New ways in RGD-peptide mediated drug targeting to angiogenic endothelium

Temming, Kai

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2007

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
In vivo evaluation of RGD-albumin
constructs as carriers for small
molecule drugs

Kai Temming\textsuperscript{1,2}, Annemiek van Loenen-Weemaes\textsuperscript{1},
Johan R. de Jong\textsuperscript{3}, Leonie Beljaars\textsuperscript{1}, Grietje Molema\textsuperscript{4},
Klaas Poelstra\textsuperscript{1}, Robbert J. Kok\textsuperscript{1,5}

\textsuperscript{1} Department of Pharmacokinetics & Drug Delivery, Groningen University Institute of
Drug Exploration, The Netherlands
\textsuperscript{2} KREATECH Biotechnology B.V., Amsterdam, The Netherlands
\textsuperscript{3} Department of Nuclear Medicine and Molecular Imaging, University Medical Center
Groningen, University of Groningen, The Netherlands
\textsuperscript{4} Medical Biology Section, Department of Pathology and Laboratory Medicine,
University Medical Center Groningen, University of Groningen, The Netherlands
\textsuperscript{5} Present affiliation: Department of Pharmaceutics, Utrecht University, The Netherlands
Introduction

Specific delivery of therapeutic entities to tumor endothelium and to tumor cells is considered an attractive strategy to battle cancer without the typical side effects of conventional cancer therapy. Cyclic RGD-peptides are popular targeting ligands for such tumor specific drug delivery [1]. This peptide binds to \(\alpha_v\beta_3\)-integrin the expression of which is upregulated on angiogenic endothelial cells as well as on several tumor cells. The restricted expression profile spurred the development of RGD-peptide based drug targeting conjugates for cell specific delivery of small molecule drugs, protein pharmaceuticals, nucleic acids, and imaging agents [1]. Here, we investigate the effect of drug-carrying RGD-albumin constructs on growth of subcutaneous tumors in mice.

We previously demonstrated the importance of multivalent interactions with the target receptor for high affinity binding and efficient uptake of drug targeting conjugates [2, 3]. Therefore, we modified human serum albumin with multiple RGD-peptides to form a biodegradable, biocompatible carrier with high \(\alpha_v\beta_3\)-specificity that can be readily modified with drugs. These RGD-albumins were harnessed with different drugs using two innovative reversible linkers. The Universal Linkage System (ULS) was applied to couple PTK787, a VEGF-R kinase inhibitor. This drug contains none of the reactive chemical groups commonly used for covalent conjugation, like e.g. carboxyl-, amino-, or sulfhydryl-groups, which demanded a new coupling strategy [4]. ULS, a platinum(II)-based linker, enabled coupling of PTK787 to HSA through a coordination linkage at one of the aromatic nitrogen atoms in the drug and subsequent attachment to methionine or histidine residues in albumin. Auristatin, an antimitotic apoptosis-inducing compound, was conjugated to albumin using a valine-citrulline linker [5, 6]. This val-cit-linker has widely been used for preparation of antibody-drug conjugates and demonstrated high stability in the circulation but is readily cleaved in the lysosomes of the target cells [7, 8]. The use of polyethylene glycol as a separate moiety or as a linker for RGD-peptides furthermore allowed fine-tuning of these drug targeting conjugates for pharmaceutical and pharmacokinetic properties. The detailed structures and constituents are described in Figure 1 and Table 1. The final drug targeting conjugates proved to bind to and to be internalized by \(\alpha_v\beta_3\)-integrin expressing cells specifically via the introduced RGD moiety. Furthermore, we demonstrated down-regulation of VEGF-induced gene expression by targeted PTK787, and killing of target cells by targeted MMAE and MMAF at low nanomolar concentration of the carrier [4-6]. In vivo we expected inhibition of angiogenesis by targeted PTK787 due to a blockade of the VEGF signaling pathway in tumor endothelial cells. Targeted auristatins shall kill these endothelial cells, induce blood coagulation, and thereby clogging of tumor blood vessels. The tumor cells which surround these clogged vessels will perish due to deprivation from oxygen and nutrients.

