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Differences in binding of ^{99m}Tc-disintegrins to integrin αvβ3 on tumor and vascular cells

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Abstract

Disintegrins which contain an Arg-Gly-Asp sequence in their binding domains are antagonists of integrins such as αvβ3. The purpose of this study was to compare a range of disintegrins with different integrin selectivities for their binding behavior in vitro to vascular endothelial cells bearing αvβ3 and to cultured tumor cells which express αvβ3.

Methods: Five disintegrins (bitistatin, kistrin, flavoridin, VLO4 and echistatin) and a cyclic pentapeptide, c[RGDyK], were radiolabeled with ^{99m}Tc and tested for binding to cells in vitro.

Results: ^{99m}Tc-Kistrin, flavoridin and VLO4 had the highest binding, ^{99m}Tc-echistatin had moderate binding, and ^{99m}Tc-bitistatin and ^{99m}Tc-c[RGDyK] had low binding to cells. The observed binding was attributed to αvβ3 to various extents: echistatin, bitistatin>kistrin>flavoridin>VLO4. Cancer cells internalized bound disintegrins after binding, but endothelial cells did not. After binding to endothelial cells, ^{99m}Tc-kistrin was not displaced by competing peptide or plasma proteins.

Conclusions: These data suggest that radiolabeled kistrin, flavoridin and VLO4 may have advantages over labeled bitistatin and small cyclic peptides for targeting αvβ3 in vivo.

Since receptor-bound radioligand is not internalized by endothelial cells, disintegrins may provide an advantage for targeting αvβ3 on vasculature because they bind strongly to surface receptors and are not readily displaced.

Keywords: Disintegrins; Integrin αvβ3; Angiogenesis; Tumors; Tc-99m; c[RGDyK]

1. Introduction

The αvβ3 integrin (vitronectin receptor) is found on many types of cells and influences cell adhesion and migration with effects on angiogenesis, restenosis, tumor cell invasion and atherosclerosis [1]. It binds ligands containing an Arg-Gly-Asp (RGD) motif, as does its closely related integrin, αIIbβ3, which is found exclusively on platelets and megakaryocytes. Binding of RGD ligands occurs at a site in the β3 subunit [2]. Both of these integrins bind the ligands fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin and others [3]. Studies have shown that the integrin αvβ3 is highly expressed on the walls (endothelium) of actively growing blood vessels. The concentration of these receptors on endothelial cells is much higher in young, actively growing blood vessels (as in a tumor), compared with mature blood vessels (as in most of the body) [4]. This makes αvβ3 a potentially useful target for radioligands for imaging and therapy of tumors. It has been shown that angiogenesis can be prevented by anti-αvβ3 antibodies or by cyclic RGD-containing peptides. Importantly, blockade of αvβ3 receptors caused apoptosis of actively proliferating vascular cells but not of preexisting vascular cells, indicating the selectivity of this targeting approach [4].

Integrin αvβ3 is also found on the surface of certain cancer cells, including melanoma, glioblastoma, breast carcinoma (including metastases) and osteosarcoma [5–8]. Other integrins have also been found on the surface of tumor cells; for example, in addition to αvβ3, melanoma cells express α1β1, α3β1 and α5β1. The expression of αvβ3, however, is highly correlated with degree of malignancy: the expression of β3 integrins on melanoma cells was restricted exclusively to

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Table 1
Amino acid sequence of ligands tested (from Refs 5[18,19,21,22])

		10	20	30	40	50	60	70	80	
		•	•	•	•	•	•	•	•	
								***		
rBitistatin	GSPPVCGNEI	LEQGEDCDCG	SPANCQDQCC	NAATCKLTPG	SQCNHGECCD	QCKFKKARTV	CRIA**RGD**WND	DYCTGKSSDC	PWNH	
Kistrin		- - -GKECDCS	SPEN- - -PCC	DAATCKLRPG	AQCGEGLCCE	QCKFSRAGKI	CRIP**RGD**MPD	DRCTGQSADC	PRYH	
Flavoridin		- - -GEECDCG	SPSN- - -PCC	DAATCKLRPG	AQCADGLCCD	QCRFKKKRTI	CRIA**RGD**FPD	DRCTGLSNDC	PRWNDL	
Echistatin					-ECESGPCCR	NCKFLKEGTI	CKRA**RGD**DMD	DYCNGKTCDC	PRNPHKGPAT	
VLO4 (dimer)			-MNSGNPCC	DPVTCKPRRG	EHCVSGPCCR	NCKFLNAGTI	CKRA**RGD**DMN	DYCTGISPDC	PRNPW	
c[RGDfV]							- - - -**RGD**fV-			
c[RGDyK]							- - - -**RGD**yK-			

cells in the vertical growth phase and metastatic lesions, the most aggressive phases of the malignant process [6].

