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LIGAND-TARGETED LIPOSOMES DIRECTED AGAINST PATHOLOGICAL VASCULATURE

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ABSTRACT

The development of liposomes targeted to angiogenic endothelial cells offers exciting prospects for intervention in cancer and inflammation. Several proteins are (strongly) over-expressed on angiogenic endothelial cells as compared to the quiescent endothelium, and could potentially serve as targets for site-specific drug delivery. In this contribution particular attention is given to the design of targeted long-circulating liposomes directed against the alpha v beta 3-integrin protein.

Key Words: Angiogenesis; Integrins; RGD-peptide; Drug targeting; Liposomes

TUMOUR ANGIOGENESIS

The formation of new blood vessels, also known as angiogenesis, is an essential process in the development of a clinically relevant tumour.[1,2] Without these newly formed vessels maximal tumour size is restricted to a few cubic millimeters as the diffusion of nutrients becomes the limiting factor for tumour growth. When tumour cells are able to stimulate angiogenesis, the supply of nutrients and the carry off of waste-products is ensured, supporting the growth of larger tumour masses.

Numerous successive events play a role in the angiogenic process.[3,4] Pro-angiogenic factors are produced and released in the tumour environment. These factors, subsequently, bind to their respective endothelial cell (EC) receptors, activate the EC and induce the degradation of the basal membrane. The EC proliferate and migrate, and after remodelling of the extracellular matrix, tubes and loops are formed. The differentiation and migration of other cell types, like pericytes, stabilises the new vessel wall.

TARGETING THE EC

Given the dependency of tumours on angiogenic blood vessels, inhibiting the formation of new capillaries or disrupting the newly formed vessels has attracted a great deal of attention.[5–7] To achieve this, one of the most attractive target cell types is the EC as they play a pivotal role in the angiogenic cascade. Other attractive properties of the angiogenic EC are: easy accessibility from the blood compartment, -tumour-type and location independent cell characteristics, -genetic stability (treatment is not expected to give rise to drug-resistant mutants), -dependency of a large number of tumour cells on a small number of EC (large amplification of the effect).

For successful application of a treatment strategy based on targeting angiogenic blood vessels, angiogenic EC need to be very specifically discriminated from the normal quiescent endothelium. Several proteins are (strongly) over-expressed on angiogenic EC as compared to the quiescent endothelium, and could potentially serve as targets for site-specific drug delivery (as has been recently reviewed by Griffioen and Molema, and shown in Table 1).[8]

One of the best-defined over-expressed angiogenic target protein and corresponding ligand is the alpha v beta 3-integrin/RGD-motif system.[9] The alpha v beta 3-integrin mediates EC adhesion to the extracellular matrix by recognising a conserved arginine–glycine–aspartic acid (RGD) sequence, which is present in several matrix proteins.[9] Synthetic cyclic RGD-peptides show a high affinity for the alpha v beta 3-integrin.[10] Unfortunately, RGD-peptides appear to have a short circulation half-life ($t_{1/2} < 10$ min) and, as a

Table 1. Proteins Over-expressed on Angiogenic EC

CD 34	endoglin
VEGF/VEGF receptor complex	Tie2
Endosialin	TNF-alpha receptor
E-selectin	CD 44
alpha v-integrins	angiostatin receptor
MMP-2	endostatin receptor
MMP-9	CM101-binding protein
30.5 kDa antigen	

result, are expected to have limited interaction with the target site.[11,12] In addition, the use of peptides as drug carriers provides limited drug transport capacity.

RGD-TARGETED PEG-LIPOSOMES

To improve circulatory half-life and drug transport capability while preserving the target affinity of the RGD-motif, RGD-peptides can be coupled to the distal end of long-circulating PEG-ylated liposomes. Moreover, the liposome may function as a platform allowing multivalent interactions with the target molecules to take place.

The coupling of RGD-peptide to PEG-liposomes (RGD-PEG-L) induced increased binding to human umbilical vein endothelial cells (HUVEC) as compared to PEG-liposomes without targeting peptide (PEG-L) illustrated in Figure 1.

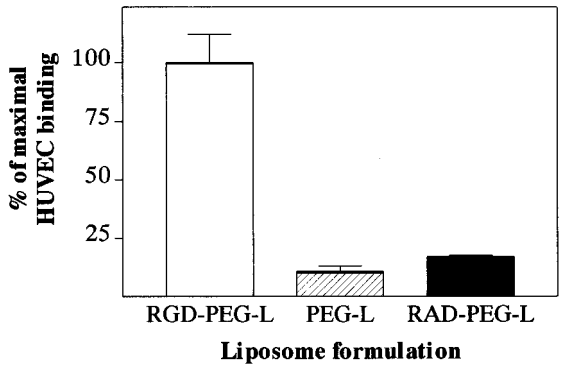

Figure 1. Binding of (peptide targeted) liposome formulations to HUVEC. DiD-labelled RGD-PEG-liposomes, control liposomes, or RAD-PEG-liposomes were incubated with 10^6 HUVEC at a concentration of 100 nmol lipid/mL. Binding was determined using FACS-analysis.