The promising results in vitro prompted us to evaluate the tumor growth inhibitory efficacy of these drug targeting conjugates in vivo in mice, bearing a subcutaneous C26 colon carcinoma. Multiple doses were administered to achieve adequate intracellular drug levels over prolonged period of time. Furthermore, we investigated pharmacokinetics and biodistribution using \(^{89}\text{Zr}\)-labeled RGD-albumin for positron emission tomography (PET) in tumor bearing mice.
Table 1.: Chemical characteristics of the in vivo tested drug targeting conjugates

<table>
<thead>
<tr>
<th>Drug Targeting Conjugate</th>
<th>Molecular weight in kDa</th>
<th>Number of RGD / HSA</th>
<th>Number of PEG / HSA</th>
<th>Number of drugs / HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTK-HSA</td>
<td>69.9</td>
<td>n.a.</td>
<td>n.a.</td>
<td>5.3</td>
</tr>
<tr>
<td>RGD-PTK-HSA</td>
<td>78</td>
<td>12</td>
<td>n.a.</td>
<td>4.4</td>
</tr>
<tr>
<td>RGD-PTK-HSA-PEG</td>
<td>93.7</td>
<td>12</td>
<td>2.5</td>
<td>6.6</td>
</tr>
<tr>
<td>RGDPEG-PTK-HSA</td>
<td>100.6</td>
<td>7</td>
<td>7</td>
<td>7.4</td>
</tr>
<tr>
<td>RGD-MMAF-HSA</td>
<td>78.3</td>
<td>8.8</td>
<td>n.a.</td>
<td>4.2</td>
</tr>
<tr>
<td>RGD-HSA</td>
<td>75.2</td>
<td>12</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>$^{89}$Zr-RGD-HSA</td>
<td>75.5</td>
<td>12</td>
<td>n.a.</td>
<td>~ 1</td>
</tr>
</tbody>
</table>

n.a.: not applicable

Materials and Methods

**Synthesis of drug targeting conjugates**

RGD-HSA, RGD-PTK-HSA, RGD-PTK-HSA-PEG, RGDPEG-PTK-HSA, and RGD-MMAF-HSA were synthesized and characterized as described previously [4, 6].

**In vivo efficacy of RGD-equipped albumins modified with PTK787 or auristatin**

**F in tumor bearing mice**

Male Balb/c mice (Harlan, The Netherlands) were housed under a 12 hour dark/light cycle, at constant humidity and temperature. Animals had free access to tap water and standard lab chow. All experiments were performed according to the national law on animal experiments and were approved by the Animal Ethics Committee of the University of Groningen. Mice were anesthetized with Forene (Abbot B.V., Hofddorp, The Netherlands) inhalation in combination with $N_2O$ (600 mL/min) and $O_2$ (300 mL/min) for injections. Tumor growth was induced by s.c. injection of $0.5 \times 10^6$ C26 murine carcinoma cells on the flank of the mice and allowed to grow to a palpable size, which occurred for at day 6 after inoculation of tumor cells. The treatment was initiated at day 7 with the first administration. For this, mice were divided into five groups (n=5) for treatment with PBS, PTK-HSA, RGD-PTK-HSA, RGD-PTK-HSA-PEG or RGDPEG-PTK-HSA. Drug targeting conjugates were administered at an equimolar dose of 5 nmol PTK787 equaling approximately 50 μg of albumin. Treatments were repeated at day 9, 11, 13, and 15. At the same time points, tumor size was measured using a digital caliper, and body weight and condition of mice were determined. Tumor volume was calculated as $0.52 \times \text{width}^2 \times \text{length}$ [mm$^3$]. Animals were sacrificed 24 hour after the last treatment. Tumor, liver, kidney, spleen, lung, and heart were retrieved for immunohistochemical evaluation and snap frozen in liquid nitrogen, and blood and plasma were harvested as well.
The treatment protocol for evaluation of RGD-albumin conjugates equipped with auristatin F was similar. Three groups of mice were treated with PBS \( (n=5) \), RGD-MMAF-HSA \( (n=6) \), and RGD-HSA \( (n=5) \). The conjugates were injected at an equimolar dose of 3.1 nmol MMAF equaling 50 μg of albumin.

