High expression of gastrin-releasing peptide receptors in the vascular bed of urinary tract cancers: promising candidates for vascular targeting applications

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Abstract

Tumoral gastrin-releasing peptide (GRP) receptors are potential targets for diagnosis and therapy using radiolabeled or cytotoxic GRP analogs. GRP-receptor overexpression has been detected in endocrine-related cancer cells and, more recently, also in the vascular bed of selected tumors. More information on vascular GRP-receptors in cancer is required to assess their potential for vascular targeting applications. Therefore, frequent human cancers ($n=368$) were analyzed using *in vitro* GRP-receptor autoradiography on tissue sections with the ^{125}I -[Tyr⁴]-bombesin radioligand and/or the universal radioligand ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6–14). GRP-receptor expressing vessels were evaluated in each tumor group for prevalence, quantity (vascular score), and GRP-receptor density. Prevalence of vascular GRP-receptors was variable, ranging from 12% (prostate cancer) to 92% (urinary tract cancer). Different tumor types within a given site had divergent prevalence of vascular GRP-receptors (e.g. lung: small cell cancer: 0%; adenocarcinoma: 59%; squamous carcinoma: 83%). Also the vascular score varied widely, with the highest score in urinary tract cancer (1.69), moderate scores in lung (0.91), colon (0.88), kidney (0.84), and biliary tract (0.69) cancers and low scores in breast (0.39) and prostate (0.14) cancers. Vascular GRP-receptors were expressed in the muscular vessel wall in moderate to high densities. Normal non-neoplastic control tissues from these organs lacked vascular GRP-receptors. In conclusion, tumoral vessels in all evaluated sites express GRP-receptors, suggesting a major biological function of GRP-receptors in neovasculature. Vascular GRP-receptor expression varies between the tumor types indicating tumor-specific mechanisms in their regulation. Urinary tract cancers express vascular GRP-receptors so abundantly, that they are promising candidates for vascular targeting applications.

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Introduction

Targeting the tumor vascular bed is a novel clinical approach for tumor imaging and therapy (Thorpe *et al.* 2003, Brack *et al.* 2004). The therapeutic strategies comprise a) occlusion or destruction of established tumor vessels with rapid shutdown of the blood supply and subsequent tumor necrosis and b) inhibition of the development of a functional neovasculature

(Narazaki & Tosato 2005) that is essential for tumor growth beyond 1–2 mm (Auguste *et al.* 2005). The most critical point in this concept is specificity, in order to avoid adverse effects to normal tissue blood supply (Narazaki & Tosato 2005). Therefore, a precondition for successfully targeting the tumor vascular bed is the identification of molecules which are specifically overexpressed in these vessels.

Peptide receptors are overexpressed in the neoplastic cells of different cancers but also in the tumor vascular bed of some cancers (Reubi *et al.* 1996, Fleischmann *et al.* 2000, 2005, 2007, Korner & Reubi 2007, Otrrock *et al.* 2007). Gastrin-releasing peptide (GRP), belonging to the family of bombesin-like peptides (Jensen *et al.* 2008), not only stimulates cancer growth through GRP-receptors in neoplastic cells (Cuttitta *et al.* 1985, Bologna *et al.* 1989, Yano *et al.* 1992) but also has been shown to be implicated in neoangiogenesis in several cancer models (Levine *et al.* 2003a,b, Bajo *et al.* 2004, Heuser *et al.* 2005, Kanashiro *et al.* 2005, 2007, Stangelberger *et al.* 2005, Kang *et al.* 2007). This may suggest that the corresponding receptor is systematically expressed in tumor vascular beds and plays an important biological function. Therefore, we explored GRP-receptor expression in a large number of different cancers from the breast, lung, prostate, kidney, colon, urinary tract, and biliary tract with *in vitro* autoradiography on human tissue sections using ^{125}I -[Tyr⁴]-bombesin and/or ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin (6–14) as radioligands for GRP-receptors. Information about prevalence and quantity of GRP-receptor expressing tumor vessels and density of expressed GRP-receptors in these frequent cancers is particularly important to determine the potential value of the numerous recently developed radiolabeled and non-radiolabeled cytotoxic bombesin analogs (Zhang *et al.* 2004, 2007, Engel *et al.* 2005, Nock *et al.* 2005, Lantry *et al.* 2006, Moody *et al.* 2006, Patel *et al.* 2006, de Visser *et al.* 2007, Waser *et al.* 2007) for *in vivo* vascular targeted clinical applications.

