

Overexpression of gastrin-releasing peptide receptors in tumor-associated blood vessels of human ovarian neoplasms

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Abstract. *Background:* Peptide receptors, overexpressed in specific cancers, represent new diagnostic and therapeutic targets. In this study, receptors for the gastrin-releasing peptide (GRP), and other members of the bombesin-family of peptides, were evaluated in ovarian neoplasms. *Methods:* 75 primary, secondary and metastatic ovarian tumors were investigated for their bombesin-receptor subtype expression, incidence, localization and density using *in vitro* autoradiography on tissue sections with the universal radioligand ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) and the GRP-receptor subtype-preferring ^{125}I -[Tyr⁴]-bombesin. *Results:* GRP-receptors were detected in 42/61 primary ovarian tumors; other bombesin-receptor subtypes (BB1, bb3) were rarely present (3/61). Two different tissue compartments expressed GRP-receptors: the tumoral vasculature was the predominant site of GRP-receptor expression (38/61), whereas neoplastic cells more rarely expressed GRP-receptors (14/61). GRP-receptor positive vessels were present in the various classes of ovarian tumors; generally, malignant tumors had a higher incidence of GRP-receptor positive vessels compared to their benign counterparts. The prevalence of such vessels was particularly high in ovarian carcinomas (16/19) and their metastases (5/5). The GRP-receptors were expressed in high density in the muscular vessel wall. Normal ovary ($n = 10$) lacked GRP-receptors. *Conclusions:* The large amounts of GRP-receptors in ovarian tumor vessels suggest a role in tumoral vasculature and possibly angiogenesis. Further, these vessels might be targeted *in vivo* with bombesin analogs for diagnosis or for therapy.

Keywords: Autoradiography, bombesin-receptors, GRP-receptors, ovary, neoplasm, tumoral vasculature

1. Introduction

Ovarian carcinomas are the major cause of death from genital tract tumors in the United States with estimated 22,220 new cases and 16,210 deaths in 2005 [1]. Currently, the treatment options for initially advanced cancers are extensive cytoreductive surgery and platinum-based combination chemotherapy usually with taxanes. However, despite of these primary interventions most of these patients relapse within 3 years and long-term survival is dismal [2]. Therefore, new therapeutic strategies are mandatory.

Targeted therapies represent new options treating tumors selectively by delivery of the therapeutic agents

directly to specific cells of the tumor tissue. For instance, receptors overexpressed by the tumor tissue may serve as targets for radiolabeled and cytotoxic agents specifically binding to them. This concept has already gained clinical application not only for therapy but also for diagnostics in e.g. neuroendocrine tumors overexpressing receptors for the peptide somatostatin [3,4].

In the last years, accumulating evidence has indicated also an important role of bombesin-like peptides and their receptors in different cancers. Bombesin-like peptides include the amphibian bombesin and its mammalian counterparts, gastrin-releasing peptide (GRP) and neuromedin B (NMB) [5]. Their actions are mediated through several receptor subtypes: the NMB-preferring receptor (BB1), the GRP-preferring receptor (BB2) and the orphan receptor subtype bb3 [6,7]. Not only have trophic effects of the peptides been observed in human cancer cell lines of various organs [8–10], but overexpression of the corresponding bombesin

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receptors has been detected in several human cancers [11–16]. Yet, little is known about bombesin-like peptides and their receptors in ovarian cancers. The few available studies suggest a significant role of these peptides, as shown by the detection of bombesin receptor mRNA in experimental human ovarian cancers and their effective growth inhibition under treatment with bombesin antagonists [17–19]. The only study evaluating the bombesin-receptor status in human ovarian cancer specimens [20] detected bombesin-receptor mRNA in the majority of ovarian cancers and functional GRP-receptors in a subset, what might qualify these often aggressive tumors as promising candidates for targeted therapies with radiolabeled and non-radiolabeled cytotoxic bombesin analogs [21,22].

The aim of this study was to evaluate the bombesin-receptor status at the protein level in carcinomas as well as in a variety of other ovarian neoplasms of all tumor categories by means of *in vitro* autoradiography on tissue sections using ^{125}I -[Tyr⁴]-bombesin and ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) as radioligands. These methods enable not only to evaluate the prevalence, density and subtype of bombesin-receptors but also allow evaluating the receptor distribution in different tumoral tissue compartments including neoplastic cells, stroma and blood vessels. The focus on tumor vessels might be particularly interesting, since angiogenesis plays an important role in primary and metastatic ovarian cancers [23]; moreover, GRP and the corresponding receptors have recently been shown to be implicated in cancer angiogenesis [15,24], and other tumors such as endometrial carcinomas showed an overexpression of GRP-receptors in blood vessels [16,25]. Exact knowledge about the bombesin-receptor status in ovarian neoplasms may, in addition to pathophysiological interest, provide information about the potential value of radiolabeled and non-radiolabeled cytotoxic bombesin analogs for diagnosis and therapy.

2. Materials and methods

2.1. Tissues

All tissue samples derived from tumors of surgically treated women and were immediately frozen after tumor excision and stored at -80°C . In general, one sample per tumor was taken. The primary ovarian tumors were classified according to the latest WHO classification [2]. Tissue samples of the following 75 pri-

mary, secondary and metastatic tumors of the ovary were available: 31 epithelial-stromal tumors (5 adenomas, 7 borderline tumors, 19 carcinomas), 20 sex-cord-stromal tumors (6 granulosa cell tumors, 8 fibrothecomas, 5 Sertoli–Leydig cell tumors, 1 Leydig cell tumor), 10 germ cell tumors (4 mature teratomas, 2 immature teratomas, 1 monodermal teratoma (carcinoid), 3 dysgerminomas), 9 metastases in the ovary including primary adenocarcinomas of the colon ($n = 4$), the stomach ($n = 1$), the lung ($n = 1$), 1 small cell lung cancer and 2 neuroendocrine carcinomas (1 from the midgut, 1 without known primary) as well as 5 intraabdominal metastases of serous ovarian carcinomas. Furthermore, 10 samples of normal pre- and postmenopausal ovaries were tested. This particular tumor population with a high proportion of the rare sex-cord-stromal tumors and secondary ovarian tumors comprises also selected samples from other centers. The study conformed to the ethical guidelines of the Institute of Pathology in Berne.

