr>
<tr>
<td>131I-CC49+C5aAP vs PBS</td>
<td>0.949</td>
<td>0.315</td>
<td>0.346</td>
<td>0.141</td>
<td>0.061</td>
<td>0.016</td>
<td>0.017</td>
<td>0.031</td>
<td>0.024</td>
<td>0.074</td>
</tr>
</tbody>
</table>

131I-CC49 vs C5aAP | 0.318 | 0.744 | 0.433 | 0.741 | 0.773 | 0.367 | 0.264 | 0.438 | 0.310 | 0.185 |
| 131I-CC49+C5aAP vs C5aAP | 0.243 | 0.822 | 0.676 | 0.331 | 0.138 | 0.034 | 0.033 | 0.019 | 0.008 | 0.012 |

131I-CC49+C5aAP vs 131I-CC49 | 0.723 | 0.616 | 0.256 | 0.237 | 0.183 | 0.130 | 0.238 | 0.049 | 0.014 | 0.033 |
Figure 5. Surviving fraction of LNCaP cells (yellow squares) and LS174T cells (purple circles) after treatment with increasing concentrations of WKYMVm peptide.

Figure 6. LNCaP tumor growth curves after indicated treatments. $^{131}$IC49 (0.25 mCi) was administered IV on day 0. Symbols indicate averages, bars indicate standard errors; n = 10.
through the interaction with formyl peptide receptor-like 1 (FPRL1). The vascular permeability is also affected by this peptide indicating that the generation of the reactive oxygen species contributes to these changes; however, the downstream signaling pathways for this peptide are distinctly different than for C5aAP peptides. Neutrophils perform a critical role in innate immune responses, including extravasation from the peripheral blood stream, migration into an infected area, and the generation of reactive oxygen species such as superoxide (Baggiolini et al., 1993; Bokoch, 1995).

Further mechanistic studies on the chemotactic migration and superoxide generation in human neutrophils as well as the activation of the respiratory burst system are in progress in vitro and in tumor-bearing and normal mice. The data gathered to date is insufficient to derive any final conclusions. However, based on the therapy data it appears that the activation of neutrophils by two different ligands i.e., C5aAP peptides and WKYMVm can induce differential cellular signaling and unique functional consequences in human neutrophils. Experiments involving both types of peptides in combination with RIT are planned to begin in the next few months. The pilot therapy studies in LNCaP-bearing mice were completed and the data is shown in Figure 6. All mice received SSKI in their drinking water 3 days before the treatment with radiolabeled antibodies. Control mice also received SSKI to assure identical handling of animals. Radioiodinated $^{131}$IICC49 was administered at a concentration of 0.25 mCi/0.2 mL PBS/mouse. Mice receiving a combination of peptide and antibodies were injected with 0.1 mg WKYMVm peptide in mixed with the dose of 0.25 mCi $^{131}$IICC49 in 0.2 mL PBS. The control mice treated with only peptide received 0.2 mg peptide in 0.2 mL PBS. The arrest of tumor growth in a combination RIT + WKYMVm peptide group is remarkable. Three days after the treatment there is a statistically significant difference between the combination therapy group and the controls. The concern is the

Table 2. Statistical analyses of the therapy data after treatment of LNCaP-bearing mice with either $^{131}$IICC49 alone or in combination with the peptide WKYMVm. The comparison was made using an unpaired t-test with Welch correction for comparison of treatment groups ($^{131}$IICC49+WKYMVm) with controls ($^{131}$IICC49+PBS, WKYMVm alone).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>day 3</th>
<th>day 5</th>
<th>day 9</th>
<th>day 14</th>
<th>day 20</th>
<th>day 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{131}$IICC49+WKYMVm vs $^{131}$IICC49+PBS</td>
<td>0.178</td>
<td>0.110</td>
<td>0.006</td>
<td>0.039</td>
<td>0.008</td>
<td>0.006</td>
</tr>
<tr>
<td>$^{131}$IICC49+WKYMVm vs WKYMVm</td>
<td>0.017</td>
<td>0.008</td>
<td>0.009</td>
<td>0.034</td>
<td>0.053</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Figure 7. Changes in body weight in mice treated with WKYMVm peptide alone (red circles) or with $^{131}$IICC49 monoclonal antibody (0.25 mCi/mouse) and WKYMVm peptide (navy squares).
loss of the body weight (Figure 7) not observed previously with the C5a-based peptides. Mice do not appear to fully recover their weight even three weeks after the treatment. The underlying causes of the weight losses are at the moment unknown and will be further explored when the benefits of including this peptide in PC3 and DU145 models are confirmed. Additional studies that also include radiotherapy using the external beam irradiation combined with the peptide treatment to confirm the role of the reactive oxygen species are also planned.

