Expression of plasminogen-related gene B varies among normal tissues and increases in cancer tissues

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Abstract We previously found that the promoter activity of plasminogen (PLG)-related gene B (PRGB) was 5-fold that of PLG. We have since examined the transcript levels of PRGB among various normal human tissues, and compared these findings with those of PLG. Reverse transcription-PCR analysis revealed that the PRGB expression varied widely among different tissues, while PLG was expressed only in the liver and kidney. RNA samples obtained from cultured cell lines also demonstrated differing PRGB expression. Furthermore, increased PRGB expression was observed in several fresh samples of cancer tissue obtained from cancer patients when compared with surrounding normal tissues.

Key words: Gene regulation; Plasminogen; Plasminogen-related gene B; Reverse transcription-polymerase chain reaction; Tissue-specific expression

1. Introduction

Plasminogen (PLG) is a key proenzyme of plasmin in the fibrinolytic and thrombolytic systems. Cleavage at the Arg-561–Val-562 bond in PLG by tissue PLG activator or urokinase results in the formation of a serine protease plasmin. Plasmin also plays an important role in processes which involve the breakdown of extracellular matrices, such as tumor cell migration, angiogenesis, and neurodevelopment [1–3].

During the course of previous studies to characterize normal and abnormal PLG genes [4–6], we identified new genes homologous to that for PLG, including PLG-related genes A, B, and C (designated PRGA, PRGB, and PRGC, respectively). These genes belong to the PLG-apolipoprotein (a) gene family [7,8]. The gene products of PRGA and PRGB are expected to be polypeptides of 8.8 kDa that correspond only to the preactivation peptide (PAP) domain of PLG. This domain plays an essential role in the conversion of the 'activation-resistant' conformation of native PLG to its 'activation-ready' form [9]. It is of interest that PRGB is expressed in the liver and, most prominently, in metastatic cancer cells [10]. Apart from the presence of transcripts of these PRGs, functions of their possible gene products remain unknown to date.

Since PLG and PRGs share an extremely high degree of sequence identity [7], these genes may be coordinately expressed; moreover, similar transcription factors may control both genes. In order to understand the expression of PRGA and PRGB, we first studied the transcriptional regulation of these genes [11]. The sequences of the 5′-flanking regions of PRGA and PRGB were established and their transcriptional activity was demonstrated to be largely dependent on a region surrounding the transcription initiation site of PLG [12]. In the present study we examine transcript levels for PRGB in various normal and tumor tissues and transformed cells, and demonstrate that it was expressed at increased levels in fresh cancer tissues.

2. Materials and methods

Venous blood was drawn from normal individuals and patients with blood dyscrasia. Tissues were obtained from patients with a tumor (or cancer) after informed consent had been obtained. These include nine bladder carcinoma (four at stage II and five at stage III), three renal cell carcinoma (one each for stage I, II, and IIIc), three stage II breast carcinoma (two papillotubular carcinoma, one solid tubular carcinoma), one gastric adeno-carcinoma (Borrman I), one ovarian carcinoma (stage Ia mucinous adeno-carcinoma), three stage I lung carcinoma (one each of small cell carcinoma, squamous carcinoma, and adeno-carcinoma), one colon carcinoma (stage I adeno-carcinoma, Rb at lower rectum), thyroid adenoma, one thymoma (hyperplasia), one stage IV malignant lymphoma (non-Hodgkin, diffuse lymphoblastic T-cell type), acute promyelocytic leukemia [APL, t(15:17)], and chronic myelocytic leukemia (CML, blastic crisis). The tissues collected during surgery were frozen immediately in liquid nitrogen and stored at −80°C until use.