2.2. Receptor autoradiography

The radioligands used were ^{125}I -[Tyr⁴]-bombesin, known to preferentially label GRP-receptors [26] and the universal radioligand ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14), which has been reported to be an outstanding universal ligand identifying all known bombesin receptor subtypes [14,27].

For autoradiography, two consecutive 20- μm thick cryostat tissue sections were processed as duplicates as described previously [14,28]. They were incubated with one of the two radioligands for 1 h at room temperature. ^{125}I -[Tyr⁴]-bombesin (2000 Ci/mmol; Anawa, Wangen, Switzerland) was added in a concentration of 100 pM in the presence or absence of 0.1 μM bombesin. The expressed bombesin-receptor subtype was evaluated in all samples by addition of 20 pM ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) (2000 Ci/mmol; Anawa) to the incubation solution in absence of any competitor peptide, as well as, in consecutive sections in presence of 50 nM of each of the following unlabeled competitors: [D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14), GRP and NMB. Furthermore, complete inhibition curves were generated for ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) in selected tissues by incubating consecutive sections in presence of increasing amounts of non-radioactive [D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14), GRP or NMB (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 bombesin-receptors was quantified with a computer-assisted image processing system, as described previously [14,28].

The incidence of GRP-receptor positive vessels was graded as follows: On each tissue slide, the tumor area with the highest number of GRP-receptor positive vessels was selected. Here, five visual fields (diameter per field 2.2 mm; overall area 19 mm²) were evaluated and given the following grades: grade 1: 1–10 GRP-receptor positive vessels; grade 2: 11–30 GRP-receptor positive vessels; grade 3: more than 30 GRP-receptor positive vessels; grade 0: no GRP-receptor positive vessel present on the slide. In order to compare the vascular GRP-receptor status of different tumor groups, we defined a score combining incidence of GRP-receptor positive vessels per tumor area and incidence per tumor group. This score was determined by dividing the sum of the grades within a given tumor group by the number of all evaluated tumors in this group. The scores are descriptive mean values for each tumor group. A statistical testing for significance is not sensible because of the small size of the tumor groups. For the same reasons we did not determine standard deviations.

3. Results

Table 1 shows that bombesin-receptors were expressed in more than two thirds of primary ovarian neoplasms (42/61). The most abundant subtype of bombesin-receptors was the GRP-receptor found in 42/61 of the cases. NMB-receptors (3/61) and bb3 (3/61) were rarely detected and only present in particular tumor types (2 teratomas, 1 Sertoli–Leydig cell tumor). GRP-receptors were found in specimens of all tumor categories. There were differences in the prevalence and quantity of GRP-receptors between the various classes of ovarian tumors comprising tumor types with a high prevalence like adenocarcinomas (17/19 cases or 89%) and those without evidence of GRP-receptors (fibrothecomas). The most important finding of the study was that the GRP-receptors were differentially expressed in the various tissue compartments of the tumors. In less than $\frac{1}{4}$ of the cases the neoplastic cells themselves were expressing GRP-receptors (14/61). Most interestingly, in more than 60% of the cases the involved compartment consisted of tumoral blood vessels (38/61).

While the density of the GRP-receptors within positive vessels was relatively constant at high level (Table 1), the incidence of these GRP-receptor positive vessels in the tested specimens was variable, ranging from none, over scattered to numerous receptor positive vessels per tumor area. These vessels were often diffusely distributed in the tumor tissue (Fig. 1A–C). But clustering of GRP-receptor expressing blood vessels in restricted tumor areas forming “hot spots” was also regularly observed (Fig. 1D–F).

GRP-receptor positive tumoral blood vessels were mostly of small size (30–120 μ m) and presented with collapsed luminal spaces; few were being medium sized (diameter up to 3 mm) and showed open lumina (Fig. 2A–C). Few of these GRP-receptor positive blood vessels presented as straight vascular segments, the longest one observed measured 2.5 mm. However, since most tumoral blood vessels run tortuously, GRP-receptor positive vessels presented predominantly as small black dots or oval structures. The GRP-receptor positive vessels were mature and had muscular walls. In general, the GRP-receptors were homogeneously, circumferentially, transmurally and strongly expressed in these muscular vessel walls (Fig. 2D–F). However, GRP-receptor positive and negative vessels could lie in close vicinity (Fig. 2G–I). The histological quality of incubated sections for autoradiography is comparable to frozen sections and reduced compared to formalin-fixed paraffin-embedded sections. GRP-receptor positive vessels were identified and correlated with autoradiography by identifying them in H&E stained sections on the basis of their particular structure including a muscular coat, which is in general well recognizable despite of this particular tissue preservation. This is true for small and mid-sized vessels, whereas capillaries cannot be identified with certainty. Due to limits of resolution of receptor autoradiography, it was not possible to evaluate with certainty whether endothelial cells either in capillaries or in mature muscular coated vessels expressed GRP-receptors as well.

GRP-receptor expressing vessels were present in all categories of ovarian tumors. Most often these vessels were observed in the category of epithelial–stromal tumors (25/31). There was a tendency to a higher incidence of these vessels with increasing malignant potential of these tumors (adenomas \rightarrow borderline tumors \rightarrow carcinomas) as illustrated and quantified in Fig. 3. The same trend was observed in sex–cord–stromal tumors ($n = 20$): while the benign sex–cord–stromal tumors, the fibrothecomas ($n = 8$), never

Table 1
Location, incidence and density of GRP-receptors per tumor class in vascular and/or neoplastic compartments of ovarian tumors

Tumor types	n	Blood vessels		Neoplastic cells	
		Incidence (positive/total cases*)	Density** dpm/mg tissue mean ± SEM	Incidence (positive/total cases*)	Density*** dpm/mg tissue mean ± SEM
A. Primary tumors					
Epithelial–stromal tumors					
Adenoma					
serous	2	0/2	–	0/2	–
mucinous	3	3/3	2468 ± 189	0/3	–
Borderline					
serous	4	4/4	1993 ± 411	0/4	–
mucinous	3	2/3	1936, 5013	0/3	–
Carcinoma					
serous	14	12/14	2520 ± 324	2/14	957, 1265
endometrioid	4	4/4	2074 ± 444	3/4	1185 ± 543
clear cell	1	0/1	–	1/1	5799
Sex–cord–stromal tumors					
Fibrothecoma	8	0/8	–	0/8	–
Granulosa cell tumor	6	4/6	1706 ± 383	1/6	1152
Sertoli–Leydig cell tumor	5	5/5	1965 ± 270	2/5	1434, 2064****
Leydig cell tumor	1	0/1	–	0/1	–
Germ cell tumors					
Teratoma	7	2/7	1630, 2940	4/7	max. 22343****
Dysgerminoma	3	2/3	1205, 1428	1/3	357
B. Metastases of ovarian serous carcinomas					
Total	5	5/5	2728 ± 608	0/5	–
C. Metastases in the ovary					
Adenocarcinoma (colon and lung)	6	4/6	1556 ± 269	2/6	6645 (colon) 995 (lung)
Neuroendocrine carcinoma	3	0/3	–	2/3	250, 2067

* Number of cases containing GRP-receptor positive vessels vs. total tested cases.