**Key Research Accomplishments to Date**

- Beneficial effects of the C5aAP peptides inclusion in the radioimmunotherapy protocols were confirmed in three prostate adenocarcinoma models: LNCaP; DU145 and PC3.
- Determined the expression of C5a receptors and effect of a peptide agonist of human C5a complement on the metabolic activities of in vitro grown prostate adenocarcinoma cells.
- Functions of the C5aAP peptides in RIT and the basic mechanism of their action were established.
- The production of reactive oxygen species was identified as a possible additional factor in the improvement of the RIT results when combined with the C5aAP treatment.
- New peptide WKYMVM that increases vascular permeability and generates superoxide was used in a RIT protocol and was shown to greatly improve the outcome of RIT indicating that the synergy/additive effects observed with C5aAP may be at least partially a result of the interaction of radiation with in situ generated reactive oxygen species.
- Using noninvasive MRI techniques, determined the C5aAP-induced changes in tumor perfusion in the experimental model of human prostate adenocarcinoma. The MRI studies of the changes in the vascular permeability are in progress.

**Reportable Outcomes**


**Conclusion**

- The inclusion of C5aAP in RIT improves the outcome of RIT of the experimental prostate adenocarcinoma grown as a subcutaneous xenografts in athymic mice. Tumors treated with C5aAP and $^{131}$CC49 antibodies grows significantly slower that tumors treated with $^{131}$ICCC49.
- C5a Receptors are expressed on some prostate cancer cells and may have an effect on the tumor growth and response to RIT.
- Increased levels of tumor oxygenation in response to stimulation of neutrophils with peptides appear to radiosensitize tumors to radioimmunotherapy.

**Abbreviations:**

- **C5aAP** response-selective peptide agonist on the human C5a complement
- **CC49** second generation monoclonal antibody that recognizes TAG72 in most of human adenocarcinomas
- **B72.3** precursor monoclonal antibody of CC49
- **nM** concentration: nanomole/liter
References:


APPENDIX A

Role of polymorphonuclear leukocytes, nitric oxide synthase, and cyclooxygenase in vascular permeability changes induced by C5a agonist peptides

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Abstract
Tumor responses to radioimmunotherapy combined with peptide agonists of human C5a anaphylatoxin such as GCGYSFKPMPLaR (C5aAP) are several-fold better than responses to radioimmunotherapy alone. The enhanced tumor vascular permeability (VP) is the key factor responsible for this improvement. These studies were designed to identify the sequence of events leading to the improved extravasation of immunoglobulin in response to C5aAP. The VP changes were measured in mice after administration of C5aAP alongside of various mediators. The depletion of circulating polymorphonuclear neutrophils (PMNs) in mice abolished the C5aAP-induced VP increase. Blocking of P-selectin also returned VP to its basal levels after the C5aAP treatment, indicating that C5aAP-induced VP changes are initiated by interactions of C5aAP with PMNs. Aminoguanidine, an inducible nitric oxide synthase (NOS) inhibitor, given before C5aAP returned VP to control levels. N®-nitro-L-arginine methyl ester, a nonselective NOS inhibitor, had a marginal effect on the activity of C5aAP. Indomethacin, a nonselective cyclooxygenase inhibitor, suppressed C5aAP-induced increases in VP, whereas N-(2-cyclohexyloxy-4-nitrophenoxy)-methanesulfonamide, a selective cyclooxygenase-2 inhibitor, was active only at high doses. While C5aAP given i.p. did not alter tumor uptake of 125I-B72.3, the i.v. administration resulted in ~40% increase, confirming the prerequisite interaction of C5aAP with PMNs. The sequence leading to the increased VP appears to be initiated by the interaction of C5aAP with C5a receptor expressed on PMNs followed by binding to endothelial cells of blood vessels. The interaction with P-selectin is responsible for the initiation of the nitric oxide cascade as evidenced by inducible NOS activation. Additionally, prostaglandins are required for expression of the full magnitude of the C5aAP activities. (Mol Cancer Ther. 2004;3(1):1–7)