1. In vivo efficacy study for PTK787 containing conjugates

![PTK-HSA RGD-PTK-HSA-PEG](image1)

![RGD-PTK-HSA RGDPEG-PTK-HSA](image2)

2. In vivo efficacy study for auristatin F (MMAF) containing conjugates

![RGD-MMAF-HSA RGD-HSA](image3)

3. PET imaging pilot study

![89Zr-RGD-HSA](image4)

**Figure 1.** Schematic representation of drug targeting conjugates tested.

*Serum and blood analysis*

Blood was analyzed for the number of white blood cells, red blood cells, and platelets, using a Microcellcounter F-800 (Sysmex-Europe, Hamburg, Germany). Serum of mice was analyzed for lactate dehydrogenase (LDH) and aspartate aminotransferase (ASAT). Immunogenicity of repeatedly dosed drug targeting conjugates was determined by analysis of antibody content against RGD-HSA and HSA in serum of mice. For this, 96-well microtiter plates (Costar) were coated for 2 h with RGD-HSA or HSA \( (1 \, \mu g/well) \), washed three times with washing buffer \( (50 \, mM \text{ Tris/HCl at pH 8, 150 mM NaCl, 0.05 % Tween 50}) \) and blocked 1 h with 3 % BSA in incubation buffer \( (50 \, mM \text{ Tris/HCl}) \).
RGD-HSA carriers in vivo

at pH 8, 0.05 % TWEEN 50). Plates were washed three times with washing buffer and incubated with 100, 300, 900, and 2700-times dilution of serum in incubation buffer for 1 h. After washing, a rabbit anti-mouse-peroxidase conjugate (DAKO) was for 1 h incubated (1:1000 dilution). After the last washing step, o-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, USA), a peroxidase substrate, was incubated for 10 min and the reaction was stopped by addition of 100 μl of 1 M H₂SO₄. Substrate conversion was measured at 490 nm using a plate reader.

**Analysis of organs**

Spleens of all animals were weighed after excision. Liver, spleen, kidney and lung were stained immunohistochemically for leukocytes by detection of CD45 expression. For this, cryostat sections (4 μm) of these organs were fixed in acetone, rehydrated and incubated with 10 % normal rabbit serum in PBS followed by 45 min of incubation with antiCD45 rat anti mouse immunoglobulin (BD Bioscience, Alphen aan den Rijn, The Netherlands). After removal of endogenous peroxidase, the staining was amplified using a peroxidase conjugated rabbit anti rat immunoglobulin (DAKO) and visualized by incubation with 3-amino-9-ethylcarbazole (AEC, Sigma). In livers, leukocyte infiltration was quantified by counting of eight to ten fields of view at 200x magnification.

**PET-study**

For PET-imaging, RGD-HSA was modified with the chelator desferal that is able to capture ⁸⁹Zirconium (⁸⁹Zr), a positron emitter with a half live of 3.5 days. Conjugation and ⁸⁹Zr-incorporation was described elsewhere [9, 10]. Radiochemical purity was determined to be 97 % by trichloroacetic acid precipitation, and specific activity was 200 MBq/mg. Two mice bearing a 100 mm³ C26 tumors (s.c.) were injected with 18 μg (3.6 MBq) of ⁸⁹Zr-RGD-HSA dissolved in PBS. A first blood sample was drawn by orbitapunction at 5 min after injection. Additional blood samples were drawn before or after scans, when the mice were still under anesthesia. Both mice were scanned simultaneously under anesthesia in one bed position in a Siemens microPET Focus 220 for 30 min. Scans were acquired 2 h, 24 h, and 48 h after injection. Each time a transmission scan with a germanium-68 point source was performed before or after the emission scan. All scans were normalized and corrected for random scatter, attenuation, and decay according to standard procedures. After the last scan (49 h) the mice were sacrificed, and organs and tissues were harvested and weighed. Radioactivity in blood samples, organs and tissues was counted on a 1282 COMPUGAMMA CS (Perkin-Elmer, Boston, MA). Based on the amount of radioactivity in the blood and the administered dosages, we calculated the concentration of drug targeting conjugate present in the blood. Pharmacokinetic parameters were derived from data of individual animals, using the non-linear curve fitting program Multifit (Dr. J.H. Proost, Department of Pharmacokinetics and Drug Delivery, University of Groningen, The Netherlands).