** At least 10 GRP-receptor positive vessels were evaluated to determine a mean density value for each case. The mean of all values (mean ± SEM; dpm/mg tissue) is given for each tumor class.

*** All GRP-receptor positive cases listed in the “incidence per tumor class” column were quantified for their receptor density in the GRP-receptor expressing tumor areas only.

**** In addition to GRP-receptors, these tumor classes expressed also NMB-receptors and/or bb3 (see text for numbers) in the neoplastic cells.

showed such vessels, nine of twelve malignant sex–cord–stromal tumors (granulosa cell tumors, Sertoli–Leydig cell tumors) had all GRP-receptor positive vessels. Furthermore, GRP-receptor positive vessels were regularly present in the malignant germ cell tumors (dysgerminomas, immature teratomas) but rarely in the benign germ cell tumors (mature teratomas). Interestingly, not only primary tumors of the ovary but also ovarian metastases of extraovarian primaries had GRP-

receptor expressing blood vessels (4/9). Conversely, also peritoneal metastases of primary ovarian carcinomas (5/5) revealed such blood vessels, usually in high incidence (Fig. 3).

Although most of our tumor samples did not include adjacent normal ovarian tissue, a few samples including one serous adenocarcinoma did contain tumor tissue bordering the normal ovary: in this sample, numerous GRP-receptor positive vessels were found in the

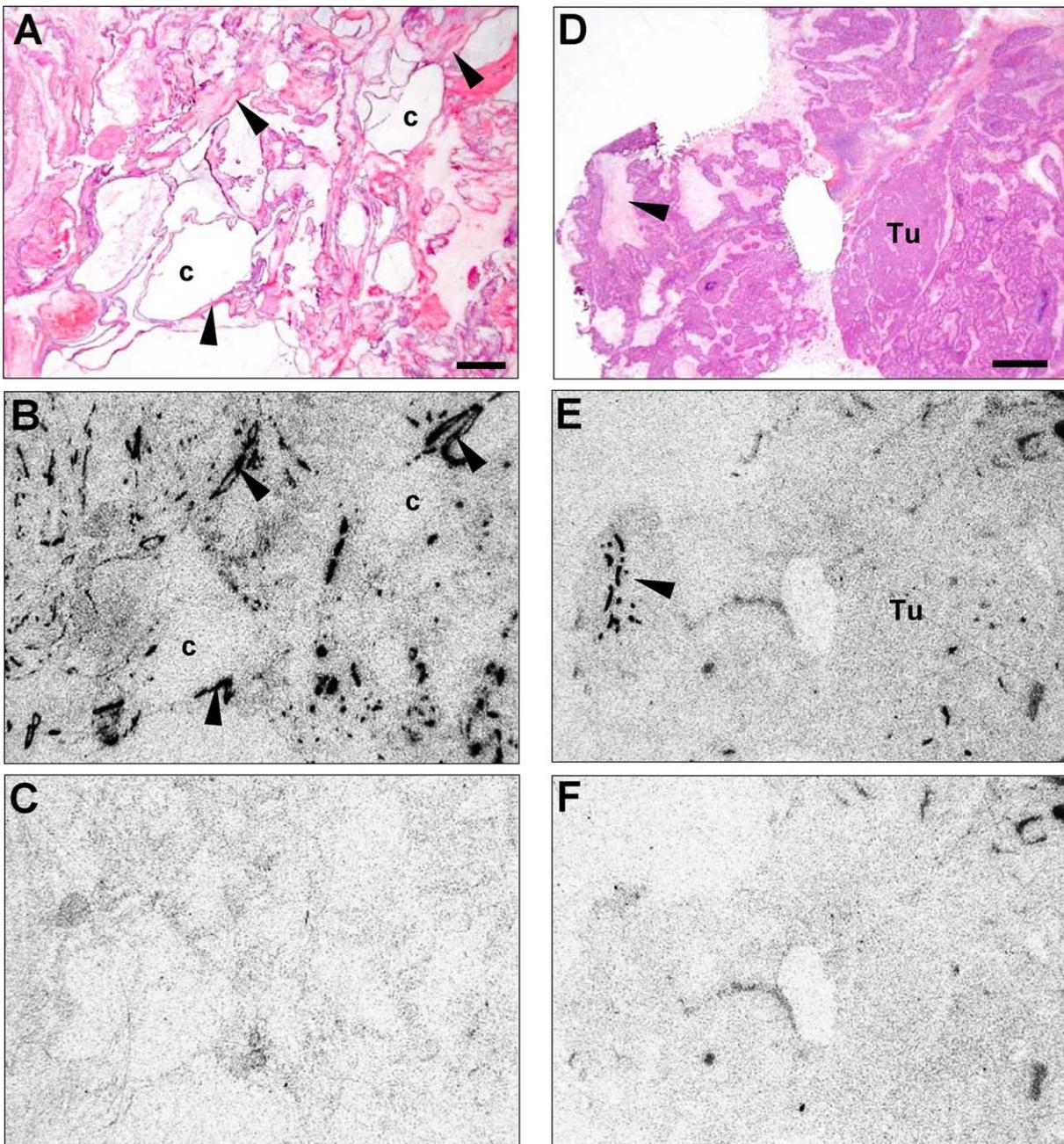


Fig. 1. Overview of two ovarian tumors with GRP-receptor positive blood vessels. A, D: H&E stained sections; bars = 1 mm. A: mucinous borderline tumor with epithelial lined cysts (c) in a tumor stroma with numerous blood vessels (three indicated by arrowheads); D: serous adenocarcinoma with solid growth of the neoplastic cells (Tu). Arrowhead indicating a circumscribed area of vascularized tumor stroma surrounded by neoplastic tissue. B, E: autoradiograms showing total binding of ^{125}I -[Tyr⁴]-bombesin. The distribution of the strongly labeled tumoral vessels is diffuse in B (three vessels indicated by arrowheads) and focal in E (arrowhead), respectively. The neoplastic cells lining the cysts (c) in B and growing solid (Tu) in E, respectively, are not labeled. C, F: control sections showing non-specific binding.

