Inhibitory effect of a synthetic prostacyclin analogue, Beraprost, on urokinase-type plasminogen activator gene expression in RC-K8 human lymphoma cells

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Summary

Plasminogen activation by urokinase-type plasminogen activator (uPA) is implicated in tumor invasion and metastasis by the breakdown of extracellular matrix. We have recently demonstrated the inhibitory effect of cAMP on uPA gene transcription in RC-K8 human lymphoma cells. Prostacyclin produced by endothelial cells is shown to increase cellular cAMP levels by activating adenylate cyclase. We, therefore, examined the effect of a stable analogue of prostacyclin, Beraprost, on uPA production in RC-K8 cells. uPA activity gradually increased in the conditioned medium with time. Beraprost (0.1 nM-1.0 μM) inhibited uPA accumulation in a dose-dependent manner without affecting cell viability. High and low molecular forms of uPA were present in the conditioned medium, but no PA inhibitor was demonstrated by fibrin zymography. All forms of uPA decreased after Beraprost-treatment. Northern blot analysis revealed that after exposure to Beraprost, uPA mRNA levels increased transiently and then rapidly decreased to below control levels. Treatment with Beraprost resulted in a rapid activation of cellular cyclic AMP-dependent protein kinase (PKA). Beraprost completely negated uPA gene expression induced by phorbol myristate acetate, an activator of protein kinase C (PKC). These results suggest that Beraprost inhibits uPA production by suppressing uPA gene expression may be through the PKA pathway and that PKA-mediated signals are dominant in uPA gene expression as compared to those medicated by PKC. This inhibition of uPA expression by a prostacyclin analogue may be an important fact to explain the mechanism of antimetastatic effects of prostacyclin.

Introduction

Invasion and metastasis are the main clinical characteristics of malignant tumors. uPA is one of the tumor-associated proteases and strongly involved in tumor cell invasion and metastasis. The invasiveness and metastatic potential appear to correlate with the PA activity on cell surface; uPA receptors expressed on the cell surface localize uPA to cell-cell and cell-substratum contact sites (1-3). A number of studies have reported this involvement in malignant tumors, for example, colorectal cancer (4, 5), gastric cancer (6, 7), breast cancer (8), lung cancer (9), and so on. Especially, tumor invasion into vascular endothelium is an indispensable step of metastasis and, therefore, endothelium is thought to be a barrier of tumor cell invasion. It has been shown that prostacyclin, derived from cyclooxygenase pathway of arachidonic acid metabolism in vascular endothelial cells, activates adenylate cyclase and increases cAMP levels (10, 11) and that prostacyclin acts not only as an antagonist of platelet aggregation (12) but also as a vasodilator via the PKA pathway (13, 14). In addition, a number of experiments have demonstrated the antimetastatic effects of prostacyclin and its analogues in vitro and in vivo (15, 16). Mechanistic studies revealed that the antimetastatic effects of prosacyclin and its analogues are more related to their interference with tumor cell-host interactions (such as tumor cell induced platelet aggregation, tumor cell adhesion to endothelial cells and subendothelial matrix, tumor cell induced endothelial cell retention, etc.) than their direct inhibition of the growth of primary tumors. However, there is no report refers to the effect of prostacyclin on uPA production in tumor cells so far.

RC-K8 is a human pre-B lymphoma cell line which constantly produces uPA in conditioned medium (17), and RC-K8 cells produce massive metastatic tumors in the lungs when injected intraperitoneally into immunosuppressed newborn hamsters (18). We found that a PKC activator, phorbol myristate acetate (PMA), stimulates uPA production in RC-K8 cells (17), whereas cAMP inhibits uPA production through the PKA pathway (19), although previous studies from other groups have generally

reported that cAMP has a stimulatory effect on uPA production (20-23). Therefore, we examined whether a stable prostacyclin analogue, Beraprost, induces PKA activation and inhibits uPA production in RC-K8 cells. The effect of Beraprost on PMA-induced uPA gene expression was also examined in this paper.