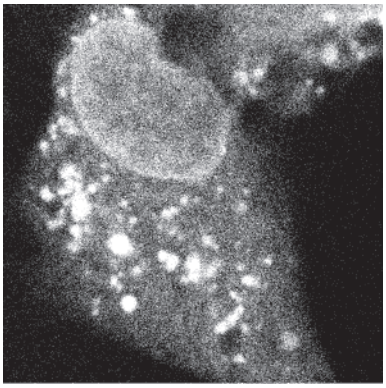

Figure 2. Incubation of doxorubicin-loaded RGD-PEG-L with HUVEC. Doxorubicin fluorescence is visible as bright fluorescent spots inside the cell, indicating uptake in endocytic vesicles.

The specificity of the interaction was illustrated by the incubation with RAD-peptide targeted PEG-liposomes (RAD-PEG-L). The single amino acid substitution (differing in a single methyl group) in the RGD, yielding the RAD-peptide, resulted in a 7-fold lower binding to the HUVEC.

In a next set of experiments, RGD-PEG-L, RAD-PEG-L and PEG-L were loaded with doxorubicin before incubation with HUVEC. Cellular distribution of doxorubicin fluorescence was examined using confocal laser scanning microscopy (CLSM). By examining the fluorescence-pattern of the encapsulated doxorubicin at different levels inside the HUVEC it was shown that RGD-PEG-L are, after binding to the cells, taken up inside endocytic vesicles, visible as bright fluorescent spots inside the cell (see Fig. 2). For RAD-PEG-L and non-targeted PEG-L hardly any intracellular doxorubicin fluorescence could be detected (data not shown).

Current studies are focusing on the in vivo behaviour of RGD-PEG-L. Preliminary data indicate that the circulation time of RGD-PEG-L after intravenous administration is substantially reduced as compared to RAD-PEG-L and PEG-L, the latter two formulations showing similar circulation kinetics. Interestingly, despite the increased clearance rate of the RGD-PEG-L, localisation at the target site in subcutaneous murine tumour models was similar. This was in contrast to expectations, as the main driving force for tumour localisation is the AUC of the liposomes after injection into the circulation. Therefore, this observation points at the occurrence of a specific interaction at the site of the tumour. Intravital microscopy experiments confirm the specific interaction at the target site. Mice bearing a dorsal skinfold window chamber in which mall tumour was implanted were intravenously injected with fluorescently labelled RGD-PEG-L, RAD-PEG-L and PEG-L. RAD-PEG-L and PEG-L liposome

administration yielded the familiar images that have been reported previously by Yuan et al.[13] PEG-liposomes were shown to extravasate into the tumour tissue and to distribute heterogeneously and to form perivascular clusters Yet, when RGD-PEG-L were administered, already at 1 h post-injection fluorescent clusters were observed inside the angiogenic blood vessels in and around the tumour tissue.

IMPLICATION OF ALPHA V BETA 3 INTEGRIN IN INFLAMMATION

Interestingly, angiogenesis is also a part of the inflammatory response.[2] In fact many inflammatory drugs, like acetyl salicylic acid and corticosteroids, may exert (part of) their effect through inhibition of angiogenesis.[14,15] Therefore, targeting to the alpha v-integrin may also be used in inflammatory conditions to achieve selective drug delivery to the EC. The EC is an attractive cell type to target in inflammatory conditions as it plays a pivotal role in the regulation of the inflammatory cascade.

In a rat model of inflammation the degree of localisation of RGD-PEG-L, RAD-PEG-L and PEG-L within the inflammatory site was evaluated. Rats received a subcutaneous injection with LPS, half an hour later followed by intravenous injection of radiolabelled liposomes and Evan's blue. Evan's blue is a marker for capillary permeability and was used to identify the exact site of inflammation. At 4 h after injection, a blue zone of approximately 1.5 cm in diameter surrounding the site of LPS-injection was visible in the skin. This zone was punched out, as well as a zone of control skin from the contralateral side of the rat. After measuring radioactivity it appeared that all liposome types localised to a higher degree in the inflamed skin than in the non-inflamed control skin. This is probably the result of the increased capillary permeability at the site of inflammation allowing local liposome extravasation.

Interestingly, a remarkable similarity with the tumour experiments was observed: the degree of localisation for the RGD-PEG-L was two-fold higher than for the RAD-PEG-L and PEG-L. The degree of localisation of the latter liposome types was similar. This finding was remarkable as the AUC of the RGD-PEG-L in these rats was approximately 4-fold lower as compared to the AUC of PEG-L and RAD-PEG-L, again pointing at the involvement of an RGD-mediated specific interaction with the pathological site.