tumor tissue, but not in the adjacent ovary. In addition, we evaluated one sample of a uterine metastasis of a serous adenocarcinoma containing adjacent nor-

mal myometrium; as shown in Fig. 4, GRP-receptor positive vessels were found restricted to the metastasis and at the interface with the surrounding host tissue,

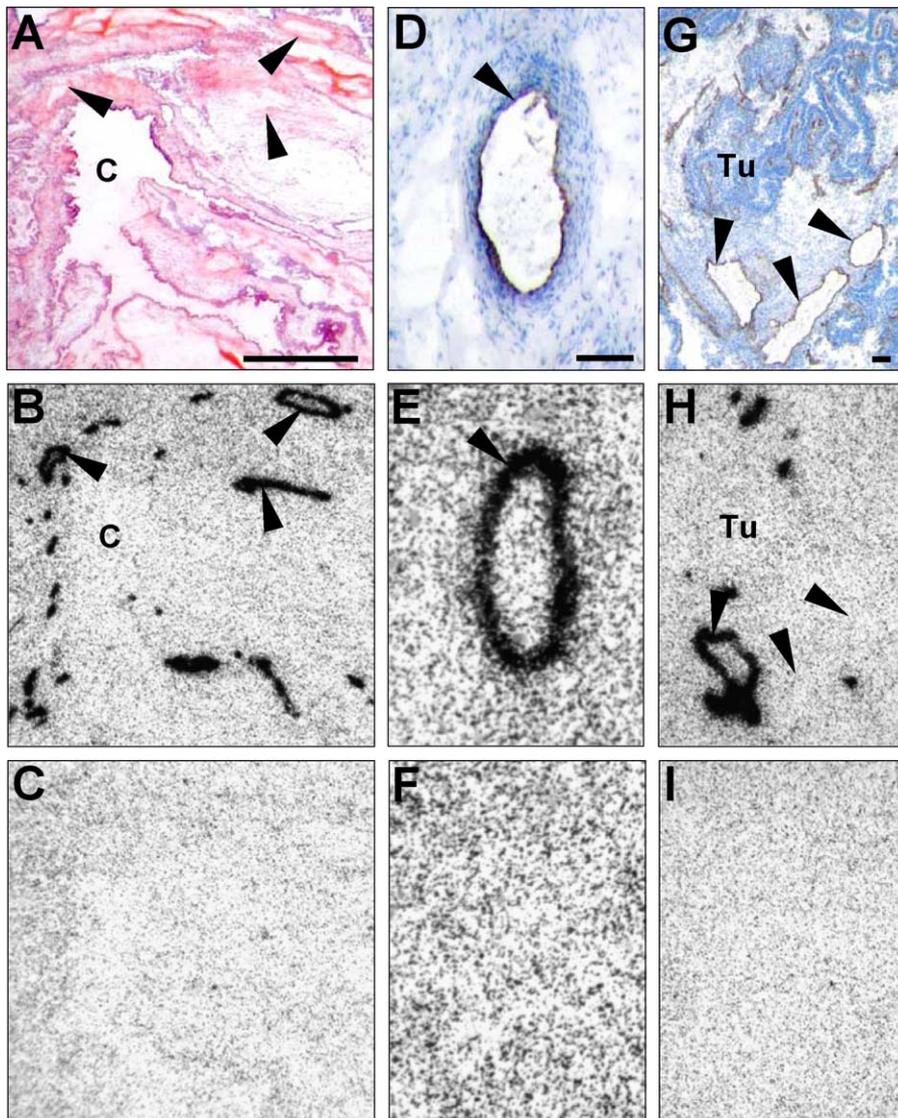
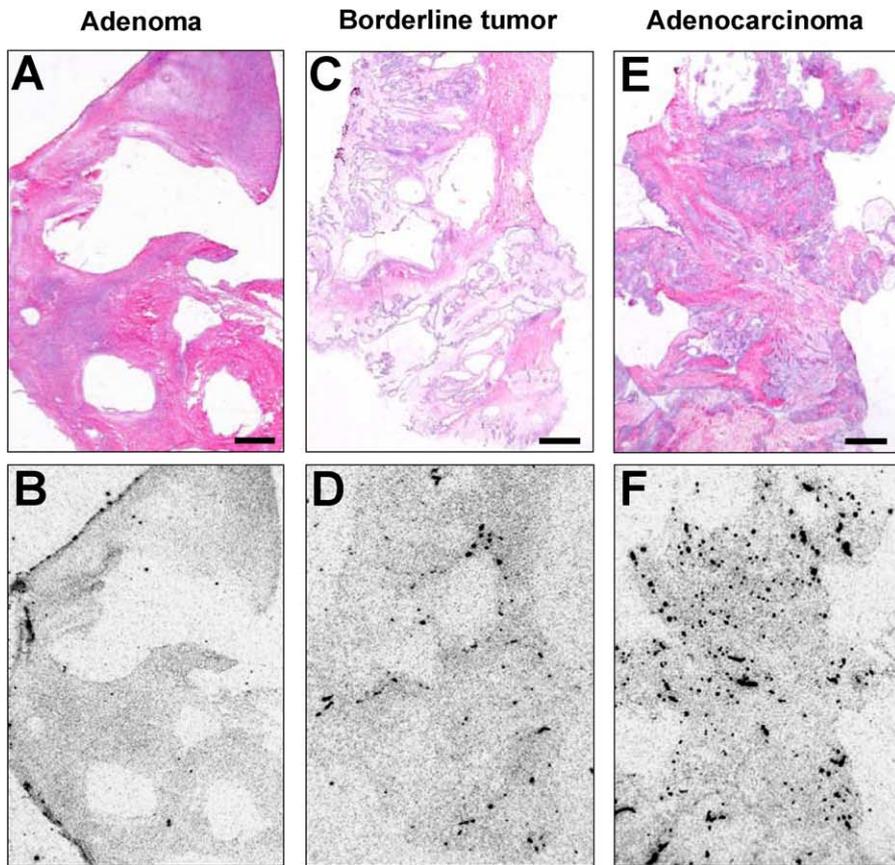