Introduction
C5a, a small activation fragment of the complement C5 protein induced by either classical or alternative pathway, is a potent proinflammatory mediator (1). It binds specifically to its receptor, C5a receptor (C5aR; CD88), from the superfamily of G protein-coupled receptors expressed on a variety of cells of myeloid and nonmyeloid origins (2, 3). On binding to CD88, C5a evokes anaphylactic and chemotactic (attractant) responses, which mediate contraction of smooth muscles, enhance vascular permeability (VP), and promote leukocyte functions such as directed chemotaxis, degranulation, mediator release, and production of superoxide anions. Of special interest to therapy of solid tumors is the ability of C5a to profoundly increase permeability of blood vessels (4), resulting in facilitated transport of macromolecular drugs into the solid tumor. However, systemic administration of C5a is contraindicated because of the possible adverse effects. A panel of C5a agonist peptides was developed to address some of these deficiencies. Based on the structure-activity study of the COOH-terminal domain of C5a, peptide agonists with varied C5aR affinities and diverse selectivity to cells expressing C5aR were synthesized (5).

One of the conformationally biased agonist peptides of human C5a, YSFKPMPLaR (C5aAP), has been reported to increase VP in the skin of guinea pig (6). Studies from our laboratories revealed that C5aAP and its GCG-modified analogue, GCGYSFKPMPLaR, improve the outcome of radioimmunotherapy (RIT) in the experimental human colorectal cancer xenografts in athymic mice by the induction of transient increases of VP (7). However, the mechanism of C5aAP-induced changes in VP and the events leading to the synergy between C5aAP and RIT are unclear. C5aAP alone has no effect on the tumor growth; therefore, the recruitment of proinflammatory cells into the tumor site is an unlikely reason for this augmented effect. Because C5a-primed polymorphonuclear neutrophils (PMNs) induce hyperpermeability and phosphorylation of adherens junction proteins in endothelial cells (8, 9) and in a similar manner C5aAP induces transient hypotension and neutropenia in rats (10), a hypothesis was put forth that C5aAP interactions with PMNs can initiate a series of events leading to the enhancement of VP. Additionally, an effort was made to identify mediators responsible for the amplification of this initial stimulus. Some of the known mechanisms of amplification include local activation of...
precursors for expanding proinflammatory cascades of plasma proteins, up-regulation by early inflammatory mediators of cell surface molecules that promote the recruitment of leukocytes (e.g., adhesion molecules), and rapid leukocyte-selective expression of inducible genes, the products of which are proinflammatory (e.g., cyclooxygenase (COX) 2, the first enzyme of arachidonic acid cascade, and nitric oxide (NO) synthase (NOS)). Inducible NOS (iNOS) has been detected in activated macrophages, PMNs, and endothelium. Moreover, NO and prostaglandins are known mediators of VP changes induced by lipopolysaccharide (11) and other VP factors (12). In these studies, the in vivo effect of C5aAP binding to C5aR expressed on PMNs, the interactions of thus primed PMNs with P-selectin, and the role of NOS and COX are considered.

Materials and Methods

Peptides, Antibodies, and Reagents

C5aAP was synthesized by a standard solid-phase method, purified, and characterized according to the previously described procedures (5). Human recombinant complement C5a peptide (rC5a) was purchased from Sigma Chemical Co. (St. Louis, MO). Human cloned C5aR was purchased from BioSignal (Montreal, Canada).

Mouse and rat IgG, N^G-nitro-l-arginine methyl ester (l-NAME), aminoguanidine hemisulfate salt (AG), and indomethacin were from Sigma. N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide (NS-398) was purchased from Biomol (Plymouth Meeting, PA). Antimouse granulocytes rat IgG2b (clone RB6-8C5) and rat antimouse P-selectin (CD62P) IgG1 (clone RB40.34) were from Leinco Technologies (St. Louis, MO) and Research Diagnostics (Flanders, NJ), respectively.

C5aR Binding Assay

Ten micrograms of rC5a were labeled with 1 mCi of Na^211 using the Iodo-Gen method and purified on a desalting column (Econo-Pac 10DG, Bio-Rad, Hercules CA) equilibrated with 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl (pH 7.4; PBS) at room temperature. Human cloned C5aR from Chinese hamster ovary (CHO) cells was incubated with 1211-I-rC5a at a final concentration of 0.05 mM NaCl. The radioactivity associated with filters was measured in the gamma counter (receptor-bound 1211-I-rC5a).

