Gastrin Releasing Peptide-Prefering Bombesin Receptors Mediate Growth of Human Renal Cell Carcinoma

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Abstract. Bombesin-like peptides typically act as neurotransmitters along the brain-gut axis and as growth factors in various human tissues. The present study demonstrates the expression of gastrin releasing peptide (GRP)-preferring bombesin receptors in human renal cell carcinoma but not in normal kidney tissue. The expression of GRP receptors was characterized at the mRNA level by reverse transcription-PCR, as well as at the protein level by binding of 125I-[Tyr4] bombesin to membranes prepared from tumor tissue (Ks 0.3 nM) and healthy kidney tissue from the same four patients. GRP receptors were also demonstrated in four human kidney carcinoma cell lines (A-498, CAKI-1, CAKI-2, and ACHN). The effects of bombesin/GRP agonists and/or antagonists on growth were investigated in vitro on CAKI-2 cells, which expressed large amounts of GRP receptors. Cell numbers stimulated by 10% fetal calf serum were significantly stimulated by interleukin-1β (control) and GRP-7 (10−7 M), both in the range of 136 to 148%; addition of the GRP receptor antagonist acetyl-GRP(20–27) (10−6 M) completely reversed this effect. Bombesin alone (10−6 M) significantly stimulated CAKI-2 cells (129%) cultured with 0.5% fetal calf serum, whereas another antagonist, D-Phe6,Leu13,(CH2NH)Leu14 bombesin(6–14) (1 μM), alone did not inhibit growth, thus excluding an autocrine mechanism. These results indicate for the first time that malignant transformation of human kidney tissue into renal cell carcinoma is accompanied by novel expression of GRP receptors. Bombesin-like peptides might act as mitogens in these carcinomas, and they might be useful as diagnostic or therapeutic tools such as tumor imaging or internal radiotherapy.

Bombesin-like peptides act as neurotransmitters mainly along the brain-gut axis and as mitogens in various tumor tissues (1–5). Molecular cloning studies identified four receptor subtypes, but only the gastrin releasing peptide (GRP)-preferring and the neurokinin B (NMB)-preferring receptor subtypes seem to be relevant in mammalian tissues (6–11). In rodents and humans, the GRP-preferring receptor subtype has been described in smooth muscle cells of the rat esophagus (13). In rats, both subtypes have been described in the central nervous system, predominantly in embryonic stages. Exogenous GRP stimulates the release of various hormones, whereas physiologic functions in humans include stimulation of intestinal secretions and motility (14–17). GRP receptors can also be found in a variety of cancer cells and in tumor tissues, where they mediate growth (18,19). In addition, bombesin-like peptides are known to be involved in autocrine growth of human small cell lung cancer (4,20). However, the mechanisms of action seem to be more complex than anticipated, inasmuch as both GRP receptor agonists and antagonists are able to stimulate and inhibit growth of similar tumor cell lines.

In the adult human kidney, bombesin receptors have not yet been characterized, but recently it was shown that bombesin might be involved in cell proliferation of the human fetal kidney (21). Preliminary results showed functional GRP receptors on COS-7 monkey kidney cells (22), and recently, growth of CAKI-1 renal adenocarcinoma cells was inhibited by a GRP receptor antagonist (23). Renal cell carcinoma, also called adenocarcinoma or hypernephroma, is the most common malignant kidney tumor and is resistant to chemotherapy and radiotherapy. Transforming growth factors (TGF α/β) have been found to be related to development of the tumor (24–26); hormonal therapies, including mainly progestational agents, failed to be effective in this disease (27).

In the present study, we investigated whether human renal cell carcinoma tissues express GRP receptors compared with normal kidney tissue of the same patients and characterized the effects of GRP receptor agonists and antagonists on growth of human renal adenocarcinoma cell lines.

Materials and Methods

Chemicals

Bombesin, [Tyr4] bombesin, and NMB were obtained from Bachem AG (Bubendorf, Switzerland). Sodium N-lauryl-sarcosinate,
bacitracin, bestatin, anastatin, diethyl pyrocarbonate (DEPC), dithio-
D,L-threitol (DTT), sodium citrate, chloroform/isoamylalcohol, 2-mer-
captopoethanol, ethylenediaminetetra-acetic acid (EDTA), and phenyl-
methylsulfonyl fluoride (PMSF) were from Fluka (Buchs, Switzerland).
Soybean trypsin inhibitor (SBTI), bovine serum albumin (BSA), trif-
louroacetic acid (TFA), mineral oil, leupeptin, DMSO, bromphenol blue,
Tris/HCl, sucrose, and glycerol were from Sigma (Buchs, Switzerland).
Nucleotide mix (dNTP-mix) was from Boehringer Mannheim (Rotkreuz,
Switzerland). Guanidinium thiocyanate (GTC) and Roti-phenol were
from Carl Roth (Reinach, Switzerland), MgCl₂, and sodium acetate were
from Merck (Dietikon, Switzerland), and oligonucleotides were from
Myrcynosynth (Balgach, Switzerland). Agarose NA and 100 Base-Pair
Ladder were from Pharmacia Biotech (Dübendorf, Switzerland). Gene-
Amp 10 × PCR buffer and Taq polymerase were from Perkin Elmer
(Rotkreuz, Switzerland), and aprotinin was from Bayer (Zürich, Switzer-
land). RNAzol was from Walk Chemie (Bad Soden, Germany), and the
Superscript cDNA synthesis kit was from Life Technologies/BRL (Eg-
genstein, Germany). Oligonucleotides were from TIP Mol Biol (Berlin,
Germany). Hybond nylon membrane and interleukin-1β (IL-1β) was from
Amersham (Freiburg, Germany). DIG Luminescent Detection Kit for Nu-
cleic Acids was from Boehringer (Mannheim, Germany). Autoradiography
film was from Kodak (Integra Biosciences, Fernwald, Germany).