Fig. 2. Detailed features of GRP-receptor positive tumoral blood vessels. A: H&E-stained section of a mucinous borderline tumor with epithelial lined cysts (c) in a tumor stroma with numerous blood vessels (three indicated by arrowheads); bar = 1 mm. B: autoradiogram showing total binding of ^{125}I -[Tyr⁴]-bombesin. Several blood vessels are labeled (three indicated by arrowheads), mostly appearing as small black dots or as black lines and tubular segments with open lumina. The neoplastic cells lining the cysts (c) are not labeled. C: control section showing non-specific binding. D: immunohistochemically (Factor VIII) stained section of a mature tumoral blood vessel (arrowhead) with a muscular wall and a brown labeled endothelium; bar = 0.1 mm. E: autoradiogram showing total binding of ^{125}I -[Tyr⁴]-bombesin. The vessel wall (arrowhead) is homogeneously circumferentially and transmurally labeled. F: control section showing non-specific binding. G: immunohistochemically (Factor VIII) stained section of a serous carcinoma (Tu) with several large tumor vessels (arrowheads) showing brown labeled endothelium; bar = 1 mm. H: autoradiogram showing total binding of ^{125}I -[Tyr⁴]-bombesin. Only one of the large vessels (arrowheads) and further smaller vessels are labeled. No labeling of the neoplastic tissue (Tu). I: control section showing non-specific binding.

whereas the deeper located myometrium vessels were GRP-receptor negative.

The other tissue compartment expressing bombesin-receptors consisted of the neoplastic cells themselves (14/61; Table 1). In the category of epithelial-stromal

tumors, the neoplastic cells expressed GRP-receptors in 6 of 19 (32%) carcinomas (1 clear cell, 2 serous and 3 endometrioid (Fig. 5A–E) type), while in adenomas ($n = 5$) and borderline tumors ($n = 7$) the neoplastic tissue component lacked GRP-receptors. Furthermore,



GRP-R positive vessels in ovarian tumors

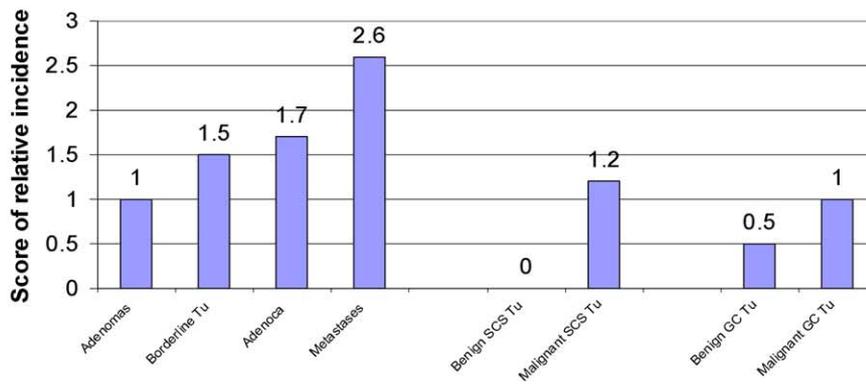


Fig. 3. Increasing incidence of GRP-receptor positive blood vessels with increasing malignant potential in tumor groups of different categories. The top part (A–F) represents GRP-receptor autoradiographies of epithelial–stromal tumors. The bottom part is a histogram representing the score of the incidence of GRP-receptors positive vessels in various tumor groups. A, C, E: H&E-stained sections of a serous adenoma (A), a serous borderline tumor (C) and a serous carcinoma (E); bars = 1 mm. B, D, F: autoradiograms showing total binding of ^{125}I -[Tyr⁴]-bombesin. Absence of GRP-receptor positive vessels in the adenoma (B), but moderate incidence in the borderline tumor (D) and high incidence of GRP-receptor positive vessels in the carcinoma (F), respectively. In all cases, non-specific binding was negligible. The score histogram combines the incidence per tumor area and the incidence per tumor group of GRP-receptor positive vessels in various tumor groups (SCS Tu: sex–cord–stromal tumors, GC Tu: germ cell tumors). The score was determined as follows: the incidence of GRP-receptor expressing vessels per tumor area was graded from 0–3. The sum of all grades in one tumor group was divided by the number of evaluated cases in this group (see method).