General Procedure for the Assessment of VP Changes

Athymic female mice, 4–6 weeks old, were used to measure cutaneous VP induced by C5aAP. Murine IgG was iodinated with Na^211 (specific activity ~2–3 mCi/mg) using the Iodo-Gen method and purified on a desalting column equilibrated with PBS. 1211-I-IgG was given i.v. via a tail vein with or without C5aAP in a total volume of 0.2 ml PBS. C5aAP doses were 20 mg/kg in the PMN depletion study and 5 mg/kg in all other studies. Thirty minutes after 1211-I-IgG administration, mice were euthanized, their blood and ears were collected and weighed, and their radioactive content was determined in a gamma counter.

PMN Depletion

Mice were treated i.p. with antigranulocyte monoclonal antibody RB6-8C5 (anti-Ly-6G) at a concentration of 0.2 mg/mouse (10 mg/kg) in 0.4 ml PBS 26 h before 1211-I-IgG administration. Control mice were treated with nonspecific rat IgG (instead of RB6-8C5) also at 0.2 mg/mouse in 0.4 ml PBS. Blood samples for leukocyte counting were taken from tail 2 h before 1211-I-IgG administration. Blood smears were stained with Wright stain for the differential counting.

Anti-P-selectin Pretreatment

Mice received i.v. 0.06 mg/mouse (3 mg/kg) doses of the antimouse P-selectin monoclonal antibody RB40.34 in 0.1 ml PBS 5 min before C5aAP and 1211-I-IgG administration. This time point was selected based on previous reports (13). Control mice were treated with nonspecific rat IgG (3 mg/kg in 0.1 ml PBS).

Mediators

To investigate the role of NOS in C5aAP-induced VP changes, two inhibitors of NOS were used: l-NAME, a nonselective, general NOS inhibitor (14, 15), and AG, a selective inhibitor of iNOS (16). Inhibitors were dissolved in PBS at 2 mg/ml l-NAME and 4 mg/ml AG. I.v. doses of 10 mg/kg l-NAME or 20 mg/kg AG both in 0.1 ml PBS were given via a tail vein 5 min before 1211-I-IgG administration. Mice were randomly divided into six groups treated as follows: (1) two control groups: sham injections of 0.1 ml PBS instead of inhibitor followed 5 min later by i.v. 1211-I-IgG either alone or in combination with 0.1 mg C5aAP; (2) two l-NAME groups: i.v. injection of l-NAME at the dose of 0.2 mg/mouse in 0.1 ml PBS followed 5 min later by 1211-I-IgG either alone or in combination with 0.1 mg C5aAP; and (3) two AG groups: i.v. dose of AG at 0.4 mg/mouse in 0.1 ml PBS followed 5 min later by 1211-I-IgG either alone or in combination with 0.1 mg C5aAP.

The involvement of COX in C5aAP-mediated changes of VP was probed using indomethacin, a nonselective COX inhibitor, and NS-398, a selective COX2 inhibitor (17). COX inhibitors were dissolved in 25% propylene glycol (PG) and given i.p. 35 min before 1211-I-IgG at a single dose of 0.1 mg/mouse (5 mg/kg) for indomethacin and at two levels of 0.002 mg/mouse (0.1 mg/kg) and 0.1 mg/mouse (5 mg/kg) for NS-398. All COX inhibitors were given in 0.2 ml 25% PG. Control mice were injected with vehicle alone using the same timing of events. Mice were divided into six groups as indicated for NOS inhibitors.

Tumor Uptake

Groups of mice with human colorectal adenocarcinoma LS174T xenografts received either an i.v. or an i.p. dose of C5aAP in 0.2 ml of 0.1% albumin in PBS. Three hours later, an i.v. or i.p. dose of 1211-I-B72.3, a monoclonal mouse
antibody specific to Tag-72, an antigen expressed by in vivo grown LS174T, in 0.1% albumin in PBS was given. Control mice were treated with 125I-B72.3 alone and a sham injection of PBS. Twenty-four hours later, mice were euthanized and necropsy was performed. Blood, lung, heart, spleen, liver, kidney, uterus, muscle, stomach, small intestine, large intestine, skin, and tumor were harvested. Radioactivity and weight of aforementioned tissues were determined.

### Statistical Analysis
All results are expressed as means ± SD unless otherwise specified. Statistical significance was determined using the unpaired, two-tailed Student’s t test.

