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 two- to four-fold better, depending on the dose of C5aAP, 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 (PMN) 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*$^{\omega}$-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-nitrophenyl)-methanesulfonamide, a selective cyclooxygenase-2 inhibitor, was active only at high doses. While C5aAP given i.p. did not alter tumor uptake of ^{125}I-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):85–91]

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

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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 2 (COX), 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^{ω}-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 $Na^{125}I$ 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 ^{125}I-rC5a at a final concentration of 0.05 nM and various concentrations of unlabeled ligands for 60 min at room temperature in the incubation buffer composed of 25 mM HEPES (pH 7.4) with 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2% BSA. The incubation mixture was filtered through GF/C filter (Whatman, England) presoaked in 0.3% polyethyleneimine in the incubation buffer. Filters were washed 9 times with ice-cold 10 mM HEPES (pH 7.4), 0.5 M NaCl. The radioactivity associated with filters was measured in the gamma counter (receptor-bound ^{125}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 $Na^{125}I$ (specific activity ~2–3 mCi/mg) using the Iodo-Gen method and purified on a desalting column equilibrated with PBS. ^{125}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 ^{125}I-IgG administration, mice were euthanized, 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 ^{125}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 ^{125}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 ^{125}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. Intravenous 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 ^{125}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. ^{125}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 i.v. ^{125}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 i.v. ^{125}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 ^{125}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 ^{125}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 ^{125}I-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. ^{125}I-rC5a binding to C5aR is inhibited by C5aAP with IC_{50} 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 ^{125}I-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 ^{125}I-IgG in skin of athymic mice and compared with the skin uptake of ^{125}I-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 indicted 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.

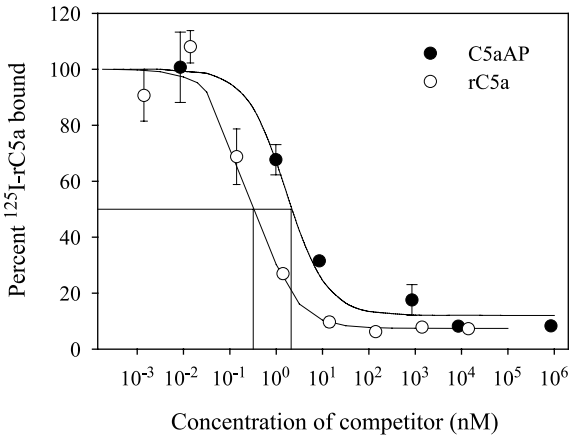

Figure 1. Competition binding profile of human rC5a, ^{125}I-rC5a, to the cloned human C5aR from CHO cells in the presence of increasing concentrations of rC5a (*open circles*) and C5aAP (*closed circles*). *Points,* mean of duplicate determinations; *bars,* SD.

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 antimouse 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 ^{125}I-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 ^{125}I-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 IgG1 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). Intravenous 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 ^{125}I-IgG. Two control groups received nonspecific rat IgG

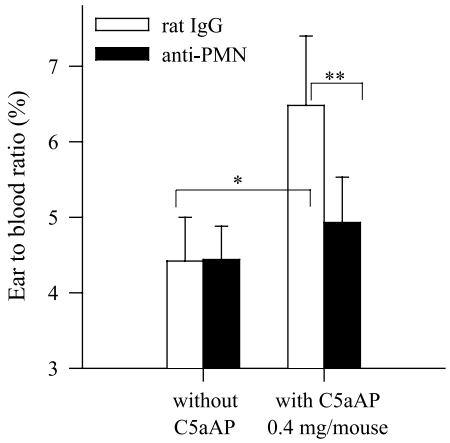

Figure 2. Effect of PMN depletion on C5aAP-induced increases in the cutaneous VP. Mice received an i.p. dose of antigranulocyte monoclonal antibody RB6-8C5, rat antimouse IgG2b (10 mg/kg in 0.4 ml PBS). Twenty-six hours later, mice were treated i.v. via a tail vein injection with ^{125}I-IgG with or without C5aAP (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. *, $P < 0.05$; **, $P < 0.01$.

in place of RB40.34 (0.06 mg/mouse) followed by either sham i.v. injection of PBS (negative control) or 0.1 mg C5aAP/mouse (positive control). Thirty minutes later, mice were euthanized and blood and ears were collected. As shown in Fig. 3, anti-P-selectin significantly inhibited C5aAP effects on VP ($P < 0.01$), indicating that the blockade of PMN rolling to the endothelial cells prevents C5aAP-induced VP increases. The cutaneous VP was 40.7± 4.6 (n = 5), 64.9 ± 13.3 (n = 5), and 42.7 ± 4.2 (n = 6) μl blood/g skin in negative control (no C5aAP treatment), positive control (C5aAP treatment), and anti-P-selectin mice (anti-PMN antibodies treatment), respectively. The P value for anti-P-selectin *versus* a positive control was $0.01 > P > 0.001$ and the P value for anti-P-selectin *versus* a negative control $P > 0.2$.