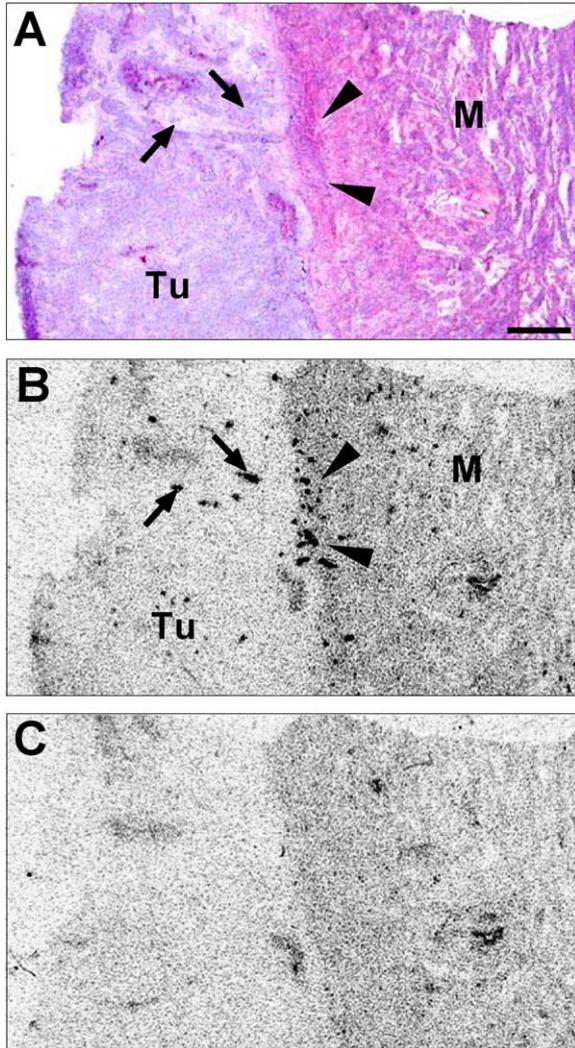


Fig. 4. GRP-receptor positive blood vessels are limited to the tumor tissue and the interface with the surrounding host tissue. A: H&E-stained section of a metastasis of a serous carcinoma (Tu) on the uterine surface adjacent to the myometrium (M); presence of blood vessels in the tumor (arrows) and the interface with the surrounding host tissue (arrowheads); bar = 1 mm. B: autoradiogram showing total binding of ^{125}I -[Tyr⁴]-bombesin. Only vessels in the tumor (arrows) and the interface (arrowheads) are labeled, whereas deeper located myometrium vessels are negative; the myometrium (M) is weakly labeled, the neoplastic cells of the tumor (Tu) are not. C: control section showing non-specific binding.

the neoplastic cells of three sex-cord-stromal tumors (1 granulosa cell tumor, 2 Sertoli-Leydig cell tumors) expressed bombesin-receptors; they consisted of GRP-receptors in all three tumors but also of NMB-receptors (density: 2939 disintegrations per minute [dmp]/mg tissue) and bb3 (density: 15724 dmp/mg tissue) focally in one Sertoli-Leydig cell tumor. Worth men-

tioning are the neuroectodermal components of two teratomas (1 immature, 1 mature) showing the concomitant distribution of all three bombesin receptor subtypes in distinct areas (Fig. 5F-K). The immature teratoma expressed an impressively high number of GRP-receptors (density: 22343 dmp/mg tissue) restricted to some of the tubular formations in the immature component, resembling primitive neuroepithelium of the embryonal neural tube. In addition, NMB-receptors (max. density: 2211 dmp/mg tissue) and bb3 (max. density: 2754 dmp/mg tissue) were detected in the well differentiated neuroectodermal tissues of both tumors. The tissue areas expressing GRP-receptors, NMB-receptors and bb3, respectively, were differentiated using the universal radioligand with selective displacers. Specifically bound universal radioligand was strongly displaced by nanomolar concentrations of [D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) and GRP but only weakly by NMB in GRP-receptor expressing areas. Foci of binding of the universal radioligand displaced by nanomolar concentrations of [D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) and NMB but not by GRP, and other areas of binding with nanomolar displacement by [D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) but neither by NMB nor GRP were identified as NMB-receptors and bb3, respectively. Moreover, 4 of 9 secondary tumors in the ovary had bombesin-receptors. Two metastases (adenocarcinomas of the colon and lung) diffusely expressed GRP-receptors in the epithelial component. The two carcinoids showed receptors for GRP and NMB.

Further, 10 normal ovaries taken from pre- and postmenopausal women were tested. Six ovaries expressed low densities of bb3 (914 ± 129 dmp/mg tissue, mean \pm SEM; Fig. 5L-P). These receptors were primarily located in the ovarian stroma, but were also rarely seen in some ovarian blood vessels. In these 6 ovaries, no difference was observed in bb3 distribution between pre- and postmenopausal conditions. No other bombesin receptor subtype was detected in any tissue component (surface epithelium, stroma, blood vessels and follicles).

Two different ligands were used for the pharmacological identification of the expressed bombesin receptor subtype in the tissues. First, we used the radioligand ^{125}I -[Tyr⁴]-bombesin; this ligand allowed to preferentially identify the GRP-receptor subtype expressed in the tumors (Figs. 1-4). Second, we used the universal radioligand ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14); competition experiments were performed in all cases in order to identify not only