2.1. RT and PCR analyses for screening

Total RNA was extracted by the standard guanidinium thiocyanate method from various samples, followed by ultracentrifugation on a cesium chloride gradient. Reverse transcription (RT) of the total RNA (10 mg) was carried out using random hexanucleotides or an oligo dT (dT18) primer and Superscript II RNase H-reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The synthesized first-strand cDNA was used for PCR in a reaction mixture of 50 ml by employing various pairs of primers for PLG or PRGB (Fig. 1); for PLG, 5′-AAGCAGCTGGGAGCAGGAAGTAT-3′ (PAP-S1 sense), and 5′-GTCTAAGCTTCTGCAGTAGTTCTCCTCCAGTC-3′ (K1b-AS3, antisense); for PRGB, 5′-ACTATGTGAATACCCAGGGGC-3′ (PRGB5 sense), and 5′-ATACATTTACTTTTCAAATAAAACGT-3′ (PRGB3 antisense); for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control, 5′-CATCACCATCTTCCAGGAGC-3′ (sense) and 5′-TAAGCAGTTGGTGGTGCAGG-3′ (antisense). After 30 cycles (for PLG and PRGB) or 23 cycles (for GAPDH), 10 ml of the reaction mixture was applied to a 2.0% agarose gel, followed by staining with ethidium bromide. The gel was subjected to quantitation of the amplified products by FLA-2000 Fluoroimage Analyzer (Fuji Photo Film, Tokyo, Japan). The fluorescence intensity of the PCR products for PRGB and PLG was normalized to that for GAPDH. When necessary, the expression levels of PRGB and PLG were then graded as follows: the value of PRGB or PLG/GAPDH in the liver sample was defined as 1.0; '−' <0.13; $0.13 \leq$ '±' < 0.25; $0.25 =$ '+' < 0.5; $0.5 \leq$ '++'.

RT-PCR analysis was carried out employing total RNA isolated from various normal tissues, tumor tissues and cultured cell lines in the following experiments, three times for each sample.

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Abbreviations: PAP, preactivation peptide; PLG, plasminogen; PRG, plasminogen-related gene; RT, reverse transcription

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2.2. Sequence analysis

DNA sequences of RT-PCR products were obtained using the dideoxy termination method with ABI sequence analyzer 373A (Perkin-Elmer, Norwalk, CT). Sequence homology searches for the DNA fragments were performed by using the BLAST Network Service in GenBank, in order to identify the PRGB gene and to confirm the exclusion of possible co-amplification of other homologous genes.

2.3. Semi-quantitative RT-PCR for PRGB in normal and cancer tissues

Using serially diluted samples of the synthesized first-strand cDNA, semi-quantitative RT-PCR was carried out by employing 3.7×10^{-2} MBq of [α-^{35}S]dCTP and the proper primers in a reaction mixture of 25 μl. After 30 cycles (for PRGB) or 23 cycles (for GAPDH), 10 ml of the reaction mixture was applied to a 9.0% polyacrylamide gel. The gel was then subjected to quantitation of the amplified products by FLA-2000 Fluoroimage Analyzer. The radioactivity of the PCR product for PRGB or PLG was normalized to that for GAPDH. Transcript levels of PRGB in normal and cancer tissues were compared using their diluted samples, which showed a linear relationship between the amounts of total RNA used and the fluorescent intensity of the radioactive bands.

2.4. Cell culture

All human cell lines were obtained from the Japanese Cancer Research Resources Bank except for HUVEC (human umbilical vein endothelial cells), which was the kind gift of Prof. Y. Sato at Tohoku University. A-172 (glioblastoma), A549 (alveolar carcinoma), G-292, HOS and MG-63 (osteosarcoma), HeLa AG (cervical carcinoma), HepG2 and Huh-7 (hepatoma), HL-60 and HLG (acute promyelocytic leukemia), HUVEC, MEG01 (megakaryoblastic leukemia), Mitten and SF-TY (fibroblast), NEC8 (testicular germ cell tumor), SCH (gastric cancer) and U937 (monocytic leukemia) cells were maintained either in Dulbecco's modified Eagle's medium, RPMI 1640, or Eagle's minimal essential medium (Nikken Biomedical Laboratory, Tokyo, Japan), supplemented with 10% fetal bovine serum (SEBAK GmbH, Germany), 50 mg/ml penicillin, 50 mg/ml streptomycin, and 100 mg/ml neomycin (PSN Antibiotic Mixture, Gibco-BRL).