Materials and methods

Tissues

All tissue samples were immediately frozen after surgery and stored at -80°C . Tissue samples of the following 368 primary tumors were analyzed: 134 breast cancers (113 invasive ductal carcinomas, 21 invasive lobular carcinomas); 57 lung cancers (29 squamous carcinomas, 17 adenocarcinomas, two large cell carcinomas, nine small cell carcinomas (SCLC)); 50 prostate cancers (acinar adenocarcinomas); 46 colon cancers (colorectal type); 32 renal cell cancers (28 clear cell carcinomas, four papillary carcinomas); 26 urinary tract cancers (invasive urothelial carcinomas; 25 from the urinary bladder, one from the renal pelvis); 23 cancers of the biliary tract (cholangiocarcinomas). In addition, samples of non-neoplastic tissues were tested (breast: 5, lung: 48, prostate: 26, kidney: 4, colon: 30, biliary tract: 3, urinary tract: 5). The tested

tumors originated either from samples investigated previously for other receptors and collected in accordance with the required international ethical guidelines or from samples collected prospectively at the Institute of Pathology, University of Bern, following the principles of the Helsinki Declaration, including informed consent and approval by the Institutional Review Board.

Receptor autoradiography

We used ^{125}I -[Tyr⁴]-bombesin as radioligand, which is known to preferentially label GRP-receptors (Vigna *et al.* 1987). For autoradiography, 20 μm thick cryostat tissue sections were processed as described previously (Reubi *et al.* 2002, Reubi & Waser 2003). They were incubated with ^{125}I -[Tyr⁴]-bombesin (2000 Ci/mmol; Anawa, Wangen, Switzerland) in a concentration of 100 pM in the presence or absence of 0.1 μM bombesin for 1 h at room temperature. Complete inhibition curves were generated in selected tissues by incubating consecutive sections in the presence of increasing amounts of non-radioactive GRP, bombesin or somatostatin-28 (Bachem, Bubendorf, Switzerland). After washing, the sections were placed in apposition to Biomax MR films (Kodak) and exposed for 7 days in X-ray cassettes. The density of the GRP-receptors was quantified with a computer-assisted image processing system, as described previously (Reubi *et al.* 2002, Reubi & Waser 2003). The reported density value for a given sample consisted of the mean of GRP-receptor determination in at least five positive vessels. In selected cases, the bombesin receptor subtype was evaluated in vessels using the universal radioligand ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6–14; 2000 Ci/mmol; Anawa) at a concentration of 20 pM. Displacement experiments were performed in consecutive sections in presence of 50 nM of each of the unlabeled competitors [D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6–14), GRP and neuromedin B (NMB) and compared with total binding (in the absence of any competitor peptide).

To compare the incidence of GRP-receptor positive vessels between the various tumor groups, a vascular score was determined as previously described (Fleischmann *et al.* 2007). Briefly, in each tumor, the region with the highest incidence of GRP-receptor positive vessels was selected and the number of GRP-receptor positive vessels was determined in a visual field of 19 mm^2 : 1–10 GRP-receptor positive vessels: grade 1; 11–30 GRP-receptor positive vessels: grade 2; more than 30 GRP-receptor positive vessels: grade 3; no GRP-receptor positive vessels present: grade 0.

The vascular score was defined as the mean of the grades from all tumors belonging to a particular tumor group.

Results

Cancers expressing GRP-receptors in their vascular bed were identified in all evaluated anatomical sites, namely in the breast, lung, prostate, kidney, colon, urinary, and biliary tract (Table 1). However, the prevalence of these GRP-receptor expressing tumor-associated vessels varied widely between these sites: it was 92% in urinary tract cancers, 88% in colon cancer, 60% in lung cancer, 57% in biliary tract cancer, 53% in renal cancer, 31% in breast cancer, and 12% in prostate cancer. A further important observation concerned the prevalence of these vessels in different tumor types of a

given anatomical site. In the breast, the prevalence of GRP-receptor expressing vessels was similar in the two different tumor types (invasive ductal carcinoma 31%, invasive lobular carcinoma 33%). By contrast, the different cancer types of the lung showed a wide variability in the prevalence of such vessels ranging from 0% in SCLC over 59% in adenocarcinomas to 83% in squamous carcinomas (Table 1).