### Results
#### C5aR Binding Assay
Affinities of C5aAP and rC5a to human cloned C5aR from CHO cells were measured in a competitive binding assay. Fig. 1 shows a typical binding profile. 125I-rC5a binding to C5aR is inhibited by C5aAP with IC50 of 1.67 ± 0.58 nM compared with 0.33 ± 0.10 nM for rC5a (0.05 > P > 0.02). C5aAP is ~5 times less effective in competing for the binding sites with 125I-rC5a than rC5a. Previous reports place this figure at ~0.2% in a binding assay conducted on isolated PMNs and peritoneal macrophages (18).

The cutaneous VP changes were measured in vivo using the uptake of 125I-IgG in skin of athymic mice and compared with the skin uptake of 125I-IgG in the absence of C5aAP as a control. The nonspecific scrambled version of the C5aAP peptide was not included as a control based on the results of structure-activity studies of a series of peptides derived from the C5a complement (6). These studies indicated that only peptides, which obey rigid structural requirements, can modify the VP. Moreover, VP changes have been shown to be dependent on the circulating C5aAP concentration (7), indicating that specific interactions of C5aAP with the receptor are required.

### Effect of PMN Depletion on C5aAP-induced VP
To confirm that the initial stimulus originates from the interaction of C5aAP with blood components, studies of the C5aAP action were conducted in PMN-depleted mice. Antigranulocyte monoclonal antibody RB6-8C5 (anti-Ly-6G), a rat antinmouse IgG2b, which selectively binds and depletes mouse neutrophils and eosinophils but not lymphocytes or macrophages, was used to deplete PMNs. This antibody after an i.p. dose of 0.2 mg/mouse produces within 24 h of administration severe peripheral neutropenia persisting for up to 96 h (19, 20). A dose of anti-PMN antibodies (10 mg/kg) was injected i.p. 26 h before the administration of C5aAP and 125I-IgG. A differential count of leukocytes in peripheral blood smears was 3879 ± 615/μl in control mice treated with nonspecific rat IgG (n = 8). This number in PMN-depleted mice (n = 8) treated with RB6-8C5 was 935 ± 442/μl (P < 0.001). Both counts were taken 24 h after i.p. administration of RB6-8C5. Two hours later, C5aAP and 125I-IgG were injected simultaneously into the tail vein and biodistribution was conducted 30 min later. Blood and skin (ears) were collected to measure cutaneous VP (Fig. 2). No changes were detected at a dose of 0.1 mg C5aAP/mouse (5 mg/kg) in either control or PMN-depleted mice (data not shown) almost certainly because the i.p. stimulation associated with the administration of anti-PMN antibodies and control rat IgG followed by the blood collection resulted in a proinflammatory reaction sufficient to mask the effect of a low dose of C5aAP. To distinguish this response from the C5aAP-induced VP changes, a higher dose of C5aAP (20 mg/kg) was used in this assay. PMN depletion had no effect on the basal level of VP; however, it resulted in a significant inhibition of C5aAP-induced VP increases (P < 0.05; Fig. 2). In control mice treated with nonspecific rat IgG in place of RB6-8C5 and 0.4 mg C5aAP, the cutaneous blood levels climbed to 64.8 ± 9.2 μl blood/g skin in mice. However, on PMN depletion with RB6-8C5 antibodies, cutaneous VP remained at normal levels of 44.4 ± 4.4 μl blood/g skin after C5aAP treatment, suggesting that the initial trigger required to induce VP changes is the interaction of C5aAP with circulating PMNs.

### Anti-P-selectin Pretreatment
Capture or tethering represents the first contact of PMN with the activated endothelium. P-selectin on endothelial cells is the primary adhesion molecule for capture and the initiation of rolling (21). Hence, the inquiry into the role of P-selectin in the C5aAP activity was the next step. The PMN-P-selectin interactions can be disrupted by an inhibition of or a competitive binding to P-selectin. Monoclonal antibody RB40.34 is a rat IgGl that can block binding of mouse P-selectin to its ligand P-selectin glycoprotein ligand-1 (PSGL-1) constitutively found on all leukocytes (13, 21, 22). i.v. dosing of anti-P-selectin monoclonal antibody RB40.34, 0.06 mg/mouse in 0.1 ml PBS (0.3 mg/kg), was followed 5 min later by an i.v. administration of 0.1 mg C5aAP and 125I-IgG. Two control groups received nonspecific rat IgG in place of

### Appendix
Blood/g skin in negative control (no CSaAP treatment), PMNs, with inhibitors of NOS. Two NOS inhibitors were tested: PA positive control was versus value for anti-P-selectin positive control (CSaAP treatment), and anti-P-selectin euthanized and blood and ears were collected. As shown mouse (positive control). Thirty minutes later, mice were...injection of PBS–6C5. Columns, mean (n = 4); bars, SD. * P < 0.05; ** P < 0.01.