3. Results and discussion

3.1. Selective amplification of PRGB

The organization of human PRGB, in terms of the locations of introns (Fig. 1) and the types of splice junctions, was identical to the gene encoding PLG [7]. The PRGB and PLG genes share more than 95% sequence identity. In order to distinguish PRGB mRNA from those for other members of the PLG-apo(A) gene family, especially PLG and PRGA, we designed a pair of gene-specific oligonucleotide primers for PRGB as described in Section 2. Using this primer pair we established the PCR condition for amplification of PRGB

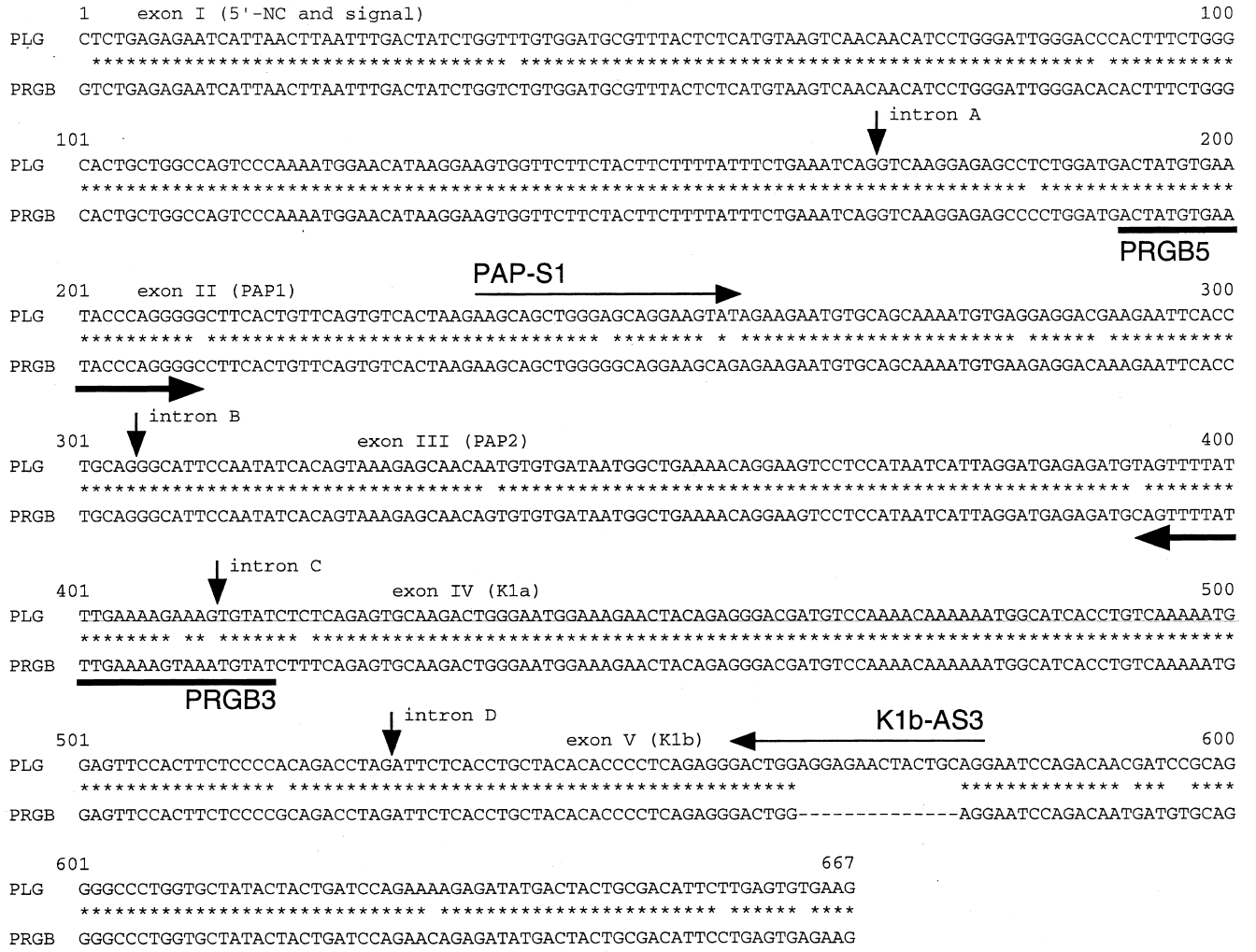

Fig. 1. Nucleotide sequence of a part of the gene and cDNA for PLG (top line) and PRGB (bottom line). Nucleotides identical in both PRGB and PLG are shown by asterisks. The arrows represent gene-specific primer pairs for PRGB and PLG, which are labeled at their 5′ ends. Short vertical arrows indicate the positions of introns. 5′-NC, 5′-noncoding region; signal, signal peptide; PAP1, 1st half of preactivation peptide; PAP2, 2nd half of PAP; K1a, 1st half of Kringle 1; K1b, 2nd half of Kringle 1.

Table 1
Detection of mRNAs for PRGB and PLG in normal organs and tissues

Organ/tissue	PRGB	PLG	Organ/tissue	PRGB	PLG
Brain (cortex)	+	−	Spleen	+	−
Tonsil	±	−	Kidney	+	+
Thyroid	+	−	Ovary	+	−
Thymus	+	−	Uterus	+	−
Lung	+	−	Prostate	±–++	−
Cardiac muscle	+	−	Testis	±	−
Breast	+	−	Placenta	+	−
Pancreas	+	−	Bladder	±	−
Liver	++	++	Skeletal muscle	±	−
Fetal liver	++	++	Leukocytes	+	−
Stomach	−	−			

PRGB or PLG/GAPDH in the liver, 1.0; '−' <0.13; $0.13 \leq$ '±' <0.25; $0.25 \leq$ '+' <0.5; $0.5 \leq$ '++'. Each tissue was examined three times by the method as described in Section 2.