While the prevalence merely reflects the percentage of tumors expressing vascular GRP-receptors within a given tumor group, the vascular score, defined as the number of GRP-receptor positive vessels per tumor area in the region of the highest incidence (see Materials and methods), is a mean for the quantitative assessment of such vessels. We also observed a wide variability of this score between the different

Table 1 Gastrin-releasing peptide-receptor expression in tumor-associated blood vessels and neoplastic cells of different tumor types in various anatomical sites: prevalence, receptor density, and vascular score

Tissues		GRP-receptors in tumor-associated blood vessels			GRP-receptors in neoplastic cells	
		Prevalence (positive/total cases (%))	Density ^a (mean $\pm$ S.E.M. (dpm/mg tissue))	Vascular score ^b	Prevalence (positive/total cases (%))	Density ^c (mean $\pm$ S.E.M. (dpm/mg tissue))
Tumor category	n					
Urinary tract cancer						
Invasive urothelial carcinoma	26	24/26 (92%)	1449 $\pm$ 122	1.69	0/26 (0%)	–
Lung cancer						
Squamous carcinoma	29	24/29 (83%)	1402 $\pm$ 115	1.28	2/29 (7%)	212, 230
Adenocarcinoma	17	10/17 (59%)	1024 $\pm$ 112	0.88	0/17 (0%)	
Large cell lung cancer	2	0/2 (0%)		0	0/2 (0%)	
Small cell lung cancer	9	0/9 (0%)		0	3/9 (33%)	1430 $\pm$ 516
Total	57	34/57 (60%)	1301 $\pm$ 94	0.91	5/57 (9%)	664 $\pm$ 335
Colon cancer						
Colorectal carcinoma	46	34/44 (88%)	1299 $\pm$ 84	0.88	2/44 (5%)	240, 335
Renal cell cancer						
Clear cell carcinoma	28	15/28 (54%)	1590 $\pm$ 266	0.82	9/28 (32%)	3013 $\pm$ 904
Papillary carcinoma	4	2/4 (50%)	2181, 578	1.0	0/4 (0%)	
Total	32	17/32 (53%)	1561 $\pm$ 243	0.84	9/32 (28%)	3013 $\pm$ 904
Biliary tract cancer						
Cholangiocarcinoma	23	13/23 (57%)	1140 $\pm$ 141	0.69	2/23 (9%)	911, 363
Breast cancer						
Invasive ductal carcinoma	113	35/113 (31%)	975 $\pm$ 61	0.39	77/113 (68%)	7276 $\pm$ 797
Invasive lobular carcinoma	21	7/21 (33%)	862 $\pm$ 93	0.38	20/21 (95%)	4910 $\pm$ 509
Total	134	42/134 (31%)	956 $\pm$ 53	0.39	97/134 (72%)	6797 $\pm$ 651
Prostate cancer						
Acinar adenocarcinoma	50	6/50 (12%)	1328 $\pm$ 201	0.14	48/50 (96%)	5315 $\pm$ 856
Ovarian cancer ^d						
Primary cancers	19	16/19 (84%)	2401 $\pm$ 262	1.7	5/19 (26%)	1155 $\pm$ 301
Metastases	6	6/6 (100%)	2728 $\pm$ 608	2.6	0/6 (0%)	–

^aVascular GRP-receptor density for each tumor was determined as a mean of the measurement of at least five positive vessels. The mean $\pm$ S.E.M. (dpm/mg tissue) of these values is given for each tumor group.

^bEach single tumor was graded according to the number of GRP-receptor positive tumor-associated blood vessels per area (0–3; see Materials and methods). The vascular score was defined as the mean of these grades in a given tumor group.

^cSolely the GRP-receptor expressing neoplastic cells were quantified for their receptor density.

^dData previously published by Fleischmann *et al.* (2007) shown for comparison.

anatomical sites (Table 1) with the highest value for cancers of the urinary tract (score 1.69). It was similar to the one previously reported for ovarian cancers (score 1.7) but lower than the score of 2.6 as determined for metastases of ovarian cancer (Fleischmann et al. 2007). Following urinary tract cancers were lung cancers (score 0.91), colon cancers (score 0.88), kidney cancers (score 0.84), biliary tract cancers (score 0.69), breast cancers (score 0.39), and prostate cancers (score 0.14). Within a given anatomical site, the vascular scores of different tumor types could be similar like in the breast (invasive ductal carcinoma: 0.39, invasive lobular carcinoma: 0.38; Table 1) or very different like in the lung (SCLC: 0, adenocarcinoma: 0.88, squamous carcinoma: 1.28; Table 1).