Tissue Procurement and Cell Culture

Five patients underwent nephrectomy, and in four the excised
kidney was histologically classified as renal cell carcinoma, also
called adenocarcinoma or hypernephroma (28). Patient information is
as follows: patient 1 (female, 82 yr), patient 2 (male, 61 yr), patient 3
(male, 79 yr), patient 4 (male, 48 yr). The kidney of patient 5 (male,
42 yr) was excised due to hydrenephrosis. Human tissues were put on
ice immediately after excision. Small pieces of tumor tissue and
healthy control tissue were removed for characterization of receptors,
and directly adjacent tissues were used for histologic examinations.
The specimens were snap-frozen in liquid nitrogen and stored at
−70°C. They were later powdered in liquid nitrogen using a pestle
and mortar and divided into two parts, one for membrane preparation
and one for extraction of total RNA.

All cell lines used in this study were maintained as a subconfluent
monolayer culture at 37°C in an atmosphere of 5% CO₂/95% O₂ by
passaging twice weekly. For counting and subculturing, cells were
in water-saturated, and 0.2 ml chloroform/isoamyl alcohol

Binding Studies with Cells and Crude Membranes

Cells were harvested with trypsin/EDTA, washed with phosphate-
buffered saline (PBS), and resuspended at a concentration of 1 to
2.5 × 10⁶ cells/ml in binding medium (Hanks’ balanced salt solution
with 25 mM Heps, 0.1% BSA, 1 mM PMSF, 0.01% SBTI, 5 μg/ml
leupeptin, and 100 μg/ml bacitracin). Cells were incubated either at 4,
22, or 37°C with 50 pM ¹²⁵I-[Tyr⁴] bombesin (total binding) or with the
addition of 1 μM unlabeled bombesin (nonspecific binding). Competition
binding experiments were performed at 22°C for 45 min with
50 pM ¹²⁵I-[Tyr⁴] bombesin and increasing concentrations of unlabeled
bombesin at concentrations from 10⁻¹² M up to 10⁻⁶ M. Duplicate samples of
150-μl cell suspension were centrifuged through silicon oil (density 1.013)
to separate bound from unbound tracer.

For crude membrane protein preparations, the frozen tissue
was resuspended in 10 ml of buffer A (20 mM Tris/HCl, pH 7.5, 0.25
M sucrose, 1 mM EDTA, 5 mM MgCl₂, 0.1 mg/ml SBTI, 1 mg/ml
bacitracin, and 1 mM PMSF), homogenized (Ultra-Turrax) for 20 s,
and centrifuged for 5 min at 500 × g. The pellets were homogenized
a second time, and both supernatants were pooled and centrifuged in
an ultracentrifuge for 45 min at 50,000 × g. After determination of
protein concentration, the pellet was stored in small aliquots at
−70°C. For binding studies with crude membranes, appropriate
amounts of protein (150 to 250 μg) were diluted in binding buffer B
(50 mM Tris/HCl, pH 7.5, 2 mM MgCl₂, 1 mM EDTA, 1 mg/ml
bacitracin, 1 mM PMSF, and 200 Kallikrein inactivating units/ml
aprotinin) and incubated at 22°C for 45 min with 50 pM ¹²⁵I-[Tyr⁴]
bombesin and increasing concentrations of unlabeled bombesin at
concentrations from 10⁻¹² M up to 10⁻⁶ M. Duplicate samples of
150-μl protein suspension were centrifuged for 5 min at 10,000 × g
separate bound from unbound tracer, washed twice with ice-cold
PBS, and counted in a gamma counter (Packard Ristar).

