In Vivo Modulation of Angiogenic Gene Expression by Acyclic Nucleoside Phosphonates PMEDAP and PMEG

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Abstract. *Acyclic nucleoside phosphonates PMEDAP and PMEG modulate expression of selected proangiogenic genes in SD-lymphoma bearing rats. Antiangiogenic efficacy of PMEDAP is relatively weak and is manifested mainly by down-regulation of vascular endothelial growth factor (VEGF) and its receptor VEGFR detectable 24 hours after treatment. Compound PMEG (an active metabolite of the prodrug GS-9219) down-regulates selected proangiogenic genes EGF, FGF, PDGF, VEGF, EGFR, FGFR, PDGFR and VEGFR much more efficiently. Its antiangiogenic potency persists and is more intensive 48 hours after treatment. Findings show that in vivo antitumour efficacy of both antimitotic acyclic nucleoside phosphonates PMEDAP and PMEG consequently affect the angiogenesis in T-cell lymphoma.*

The anticancer effects of compounds PMEDAP, PMEG and PMEDAP N^6-substituted derivatives (Me_2Net-PMEDAP, allyl-PMEDAP, Me_2-PMEDAP and cypr-PMEDAP) have been studied in detail in a spontaneous T-cell lymphoma model in inbred SD/cub rats (1) which is relevant to human pathology (non-Hodgkin's lymphoma) (2). A significant therapeutic effect has been recorded after treatment with PMEDAP (3-5). Decrease of the lymphoma weight during PMEDAP administration was accompanied by suppression of mitotic activity of neoplastic cells and increased chromatin condensation as well as by increased nuclear DNA fragmentation resulting in induction of apoptosis in *in vivo* growing lymphomas (4). Another study has shown that the therapeutic effect of PMEDAP depends on the phenotype of the individual neoplasm (5). Unlike PMEDAP, its N^6-mono- and disubstituted congeners were less effective or exhibited the same antitumor efficacy as PMEDAP (6). PMEG and cypr-PMEDAP were also potent but significantly reduced the survival of treated lymphoma-bearing rats due to toxicity (7).

Recent studies have shown that the antiproliferative effect of PMEDAP N^6-substituted derivatives is evoked by intracellular transformation to PMEG catalyzed by methylAMP aminohydrolase (8, 9). PMEG is the active compound of a novel prodrug GS-9219, which possesses an excellent therapeutic efficacy *in vivo* against spontaneous non-Hodgkin's lymphoma in dogs (10). PMEG and PMEDAP are phosphorylated by cellular kinases to form diphosphates (analogs of nucleoside 5' triphosphates) (11, 12), which inhibit replicative DNA polymerases (13, 14) while the prodrug GS-9219 is first hydrolyzed to cyprPMEDAP and thereafter deaminated to PMEG (10). This ability is believed to account for the cytostatic activity of the parental compound. Moreover, the latest findings show that the diphosphates of PMEG and PMEDAP inhibit activity of human telomerase *in vitro* (15). These results are consistent with cytostatic properties of both compounds. It is highly probable that DNA damage will affect signaling pathways associated not only with cell proliferation and apoptosis but also with angiogenesis.

Tumor angiogenesis is a complicated process stimulated by the secretion of various signaling molecules to promote neovascular formation (16, 17). Angiogenic factors and their

Abbreviations: Acyclic nucleoside phosphonates (ANP): 9-[2-(phosphonomethoxy)ethyl]-guanine (PMEG), 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP), 9-[2-(phosphonomethoxy)ethyl]-N^6-(2-dimethylaminoethyl)-2,6-diaminopurine (Me_2NEt-PMEDAP), 9-[2-(phosphonomethoxy)ethyl]-N^6-allyl-2,6-diaminopurine (allyl-PMEDAP), 9-[2-(phosphonomethoxy)ethyl]-N^6-dimethyl-2,6-diaminopurine (Me_2-PMEDAP) and 9-[2-(phosphonomethoxy)ethyl]-N^6-cyclopropyl-2,6-diaminopurine (cypr-PMEDAP); SD-lymphoma, spontaneous transplantable T-cell lymphoma of Sprague-Dawley rats; SD/Cub, Sprague-Dawley inbred rats/Charles University Biology; RQ-RT-PCR, real-time quantitative reverse transcriptase-polymerase chain reaction.

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activity are well described in solid tumors but have not yet been completely established in hematopoietic neoplasms (18). Increasing evidence suggests that neovascularization is also important in hematological malignancies (19).