Morphological characteristics, distribution, and localization of GRP-receptor expressing tumor-associated blood vessels are summarized in Table 2. An overview of a receptor autoradiography of a typical case, an urothelial carcinoma, is shown in Fig. 1: most GRP-receptor positive blood vessels crossed the cut plane and appeared autoradiographically as black dots, oval structures or rings; occasionally, GRP-receptor positive blood vessels presented as straight vascular segments in the section. Notably, the GRP-receptor expressing vessels possessed a muscular wall; many of the GRP-receptor positive vessels were tortuous and showed no regularly layered wall, being therefore compatible with neoangiogenic tumor vessels (Fig. 2A–C); only few of the GRP-receptor positive vessels had well-organized vessel walls that would be

compatible with pre-existing small veins (Fig. 2D–F). The muscular wall of the vessels was the principle site of GRP-receptor expression, with a moderate to high receptor density (Table 1) and with a homogeneous, circumferential, transmural distribution of the receptor. However, the limited resolution of the receptor autoradiography did not permit to conclusively evaluate a possible GRP-receptor expression of the bordering endothelial cells. In general, GRP-receptor positive vessels were small (diameter 30–120 µm) and their lumina were collapsed; few were medium sized (diameter up to 2.5 mm). The distribution of GRP-receptor expressing tumor-associated vessels was predominantly diffuse in the whole tumor sample, rarely restricted to circumscribed tumor areas in the form of ‘hot spots’. When vascular GRP-receptors were present in a sample, only a part of the tumor-associated vessels expressed the receptors and positive and negative vessels could lie in close vicinity (Fig. 3A–C). GRP-receptor positive vessels were present within the tumor (Fig. 3D–F) and in the interface with the surrounding host tissue (Fig. 3G–I) within a rim of up to 3 mm. However, as the tumor/host border is often poorly defined and only a minority of the samples evidently included peritumoral host tissue the accurate incidence of peritumoral GRP-receptor positive vessels could not be determined.

Inhibition curves were generated in successive sections using the radioligand ¹²⁵I-[Tyr⁴]-bombesin in presence of increasing amounts of various related (bombesin, GRP) and non-related (somatostatin-28)

Table 2 Morphological characteristics, distribution, and localization of gastrin-releasing peptide-receptor expressing tumor-associated blood vessels

Characteristics of GRP-receptor expressing tumor-associated blood vessels	
Vessel type	In principle vessels with a muscular wall Tortuous, poorly organized vessels, compatible with neoangiogenic vessels ⁽⁺⁺⁺⁾ Typical veins ⁽⁺⁾ Typical arteries ⁽⁻⁾
Vascular compartment of GRP-receptor expression	Muscular vessel wall with homogeneous, circumferential and transmural GRP-receptor expression
Vessel size	Vessel diameter: in general 30–120 µm (rarely up to 2.5 mm) Longest vessel segment identified with GRP-receptor expression: 5 mm
Distribution of vessels	Mostly diffuse in whole sample, in rare cases in circumscribed areas as ‘hot spots’ GRP-receptor positive and negative vessels can lie in close vicinity
Localization of vessels	Intratumorally Peritumorally (up to 3 mm from the tumor) at the interface with the surrounding host tissue

⁽⁺⁺⁺⁾Frequent type of GRP-receptor positive vessels.

⁽⁺⁾Rare type of GRP-receptor positive vessels.

⁽⁻⁾GRP-receptor negative vessels.

Figure 1 GRP-receptor expressing tumor-associated blood vessels in invasive urothelial carcinoma of the urinary tract. (A) H&E stained section (bar=1 mm) of an invasive urothelial carcinoma with sheets of neoplastic cells (Tu) and tumor stroma (st) containing blood vessels (three vessels indicated by arrowheads). (B) Autoradiogram showing total binding of ^{125}I -[Tyr⁴]-bombesin. Strongly labeled tumor-associated blood vessels presenting as black dots, lines, and tubular segments are present in the tumor. The neoplastic cells (Tu) are not labeled. (C) Control sections with non-specific binding.

unlabeled ligands for the pharmacological characterization of the GRP-receptor subtype in tumor-associated blood vessels. As shown in Fig. 4, the rank order of potencies at the receptor is characteristic for the GRP-receptor. Moreover, using the universal radioligand ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6–14), displacement in the nanomolar range was always obtained with GRP, but not with NMB, further confirming the sole GRP-receptor expression in these vessels.