Isolation of Total RNA

Total RNA was isolated from frozen tissues by the GTC method
(A). Briefly, powdered tissue was resuspended 1/10 (wt/vol) in GTC
stock solution (4 M GTC, 25 mM sodium citrate, pH 7.0, 0.5%-
N-lauryl-sarosinate) supplemented with mercaptoethanol (7.2 μl/ml)
and homogenized using a syringe with a 25-gauge needle. Per 1 ml of
homogenized solution, 0.1 ml sodium acetate (2 M, pH 4.0), 1 ml
phenol (water-saturated), and 0.2 ml chloroform/isoamyl alcohol
(49:1) were added (vortex after each step) and incubated for 15 min on
ice. Solutions were centrifuged at 20,000 × g for 20 min at 4°C, the
pellets were resuspended in 100 to 200 μl of GTC solution, incubated
for 15 min on ice, and again precipitated with 1 vol of isopropanol for at least 1
hr at −20°C. After centrifugation at 20,000 × g for 20 min at 4°C, the
pellet was washed with 75% ethanol and dried in a Speed-vac, the
RNA was resuspended in DEPC-treated water, and the RNA concen-
tration was determined spectrophotometrically (OD₂₆₀nm/OD₂₈₀nm
between 1.8 and 2.0).
Cell lines were grown according to techniques described previously and washed with PBS immediately before RNA isolation using the RNAzol technique (Walk Chemie, Bad Soden, Germany). In brief, cells were grown in 175-cm² cell culture flasks (Nunc, Wiesbaden, Germany) at a density of 400,000 cells/plate and resuspended in 10 ml of RNAzol after mechanical mobilization using a cell scraper. After homogenizing the cell solution using a mechanical homogenizer, 0.1 vol/ml chloroform was added (vortex after each step) and incubated for 15 min on ice. Solutions were centrifuged at 15,000 × g for 15 min at 4°C, the supernatant was transferred into fresh tubes, and isopropanol precipitation steps were performed as described above. The RNA concentration was determined photospectrometrically (OD260nm/OD280nm between 1.8 and 2.0).

Revers Transcription of Total RNA

Total RNA was reverse-transcribed using the First-Strand cDNA Synthesis Kit from Pharmacia (Uppsala, Sweden). Briefly, from each sample, 5 μg of heated (65°C for 10 min) total RNA solution was used, together with 1 μl of DTT, 1 μl of oligo(dt) primer (1:25 diluted), and 5 μl of Bulk First-Strand Reaction Mix (containing cloned FPL-pure® Murine Reverse Transcriptase, RNAGuard, RNase/DNase-Free BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer) in a total volume of 15 μl, which was mixed and incubated at 37°C for 1 h and stored at −20°C.

Total RNA of the cell lines was reverse-transcribed using the Superscript preamplification kit for cDNA synthesis (Life Technologies-BRL). Briefly, from each cell line, 4 μg of heated (65°C for 15 min) total RNA solution was used, together with 1 μl of DTT, 1 μl of oligo(dt) primer (0.5 μg/ml), and 7 μl of the reaction mix supplied by the manufacturer (containing 200 U/μl cloned Superscript II Reverse Transcriptase [Life Technologies-BRL], RNase/DNase-free DEPC-treated water; dATP, dCTP, dGTP, and dTTP in aqueous buffer; and 25 nm MgCl₂) in a total volume of 20 μl, which was then mixed and incubated for 50 min at 40°C, 15 min at 70°C, and 5 min at 4°C. Subsequently, 1 μl of RNaseH (2 U/μl) was added, followed by a subsequent incubation for 15 min at 37°C.

Reverse Transcription- and Nested-PCR Analysis

Several concentrations of human pancreatic cDNA (Stratagene, La Jolla, CA) in the range of 0.1 ng to 0.1 pg were used as templates in separate PCR reactions using human GRP receptor-specific oligonucleotides (access no: NM_005314) and human β-actin-specific oligonucleotides (access no: X00351 J0074 M10278). The following primers were used: GRP-R S1: 5'-GAA CGA TGA CTT GTC CCA CCC GGG-3' and GRP-R AS1: 5'-TGG AAG GGA TGG AGG TCA GAA-3'; human β-actin S2: 5'-ATC TGG CAC ACA ACC TTC TAC A-3' and human β-actin AS2: 5'-GCT CGT TGT TGC CAA TGG TGA TGA C-3'. For PCR amplification, 2 μl of each cDNA template, 1 μl of 2.5 mM dNTP, 2.5 μl of 99.9% DMSO, 1 μl (20 to 30 pmol) of each primer, and 5 μl of 10× PCR buffer were added, adjusted to 50 μl final volume with H₂O in a sterile 0.5-ml microcentrifuge tube. Immediately before starting the reaction in a preheated (95°C) thermocycler, 0.4 μl of Taq polymerase was added to each reaction tube, together with one drop of mineral oil. Cycle conditions were 94°C for 45 s; 58°C for 45 s; and 72°C for 1 min for 25 cycles. The following nested primers for the constitutive human β-actin messages were used: nesS2: 5'-TCC CTG TAT GCC TCT GTG CGT-3' and human β-actin nesAS2: 5'-GGT CTT GTC TGG CAA TAG TGA TGA C-3'. All PCR products were analyzed by separation on an agarose gel (1.5% agarose in TBE), compared with a standard DNA marker (100-bp ladder), and examined by sequencing using fmol™ DNA sequencing system from Promega (Lausanne, Switzerland).