Materials and Methods

Reagents

Beraprost was a generous gift of Kaketsukenn (Kumamoto, Japan). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma Chemicals (St. Louis, MO, USA). A random primed labeling kit was form Boehringer Mannheim Biochemica (Mannheim, Germany).

Cell culture and Cell Growth Studies

RC-K8 and PL-21 cells were grown in RPMI-1640 culture medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Whittaker Bioproducts, Walkersville, MD USA), 100 U/mL penicillin, and 100 μg/mL streptomycin as previously described (17, 24). To examine the effect of Beraprost on uPA production, cells were cultured for 48 h in serum-free RPMI-1640 medium containing various concentrations (0.1 nM-1 μM) of Beraprost. Every experiment was performed in triplicate. Cell viability was determined by trypan blue dye exclusion under bright-field microscopy . The number of surviving and proliferating cells was measured by the colorimetric MTT assay for mitochondrial dehydrogenase enzyme activity as described by Mosmann (25). The correlation curve between cell numbers, counted by the trypan blue dye exclusion method, and the amount of MTT formazan generated was used to calculate the cell number. The assay was conducted with cells growing in 0.1 ml medium in triplicate.

uPA activity

uPA activity in conditioned medium was quantitatively measured using chromogenic substrate S-2444 (KABI Diagnostica, Stockholm, Sweden) (26). Briefly, the reaction was started by adding 50 μL of S-2444 (1 mg/ml) to each well of a 96-well microplate containing 50 μL test sample. Increases in absorbance at 405 nm over a 20-min period were measured using a micro-ELISA-reader. The results were compared to those obtained using serially diluted standard uPA (The Green Cross, Osaka, Japan).

SDS-PAGE and Fibrin-Zymography

RC-K8 conditioned medium (20 μL) mixed with electrophoresis buffer (0.1 M Tris HCl pH 6.8, 2% SDS, 20% glycerol) was incubated at 37°C for 2 h and then electrophoresed in 6-15 % lineal gradient polyacrilamide slab gel using the Laemmli's buffer system. After electrophoresis, SDS in the gel was removed by soaking in 2.5% Triton X-100 solution for 2 h and PA activity in the gel was visualized by fibrin-zymography (27).

RNA preparation and Northern blot analysis

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (28). Total RNA (10 μg) was subjected to Northern blot analysis as described (29). uPA cDNA (461 bp encoding amino acid residues 165-301, a gift of The Green Cross) and β-actin cDNA (Wako Pure Chemicals, Osaka, Japan) were labeled with [^{32}P]-dCTP (ICN Biomedicals, Irvine, CA, USA) by the random primer method. mRNA levels were quantitatively measured by counting radioactivity using the BAS 2000 Imaging analyzer (Fuji film, Tokyo, Japan)

Detection and quantitation of PKA activity in RC-K8 cells after stimulation with Beraprost

PKA activity in cell lysates was measured using a non-radioactive method developed by Promega (Madison, WI, USA). After stimulation with Beraprost, cells

were washed with phosphate-buffered saline and resuspended in 100 μl of cell lysis buffer (Tris-HCl pH 7.5 150 mM, EDTA 0.5 mM, EGTA 0.5 mM, 2-mercaptoethanol 10 mM, leupeptin 20 μg/mL, PMSF 0.5 mM and Triton X-100 0.5%). Reaction mixtures containing 10 μL of cell lysate, 5 μL ATP, 5 μL PepTag ^{TM} A1 peptide and 5 μL (5 ×) reaction buffer were incubated for 30 min at 30℃. Phosphorylated peptides were separated from non-phosphorylated peptides by agarose gel electrophoresis according to the manufacturer's instructions. Protein kinase activities were quantified using a spectrofluorometer (Shimazu, Kyoto, Japan) as follows. Negatively-charged phosphorylated bands were excised from the gel and melted according to the manufacturer's instructions. Melted gels were diluted with 2.5 mL distilled water, and the intensity of the emission wavelength (592 nm) was measured using the spectrofluorometer. Due to the unreliability of the PKA standard included in the kit, absolute PKA levels in cell lysates could not calculated; therefore PKA activity was expressed as relative values.