alone. Sequencing analysis together with a computer-assisted homology search confirmed that PRGB was specifically amplified by the current method: no genes homologous to PRGB were identified in the amplified products. Specific amplification of PRGB was also confirmed by restriction fragment length polymorphism (RFLP) analysis with *Ear*I (data not shown), since an *Ear*I site is present only in PRGB but not in PRGA or PLG. In addition, we established the PCR conditions necessary for amplification of PLG alone. Specific amplification of PLG was confirmed by sequencing analysis and digestion with *Ear*I and *Mbo*II, since two *Mbo*II sites exist in PLG and PRGB, while only one is found in PRGA.

3.2. *Differing in vivo expression of PRGB in various normal tissues*

In order to identify the site of PRGB expression in vivo, we screened a number of tissues and organs by RT-PCR using PRGB-specific primers. DNA bands were observed for various normal human tissues. Selected results are demonstrated in Fig. 2. When we graded the expression levels of PRGB by scoring the fluorescence intensity of its DNA bands, adult liver, fetal liver, and prostate scored '++' (Fig. 2, top), while stomach scored '−' (Table 1). The scores of many other organs/tissues ranged from '+' to '±'. Although Weissbach and Treadwell reported that no PRGB mRNA was detected in normal breast tissue [10], our results clearly demonstrated the presence of PRGB mRNA in the sample isolated from a healthy (normal) breast. This may be due to the difference in sensitivity of the methods employed in both studies. It was found for the first time that PRGB expression varies widely among normal extrahepatic tissues, while GAPDH mRNA is ubiquitously expressed. In contrast to PRGB, PLG was expressed only in the liver and kidney (Fig. 2, bottom). These results suggest that although the PRGB and PLG genes are highly homologous [7], their gene regulation differs markedly [11].

It is interesting that the prostate obtained from two individuals scored '++', while that obtained from a third scored '±' (Table 1), indicating the presence of a deviation in PRGB expression among individuals. However, no significant difference in PRGB mRNA levels was observed in other tissues, including the liver, breast and kidney (described later).