Southern Blot Hybridization

For Southern blot analysis, the PCR products were electrophoresed in 2.0% agarose gel. The gel was subsequently denatured (0.5 M NaOH/1.5 M NaCl) and washed (1.5 M NaCl/1 M Tris, pH 8.0) both for 30 min at room temperature. The DNA was transferred to a positively charged nylon hybridization transfer membrane (Hybond N⁺ membrane; Amersham) equilibrated in 20× SSC. The membrane was cross-linked using ultraviolet radiation (Stratalinker; Stratagene, Heidelberg, Germany) and hybridized overnight at 60°C under high stringency conditions with a digoxigenin-labeled oligonucleotide probe of the human GRP-preferring bombesin receptor subtype coding region (30-mer, 5'-DIG-GGG GAT CTT TGT GCC AAC AAT CCT GCC AAA-3'; TIB-Molbiol). The membranes were rinsed in a washing buffer (2× SSC/0.1% sodium dodecyl sulfate, 60°C) and incubated for 2.5 h at 60°C in prehybridization buffer (Easy Hyb Buffer; Boehringer Mannheim). The hybridization was performed in the same buffer that included the oligonucleotide probe (140 pmol). The membranes were washed (2× SSC/0.1% sodium dodecyl sulfate, 60°C) and air-dried. The detection was performed using the DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim), according to recommendations of the manufacturer. The blots were autoradiographed for 5 to 30 min (Kodak; Integra Biosciences).

Growth Assays

The human kidney carcinoma cell line CAKI-2 was plated on day 0 in 12-well culture dishes (Nunc) in the appropriate medium containing 10% FCS and 1% penicillin and streptomycin at a density of 15,000 cells/well. Cells were incubated in a humidified 5% CO₂/95% air atmosphere at 37°C for 12 h, and then washed twice with PBS and synchronized overnight in serum-free medium. On day 1, cells were incubated in medium containing 10% FCS with or without GRP-7 (10⁻⁷ M), GRP antagonist acetyl GRP(20-26) (10⁻⁶ M) or as a positive control IL-1β (10 ng/ml) (Amersham) for an additional 144 h up to day 6. From day 1 to day 6, GRP-7, GRP antagonist, or IL-1β was added daily. On each day, cells were trypsinized and cell viability was determined using the trypan blue method. All viable cells were counted in a Neubauer counting chamber in a blinded manner by two different investigators.

In a second growth assay, CAKI-2 cells were plated on day 0 in 10-cm culture dishes in the appropriate medium containing 10% FCS at a density of 200,000 cells/plate. Cells were incubated in a humidified 5% CO₂/95% air atmosphere at 37°C for 12 h, and then washed twice with PBS and synchronized overnight in serum-free medium. On day 1, cells were incubated in medium containing 0.5% FCS with or without bombesin (1 μM) or antagonist (D-Phe₆,Leu₁₃,CH₂NH₆,Leu₁₄ bombesin(6–14))(1 μM) for an additional 72 h up to day 4. On each day, cells were trypsinized and cell numbers were determined using a Coulter counter model ZM (Coulter Electronics, Luton Beds, United Kingdom). From day 1 to day 4, bombesin or antagonist was added daily to the medium to diminish the effect caused by degradation of the peptides in the medium.

Results

Characterization of GRP-Preferring Bombesin Receptors in COS-7 Cells

Total binding of $^{125}$I-$[\text{Tyr}^4]$ bombesin increased within 15 min at 22°C and 37°C and reached a plateau between 30 and 90 min, and slowly decreased at 120 min (Figure 1, top panel). At 4°C, binding increased very slowly and reached only approximately 50% of binding detected at 22 or 37°C. Addition of 1 μM unlabeled bombesin decreased binding of $^{125}$I-$[\text{Tyr}^4]$ bombesin by at least 90% at 22°C and at 37°C, respectively. Binding of $^{125}$I-$[\text{Tyr}^4]$ bombesin to COS-7 cells was inhibited in a dose-dependent manner by unlabeled bombesin; the inhibition produced by NMB was much less prominent (Figure 1, bottom panel). Computer analysis of the ability of bombesin or NMB to inhibit binding of $^{125}$I-$[\text{Tyr}^4]$ bombesin demonstrated a single class of binding sites. Bombesin had a high affinity with a dissociation constant ($K_d$) of $0.21 \pm 0.05$ nM, whereas NMB was 263 times less potent with a $K_d$ of $0.055 \pm 0.019$ mM (Figure 1, bottom panel). This pattern is typical for a GRP-preferring bombesin receptor subtype and is similar to results observed in other tissues expressing this bombesin receptor subtype. COS-7 cells showed a high specific binding of $^{125}$I-$[\text{Tyr}^4]$ bombesin, which was time- and temperature-dependent.