Statistical analysis

Statistical analysis was done on a Macintosh IIsi (Apple Computer, Inc., Cupertino, CA, USA). Results were expressed as mean ± SE. Statistical significant was determined using the Student *t*-test to compare unpaired data.

Results

Effect of Beraprost on uPA accumulation in RC-K8 conditioned medium

Approximately 45 IU/mL uPA was accumulated in RC-K8 conditioned medium 48 h after replacement of culture medium with FBS-free RPMI-1640 medium. Beraprost inhibited uPA accumulation in a dose-dependent manner (Fig. 1) without affecting cell viability, checked by the MTT method. It was further confirmed by the fibrin-zymography as shown in Fig. 2. Although different sized PAs are present in the

conditioned medium, all of them belong to uPA since the PA activity was specifically inhibited by anti-uPA antiserum but not by anti-tissue-type PA antiserum (data not shown). Therefore, the smaller sized forms are thought to be degraded uPAs, compatible to the low molecular forms of two chain uPAs and the largest band coincided with the high molecular form of uPA (30). Neither PA inhibitor nor PA/PA inhibitor complex was present in RC-K8 conditioned medium. Beraprost itself showed no effect on uPA activity when directly mixed with the standard uPA or RC-K8 conditioned medium.

PKA activation by Beraprost

We examined whether Beraprost actually induces PKA activation in RC-K8 cells, because prostacyclin is known to be an activator of adenylate cyclase and increases cellular cAMP levels (10, 11). Beraprost certainly induced PKA activation in a dose- and time-dependent manner as shown in Fig. 3, panel A and B. We did not measure intracellular cAMP levels, but Beraprost must elevate cAMP levels through the adenylate cyclase pathway in RC-K8 cells.

Northern blot analysis

After treatment with Beraprost, uPA mRNA levels transiently increased and then rapidly decreased below control levels (Fig. 4). This time-course is very similar to that induced by cAMP shown in our previous report (19). As well as PKA, PKC is an important effector molecule that controls uPA gene expression (31-33). We know that addition of PMA, a tumor promoter and PKC activator, to RC-K8 cell culture medium induces uPA production (17). Therefore, effect of Beraprost on PMA-induced uPA gene expression was also investigated. uPA mRNA levels significantly increased from 2 to 9 hours after exposure to 1 μM PMA and the peak induction was observed after 5 hours (data not shown). Interestingly, Beraprost completely inhibited the PMA-induced uPA expression as shown in Fig. 5.

Discussion

Our results clearly demonstrate the inhibitory effect of Beraprost on uPA production in RC-K8 cells, supporting our previous findings; cAMP inhibits uPA expression via the PKA pathway (19). In addition, Beraprost partly inhibited uPA gene expression in 5 out of 6 esophageal carcinoma cell lines examined (data not shown). Therefore, this inhibitory effect of Beraprost on uPA gene expression seems to be not restricted in RC-K8 cells. However, it has been generally shown that cAMP has a stimulating effect on uPA production (20-23). The mechanism of cAMP-induced uPA gene expression in LLC-PK1 porcine kidney cells has been extensively investigated by Nagamine and coworkers, who have identified a cAMP-responsive region 3.4 kb upstream of the transcription initiation site (34). PKC as well as PKA plays an important role in cellular signal transduction. It has been also shown that uPA is induced upon the activation of PKC in a number of cells (17, 31-33), and it appears to be independent of PKA (31, 32, 35). Interestingly, Beraprost completely inhibited PMA-induced uPA expression, suggesting that cAMP-mediated signals are dominant in uPA gene expression as compared to those by PKC. Similar inhibitory effect of cAMP on PMA-induced uPA gene expression was also observed in human glomerular epithelial cells and the MDCK cells (32). As shown in our previous report (19), the correlation between PKA activation and down-regulation of uPA production after cAMP stimulation suggests that phosphorylated factors by PKA are implicated in the down-regulation process. Recent studies have identified different isoforms of the cAMP-responsive element (CRE) binding protein. Among them, CREM isoforms appear to regulate CRE-mediated transcription both positively and negatively (36). Therefore, such CREM may act as a uPA gene expression inhibitor in RC-K8 cells.