In contrast to PRGB and PLG, judging from the PCR-RFLP pattern the PRGA transcript was undetectable in all

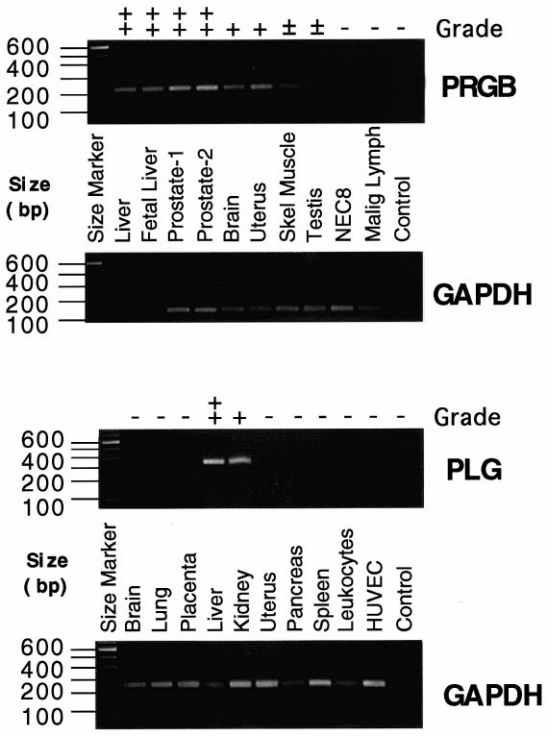

Fig. 2. Expression of PRGB (top) and PLG (bottom) detected by RT-PCR. The expression levels of PRGB and PLG are relatively graded as described in Section 2. Each tissue was examined three times by the method as described in Section 2.

Table 2
Detection of mRNAs for PRGB and PLG in cultured cell lines

Cell line	PRGB	PLG	Cell line	PRGB	PLG
A-172	±	−	HL-60	±	−
A549	±	−	HLG	±	−
G-292	+	−	HUVEC	±	−
HOS	±	−	MEG01	+	−
MG-63	+	−	Miten	±	−
HeLa AG	−	−	NEC8	−	−
HepG2	+	+	SCH	−	−
Huh-7	±	+	U-937	±	−

Ca, carcinoma; A-172, glioblastoma; A549, alveolar cell ca; G-292, HOS, MG-63, osteosarcoma; HeLa AG, cevical ca; HepG2, hepatocellular ca; Huh7, hepatoma; HL-60, HLG, promyelocytic leukemia; HUVEC, umbilical vein endothelial cell; MEG01, megakaryoblastic leukemia; Miten, fibroblast; NEC8, testicular teratoma; SCH, gastric cancer; SF-TY, fibroblast; U-937, histiocytic lymphoma. Each tissue was examined three times.

Table 3
Detection of mRNAs for PRGB and PLG in neoplastic and normal tissues (a total of 26 neoplastic samples)

Tissue	PRGB		PLG	
	Tumor	Normal	Tumor	Normal
Thyroid (adenoma)	+	+	−	−
Thymoma	+	+	−	−
Lung ca (small cell ca)	+	+	−	−
Lung ca (squamous cell ca)	+	+	−	−
Lung ca (adeno-ca)	+	+	−	−
Breast ca (papillotubular ca)	+	+	−	−
Breast ca (solid tubular ca)	++	+	−	−
Gastric ca (adenoma)	+	−	−	−
Colon ca (adenoma)	+	±	−	−
Ovarian ca (mucinous adenoma)	+	+	−	−
Bladder ca (transitional cell ca)	+	±	−	−
Renal ca (renal cell ca)	±–++	±–+	+	+
Malignant lymphoma*	−	NA	−	NA
APL	+	NA	−	NA
CML	+	NA	−	NA

ca, carcinoma; NA, not available; *, diffuse lymphoblastic T-cell type; APL, acute promyelocytic leukemia; CML, chronic myelocytic leukemia (blastic crisis). Each tissue was examined three times.

tissue samples including the liver examined in the present study (Tateno and Ichinose, unpublished data).