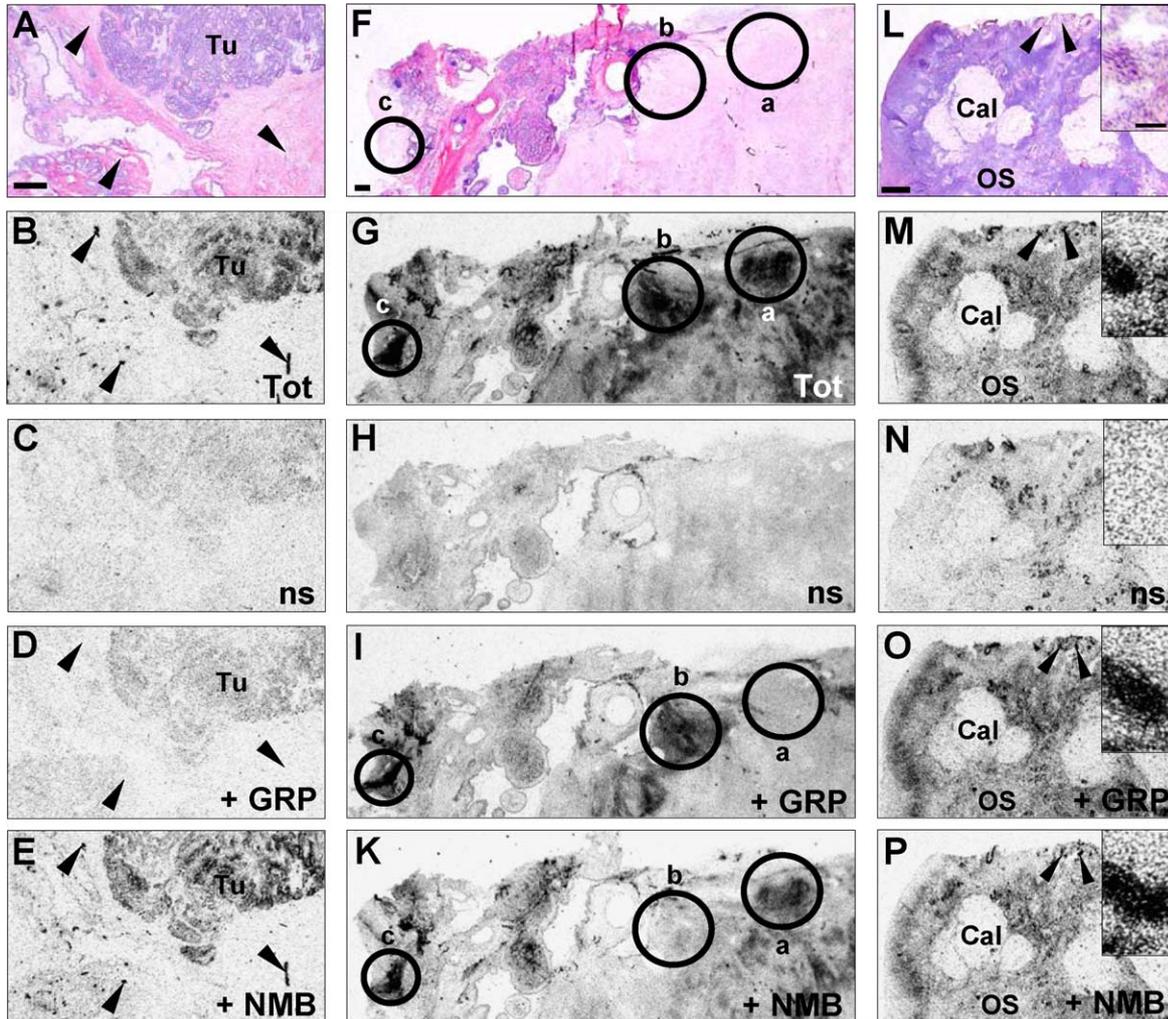


Fig. 5. Autoradiographic evidence for different bombesin-receptor subtypes in an endometrioid adenocarcinoma (left; A–E), a mature teratoma (middle; F–K) and a normal ovary (right; L–P) using the universal radioligand ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) with selective competitors. A, F, L: H&E-stained sections; bars = 1 mm. A: endometrioid adenocarcinoma with neoplastic cells (Tu) and abundant stroma with blood vessels (arrowheads). F: mature teratoma with an abundant histologically homogeneous neuroectodermal component (three foci circled) and a minor component of ectodermal tissue (middle-left). L: normal ovary with blood vessels (arrowheads) and corpora albicantia (cal) in ovarian stroma (os); bar = 1 mm. Insert showing a higher magnification of a blood vessel; bar = 0.1 mm. B, G, M: autoradiograms showing total binding of ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14). B: the neoplastic cells (Tu) and numerous vessels (arrowheads) of the adenocarcinoma are labeled. G: focal labeling of the neuroectodermal (three foci circled) and ectodermal component. M: only the ovarian stroma (os) and some vessels (arrowheads; insert) are labeled. C, H, N: control sections showing non-specific binding. D, I, O: autoradiograms showing ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) binding in presence of 50 nM GRP. D: the radioligand is displaced in the neoplastic cells (Tu) and vessels (arrowheads) of the adenocarcinoma. I: the radioligand is displaced in some areas (e.g. circle a), but not in others (e.g. circles b and c). O: no displacement of the radioligand is observed in the ovarian stroma (os) and the vessels (arrowheads; insert). E, K, P: autoradiograms showing ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) binding in presence of 50 nM NMB. E: virtually no displacement is observed in the neoplastic cells (Tu) and the vessels (arrowheads). Considering the displacement by GRP in D this pharmacological profile is characteristic for GRP-receptors. K: foci of complete displacement of the radioligand (e.g. circle b) by NMB without displacement by GRP in I indicate expression of NMB-receptors. In addition, areas without displacement by NMB (e.g. circles a and c). Some of them with (e.g. circle a) others without (e.g. circle c) displacement by GRP in I, indicating expression of GRP-receptors and bb3, respectively. P: likewise in O, no displacement of the radioligand is observed in the ovarian stroma (os) and the vessels (arrowheads; insert). This pharmacological profile is characteristic for the bombesin-receptor subtype bb3. No other bombesin-receptor subtype is identified.

GRP-receptors but also NMB and bb3 receptor subtypes (Figs 5 and 6). Inhibition curves were generated with this radioligand in successive sections in presence of increasing amounts of non-radioactive ligands ([D-Tyr⁶, β-Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14), GRP and NMB, respectively). In the examples of Fig. 6, their rank order of potencies at the receptor was characteristic for the GRP-receptor.

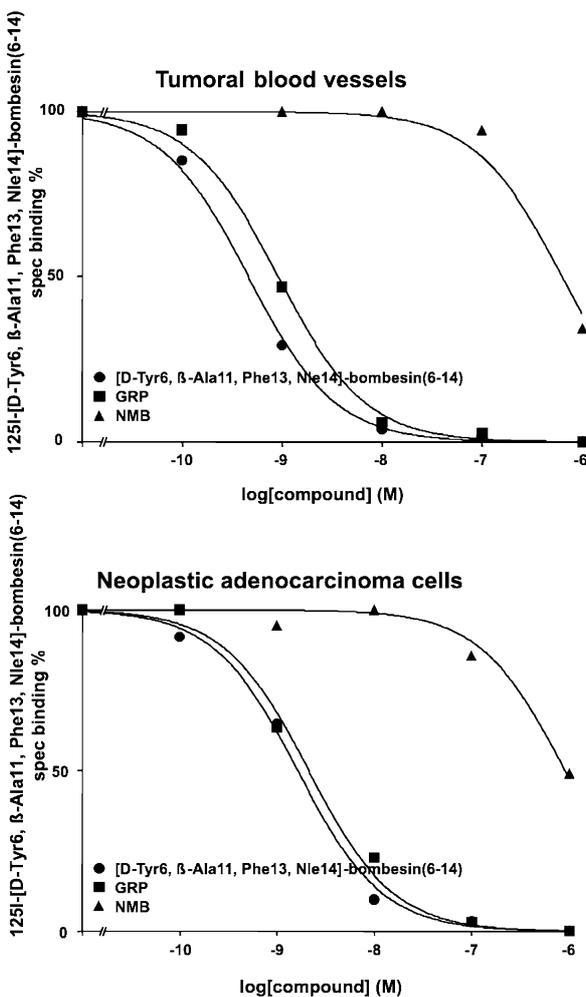


Fig. 6. Competition curves in representative samples of tumoral vessels and ovarian clear cell adenocarcinoma using ¹²⁵I-[D-Tyr⁶, β-Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) as radioligand. Successive tissue sections were incubated with the radioligand and increasing concentrations of [D-Tyr⁶, β-Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) (●) and unlabeled GRP (■) and NMB (▲). High-affinity displacement of the tracer was found with [D-Tyr⁶, β-Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) and GRP, whereas neuromedin B 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.