A number of experiments have demonstrated the antimetastatic effects of prostacyclin and its analogues in vitro and in vivo; many mechanisms are proposed in the antimetastatic effects of prostacyclin, among which are direct anti-proliferation effect, inhibition of tumor cell-induced platelet aggregation and inhibition of tumor cell

adhesion to endothelial cells (15, 16). However, there is no previous report refers to the effect of prostacyclin on uPA production in tumor cells so far. The inhibition of uPA expression by a prostacyclin analogue may be an important fact to explain the mechanism of antimetastatic effects of prostacyclin. Any way, vascular endothelium must function as a physical and biological barrier to tumor invasion and metastasis. As shown in our previous papers (37, 38), cAMP and PMA synergistically induce plasminogen activator inhibitor type 2 (PAI-2) expression in PL-21 human malignant cells. Beraprost virtually enhanced PMA-induced PAI-2 gene expression in PL-21 cells (data not shown). These results suggest that the stimulation which gives rise to an increase in cAMP levels appears to inhibit plasminogen activation in tumor cells and prevent the tumor cell infiltration and invasion. We know that RC-K8 cells express uPA receptors on the cell surface, checked by the flow cytometry using a monoclonal antibody against the uPA receptor, and produce massive metastatic lung tumors when injected into immuno-suppressive newborn hamsters (18). Further investigations will undoubtedly help our understanding of the role of uPA modulation by prostacyclin on tumor invasiveness and metastatic potential of this cell line.

Acknowledgments

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Abbreviations

Abbreviations used are: uPA, urokinase-type plasminogen activator; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PMA, phorbol myristate acetate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; FBS, fetal bovine serum; CRE, cAMP-responsive element.

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Figure Legends

Fig. 1 Effect of Beraprost on uPA accumulation in RC-K8 conditioned medium. Cells were cultured in the presence of various concentrations of Beraprost (0.1-1000 nM) for 48 h, and PA activities in the conditioned medium were measured using S-2444. Lane 1, control; 2, 0.1 nM Beraprost; 3, 1.0 nM; 4, 10 nM; 5, 100 nM; 6, 1000 nM. Results are the mean ± SE of 3 determinations. *P<0.05; **P<0.01 compared with control values.