The purpose of the present study was to examine an expression of selected genes associated with angiogenesis in the spontaneous SD-lymphoma during *in vivo* treatment with anticancer acyclic nucleoside phosphonates PMEDAP and PMEG. Expression of genes coding epidermal growth factor (*EGF*), fibroblast growth factor (*FGF*), platelet-derived growth factor (*PDGF*), vascular endothelial growth factor (*VEGF*) and their receptors was examined using quantitative real time PCR.

Materials and Methods

Experimental animals. Two- to three-month-old males of the Prague subline of Sprague-Dawley inbred rats (SD/Cub) were used for the experiments. The rats were bred under conventional conditions with commercial granule food (Bergman TM2-CH) and tap water *ad libitum*. Fifteen untreated rats served as an independent group for evaluation of lymphoma growth dynamics. Six rats were included in each treatment and control group for evaluation of the effect of PMEDAP and PMEG (for treatment; see *Drug administration*). Animals of the same age were used in treatment and control groups. Animal handling and experimental protocols were undertaken as per guidelines of the Institutional Ethical Committee for Animal use.

Tumor. Spontaneous transplantable T-cell lymphoma is a highly genetically defined model of spontaneous hematological malignancy (2). In this study, lymphoma SD 10/96 (74th passage) was used. In all experiments, a suspension of 10^6 lymphoma cells in PBS (saline) was injected subcutaneously (*s.c.*) into the right flank of the anesthetized rat.

Lymphatic nodes. Submandibular lymphatic nodes of young healthy SD/Cub rats were used for isolation of total RNA, which served as a calibrator rat sample.

Angiogenesis. Samples of lymphoma tissue were scrutinized by (real-time) RQ-RT-PCR for expression of genes *EGF* (Rn 00563336_m1), *FGF* (Rn 00563362_m1), *PDGF* (Rn 00709363_m1), *VEGF* (Rn 00582935_m1), *EGFR* (Rn 00580398_m1), *FGFR* (Rn 00577234_m1), *PDGFR* (Rn 00709573_m1) and VEGFR (*Flt4*; Rn 00586429_m1), which could be associated with angiogenesis in lymphoma tissue. Three rats were killed and autopsy performed at three-day intervals, starting 14 days after the inoculation of tumor cells and continued over 12 days (26 days after lymphoma cell inoculation). Lymphoma tissue weight was measured at autopsy. Submandibular lymphatic node cells from the autopsied animals were also examined. Expression of the afore mentioned genes was examined after PMEDAP or PMEG treatment of SD/Cub lymphomas (see *Drug administration*).

Compounds. Acyclic nucleoside phosphonates 9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine (PMEDAP) and PMEG - 9-[2-(phosphonomethoxy)ethyl]guanine, were prepared according to Holý *et al*. (20).

Drug administration. In the *in vivo* experiments PMEDAP was injected intraperitonealy (*i.p.*) in each animal in the treated group at a dose of 5 mg kg^{-1}; PMEG at a dose of 0.3 mg kg^{-1}. Five doses were administered at three-day intervals, once daily (1 x d). Administration of the drugs started 4 days after the inoculation of tumor cells and continued during the next 12 days. An autopsy was performed 24 hours and 48 after the last injection of PMEDAP and PMEG, respectively; lymphoma weight was determined and the sample of each lymphoma tissue was scrutinized by RQ-RT-PCR for expression of genes associated with tumor angiogenesis.

Relative quantification of gene expression – evaluation by RQ-RT-PCR. Total RNA was extracted from rat tissues by RNeasy Mini Kit (Qiagen, MD, USA) according to the manufacturer's recommendations. RNA integrity was determined by gel electrophoresis in 2% agarose gel stained with ethidium bromide. The purity of the RNA was assessed by the ratio of absorbance at 260 nm and 280 nm. The purity was in the range of 1.8 to 2.2. Total RNA concentration was estimated by spectrophotometric measurements at 260 nm assuming that 40 μg of RNA per milliliter was equivalent to one absorbance unit. RNA was stored in aliquots at –20˚C until used for reverse transcription. Isolated mRNA was converted to cDNA using reverse transcription reagents (Applied Biosystems, CA, USA), according to the manufacturer's instructions.