Integrin αvβ3 is being actively investigated as a target for radiotracers in order to image tumor angiogenesis. When radiolabeled ligand for αvβ3 is injected into the bloodstream of a cancer patient, the radioligand would be expected to bind to αvβ3 receptors in actively growing vasculature of the tumor, permitting external detection of the higher concentration of radiotracer in the tumor compared with surrounding tissue. An advantage of this approach is that the radioligand would accumulate in the vasculature of virtually any type of solid tumor and provide a method for locating a wide variety of cancer types.

Potential ligands for αvβ3 include antibodies [9], synthetic peptides and their conjugates [10], and disintegrins [11–13]. Disintegrins, the most potent known inhibitors of integrin function, were first identified as a family of αIIbβ3 antagonists found in the venoms of various snakes [14]. They comprise a class of cysteine-rich polypeptides which are constrained by internal disulfide linkages into multi-loop structures. An RGD or analogous sequence is found in many disintegrins, located in a mobile loop which projects 14–17 Å from the protein core [15], and is critical for the disintegrins' binding to β3 integrins.

Studies by Juliano et al. [11] showed that several disintegrins (kistrin, echistatin and flavoridin) bind with high affinity to αvβ3 in a solid-phase assay and that they inhibited binding of cultured endothelial cells to vitronectin. Other disintegrins tested had lower binding to αvβ3. Cultured endothelial cells also bound to the disintegrins bitistatin, echistatin, kistrin, flavoridin and the RGD-containing nondisintegrin mambin.

Bitistatin is the largest known monomeric disintegrin. Radiolabeled bitistatin is currently being investigated for imaging platelet deposits in vivo [16]. It also showed initial promise for imaging tumors in mice, with tumor uptake of 12 %ID/g [17]. Other disintegrins appear to have better selectivity for αvβ3 and, thus, may be better candidates for imaging tumor angiogenesis.

The purpose of this study was to compare ^{99m}Tc-bitistatin with a range of disintegrins that have different integrin selectivities, all radiolabeled with ^{99m}Tc, for their binding in vitro to vascular cells bearing αvβ3 and to cultured tumor cells which express αvβ3.

2. Methods

2.1. Source of ligands

Table 1 lists the amino acid sequences of polypeptides used in this study. Bitistatin was produced as a recombinant product [18]. Other disintegrins were produced by reversed-phase (RP) HPLC purification from freeze-dried natural snake venom (Miami Serpentarium, Punta Gorda, FL, USA): kistrin (from *Calloselasma rhodostoma* venom) [19]; echistatin (from *Echis carinatus*) [20]; flavoridin (from *Trimeresurus flavoviridis*) [21]; and VLO4 (homodimer from *Vipera lebetina obtusa*) [22]. The active component was identified by its bioactivity (e.g., ability to inhibit platelet aggregation in human platelet-rich plasma, or ability to inhibit adhesion of cultured cells [22]). The active material was purified to a single peak on RP-HPLC. Protein purity was tested by SDS/PAGE and matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS), performed at the Wistar Mass Spectrometry Facility, Philadelphia, PA, USA. Protein concentrations were determined by Lowry assay (Pierce, Rockford, IL, USA). Synthetic cyclic peptides c[RGDyK] and c[RGDfV] were obtained from Bachem Bioscience, King of Prussia, PA, USA.

2.2. Radiolabeling

Disintegrins or cyclic peptide was prepared for ^{99m}Tc labeling by coupling hydrazinonicotinate (HYNIC) groups to lysine sidechains using succinimidyl-hydrazinonicotinamide (SHNH) [23]. A 20:1 molar ratio of SHNH/polypeptide in borate buffer pH 8.5 was used. The products were purified by reversed-phase HPLC on a C_{18} column (300 Å pore size) (Varian, Palo Alto, CA, USA) using a linear gradient of 0–50% acetonitrile in 0.1% TFA over 25 min and monitoring UV absorbance at 280 nm (Waters, Milford, MA, USA). The collected peak was freeze-dried from mobile phase in 10-μg aliquots, which were then stored at −70°C. The number of HYNIC linkers attached per molecule was determined by hydrazone assay [24] and the protein concentration was determined by Lowry assay.

Aliquots were labeled by mixing with a ^{99m}Tc-tricine intermediate [25]. Lyophilized kit vials containing 54 mg tricine and 75 μg $SnCl_2 \cdot 2H_2O$, pH 7.3, were prepared and stored at 4°C. For labeling, 1850 MBq of ^{99m}Tc sodium pertechnetate solution (Cardinal Health, Sharon Hill, PA,