Detection of GRP Receptor mRNA and Protein in Human Renal Cell Carcinoma Tissue

Based on the fact that a monkey kidney cell line expresses specific GRP-preferring bombesin receptors, we examined human kidney tissues from patients undergoing tumor nephrectomy for the expression of human GRP receptors. Five patients underwent nephrectomy, four of them due to renal cell carcinoma and one due to hydronephrosis. First, total RNA of GRP-R was analyzed by a combination of reverse transcription (RT)-/nested-PCR using specific GRP-R oligonucleotides. A standard curve with various concentrations (0.1 ng to 0.1 pg) of cDNA used as templates in separate nested PCR reactions for GRP-R and β-actin confirmed that the PCR product varies linearly with the amount of input cDNA (Figure 2). As shown in Figure 3 (top panel), three out of four patients with renal cell carcinoma expressed high amounts of GRP-R mRNA in the tumor tissue, whereas GRP-R mRNA in normal tissue from the same kidney could hardly been detected. As control for equal amounts of cDNA used for the combined RT-/nested-PCR, oligo(dT) reverse-transcribed cDNA of each sample was used for amplification of the constitutive β-actin message (Figure 3, top panel). As negative control, DNA free water instead of cDNA as template for reverse transcription was used under the same RT-PCR conditions. As positive control, we used cDNA reverse-transcribed from smooth muscle tissue of the human colon (Figure 3, top panel). The amplified GRP-R DNA bands were excised from the gel and verified by automatic sequencing using the fmol™ DNA sequencing kit from Promega (data not shown). In the kidney tissue of patient 5 (hydronephrosis), GRP-R mRNA was not clearly detectable (data not shown), and no further studies have been performed with these tissues.

Figure 1. (Top) Time and temperature dependence of binding of $^{125}$I-$[\text{Tyr}^4]$ bombesin to COS-7 cells. Cells were incubated at indicated temperatures with 50 pM $^{125}$I-$[\text{Tyr}^4]$ bombesin without (closed symbols) or with (open symbols) 1 μM unlabeled bombesin. Data shown are means ± SEM of four experiments, and in each experiment values were determined in duplicate. (Bottom) Ability of bombesin or neuromedin B (NMB) to inhibit binding of $^{125}$I-$[\text{Tyr}^4]$ bombesin to COS-7 cells. Cells were incubated for 45 min at 22°C with 50 pM $^{125}$I-$[\text{Tyr}^4]$ bombesin plus indicated concentrations of unlabeled peptides. Binding is expressed as percentage of radioactivity that was saturable bound in the absence of nonradioactive peptides. Data shown are means ± SEM of four experiments, and in each experiment values were determined in duplicate.

To determine whether renal cell carcinoma tissue also expresses higher protein levels of GRP receptors compared with normal kidney tissue, we performed binding studies with crude membranes isolated from tumor tissue. As a positive control of $^{125}$I-$[\text{Tyr}^4]$ bombesin binding to membranes, we used rat pancreatic membranes from an earlier study, in which we demon-
strated the expression of GRP-preferring bombesin receptors (30) (Figure 3, bottom panel). Binding of 125I-[Tyr\textsuperscript{4}] bombesin to freshly isolated renal cell carcinoma membranes was inhibited in a dose-dependent manner by increasing amounts of unlabeled bombesin (K\textsubscript{d} 0.3 ± 0.05 nM, B\textsubscript{max} 320 ± 35 fmol/mg membrane protein), whereas binding studies with membranes from normal kidney tissue of the same patients showed no specific binding.

Detection of GRP-R mRNA in Monoclonal Human Kidney Cancer Cell Lines

To have the tools to test GRP-mediated growth effects, several human kidney cancer cell lines were examined for the expression of GRP-R mRNA by RT-PCR and Southern blot analysis (Figure 4). RT-PCR from RNA isolated from the tumor cell lines A-498, CAKI-1, CAKI-2, ACHN, and normal pancreas as a positive control revealed GRP-preferring bombesin receptor subtype-specific amplificates (392 bp) after two rounds of PCR according to a protocol using the nested-PCR technique (Figure 4, top panel). The amplified GRP-R DNA bands were excised from the gel and sequenced by automatic sequencing, using the fmol\textsuperscript{TM} DNA sequencing kit from Promega (data not shown) or characterized by restriction enzyme analysis. cDNA sequence analysis of the amplified fragments showed a 100% sequence homology to the GRP-preferring
All GRP-preferring bombesin receptor subtype-positive fragments hybridized with a homologous oligonucleotide. To rule out false negative results, all RNA were checked for integrity by \( b \)-actin RT-PCR (340 bp). PCR products amplified from the same four human kidney cell lines (A498, CAK1-1, CAKI-2, ACHN) and human pancreas were analyzed by the Southern blot technique (Figure 4, bottom panel). After RT-PCR and gel electrophoresis, the human GRP receptor gene-specific cDNA amplificates were transferred to a membrane and hybridized with a human GRP receptor-specific oligonucleotide. All DNA fragments that were amplified from kidney cell lines and the positive control were hybridized with the gene-specific oligonucleotide.