**Statistics**

Statistical analysis was performed using Student’s two-tailed t-test assuming equal variances. Differences were considered to be significant when p < 0.05 unless otherwise stated.
Figure 2. Elevated ASAT levels and reduced white blood cells (WBC) counts were found in mice that suffered an anaphylactic shock. Similar findings were made in RGD-MMAE-HSA treated mice. (A) ASAT levels in serum and (B) WBC counts in blood of mice treated with PTK787 containing drug targeting conjugates. (C) LDH and (D) ASAT levels in serum of RGD-MMAF-HSA treated mice and (E) white blood cell counts in blood of mice treated with RGD-MMAF-HSA or RGD-HSA. Increase in ASAT levels and decrease in white blood cells are evident for the RGD-PTK-HSA-PEG and the RGD-MMAF-HSA treated group. Bars and error bars represent mean with standard deviation.

*: Anaphylactic shock made withdrawal of sufficient blood difficult or even impossible in RGD-HSA, RGD-PTK-HSA, and RGD-PTK-HSA-PEG treated mice.

**: p < 0.01

Results

Effects of RGD-equipped albumin conjugates with PTK787 or MMAF in tumor bearing mice.

Tumor growth inhibitory efficacy of drug targeting conjugates was evaluated in a s.c. C26 colon carcinoma model in balb/c mice. One day after the tumors became palpable, the treatment was started with administration of different PTK787 containing drug targeting conjugates every other day for 10 days. Upon the fifth injection (day 15), several unexpected observations were made in the groups of mice treated with RGD-PTK-HSA and RGD-PTK-HSA-PEG. The mice recovered from the inhalation anesthesia during injection, but after 15 min their movement slowed down. The extremities turned cold and blue, tachycardia and hyperpnoea were observed. Animals of these two groups were
RGD-HSA carriers in vivo

immediately anesthetized and sacrificed to prevent further suffering. Blood retrieval by heart puncture was difficult in comparison to healthy mice, suggesting that animals suffered from hypotensive shock-like syndrome. Consequently, blood and serum analysis was impossible for 7 out of 10 mice in these two groups. Thus for analysis of ASAT, white blood cell count and IgG formation (ELISA) only samples of two respectively three mice of the RGD-PTK-HSA-PEG group were available and no samples were available for RGD-PTK-HSA treated mice.

Figure 3. Antibodies against RGD-HSA were formed after five consecutive injections of drug targeting conjugate. (A,C) RGD-HSA coated ELISA plates were used to determine formation of IgG against RGD-HSA in the serum of treated mice. (B,D) Formation of IgG against HSA was determined using HSA coated plates. The antibody formation against PTK787 containing drug targeting conjugates is shown in (A) and (B), while data for auristatin containing drug targeting conjugates are shown in (C) and (D).

*: Anaphylactic shock made withdrawal of sufficient blood difficult or even impossible in RGD-HSA, RGD-PTK-HSA, and RGD-PTK-HSA-PEG treated animals.

*: p < 0.001
The effect of RGD-MMAF-HSA on tumor growth was compared to treatments with PBS or with the drug carrier RGD-HSA. This experiment was performed with an identical schedule as described above and again the same severe adverse events were observed at the fifth dose of the treatment. The adverse event occurred only in the RGD-HSA treated control group. We were not able to recover serum from these animals. RGD-MMAF-HSA treated animals displayed none of these adverse events, yet their livers were macroscopically different from normal livers, as they had no homogeneous color but were slightly freckled and clearly damaged.

**Figure 4.** (A) Quantification of CD45 positive cells in the liver. (B) Photomicrographs of CD45 stained liver sections of (1) PBS, (2) PTK-HSA, (3) RGD-PTK-HSA, (4) RGD-PTK-HSA-PEG, and (5) RGDPEG-PTK-HSA treated mice (200x magnification). Most leukocytes could be found in livers of RGD-PTK-HSA and RGD-PTK-HSA-PEG treated animals. Color picture can be found in the appendix.