3.3. Expression of PRGB in transformed cell lines

We also screened for the transcripts of PRGB and PLG in various transformed cell lines by RT-PCR employing the same conditions as used to examine normal tissues/organs (Fig. 2). The PRGB transcript was observed in osteosarcoma cell lines (G-292, MG-63 and HOS in Table 2), a finding consistent with the report that PRGB expression was detected in MG-63 [10]. It was of interest that PRGB expression in a megakaryoblastic leukemia cell line (MEG01) was relatively high. In contrast, PLG was expressed only in hepatoma cell lines (HepG2 and Huh-7); its mRNA was not detected in any other cultured cell lines. These results are in good agreement with the fact that a high expression level of PLG was found only in the liver and kidney, as described in the preceding section. Thus, it may be said that since the expression activity of PRGB is much higher than that of PLG [11], PRGB can be transcribed efficiently in various tissues and cell lines. The liver must be very rich in transcription factors which drive a relatively weak PLG promoter. These tissue and cell distribution studies demonstrate a much wider extrahepatic dispersion of PRGB mRNA than PLG mRNA, and support the idea that the two genes are expressed differently.

3.4. Increased PRGB transcripts in cancer tissues

Since several transformed cell lines contained increased amounts of the PRGB transcript as described above, we next employed the RT-PCR assay to examine primary cancer tissues obtained from cancer patients for the presence of the PRGB transcript. PRGB transcripts appeared to be increased in some cancer tissues when compared with their surrounding normal tissues (Table 3), while in other cancer tissues they remained unchanged. When the fluorescent intensity of the amplified products was normalized to that of GAPDH, the ratio of PRGB transcript in tumor tissue to that in normal tissue ranged from 1.5–5.6 in breast carcinoma, gastric carcinoma, colon carcinoma, bladder carcinoma and renal carcinoma (Fig. 3), indicating that PRGB expression was increased in these tissues. The levels of PLG mRNA, however, remained unchanged (data not shown).

In order to substantiate these findings, we next carried out semi-quantitative RT-PCR using a radiolabeled nucleotide. Under experimental conditions, a linear relationship was observed between the amounts of total RNA employed and the radioactivity of these bands (Fig. 4, right: cancer, Robson's stage II renal cell carcinoma; normal, the surrounding normal tissue). The relative ratio of PRGB/GAPDH was then calculated. Among the six samples, a renal cell carcinoma obtained from patient 3 (stage IIIc by Robson's classification) expressed PRGB most (PRGB/GAPDH ratio, 19.6), that from patient 1 (stage I) least (1.4), and that from patient 2 (stage II) yielded a level of expression between that of patient 3 and patient 1 (4.0), when compared to the patients' normal tissues (Table 4). PRGB mRNA levels in normal kidney tissue were fairly constant in these cases. Thus, the increase in PRGB expression varied from one case to another even within the same type of cancer. The reasons for this deviation in PRGB expression are unknown at present. It is possible that the variable PRGB expression is due to different stages of the

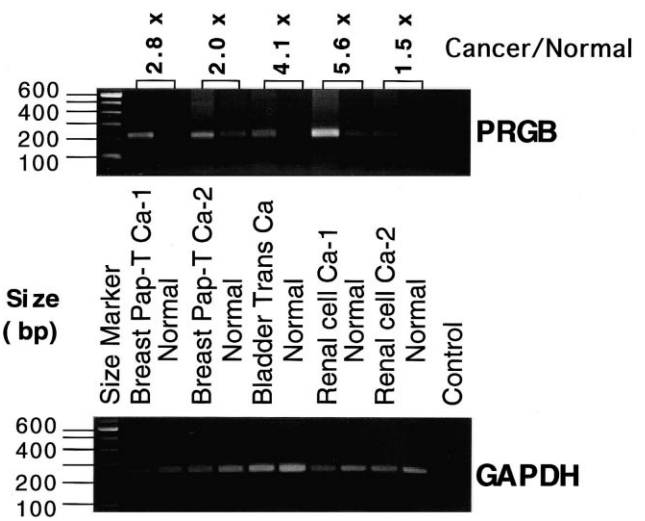

Fig. 3. Increased PRGB expression in cancer tissues. mRNA samples were extracted from cancers and their surrounding normal tissues. The results for five selected samples are shown. Each tissue was examined three times by the method.