PERSPECTIVE

The development of liposomes targeted to angiogenic EC offers exciting prospects for tumour eradication or inflammation modulation. Yet, many

hurdles remain. The specificity of the interaction should be sufficiently high, especially when destruction of angiogenic blood vessels is the objective, because several of the mentioned potential target proteins are also expressed (albeit at lower levels) on the normal quiescent endothelium.

In view of the heterogeneity of the tumour micro-environment it is anticipated that several target receptors need to be targeted for successful inhibition of growth in the various tumour regions. For this purpose we are currently developing various other targeting ligands to be utilised for liposome targeting to angiogenic EC. Hopefully, the recent discoveries in angiogenesis research together with the increased possibilities for target molecule selection will allow the further development of this new class of anti-angiogenic/anti-inflammatory agents.

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REFERENCES

1. Folkman, J. Angiogenesis in Cancer, Vascular, Rheumatoid and Other Disease. Nat. Med. **1995**, *1*(1), 27–31.
2. Carmeliet, P.; Jain, R.K. Angiogenesis in Cancer and other Diseases. Nature **2000**, *407*(6801), 249–257.
3. Carmeliet, P. Mechanisms of Angiogenesis and Arteriogenesis. Nat. Med. **2000**, Apr; *6*(4), 389–395.
4. Michiels, C.; Arnould, T.; Remacle, J. Endothelial Cell Responses to Hypoxia: Initiation of a Cascade of Cellular Interactions. Biochim. Biophys. Acta **2000**, *1497*(1), 1–10.
5. Deplanque, G.; Harris, A.L. Anti-angiogenic Agents: Clinical Trial Design and Therapies in Development. Eur. J. Cancer **2000**, *36*(13), 1713–1724.
6. Cherrington, J.M.; Strawn, L.M.; Shawver, L.K. New Paradigms for the Treatment of Cancer: the Role of Anti-angiogenesis Agents. Adv. Cancer Res. **2000**, *79*, 1–38.
7. Molema, G.; Meijer, D.K.; de Leij, L.F. Tumor Vasculature Targeted Therapies: Getting the Players Organized. Biochem. Pharmacol. **1998**, *55*(12), 1939–1945.
8. Griffioen, A.W.; Molema, G. Angiogenesis: Potentials for Pharmacologic Intervention in the Treatment of Cancer, Cardiovascular Diseases, and Chronic Inflammation. Pharmacol. Rev. **2000**, *52*(2), 237–268.
9. Horton, M.A. The Alpha v Beta 3 Integrin. Vitronectin Receptor. Int. J. Biochem. Cell Biol. **1997**, *29*(5), 721–725.
10. Koivunen, E.; Wang, B.; Ruoslahti, E. Phage Libraries Displaying Cyclic Peptides with Different Ring Sizes: Ligand Specificities of the RGD-Directed Integrins. Biotechnology (NY). **1995**, *13*(3), 265–270

11. van Hagen, P.M.; Breeman, W.A.; Bernard, H.F.; Schaar, M.; Mooij, C.M.; Srinivasan, A.; Schmidt, M.A.; Krenning, E.P.; de Jong, M. Evaluation of a Radiolabelled Cyclic DTPA-RGD Analogue for Tumour Imaging and Radionuclide Therapy. Int. J. Cancer **2000**, *90*(4), 186–198.
12. Noiri, E.; Goligorsky, M.S.; Wang, G.J.; Wang, J.; Cabahug, C.J.; Sharma, S.; Rhodes, B.A.; Som, P. Biodistribution and Clearance of 99 mTc-labeled Arg-Gly-Asp (RGD) Peptide in Rats with Ischemic Acute Renal Failure. J. Am. Soc. Nephrol. **1996**, *7*(12), 2682–2688
13. Yuan, F.; Leunig, M.; Huang, S.K.; Berk, D.A.; Papahadjopoulos, D.; Jain, R.K. Microvascular Permeability and Interstitial Penetration of Sterically Stabilized (Stealth) Liposomes in a Human Tumor Xenograft. Cancer Res. **1994**, *54*(13), 3352–3356.
14. Colville-Nash, P.R.; Alam, C.A.; Appleton, I.; Brown, J.R.; Seed, M.P.; Willoughby, D.A. The Pharmacological Modulation of Angiogenesis in Chronic Granulomatous Inflammation J. Pharmacol. Exp. Ther. **1995**, *274*(3), 1463–1472.
15. Jones, M.K.; Wang, H.; Peskar, B.M.; Levin, E.; Itani, R.M.; Sarfeh, I.J.; Tarnawski, A.S. Inhibition of Angiogenesis by Nonsteroidal Anti-Inflammatory Drugs: Insight into Mechanisms and Implications for Cancer Growth and Ulcer Healing. Nat. Med. **1999**, *5*(12), 1418–1423.