*: p < 0.01
#: p < 0.01 in comparison to both control and RGD-PTK-HSA-PEG groups and p < 0.05 in comparison to RGD-PTK-HSA group.
Serum and blood analysis

Serum analysis revealed an increased ASAT-level for the two mice of the RGD-PTK-HSA-PEG group (Figure 2A) and blood analysis demonstrated a reduction in white blood cells in the circulation (Figure 2B). An ELISA for antibodies against RGD-HSA and HSA demonstrated the formation of IgG against RGD-HSA in the RGD-PTK-HSA-PEG group (Figure 3A) and interestingly some IgG against HSA in the heavily PEGylated RGDPEG-PTK-HSA treated group (Figure 3B).

In serum of RGD-MMAF-HSA treated mice, increased LDH and ASAT levels were found (Figure 2C-D). Serum of this group contained antibodies against RGD-HSA and to a lower extent against HSA as well (Figure 3C-D). Leukocytes in the circulation were clearly reduced in the RGD-MMAF-HSA treated group and even further reduced in the RGD-HSA treated control group (Figure 2E).

Analysis of organ sections

Various organs were examined for infiltrating CD45-positive leukocytes to determine whether the treatments induced an inflammatory response. Elevated numbers of CD45-positive cells were detected in particular in livers. Figure 4 illustrates a massive influx of such cells into the liver tissue with highest amounts in groups treated with RGD-PTK-HSA and RGD-PTK-HSA-PEG. Interestingly, a sinusoidal staining pattern was observed in the livers of these two groups, as illustrated by Figure 4B3 and 4B4.

Also mice treated with RGD-MMAF-HSA and RGD-HSA displayed anomalies in their organs. E.g., RGD-MMAF-HSA treated animals had an increased spleen weight (Figure 5). Significant differences for the influx of CD45 positive cells were found in liver and lungs (Figure 6). RGD-HSA treated mice showed the most prominent influx of CD45 positive cells in livers and also the positively stained sinusoidal endothelium, comparably to animals treated with RGD-PTK-HSA and RGD-PTK-HSA-PEG in the first in vivo experiment (Figure 4). Less influx of immune cells was detected in livers of RGD-MMAF-HSA treated mice, but these animals showed most CD45 positive cells in the lungs.

![Figure 5](image.png)

Figure 5. RGD-MMAF-HSA treated animals demonstrated aberrant spleen weight. Data are presented as mean of five to six mice with standard deviation.

*: p < 0.001 in comparison to all groups
Figure 6. (A) Quantification of CD45 positive cells in the liver. (B) CD45 stained liver and lung sections of (1) PBS, (2) RGD-MMAF-HSA, and (3) RGD-HSA treated mice. Most CD45 positive cells could be found in livers of RGD-HSA treated animals and lungs of RGD-MMAF-HSA treated animals (200x magnification). *: p < 0.001 in comparison to all groups

Color picture can be found in the appendix.

Inhibition of tumor growth

Experiments had been designed to determine effect of targeted PTK787 or auristatin on tumor growth. Tumors of murine C26 carcinoma cells were palpable after 6 days and grew rapidly to sizes of approximately 550 mm$^3$. Groups treated with RGD-containing PTK787 conjugates showed a slower tumor growth in comparison to the two control groups (Figure 7A). This difference was significant for the mice treated with RGD-PTK-HSA and RGD-PTK-HSA-PEG from day 9 to 15 in comparison with PBS and PTK-HSA treated mice.

Upon studying anti-tumor effects of RGD-HSA and RGD-MMAF-HSA, we observed tumor growth inhibition by RGD-HSA but not by RGD-MMAF-HSA. The differences in tumor size between RGD-HSA treated animal and the other two groups were statistically significant from day 11 to day 15 (Figure 7B).
Figure 7. Effect of RGD-equipped albumins conjugated with either PTK787 (A) or MMAF (B) on tumor growth. Mice were injected with C26 carcinoma cells for tumor inoculation. After the tumors became established (day 7), animals were administered drug targeting conjugate at a concentration of 5 nmol PTK787 or 3.1 nmol MMAF per injection, every other day, for five consecutive times as indicated by arrows. Tumor size is presented as mean with standard error.