The other tumor tissue compartment with GRP-receptor expression was the neoplastic cell itself (Table 1). This compartment also revealed great differences in the prevalence of GRP-receptor expression between the different anatomical sites. While, as reported previously (Gugger & Reubi 1999, Markwalder & Reubi 1999), the vast majority of prostate and breast cancers tested in this study showed neoplastic cells overexpressing GRP-receptors (96 and 72% respectively), the neoplastic cells of urinary tract cancers completely lacked detectable GRP-receptors. Moderate expression of GRP-receptors was found in tumor cells of kidney cancers (28%), while very low expression was found in biliary tract (9%) and colon (5%) cancers. Furthermore, differences in the prevalence of GRP-receptor positive neoplastic cells could be observed between tumor types of a given anatomical site. For instance, no GRP-receptors were found in adenocarcinomas of the lung while they were present in 33% of the SCLC (Table 1).

Most importantly, vascular GRP-receptors were not expressed in non-neoplastic tissues of the breast, prostate, kidney, colon, biliary tract, and urinary tract. However, single GRP-receptor positive vessels were present in non-neoplastic lung tissue (4/48) where they were associated with foci of inflammation. Additionally, as reported previously, GRP-receptors were expressed focally and in small amounts in the prostate stroma (Markwalder & Reubi 1999), in the muscular layers of the colon (Rettenbacher & Reubi 2001) and urinary tract at variable levels and in the neural plexus of the colon (Rettenbacher & Reubi 2001). Also the epithelial compartment in prostatic intraepithelial neoplasia (Markwalder & Reubi 1999) and in some cases of non-neoplastic prostate and breast (Gugger & Reubi 1999) showed focal GRP-receptor expression while the epithelial components in the remaining non-neoplastic tissues (lung, kidney, biliary tract, and urinary tract) lacked GRP-receptors.

Discussion

The tumor vascular bed is important for tumor progression because tumors exceeding 1–2 mm need their own blood supply for further growth (Auguste *et al.* 2005). Development and function of the tumor vascular bed are regulated by a large number of mechanisms characterized recently at the molecular level (Auguste *et al.* 2005). Newly, also GRP and its receptor have been shown to play a role in the vascular bed in specific cancer models (Levine *et al.* 2003a,b, Bajo *et al.* 2004, Heuser *et al.* 2005, Kanashiro *et al.* 2005, 2007, Stangelberger *et al.* 2005,

Figure 2 GRP-receptor expressing tumor-associated blood vessels possess a muscular wall. Many are vascular structures compatible with neoangiogenic vessels (A–C); rarely are they pre-existing small veins (D–F). (A and D): H&E-stained sections (bars=0.1 mm) showing detailed features of tumor-associated blood vessels. (A): a colon cancer-associated blood vessel of medium size without well organized, layered muscular wall, compatible with a neoangiogenic vessel. (D): a breast cancer-associated vein with a well organized, layered muscular vessel wall. (B and E): autoradiograms showing total binding of ^{125}I -[Tyr⁴]-bombesin. Strong labeling of the muscular vessel walls in B and in E. (C and F): control sections with non-specific binding.

Kang *et al.* 2007), suggesting a putative role in human primary cancers as well. The present report is the first study evaluating systematically a large number of common human cancers from various anatomical sites specifically for GRP-receptor expression in their tumor vascular beds. A main message of this study is that tumors expressing GRP-receptors in their vascular bed can be detected in all evaluated anatomical sites. Importantly, however, the incidence of these receptor positive vessels, reflected by their prevalence and the vascular score, varied broadly between the anatomical sites: urinary tract cancer had the highest prevalence (92%) and vascular score (1.69) while prostate cancer displayed the lowest values (prevalence: 12%; score: 0.14). These values could also vary between different tumor types within a given anatomical site as shown in the lung for SCLC (prevalence: 0%; score: 0), adenocarcinomas (prevalence: 59%; score: 0.88) and squamous carcinomas (prevalence: 83%; score: 1.28). Together, these data suggest that GRP-receptor expression in the tumor vascular bed may be a phenomenon related to the specific tumor type rather than a universal and uniform regulatory mechanism in the tumor vascular bed *per se*. Further functional evaluation of vasculotropic effects of GRP in tumors should therefore consider these inter-tumoral differences and preferentially choose tumor models

displaying the highest amounts of vascular GRP-receptors related to urothelial cancer or, as previously shown by our group, to ovarian cancers (Fleischmann *et al.* 2007).