**Bombesin Binding to Human Kidney Cancer Cell Lines**

Two human kidney cancer cell lines (CAKI-2, and ACHN) were further examined by binding studies with \(^{125}\text{I}-\text{Tyr}^4\) bombesin for the expression of bombesin receptors. ACHN and CAKI-2 cells showed specific \(^{125}\text{I}-\text{Tyr}^4\) bombesin binding, which was time- and temperature-dependent (Figure 5, top/center panel). As shown in Figure 5 (bottom panel), both cell lines show a characteristic competitive inhibition of \(^{125}\text{I}-\text{Tyr}^4\) bombesin binding by adding increasing amounts of unlabeled bombesin at concentrations from \(10^{-12}\) M up to \(10^{-6}\) M, demonstrating that binding to the bombesin receptor is specific. Computer analysis of the binding data calculated \( K_d \) values of 0.12 ± 0.02 nM for ACHN cells and 0.22 ± 0.09 nM for CAKI-2 cells, respectively (Figure 5, bottom panel).

**Growth Effects of Bombesin Agonists and Antagonists on Human Kidney Cancer Cells (CAKI-2)**

Bombesin-mediated growth effects on CAKI-2 cells were examined using different culturing conditions and bombesin-like peptide agonists and antagonists. First, CAKI-2 cells were grown in medium containing 10% FCS for 6 d, and cell number was determined by cell counting every other day (Figure 6, top panel). IL-1\( \beta \) (10 ng/ml) was used as a positive control, inducing a significant stimulation of growth (138 to 148%) between day 2 and day 6, compared with 10% FCS alone. The addition of GRP-7 (10\(^{-7}\) M) had a similar effect, inducing a significant stimulation of cell numbers (136 to 146%) compared with control. When 10\(^{-6}\) M acetyl-GRP(20–27) (a potent and specific GRP-prefering bombesin receptor antagonist) was added, together with GRP-7 (10\(^{-7}\) M), cell numbers were not different from control, indicating that the effect was mediated by the GRP-prefering bombesin receptor subtype expressed by CAKI-2 cells (Figure 6, top panel). To more completely characterize the relevance of bombesin-mediated growth effects and to test whether an autocrine loop, involving secretion of bombesin-like peptides by CAKI-2 cells might be functional, cell growth was analyzed using different culture conditions. (Figure 6, bottom panel). As control, cell numbers were quantified daily for 4 d with medium containing 0.5% FCS. Addition of bombesin (1 \( \mu \)M) induced a significant stimulation of growth (129% of control), D-Phe\(_6\),Leu\(_{13}\),Leu\(_{14}\) bombesin(6–14) (1 \( \mu \)M), another potent and specific GRP-prefering bombesin receptor antagonist, also slightly stimulated cell growth (109% of control); however, this effect was not significant (Figure 6, bottom panel). These data indicate that no autocrine loop is involved in bombesin-stimulated growth effects in CAKI-2 cells.
Figure 5. Time and temperature dependence of binding of \(^{125}\text{I}[-\text{Tyr}^4]\) bombesin to CAKI-2 cells (top panel) and ACHN cells (middle panel). Cells were incubated at indicated temperatures with 50 pM \(^{125}\text{I}[-\text{Tyr}^4]\) bombesin without (closed symbols) or with (open symbols) 1 \(\mu\text{M}\) unlabelled bombesin. Data shown are means \(\pm\) SEM of three experiments, and in each experiment values were determined in duplicate. (Bottom) Ability of bombesin to inhibit binding of \(^{125}\text{I}[-\text{Tyr}^4]\) bombesin to CAKI-2 and ACHN cells. Cells were incubated for 45 min at 22°C with 50 pM \(^{125}\text{I}[-\text{Tyr}^4]\) bombesin plus indicated concentrations of unlabelled peptides. Binding is expressed as percentage of radioactivity that was saturable bound in the absence of nonradioactive bombesin. Data are means \(\pm\) SEM of three experiments, and in each experiment values were determined in duplicate.