*: p < 0.05 in comparison to control groups (PBS, PTK-HSA)

Distribution of $^{89}$Zr-RGD-HSA in vivo

Previously we showed tumor homing of RGD-albumins using immunohistoc hemical techniques [5]. Here, $^{89}$Zr-RGD-HSA, administered to tumor bearing mice, was used to corroborate these findings and to give quantitative information on plasma disappearance and whole body distribution. In a pilot study, $^{89}$Zr-RGD-HSA was administered to two tumor bearing mice, microPET scans were acquired 2, 24, and 48 h after injection, and organs and tissues were harvested thereafter for assessment of accumulated radioactivity. Figure 8A illustrates that $^{89}$Zr-RGD-HSA was rapidly eliminated from the blood with a $t_{1/2}$ of less than 20 min (Figure 8B), from which we conclude that it has not retained the long circulating properties of parental HSA.

$^{89}$Zr-RGD-HSA accumulated mainly in liver, spleen and kidneys 49 h after injection (Figure 8B). Already after 2 h, nearly all of the drug carrier accumulated in liver and spleen (Figure 9). The scan furthermore failed to show the heart, which corroborates the fast elimination which was observed in the blood samples. Bladder and some joints could already be seen after 2 h. It cannot be ruled out that $^{89}$Zr-RGD-HSA was rapidly degraded in liver and spleen, since intact drug carrier can not be filtrated in the kidney and only free $^{89}$Zirconium will accumulate in the bones. However, $^{89}$Zr and its chelator desferal are stable, designed to reside inside the cell once they are internalized, and therefore should not accumulate in bones and joints [10]. Another possibility is selective recognition of osteoclast by $^{89}$Zr-RGD-HSA. Osteoclasts are known for a high expression of $\alpha_\beta_3$-integrin and have been imaged using a $^{64}$Cu-labeled RGD [11-13]. Thus it is possible that $^{89}$Zr-RGD-HSA selectively bound to these osteoclasts in the joints.

MicroPET scans at 24 and 48 h were not significantly different from the shown scan at 2 h after injection.
Figure 8. Plasma disappearance and whole body distribution of RGD-HSA was studied using a positron emitting label. For this, $^{89}$Zr-RGD-HSA was injected into tumor bearing mice. (A) $^{89}$Zr-RGD-HSA is rapidly eliminated from the blood. (B) Pharmacokinetic parameters with standard error as calculated using Multifit assuming a two compartment model with elimination from the peripheral compartment. (C) 49 h after injection, mice were sacrificed and organs and tissues were harvested and counted for $^{89}$Zirconium. Whole body distribution is expressed as percent injected dose per gram of tissue (% ID/g). Data are presented as mean of two mice with standard deviation.

Discussion

Specific delivery of antiangiogenic or apoptosis inducing drugs to $\alpha_v\beta_3$-integrin expressing cells is a relevant approach for cancer therapy since $\alpha_v\beta_3$-integrin over-expression can be found on angiogenic endothelial cells and on many human tumors [1, 14-16]. Previously we developed drug targeting conjugates with promising in vitro efficacy for the cell specific delivery of PTK787 or
RGD-HSA carriers in vivo