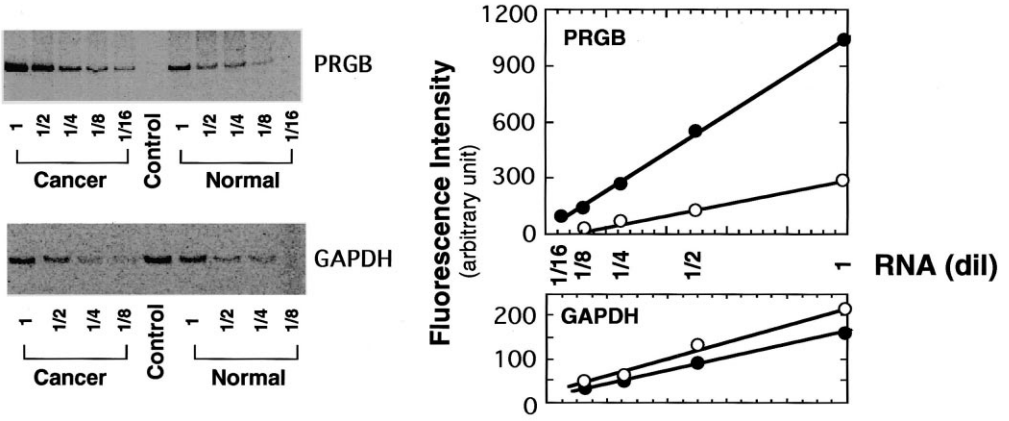

Fig. 4. Semi-quantitative RT-PCR assay for the mRNAs of PRGB and GAPDH. A radioactive nucleotide ([α-^{35}S]dCTP) was incorporated into PCR products to estimate the amounts of mRNAs for both genes. Left: Four lanes ('cancer' tissue of Robson's stage II renal cell carcinoma) on the left of both panels contain amplified products of diluted (1/8 or 1/16) samples from a cancer tissue. 'Normal' stands for a sample from the surrounding normal tissue of the same patient. Right: Linear relationship between the amounts of total RNA employed and the fluorescence intensity of the radioactive bands. Amounts of the amplified product roughly represent the amounts of mRNAs for PRGB (top) and for GAPDH (bottom), respectively. Each tissue was examined three times.

cancer's progression. It was observed in this study that PRGB expression in renal cell carcinoma increased indeed at rates echoing the cancer's own advancement as described above.

In contrast to renal cell carcinoma, among breast cancers PRGB expression was high in a sample of solid tubular carcinoma (PRGB/GAPDH ratio, 10.3), but intermediate in two samples of papillotubular carcinoma (4.1 and 2.3). All three patients were in stage II. PRGB mRNA levels in the normal breast tissue were constant ('+') in these cases. Therefore, it may be said that the difference in PRGB expression in breast cancers is attributed to a difference in cell types.

In summary, we found that PRGB expression varied widely among different normal tissues and transformed human cell lines. Furthermore, greatly increased PRGB expression was observed in several cancer tissues obtained fresh from cancer patients, when compared with surrounding normal tissues. The pathophysiological function of PRGB, however, remains unknown. The PAP domain of PLG interacts with the aminohexyl site of the PLG molecule [13] and is directly involved in maintaining the activation-resistant conformation [14]. Thus, the PAP domain plays an essential role in its activation and fibrin binding, which results in the modulation of plasmin generation. Accordingly, increased levels of the gene product of PRGB composed of the PAP domain alone may affect the activation of PLG to plasmin, and may play a role in the invasion and metastasis of cancer cells, such as extracellular matrix destruction [3,15]. This assumption is analogous to the fact that a 38 kDa fragment of PLG (K1–3), named angiostatin, potently inhibits neovascularization, growth of tumors and their metastases [16,17]. This hypothesis will be examined in the future by the expression and characterization of recombinant PRGB in mammalian cells.

Table 4
Semi-quantitation of mRNA for PRGB in cancer and normal tissues

Tissue	stage	tumor/normal
Breast solid tubular ca	stage II	10.3
Breast papillotubular ca-1	stage II	4.1
Breast papillotubular ca-2	stage II	2.3
Renal cell ca-1	stage I	1.4
Renal cell ca-2	stage II	4.0
Renal cell ca-3	stage IIIc	19.6

Each tissue was examined three times.

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