Mediators

Two potential mediators of VP were evaluated in these studies: NO and prostaglandins. To examine the role of NO, a chemical messenger produced by a family of NOS, the cutaneous VP was measured in mice treated with inhibitors of NOS. Two NOS inhibitors were tested: L-NAME, a competitive, nonselective inhibitor of all NOS (14, 15), and AG, which selectively inhibits iNOS with IC_{50} = 250 μM for iNOS and IC_{50} = 526 μM for constitutive NOS (16). Mice were treated i.v. with selected NOS inhibitors 5 min before the administration of ^{125}I-IgG alone or in combination with C5aAP. Changes in VP were assessed 30 min after administration of the radioactive tracer. The regulation of NOS activity with L-NAME had only a marginal effect on basal VP in a control group with the average increase registered at 6 μl blood/g skin ($0.1 > P > 0.05$). Similarly, in the presence of AG, basal levels of cutaneous VP remained basically unaffected (Table 1). When C5aAP was included in the treatment scheme, L-NAME, a rapidly reversible inhibitor of iNOS (15), had no effect on VP, whereas AG, a selective iNOS inhibitor, abolished C5aAP activity and returned VP nearly to control levels ($P < 0.05$), giving a clear indication that iNOS plays a significant role in the C5aAP activities.

The synthesis of prostaglandins, important mediators of VP, is regulated by COX1 and COX2. COX1 is expressed constitutively and is present in a wide variety of cell types where it influences the physiological functions of prostaglandins, whereas COX2 is an inducible enzyme involved in those aspects of the inflammatory process that are mediated by prostaglandins. The role of these two enzymes in the C5aAP-induced VP changes was evaluated via the use of nonspecific and specific COX inhibitors in combination with C5aAP. Because the i.p. administration was required for both inhibitors, the experimental design was modified to produce a steady-state circulating concentration of the systemic inhibitor. All injections were given 35 min before the i.v. administration of ^{125}I-IgG alone or in combination with 0.1 mg C5aAP. The net effect of C5aAP in control mice was lesser after i.p. than i.v. administration of inhibitors (Table 2), indicating again that a peritoneal stimulation masks to some degree the effects of C5aAP. Pretreatment with indomethacin (5 mg/kg), a nonselective COX inhibitor with IC_{50} = 0.74 μM for COX1 and 0.97 μM for COX2 (17, 23), had no effect on the basal level of VP but abolished VP gains induced by C5aAP ($P < 0.05$). In contrast, the effect of NS-398, a selective COX2 inhibitor (IC_{50} = 1.77 μM for COX2 and 75 μM for COX1), was equivocal. At low dose, NS-398 influenced neither the basal level nor the C5aAP-induced increase in VP. At the higher dose expected to produce steady-state levels of the inhibitor well within the range of COX1 inhibition, the normally observed C5aAP-induced VP increases were reduced to levels practically corresponding to control values. Although peritoneal stimulation appeared to interfere with COX-related VP effects, there is strong evidence that inducible COX2 is probably not involved in C5aAP activity, but the activity of COX1, a constitutively

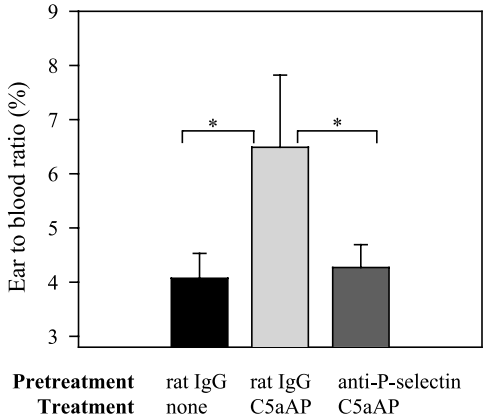

Figure 3. Results of antimouse P-selectin monoclonal antibody RB40.34 pretreatment on C5aAP-induced changes in cutaneous VP. Mice were treated i.v. with anti-P-selectin at a concentration of 3 mg/kg in 0.1 ml PBS 5 min before ^{125}I-IgG administration. *Columns,* mean (n = 5); *bars,* SD. *, $P < 0.01$.