auristatin F [4, 6]. Here we studied the efficacy of RGD-albumin conjugates in tumor bearing mice. However, in order to understand the underlying effect of the here reported tumor growth inhibition, we first have to evaluate the observed side effects. Three of the repeatedly administered drug targeting conjugates displayed identical and fatal adverse events (RGD-HSA, RGD-PTK-HSA, RGD-PTK-HSA-PEG). The cold and blue extremities and the tachycardia and the fact that the heart was scarcely pumping blood were indications of an anaphylactic shock. Unfortunately only three of these 15 mice could be tested for IgG formation against RGD-HSA, but these three showed high antibody titers (Figure 3), supporting the premise of an anaphylactic shock. Often immunogenicity and antibody formation is found for aggregated proteins but the drug targeting conjugates shown here had the same grade of aggregation as parental albumin (ca. 5%) [17] and, although having the same grade of aggregation, PTK-HSA did not induce antibody formation. Thus aggregation can be ruled out as cause for the immune response. The RGD-peptide and the human serum albumin can be the cause for the response, since only these two moieties can be found in the therapeutics administered to all three groups that suffered from the anaphylactic shock. ELISAs proved that most of the antibodies formed recognized RGD-HSA, few recognized HSA alone. From these results we concluded that RGD-modified albumins are immunogenic, regardless of further modifications with drug or even with PEG (e.g. RGD-PTK-HSA-PEG). The first four injections sensitized the animals and the fifth injection induced an anaphylactic shock. Furthermore, these three groups stand out by a decreased white blood cell count and a striking influx of CD45 positive cells in the liver. Of course, one question arises immediately: Why did RGD-MMAF-HSA treated mice not suffer a shock? A possible explanation is that the immunogenicity is dependent on the amount of RGD-peptides per albumin. As can be derived from Table 1, RGD-HSA, RGD-PTK-HSA, and RGD-PTK-HSA-PEG all contain 12 peptide moieties per albumin whereas RGD-MMAF-HSA contains only 8. Nevertheless, RGD-MMAF-HSA treated animals were also sensitized for an RGD-albumin allergen, as can be appreciated from Figure 3C. We therefore speculated that further injections could have eventually induced an anaphylactic shock as well. RGD-MMAF-HSA treated mice differed from all other animals by a number of observations. First, they had an abnormal appearance of the liver, second, the spleen was significantly enlarged, and third, not only liver but also lungs were found to be heavily infiltrated with leukocytes. In addition, high LDH and ASAT values indicated tissue and especially liver damage. The distribution data of $^{89}$Zr-RGD-HSA revealed fast accumulation in spleen and liver. Assuming a similar distribution for RGD-MMAF-HSA, we may have delivered MMAF, a potent apoptosis inducing drug, directly into these two organs. Since MMAF toxicity will not discriminate between neoplastic or normal cell types, such an intracellular accumulation of the conjugate may have caused the organ damage. Although it probably accumulated in the same organs, targeted PTK787 did not induce liver damage, since this VEGFR kinase inhibitor is not directly toxic.

Integrating the data of both in vivo experiments, two modes of side effects can be separated. All RGD-albumin conjugates induced antibody formation but only conjugates with the highest number of RGD-peptides per albumin appeared to be capable of inducing a fatal anaphylactic shock at the fifth injection. Increased ASAT levels or other signs of liver damage in these three groups are likely a symptom of the shock rather than a direct effect of the drug targeting conjugate. The second mode of side effect can in theory be related to a failed targeting approach of MMAF, which, once delivered to the wrong cell type, caused damage to at least the liver and spleen.
Figure 9. MicroPET scans provided a three-dimensional image of the distribution of the $^{89}$Zr-label. Here we show a coronal section (A), a sagittal section (B) and 10 transverse sections (C) of a tumor bearing mice, injected with $^{89}$Zr-RGD-HSA. (D) displays the positioning of coronal, sagittal, and transverse planes. The distinct position of each of the transverse sections are marked in A and B. The cross in each section marks the position of the sections in the corresponding dimensions. The microPET scan was acquired 2 h after injection of $^{89}$Zr-RGD-HSA. The color picture can be found in the appendix.

The strong immunogenic reaction against RGD-peptide modified albumin was surprising, knowing that conjugates of ovalbumin or TNF with a similar cyclic vascular targeting peptide (CNGRC) elicited no or little immune response, even when given over a prolonged period of time with complete Freund’s adjuvant [18]. The authors of this study argued that the CNGRC peptide is not immunogenic since it mimics a self-structure, the GNGRG loop of fibronectin. However, although the RGD-sequence can be found in many extracellular matrix proteins (e.g. fibronectin,
RGD-HSA carriers in vivo

laminin, thrombospondin, osteopontin) [19-22], known to bind to integrins as well, it elicited a strong immunogenic response. It is known that folding of the RGD-peptide, and thereby its binding specificity and affinity, is influenced by flanking sequences of the RGD. The used c(RGDfK)-peptide might be folded into a structure unknown to the immune system but still very suited for binding to \( \alpha_v \beta_3 \)-integrin. The fact, that RGD-peptides have been widely used as a targeting agent makes one wonder why such adverse events have not been observed elsewhere. Several points may explain it:

1. Apart from the c(RGDfK) sequence, RGD4C and RGD10 are other variants of the RGD peptide that are widely used. It is unclear whether other RGD-peptides feature the same immunogenicity profile.