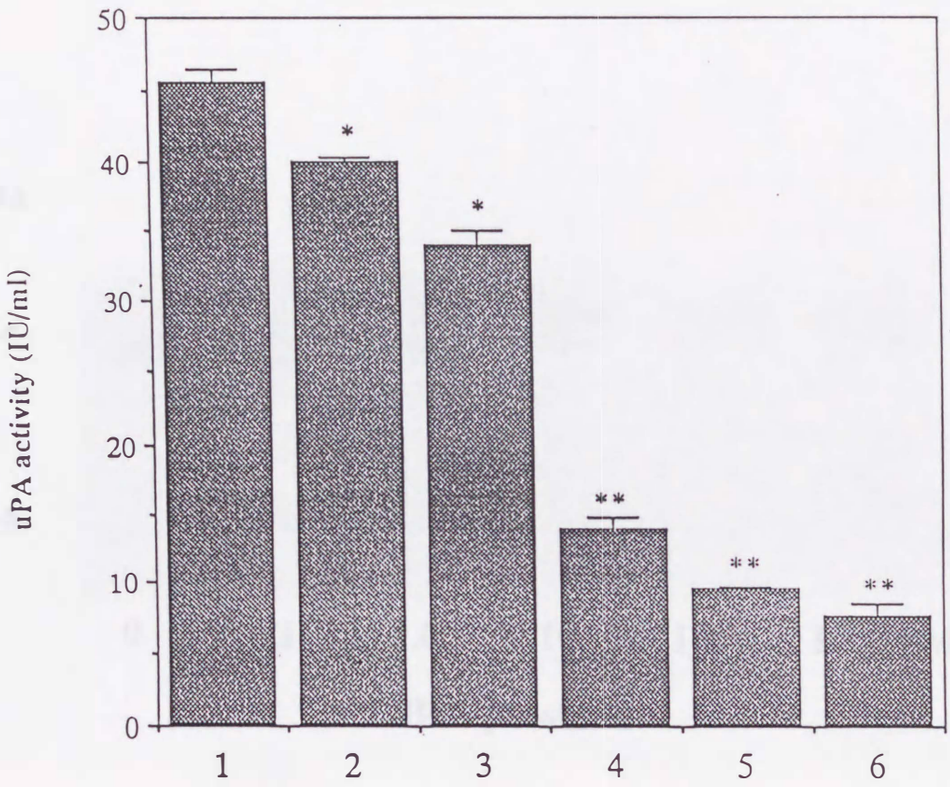
Fig. 2 Fibrin zymography of conditioned media from Beraprost-treated RC-K8 cells. Cells were cultured in the presence of various concentrations of Beraprost (0.1-1000 nM) for 48 h. Twenty μL of the conditioned medium was subjected to SDS-PAGE and zymography was performed. High (50-54 kDa) and low molecular forms (34 kDa) of uPA were observed in the standard uPA preparation and RC-K8 conditioned medium.

Fig. 3 Dose- and Time-dependent PKA activation by Beraprost. RC-K8 cells were cultured in RPMI-1640 containing various concentrations of Beraprost for 10 min in panel A and 100 nM Beraprost for indicated times shown in panel B. PKA activities in cell lysates were measured as described in Materials and Methods. The mean ± SE of triplicate values are shown.

Fig. 4 Time course of uPA mRNA modulation by Beraprost. The autoradiograph shown is representative of 3 Northern blot analyses. The blot was first hybridized with a uPA cDNA probe and subsequently hybridized with a β-action cDNA probe to control for RNA loading.

Fig. 5 Inhibitory effect of Beraprost on PMA-induced uPA gene expression in RC-K8 cells. Shown is a representative autoradiograph of Northern blots of uPA and β-actin 5

hours after treatment with either 100 nM Beraprost (lane 2), 1 μM PMA (lane 3) or both (lane 4).