4. Discussion

This is the first study evaluating the bombesin-receptor protein status in normal and neoplastic ovarian tissues using a morphological method. It shows that the GRP-receptor protein is frequently expressed in various ovarian neoplasms of all tumor categories, while the normal ovary expresses low densities of the bombesin-receptor subtype bb3. This is in agreement with previous data showing the abundance of GRP-receptor mRNA in ovarian carcinoma samples of xenograft models and humans [17–20]; the present study, however, considerably extends these data since the morphology-based method of autoradiography enables us to detect the tumoral vasculature as the preferred site of GRP-receptor protein expression in ovarian carcinomas. Blood vessels in 84%, but neoplastic cells in only 32% of the carcinomas express the GRP-receptor protein.

GRP-receptor expressing blood vessels are limited to the tumors and the tumoral interface. These vessels are mainly of small size (30–120 μm) and possess a muscular coat indicating mature vessels; the GRP-receptors are usually expressed in high densities, and homogeneously and transmurally distributed in the muscular wall of the vessels. As muscle cells have the ability to contract, these vascular receptors might play a role in hemodynamics influencing tumor progression by alteration of the blood flow in the tumoral vasculature. Indeed, bombesin and GRP have been shown to influence the vascular tone under physiological conditions [29–31]; treatment with GRP-receptor antagonists can alterate the microvascular perfusion of a tumor in a xenograft model [15]. Alternatively, the overexpression of GRP-receptors in this site might be implicated in blood vessel maturation, defined as the process of recruitment of cells (pericytes, smooth muscle cells) to form the vessel wall, which is an important step in neoangiogenesis. This step involves distinct mechanisms like cell proliferation, migration and morphogenesis, all known to be mediated by GRP and the corresponding receptor [5,32–34]. The results of recent functional studies propose GRP to be a new angiogenic molecule and link GRP and its receptor to tumor-neoangiogenesis. For instance, GRP up-regulates the pro-angiogenic vascular endothelial growth factor A (VEGF-A) *in vitro* in prostate and endometrial cancer cell lines [35,36]. In addition, GRP promotes migration and cord formation of endothelial cells *in vitro* as well as angiogenesis *in vivo* [37]. Conversely, the application of GRP antagonists induces anti-angiogenic

effects: on molecular level, a substantial reduction of the pro-angiogenic growth factors VEGF-A, fibroblast growth factors (FGF) and insulin-like growth factors (IGFs) has been reported for breast carcinoma cell lines *in vitro* and *in vivo* [38]; on tissue level, GRP antagonists reduce the blood vessel density in xenograft models of renal cell [15] and breast [38] carcinomas. The GRP-receptor protein overexpression in blood vessels of ovarian tumors and particularly in carcinomas therefore may give new dimension to the newly discussed role of GRP and the corresponding receptor in tumor-neoangiogenesis [15,24].

GRP-receptor positive blood vessels are present in all tumor categories with a tendency for higher incidence of GRP-receptor positive vessels in malignant tumors compared with benign or low grade malignant tumors. This finding might reflect the different conditions of vascular development in various tumors with variable growth velocities. Most interestingly, these GRP-receptor positive vessels are furthermore detected in tumors of ovarian origin that are usually fatal, namely the metastases of primary ovarian carcinomas. This phenomenon points to GRP-receptor overexpression in tumor vasculature as a mechanism independent of the primary site of tumor genesis. Interestingly, these vessels are present in very high numbers and with high GRP-receptor densities in these metastases, potentially reflecting tumor clones with uppermost angiogenic properties.

The other ovarian tumor tissue component that expresses GRP-receptors is the neoplastic cell itself. The prevalence, however, varies among the different tumor types in our population. GRP-receptors are frequently found in the endometrioid subtype of ovarian carcinomas, what is in accordance with our previous study showing an overexpression of GRP-receptors in a substantial part of endometrioid carcinomas of the uterus [16]. Conversely, GRP-receptors are rarely and only focally detected in the neoplastic cells of our serous carcinomas. A particularly high density of GRP-receptors is present in the neoplastic cells of the one clear cell carcinoma. A definitive pathophysiological assessment of these findings is not possible due to the limited number of evaluated cases per tumor group. However, neither a tumor subtype-associated GRP-receptor expression pattern nor a contribution of the hormonal status of the patient can be excluded and consequently a tumor subtype-specific analysis is advised when performing receptor analysis.

Pharmacological evidence for a preferential expression of the GRP-receptor subtype in the tested tissues is

achieved using the universal radioligand ^{125}I -[D-Tyr⁶, β -Ala¹¹, Phe¹³, Nle¹⁴]-bombesin(6-14) with receptor subtype-selective competitors. With this method, the tumor vessels are found to express GRP-receptors, whereas the non-neoplastic ovarian tissue expresses bb3 receptors in the stroma and in rare vessels. In all but three bombesin-receptor positive tumors, the GRP-receptor is the predominantly expressed subtype.

Our findings might be clinically significant. The majority of ovarian carcinomas are diagnosed as advanced-stage disease [2] and despite surgery and chemotherapy most of these patients relapse and die of the disease. In this hopeless situation novel treatment strategies are mandatory. Anti-vascular therapies are innovative strategies comprising two concepts [39]: (a) vascular targeting aiming at the destruction of already established tumoral vessels and inducing tumor necrosis and (b) anti-angiogenesis aiming at the inhibition of neovascularisation with subsequent control of tumor progression. The marked overexpression of GRP-receptors in tumoral vessels in primary and metastatic ovarian cancers might represent an optimal molecular basis for vascular targeting with radiolabeled and non-radiolabeled cytotoxic GRP analogs [21,22], destroying already established vessels or, alternatively, for targeted anti-angiogenic therapies with GRP antagonists, preventing the new formation of blood vessels. First experimental data indicating inhibition of tumor angiogenesis and tumor control in breast cancer xenografts treated with GRP antagonists confirm this notion [38]. In addition, the GRP-receptor expressing neoplastic cells of certain tumors, such as ovarian clear cell and some endometrioid carcinomas, might serve as targets for such a therapy which has been shown to have virtually no systemic side effects [40]. Finally, *in vivo* diagnostic might expand the clinical applications. Vascular and/or neoplastic cell targeting with radiolabeled GRP analogs to detect primaries and/or metastases may be used for initial tumor staging as well as follow-up.

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