Figure 6. Effects of bombesin-like peptides on growth of CAKI-2 cells in vitro. (Top) Growth of CAKI-2 cells with medium containing 10% fetal calf serum (FCS) alone (control) or with interleukin-1\(\beta\) (IL-1\(\beta\)) (10 ng/ml) as positive control, which significantly stimulated cell numbers compared with control during the whole 6-d period. Addition of GRP-7 (0.1 \(\mu\text{M}\)) alone significantly stimulated cell growth, whereas this effect was abolished by concomitant addition of the GRP receptor antagonist acetyl-GRP(20–26) (1 \(\mu\text{M}\)). Cell numbers were counted every 48 h. Data are means \(\pm\) SEM of 10 experiments. *\(P\), 0.05 compared with control. (Bottom) Growth of CAKI-2 cells in medium containing 0.5% FCS (control). Bombesin alone (1 \(\mu\text{M}\)) significantly stimulated cell number compared with control. The GRP receptor antagonist D-Phe6,Leu13,(CH2NH)Leu14 bombesin(6–14) alone (1 \(\mu\text{M}\)) did not significantly alter FCS-induced growth effects, thus excluding a possible autocrine mechanism. Cell numbers were determined every 24 h for 4 d. Data are means \(\pm\) SEM of four experiments, and in each experiment values were determined in triplicate. *\(P\), 0.05 compared with control.
Discussion

In this study, we demonstrate for the first time that human renal cell carcinoma tissue, but not normal kidney tissue, expresses GRP-preferring bombesin receptors. Furthermore, we were able to show that bombesin-like peptides stimulate growth of human renal cell carcinoma cells (CAKI-2). Recently, the kidney was discovered as a target organ for gastrointestinal hormones such as cholecystokinin and gastrin; the receptors for these peptides have been characterized by pharmacologic and molecular tools (31,32). The distribution of bombesin-like peptides or its receptors in normal human kidney tissue has not been described previously. Moreover, these peptides have not been associated with the relatively frequent and highly malignant human renal cell carcinoma. Preliminary data indicated that COS-7 cells derived from monkey kidney fibroblasts express GRP receptors; however, these data were generated before the different bombesin receptors were cloned (22). Our present data confirm and extend these findings by demonstrating that COS-7 cells express large amounts of GRP-preferring bombesin receptors (Figure 1). In addition, we performed growth studies with COS-7 cells, but bombesin at a concentration of 1 μM did not significantly alter FCS-stimulated growth of this cell line in vitro (data not shown). There are various theories that may explain the lack of GRP-mediated growth effects in COS-7 cells. It might be due to differences in species, kidney cell types, or state of differentiation. A defect in the signaling pathway downstream of the GRP receptor in COS-7 cells might be another cause. Bombesin-like peptides act as neurotransmitters, mediating many physiologic functions, but the dramatic effects on growth of Swiss 3T3 cells or small cell lung cancer cells that are often cited represent the exception rather than the rule. Very recently, it was shown that the growth of CAKI-1 cells, derived from a renal adenocarcinoma, xenografted into nude mice was inhibited by a bombesin antagonist (23). Along this line of investigation, we began to quantify GRP receptors in tumor tissues compared with adjacent healthy control tissue.

Along the same line, we have demonstrated that three out of four tissues from patients with renal cell carcinoma expressed high amounts of GRP-R mRNA, whereas in unaffected tissue from the same kidney, GRP-R mRNA was hardly detectable. There were no obvious histologic or morphologic differences between the four tumors that could explain the lack of GRP-R expression in one tissue. To determine whether GRP-R mRNA is translated to GRP receptor protein in normal and tumor tissue, we characterized GRP receptor expression by the ability of crude membranes to bind 125I-[Tyr4] bombesin. Binding of radiolabeled bombesin was dose-dependently inhibited by unlabeled bombesin (Kd 0.3 nM) in tumor tissue, which is typical for a GRP-preferring bombesin receptor subtype and comparable to rat pancreatic membranes, which were used as control. Membranes prepared from healthy kidney tissue of these patients did not specifically bind radiolabeled bombesin.

These binding studies are extraordinary inasmuch as they were only possible with renal cell carcinoma, but not gastrointestinal tumor tissues. We have previously studied a large number of gastrointestinal tumors for the expression of bombesin receptors, and we were able to show that GRP receptor mRNA is always present, both in the tumor tissue and in the adjacent healthy tissue (data not shown). However, it was never possible to demonstrate specific binding of radiolabeled bombesin to any of these gastrointestinal tumor tissues. There are several possibilities for the failure of ligand binding to crude membranes of solid human tumor tissues. First, despite high amounts of GRP-R mRNA on the level of transcription, the amount of translated GRP-R proteins expressed on the cell surface is not high enough for detection in binding assays. Second, GRP-R proteins could be degraded during preparation of membranes; however, this is unlikely because we prepared human tumor membranes in the same way as rat pancreatic membranes, which contain large amounts of proteolytic enzymes. Furthermore, tissues were collected immediately after excision and kept at 4°C during subsequent procedures. Third, peptidic ligands could be degraded by membrane-bound endopeptidases during the binding experiment (33,34). This led us to determine the stability of the tracer during binding experiments. Incubation of radiolabeled bombesin, together with crude membranes from human gastrointestinal tumor tissues and subsequent HPLC analysis, confirmed that the peptide was degraded within 2 min. It is known that neutral endopeptidase (EC 3.4.24.11) degrades bombesin and other peptides that might be inhibited by phosphoramidon (33,34); however, in our studies investigating gastrointestinal tumors, rapid degradation of bombesin was not prevented even by a cocktail of peptidase inhibitors (phosphoramidon, bestatin, anastatin, aprotinin, PMSF, SBTI, bacitracin). We conclude that renal cell carcinoma does not express large amounts of membrane-bound endopeptidases.