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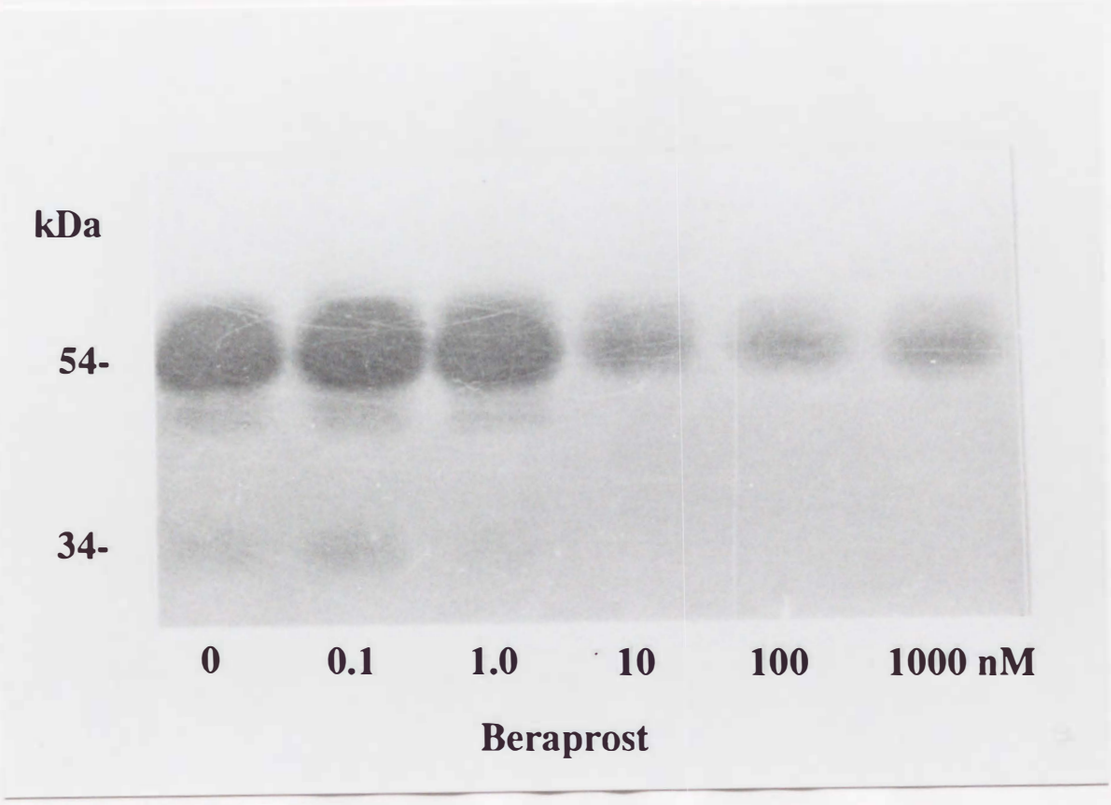
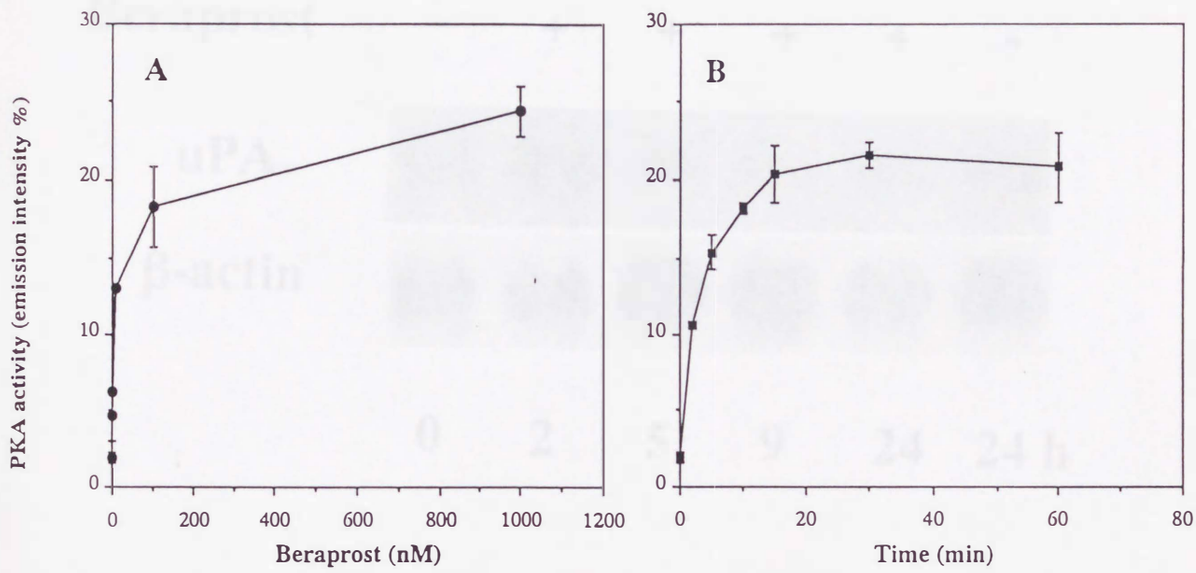