Blood vessels in the tumor bed are of different origins including preexisting veins and arteries incorporated in the growing tumor mass and newly formed neoangiogenic blood vessels with abnormally organized walls (Jain 2003, Auguste *et al.* 2005, Dome *et al.* 2007). In our study, GRP-receptor positive blood vessels were mostly small and characteristically had a muscular wall. In general, these tumor-associated vessels ran tortuous, had no specific structure of the wall and were morphologically compatible with neoangiogenic vessels. Notably, the muscular wall of these vessels was the site of GRP-receptor expression with moderate to high receptor densities in a homogeneous, transmural receptor distribution. Only rarely did GRP-receptor positive vessels present morphologically as typical small veins. What may be the function of these tumoral vessels expressing GRP-receptors? First, tumor-associated GRP-receptor positive vessels might be involved in tumoral hemodynamics. Data in line with this concept are that bombesin and GRP physiologically regulate the vascular tone (Bjennings *et al.* 1991, Luu *et al.* 1993, Clive *et al.* 2001) and that GRP-receptors are expressed

Figure 3 Distribution and localization of GRP-receptor positive vessels in the tumor vascular bed. (A–C): GRP-receptors are expressed only in a part of the tumor-associated blood vessels. (D–I): GRP-receptor expressing tumor-associated blood vessels are present intratumorally (D–F) and/or at the interface with the surrounding host tissue (G–I). (A): invasive urothelial carcinoma (Tu) showing immunohistochemically (CD34) brown stained endothelium of blood vessels (three indicated by arrowheads), which run within narrow stromal septa; bar=0.1 mm. (B): autoradiogram showing total binding of ^{125}I -[Tyr⁴]-bombesin. The vessels (three indicated by arrowheads) are only labeled in the lower part of the sample but not in the upper part. The neoplastic cells of the tumor (Tu) are not labeled. (C): control section showing non-specific binding. (D and G): H&E stained sections; bars=1 mm. (D): invasive urothelial carcinoma (Tu) composed of solid growing neoplastic cells and intratumoral blood vessels (three vessels indicated by arrowheads); (G): clear cell renal carcinoma (Tu) showing solid growing neoplastic cells (two histopathologically unremarkable areas indicated by arrows). The tumor is well demarcated from the surrounding host tissue (ht) and there are numerous blood vessels at the tumor/host interface (three indicated by arrowheads). (E and H): autoradiograms showing total binding of ^{125}I -[Tyr⁴]-bombesin. Strongly labeled tumor-associated blood vessels presenting as black dots, lines or tubular segments are present intratumorally in E and at the interface of the tumor (Tu) with the host tissue (ht) in H (three vessels of each sample indicated by arrowheads). The neoplastic cells are focally labeled in H (arrows) but not in E. (F and I): control sections with non-specific binding.

Figure 4 Competition curves in a representative GRP-receptor expressing tumor-associated vessel in colon cancer using ^{125}I -[Tyr⁴]-bombesin as radioligand. Successive tissue sections were incubated with the radioligand and increasing concentrations of unlabeled GRP (●), bombesin (■), and somatostatin-28 (SS-28; ▲). High-affinity displacement of the tracer was found with GRP and bombesin, whereas SS-28 induced only displacement at lower affinity. Non-specific binding was subtracted from all values. The observed rank order of potencies of these analogs is compatible with the GRP-receptor subtype.