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

	Vascular leakage (μl blood/g skin)	
	Without C5aAP	Treated with C5aAP
PBS control	41.0 ± 3.7	93.1 ± 28.9[a]
L-NAME (10 mg/kg)	48.7 ± 6.5	72.3 ± 23.8
AG (20 mg/kg)	41.1 ± 1.8	53.9 ± 13.1[b]

Note: Mice were treated i.v. with L-NAME (0.2 mg/mouse) or AG (0.4 mg/mouse) 5 min before ^{125}I-IgG administration. Each value represents the mean ± SD of four mice.

expressed isoform, 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.

Tumor Uptake

The site of injection of C5aAP (*i.e.*, a tail veil for i.v. administrations and i.p. injections) had a marked affect on the tumor uptake of ^{125}I-B72.3 and allowed to attribute, at least partially, the overall effect of C5aAP to its initial interaction with blood components. Fig. 4 shows the results of biodistribution conducted 24 h after administration of ^{125}I-B72.3 in mice bearing s.c. human colorectal adenocarcinoma. C5aAP given i.p. had no effect on tumor uptake: ^{125}I-B72.3 dose accumulated in LS174T tumors was 11.93 ± 0.68% of injected dose/g (%ID/g) in control mice treated with an i.v. dose of ^{125}I-B72.3 compared with 11.39 ± 0.52%ID/g and 11.69 ± 1.70%ID/g in mice treated with an i.v. dose of ^{125}I-B72.3 in combination with an i.p. dose of C5aAP (5 mg/kg) and with both drugs given i.p., respectively ($P > 0.2$). When C5aAP was given via a tail vein as an i.v. dose, the uptake into the tumor increased by ~40% regardless of the route of injection of ^{125}I-B72.3 and was nearly identical for i.v. (15.98 ± 0.21%ID/g) *versus* i.p. (15.05 ± 0.68%ID/g) administration of ^{125}I-B72.3. Similar to cutaneous VP changes, the full effect of C5aAP on tumor's VP appears to require an initial interaction of C5aAP with PMNs.

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

	Vascular leakage (μl blood/g skin)	
	Without C5aAP	Treated with C5aAP
25% PG (control)	38.8 ± 4.6	60.7 ± 12.4[a]
Indomethacin (5 mg/kg)	35.1 ± 2.7	45.9 ± 2.7[b]
NS-398 (0.1 mg/kg)	45.2 ± 4.8	49.2 ± 13.6
NS-398 (5 mg/kg)	ND	44.3 ± 2.1[b]

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

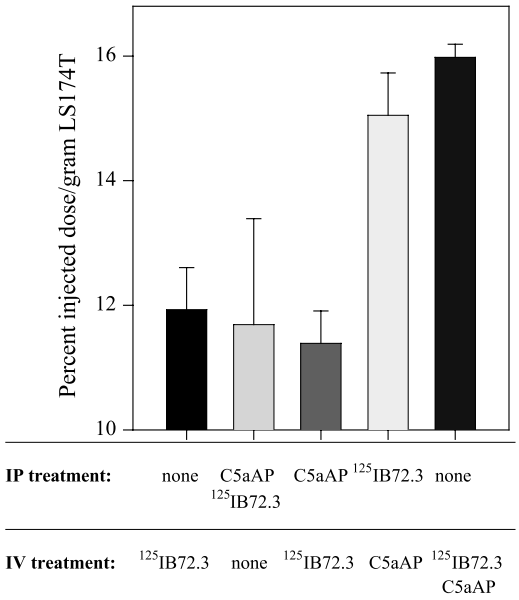

Figure 4. Tumor uptake of ^{125}I-B72.3 in LS174T-bearing athymic mice after either an i.v. or an i.p. treatment with a single dose of C5aAP. Mice were treated with a dose of C5aAP followed 3 h later by ^{125}I-B72.3. Biodistribution was conducted at 24 h post-^{125}I-B72.3. *Columns,* mean ($n = 4$); *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 RIT 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. Inducible NOS 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 prostanoids, histamine, and PMNs in rabbits (32) concluded that indomethacin does not alter the C5a-induced neutropenia but normalizes plasma prostanoid 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.

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