Fig 2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PKA activity (emission intensity %)
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Fig 3

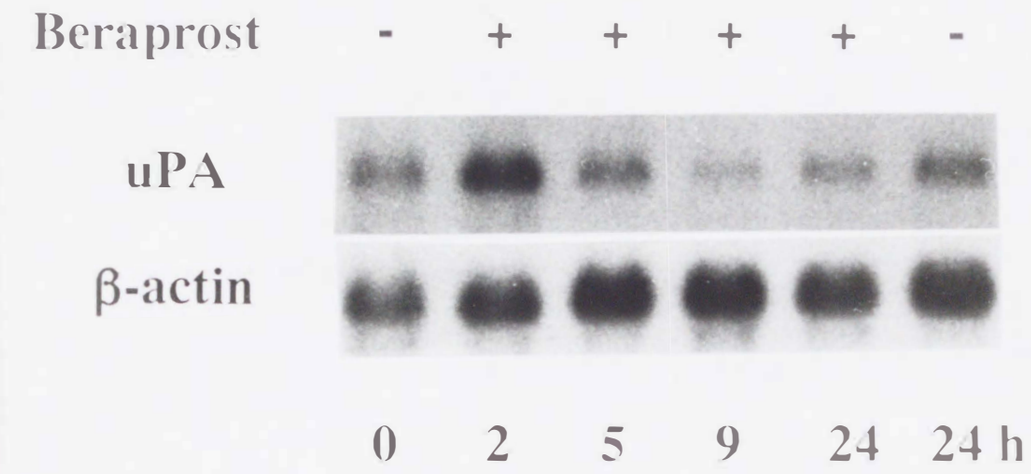


Fig 4

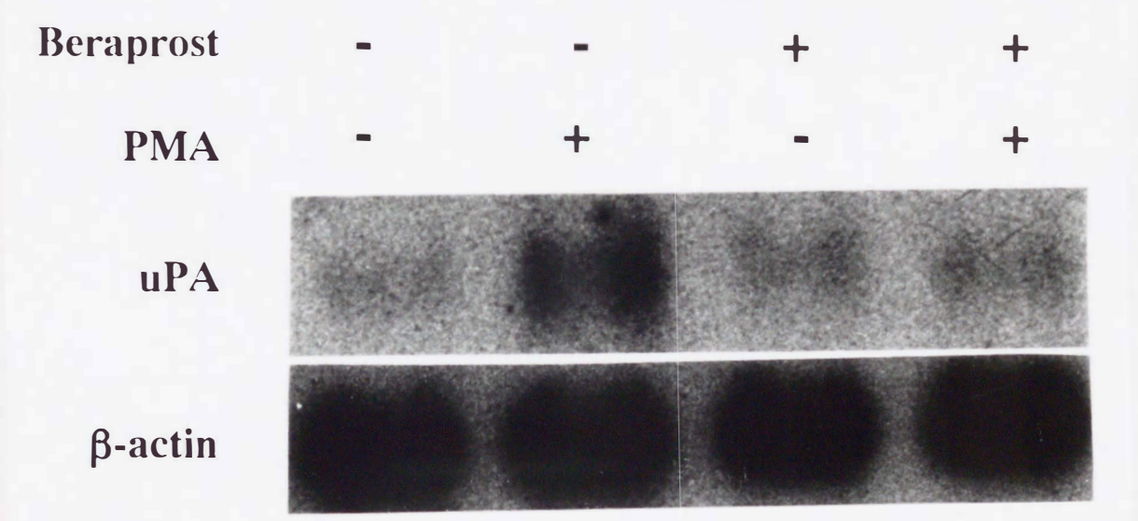
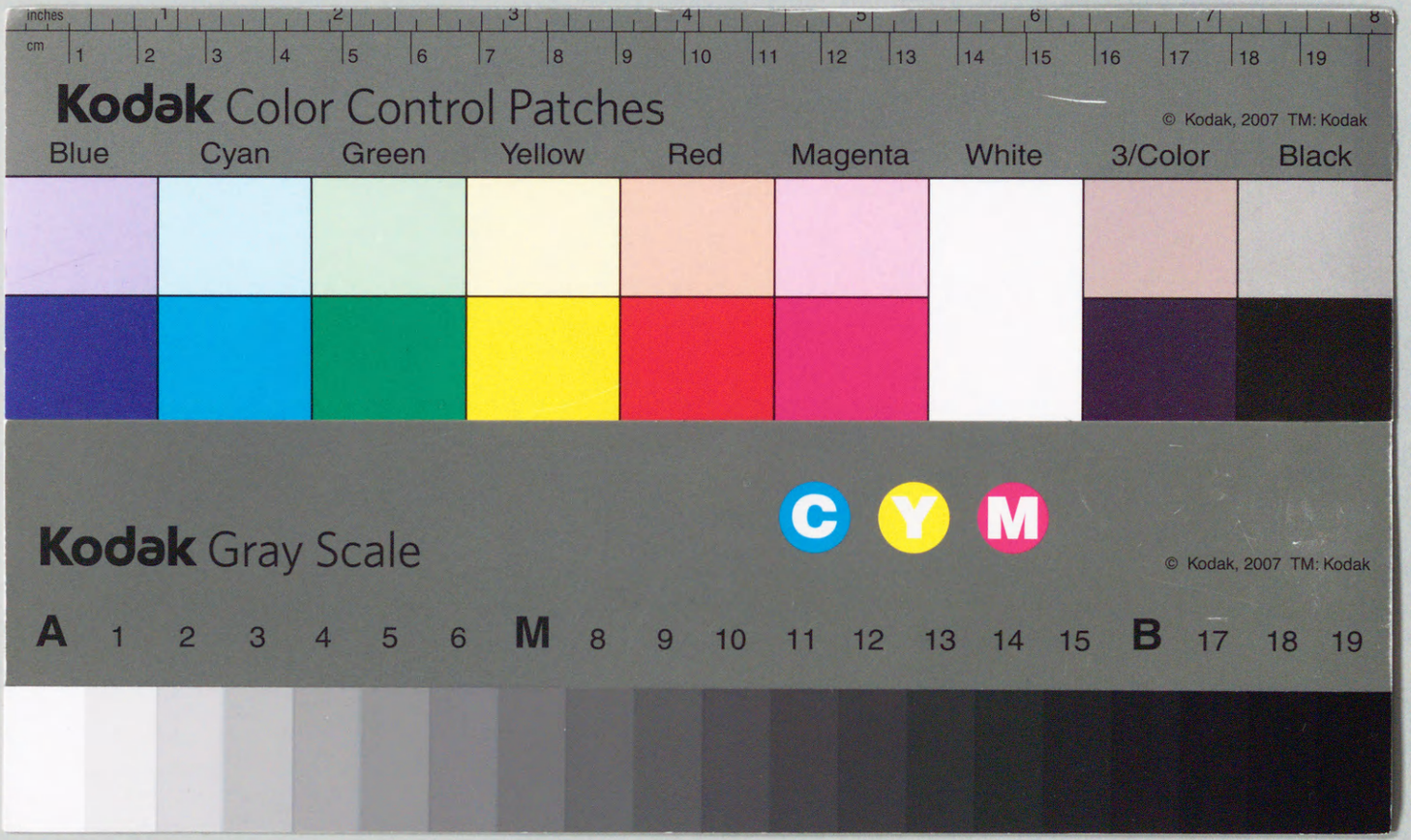


Fig 5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