**Figure 2.** Effect of PMN depletion on CSaAP-induced increases in the cutaneous VP. Mice received an i.p. dose of antigranulocyte monoclonal antibody RB6-8C5, rat antimouse IgG2b (10 mg/kg in 0.4 ml PBS). Twenty-six hours later, mice were treated i.v. via a tail vein injection with 125I-IgG with or without CSaAP (0.4 mg/mouse). Control mice received an i.p. dose of nonspecific rat IgG (10 mg/kg in 0.4 ml PBS) in place of RB6-8C5. Columns, mean (n = 4); bars, SD. *, P < 0.05; **, P < 0.01.

**Figure 3.** Results of antimouse P-selectin monoclonal antibody RB40.34 pretreatment on CSaAP-induced changes in cutaneous VP. Mice were treated i.v. with anti-P-selectin at a concentration of 3 mg/kg in 0.1 ml PBS 5 min before 125I-IgG administration. Columns, mean (n = 5); bars, SD. *, P < 0.01.

**Appendix**
Table 1. Effects of NOS inhibitors on C5aAP-stimulated VP changes

<table>
<thead>
<tr>
<th>Vascular leakage (μl blood/g skin)</th>
<th>Without C5aAP</th>
<th>Treated with C5aAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS control</td>
<td>41.0 ± 3.7</td>
<td>93.1 ± 28.9*</td>
</tr>
<tr>
<td>i-NAME (10 mg/kg)</td>
<td>48.7 ± 6.5</td>
<td>72.3 ± 23.8</td>
</tr>
<tr>
<td>AG (20 mg/kg)</td>
<td>41.1 ± 1.8</td>
<td>53.9 ± 13.1*</td>
</tr>
<tr>
<td>Note: Mice were treated i.v. with i-NAME (0.2 mg/mouse) or AG (0.4 mg/mouse) 5 min before [125]I-lgG administration. Each value represents the mean ± SD of four mice.</td>
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</table>

activity, but the activity of COX1, a constitutively expressed isoenzyme, is clearly necessary for the C5aAP-induced VP. It follows that there is a connection between COX1-catalyzed synthesis of prostaglandin and C5aAP biological activities, although the COX1 levels remain essentially unaffected by factors responsible for COX2 induction.

Table 2. Effects of COX inhibitors on C5aAP-induced VP changes

<table>
<thead>
<tr>
<th>Vascular leakage (μl blood/g skin)</th>
<th>Without C5aAP</th>
<th>Treated with C5aAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% PG (control)</td>
<td>38.8 ± 4.6</td>
<td>60.7 ± 12.4*</td>
</tr>
<tr>
<td>Indomethacin (5 mg/kg)</td>
<td>35.1 ± 2.7</td>
<td>45.9 ± 2.7*</td>
</tr>
<tr>
<td>NS-398 (0.1 mg/kg)</td>
<td>45.2 ± 4.8</td>
<td>49.2 ± 13.6</td>
</tr>
<tr>
<td>NS-398 (5 mg/kg)</td>
<td>ND</td>
<td>44.3 ± 2.1*</td>
</tr>
</tbody>
</table>

Note: Mice were treated i.p. with indomethacin or NS-398 35 min before [125]I-lgG administration. Each value represents the mean ± SD of four to six mice. *Significantly different from basal VP (P < 0.05). **Significantly different from controls treated with C5aAP (P < 0.05).

Figure 4. Tumor uptake of [125]I-B72.3 in LS174T-bearing athymic mice after either an i.v. or an i.p. treatment with a single dose of C5aAP. Mice were treated with a dose of C5aAP followed 3 h later by [125]I-B72.3. Biodistribution was conducted at 24 h post [125]I-B72.3. Columns, mean (n = 4); bars, SD.