in the blood pressure controlling vascular muscle cells. Second, these GRP-receptors might play a role in tumor neoangiogenesis. Indeed, previous functional studies have been able to link GRP to angiogenesis: at the molecular level, GRP up-regulates pro-angiogenic factors such as vascular endothelial growth factor (VEGF) in prostate and endometrial cancer *in vitro* (Levine *et al.* 2003a,b) and in neuroblastoma *in vivo* (Kang *et al.* 2007) while GRP antagonists reduce such factors in glioblastoma (Kanashiro *et al.* 2005), prostate (Stangelberger *et al.* 2005), breast (Bajo *et al.* 2004), and non-small cell lung (Kanashiro *et al.* 2007) cancer. Correspondingly, on cellular and tissue level, GRP stimulates endothelial cell migration and cord formation *in vitro* as well as angiogenesis *in vivo* (Martinez *et al.* 2005), whereas GRP-receptor antagonists reverse these effects and furthermore diminish the tumoral blood vessel density in animal models of breast (Bajo *et al.* 2004) and renal cell (Heuser *et al.* 2005) cancer. Although speculative, the particular GRP-receptor expression in the muscular coat of tumor-associated vessels might trigger a crucial process in neoangiogenesis, namely the vessel wall formation by recruitment of their cellular elements, the pericytes/smooth muscle cells (Jain 2003). The important cellular actions in this process are proliferation, migration, and morphogenesis, and, notably, these cellular properties have been shown to be stimulated by GRP (Bunnett 1994, Kim *et al.* 1997,

Yule & White 1999, Jensen *et al.* 2001). Consequently, the detection of this particular GRP-receptor expression in the muscular coat of tumor-associated blood vessels might open the way for new insights in tumoral hemodynamics or alternatively add new aspects to the pro-angiogenic properties of GRP (Heuser *et al.* 2005, Martinez 2006), in particular to the understanding of neoangiogenic blood vessel maturation.

The other tumor tissue component expressing GRP-receptors investigated in the present study was the neoplastic cell itself. Of all tested tumors, only prostate and breast cancers expressed large amounts of GRP-receptors in this component, as previously reported (Gugger & Reubi 1999, Markwalder & Reubi 1999). Those, however, had very few vascular GRP-receptors. Conversely, urinary tract tumors, with a high expression of GRP-receptors in vessels, had none in the neoplastic cells. One can therefore observe a trend for GRP-receptor deficient tumors (e.g. urinary tract and serous ovarian (Fleischmann *et al.* 2007) cancers) to express large amounts of GRP-receptors in their tumor vascular bed, and for GRP-receptor expressing tumors (e.g. prostate and breast cancers) to lack GRP-receptors in their vascular bed. Consequently, caution should be given when assessing the cellular origin of GRP-receptors detected on mRNA or protein level in homogenates of tumor samples.

The results of our study may be of potential clinical relevance. Cancer targeting in general and GRP-receptor targeted cancer imaging and therapy in particular has attracted a considerable amount of interest over the last years (de Visser *et al.* 2008). These approaches primarily focused on the neoplastic cancer cells themselves. However, alternative strategies have recently been explored by evaluating in particular the targeting of the tumor vascular bed (Thorpe *et al.* 2003, Brack *et al.* 2004). The key to successful selective molecular vascular targeting is the identification of target molecules being considerably expressed in the tumoral vasculature. Vascular GRP-receptors in urothelial cancers may meet these criteria as would also ovarian cancers (Fleischmann *et al.* 2007). Those cancers should be the first to be selected for *in vivo* targeting in patients. Whether other cancers with less GRP-receptor positive tumoral vessels (lung, kidney or colon cancers) would also apply for such an approach, should be evaluated in a second step. For angi destructive therapies, radiolabeled or cytotoxic bombesin receptor agonists (Zhang *et al.* 2004, 2007, Engel *et al.* 2005, Nock *et al.* 2005, Lantry *et al.* 2006, Moody *et al.* 2006, Patel *et al.* 2006, de Visser *et al.* 2007, Waser *et al.* 2007) and their antagonists

(Cescato *et al.* 2008) may be delivered specifically to tumor vessels in order to damage them, causing rapid shutdown of vascular function and leading to tumor necrosis with subsequent tumor control. In fact, a similar concept has already been successfully applied in animal models using annexin A1 as a specifically expressed vascular target for radioimmunotherapy in tumors (Oh *et al.* 2004). Alternatively, bombesin receptor antagonists might be used for angiostatic therapies preventing tumor progression by inhibition of neovascularization. Such anti-vascular effects of bombesin antagonists have recently been reported in animal models of breast (Bajo *et al.* 2004) and renal cell (Heuser *et al.* 2005) cancer. Even more powerful, a dual vascular targeting may be conceivable, supplementing and optimizing anti-VEGF directed anti-vascular therapies in urinary tract cancer (Mitra *et al.* 2006) by synergistic effects of a vascular GRP-receptor targeting. If feasible, *in vivo* imaging of the tumor vascular bed might in future expand the clinical applications of vascular GRP-receptor targeting.

Declaration of interest

No conflict of interest to declare.

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