The mechanisms that promote growth of the highly malignant renal cell carcinoma are poorly understood, but there is some evidence that regulatory peptides such as TGF-α and β, somatostatin, epidermal growth factor, and tumor necrosis factor-α might be involved (24–27). The new finding that renal cell carcinoma, but not healthy kidney tissue, expresses a high number of GRP receptors makes it tempting to speculate that GRP might serve as a growth factor in this type of cancer. We were able to demonstrate that CAKI-2 cells, derived from a human clear cell kidney carcinoma (35), express high amounts of GRP receptors. Radiolabeled bombesin binding to these cells is saturable and time- and temperature-dependent. In addition, the receptors possess a high affinity for this peptide, as binding of radiolabeled bombesin is half-maximally inhibited by unlabeled bombesin in the nanomolar range (Figure 5). To test the ability of bombesin-like peptides to mediate growth of this cell line in vitro, we quantified cell numbers over various periods of time using different GRP receptor agonists and antagonists alone or in combination. The agonists bombesin and GRP-7 both significantly enhanced growth of cells stimulated with high (10%) or very low (0.5%) FCS concentrations (Figure 6). The stimulatory effect was in the same range as observed with IL-1β, which is known to stimulate this cell line. The effect of GRP-7 (0.1 μM) was completely inhibited by concomitant application of the specific GRP receptor
antagonist acetyl-GRP(20–26) at 1 μM concentration, indicating that the effect is specifically mediated by a specific receptor (Figure 6, top panel). Bombesin-like peptides are not circulating hormones, but act in a paracrine or autocrine manner to stimulate growth, an effect that is well established for certain tumors such as small cell lung cancer (4). In this study, we have tested this hypothesis by incubating CAKI-2 cells with 1 μM of the potent GRP receptor antagonist D-Phe6,Leu13,(CH2NH)Leu14 bombesin(6–14), but growth was not significantly altered compared with control, which indicates that CAKI-2 cells do not release bombesin-like peptides into the medium, thus excluding an autocrine growth mechanism.

All four human kidney carcinoma cell lines (A-498, CAKI-1, ACHN, CAKI-2) that proved to be positive for GRP receptor mRNA were further characterized for the expression of GRP receptor protein and potential mediation of growth effects. On the protein level, A-498 and CAKI-1 cells did not express relevant numbers of receptors in terms of 125I[Tyr3] bombesin binding; therefore, growth studies were not performed with these two cell lines. ACHN cells specifically bound radiolabeled bombesin (Figure 5); however, the receptor density was smaller compared with CAKI-2 cells. FCS-stimulated growth of ACHN cells in vitro was not significantly altered either by bombesin agonists or antagonists (data not shown). Recently, it has been shown that growth of CAKI-1 cells xenografted into nude mice was inhibited by the bombesin antagonist RC-3940-II. However, bombesin receptors have not been demonstrated on this cell line, and it has been speculated that this effect might be indirectly mediated by altering epidermal growth factor receptors (23). In our study, we could detect GRP-R mRNA by RT-PCR and Southern blot analysis, respectively, but not the receptor protein by binding studies, indicating that CAKI-1 cells either inconstantly express GRP receptors or express them at a very low level. The inconsistency between mRNA and protein expression of GRP-R was only seen with cell lines and might be due to a lack of translation, as the conditions of the PCR reaction were tested to yield linear amplification of GRP-R and β-actin products over a wide range. Nevertheless, we would suggest quantifying GRP-R in renal cell carcinoma by using radioligand studies rather than quantitative PCR.

Our data indicate that malignant transformation of kidney tissue into renal cell carcinoma is accompanied by novel expression of GRP receptors. We and others have shown that bombesin-like peptides mediate growth of human hypernephroma cell lines. Growth of CAKI-2 cells is stimulated by GRP receptor agonists in vitro, and xenotransplanted CAKI-1 cells were inhibited by a potent GRP antagonist in vivo (23). Additional in vivo studies with primary renal cell carcinoma-derived cells might clarify the relevance of GRP receptor antagonists. In the future, isotope-labeled bombesin-like peptides might be useful as diagnostic or therapeutic tools, such as tumor imaging or internal radiotherapy, as has been demonstrated with somatostatin analogs such as Octreoscan® or DOTA-Tyr-octreotide (36–38).

References


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