2. We concluded that the number of RGD per carrier defines the grade of immunogenicity. Many drug targeting conjugates do not possess multiple RGD-peptides, leaving only liposomes, nanoparticles, proteins and polymers as drug carriers with multiple RGD peptides.

3. Many in vivo studies were performed with only a single injection or in nude mice. In both cases the formation of antibodies and an anaphylactic shock are unlikely to occur. Here, balb/c mice, which are known for a strong humoral immune response, have been used for in vivo studies with five consecutive injections.

The most similar drug targeting conjugate, a HPMA-copolymers modified with 11 c(RGDfK)-peptides per polymer, was thus far only used for imaging studies using a single injection [23]. It would be of interest to study whether such a construct also sensitizes Balb/c mice for the RGD-peptide.

RGD-PTK-HSA and RGD-PTK-HSA-PEG treated mice displayed a significant inhibition of tumor growth. This inhibition, however, was also found in RGD-HSA but not in RGD-MMAF-HSA treated mice, indicating that tumor growth was inhibited by albumin carriers modified with at least 12 RGD-peptides but probably not due to the attached drug. Tumor growth inhibition can either be attributed to a direct effect of RGD-HSA on tumor endothelium or tumor cells, or to a “side effect” of the activation of the immune system. The latter seems to be more likely when the fast clearance of \(^{89}\)Zr-RGD-HSA in the blood was taken into account \((t_{1/2} = 19 \text{ min})\) which made a direct effect of RGD-HSA on tumor growth is unlikely. If we assume a similar distribution profile, RGD-MMAF-HSA and also the other drug targeting conjugates had neither sufficient residence time in the blood for tumor accumulation by active receptor-mediate uptake nor for passive tumor accumulation by enhanced permeability and retention effect.

RGD-modification of proteins has been shown earlier to lead to a reduced the half-life. Schraa and co-workers modified antibodies with c(RGDfK) and c(RADfK)-peptides and demonstrated that RGD-modification reduced the half-life by 70% while RAD modification had no impact on the circulation time [24]. Mitra and co-workers seem to have overcome the problem of fast elimination with HPMA-c(RGDfK) constructs. HPMA-c(RGDfK) accumulated in a tumor in the course of 48 h (ca. 5 %ID/g) with only very limited tumor accumulation after 1 h (ca. 1 %ID/g) [23, 25]. In comparison, RGD-HSA was at the 1 h - time point nearly completely eliminated from the circulation and thus could not accumulate in the tumor. The fast elimination of RGD-HSA in our study can mainly be attributed to fast uptake in spleen and liver. These organs were clearly visible in the microPET scan after two hours. Increased uptake of RGD-modified macromolecules in spleen and liver was also observed for RGD-modified antibodies [24] and HPMA copolymers [25] in
comparison to the unmodified antibody and polymer respectively, but the extent and speed of accumulation of RGD-HSA in these two organs is unmatched.

In summary, the in vivo evaluation of RGD-albumin drug carriers revealed high immunogenicity of c(RGDfK)-albumin conjugates. It was furthermore shown that the targeting approach was hampered by a fast elimination from the blood stream and accumulation in liver and spleen. The latter were damaged due to the local delivery of apoptosis inducing MMAF. In spite of the promising in vitro data, we concluded that RGD-albumin conjugates are not suitable for targeted drug delivery.

Acknowledgments

Prof. G. A. M. S. van Dongen and Ir. L. R. Perk of the VU medical center are kindly acknowledged for providing the desferal labeling technology. This work was made possible by grants from Marie Curie (HPMI-CT-2002-00218) and SenterNovem (TSGE1083).

Reference List


(22) Pytel R. Amino acid sequence of the murine Mac-1 alpha chain reveals homology with the integrin family and an additional domain related to von Willebrand factor. EMBO J 1988 May;7(5):1371-1378.