Discussion

Monoclonal antibodies significantly improved the targeted delivery of therapeutic radioisotopes to tumors. However, gains in selectivity are strongly counteracted by problems related to the heterogeneity of structure and physiology of solid tumors resulting in minimal radiolabeled monoclonal antibody localization at the tumor site. The accretion of radiolabeled antibodies and consequently the radiation doses deposited in tumors rely on the tumor blood flow, the tumor vascular volume, and the VP of tumor vessels to macromolecules. Methods to transiently change tumor VP have been suggested as a means to increase access of RTT to tumors (24-26). This approach was substantiated in a recent study of the C5aAP-augmented RIT in a mouse model of human colorectal adenocarcinoma (7). The improved tumor responses were attributed to the improved penetration of RIT into the tumor after the C5aAP-induced transient increase of VP. The translation of this approach to the clinic requires a comprehensive knowledge of mechanisms involved in the generation of these VP changes. Because of the inherent variability of xenograft physiology, particularly in large tumors required for the assessment of the VP changes (e.g., compromised vascular structure, variable tumor vascular volumes, impaired local blood flow, variable sizes of the necrotic fraction, heterogeneous penetration of macromolecules into xenograft, etc.), the VP changes were measured in mouse skin (11, 12, 27).

The VP responses observed after C5aAP activation suggest that binding of C5aAP to the C5aR followed the magnification of this initial signal by endogenous, humoral, and cell-derived amplification systems that initiate the
production of secondary messengers. Based on our data, the first step in the activation process involves binding of C5aAP to C5aR expressed on PMNs or endothelial cells followed by the activation of iNOS. Concurrently, C5aAP-primed leukocytes express PSGL-1, sialyl Lewis X, or a closely related oligosaccharide (28, 29). Transient interactions between P-selectin and PSGL-1 allow leukocytes to roll along the endothelium, ultimately resulting in an enhanced VP (8, 13). It has been reported that antihistamine inhibits the C5aAP-induced VP increase in guinea pig skin (6) after intradermal injection of C5aAP. In this instance, the most likely course of events involved a local response at the level of dermal mast cells, which express functional C5aR, followed by the secretion of histamine. It is doubtful however that after a systemic administration of C5aAP, scarce circulating basophils, <1% of total leukocytes, are the paramount cell population contributing to VP changes inasmuch as the PMN depletion attenuates the C5aAP-induced hyperpermeability. The absence of C5aAP-stimulated VP changes in PMN-depleted mice after the P-selectin blockade indicates that the activation of PMNs is the most plausible pathway for the C5aAP-induced VP changes.

Parallel or alternative pathways to VP enhancements involve the expression of signal amplifying mediators. iNOS on activation may produce NO at the site of adhesion. It is unclear which cells are the principal source of NO (i.e., PMNs or endothelial) and at which point of the amplifying cascade NO becomes a predominant factor in VP changes. It is evident however that iNOS plays a significant role in the C5aAP-induced enhancement of VP (i.e., the inclusion of iNOS inhibitors in the treatment scheme abolishes all VP changes mediated by C5aAP). The existing published data are somewhat ambiguous in this context. For example, it is reported that C5a induces a dose-dependent vasodilation mediated by NO in the small intestine microvessels (30). Conversely, neutrophils exposed to C5a fail to show increases in intracellular cyclic GMP, an indicator of NO production (31). Therefore, other factors such as degranulation and release of chemical mediators such as histamine, serotonin, interleukin (IL)-1, IL-6, tumor necrosis factor, and IL-8 from mast cells, platelets, PMNs, or monocytes at the site of adhesion may also play a role in C5aAP signaling of VP modification.

The metabolic effects of C5aAP are also impaired by indomethacin, a prostanoid synthesis inhibitor. Studies on the involvement of prostanooids, histamine, and PMNs in rabbits (32) concluded that indomethacin does not alter the C5a-induced neutrophilia but normalizes plasma prostanoind levels. C5aAP effects on VP are largely abolished by indomethacin, also indicating that COX products play an important role in the C5aAP-induced VP changes. However, in these studies, the coadministration of C5aAP with prostaglandin E2 (data not shown) had no measurable effect on VP. The role of COX is further complicated by the apparent resistance of C5aAP-stimulated VP increases to COX2 inhibition, suggesting that C5aAP does not regulate COX2 expression.

In conclusion, the C5aAP-induced VP increases appear to originate from the binding of this peptide to C5aR, activation of PMNs amplified by two apparently independent signals: an increased synthesis of iNOS and COX metabolic products. The net effect is an improved uptake of radiolabeled antibodies into the tumor mass, increased radiation doses, and thereby improved tumor responses to RIT.

References

Appendix