Real-time PCR was performed in the 7300 real-time PCR system (Applied Biosystems) using standard conditions. Every 25 μL reaction mix for real-time PCR was made with 12.5 μL TaqMan Universal PCR master mix (Applied Biosystems), 100 ng of cDNA, RNase free water and specific TaqMan probes for *EGF, FGF, PDGF, VEGF, EGFR, FGFR, PDGFR* and *VEGFR* (all Applied Biosystems). Beta-2-microglobulin was used as an endogenous control (Applied Biosystems). Expression of all genes was normalized to mRNA loading for each sample and used as an internal standard. The quantity of mRNA was given as $2^{-\Delta\Delta ct}$. $\Delta\Delta ct$ was calculated as follows: $\Delta\Delta ct=\Delta ct$ (sample of interest) – Δct (calibrator) and $\Delta ct=ct$ (target gene) – ct (endogenous control). Mixed sample of mRNA from healthy SD/Cub rats was used as the calibrator (its value was assumed to equal 1). The gene expressions in rats after treatment were given as relative values referred to the value of the calibrator. Levels of the gene transcripts were quantified in the quadruplex PCR reaction.

Statistics. The Student's *t*-test was used for the statistical comparison and *p*-values ≤0.05 were considered statistically significant.

Results and Discussion

Relative expression of genes *EGF, FGF, PDGF, VEGF, EGFR, FGFR, PDGFR* and *VEGFR* was examined in SD-lymphoma cells in relation to the tumor weight of the experimental animals during a period of 26 days (Figures 1, 2). Samples for RQ-RT-PCR were recovered on days 14, 17, 20, 23 and 26 after *s.c.* inoculation of tumor cells.

The data show that the expression of both fibroblast growth factor (*FGF*) and its receptor (*FGFR*) genes was significantly lower in the lymphoma cells compared to the expression in lymph node cells of healthy animals (*FGF* – day 14: $p<0.002$; day 17: $p<0.02$; day 20: $p<0.03$; day 23:

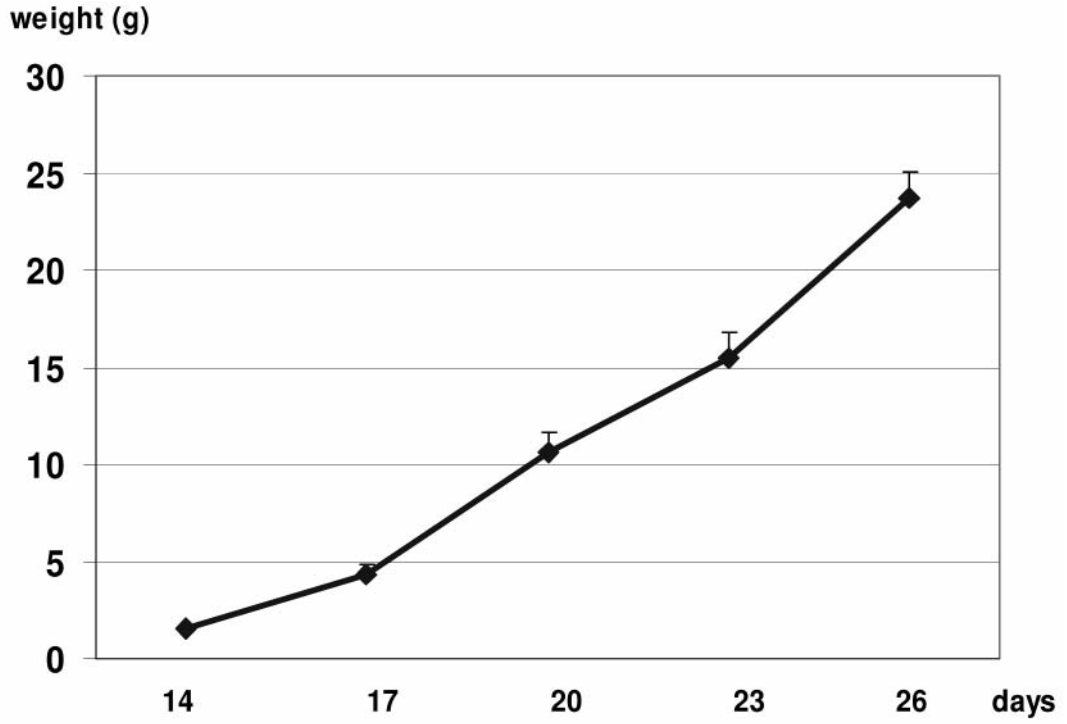

Figure 1. *Mean lymphoma weight during the period of 26 days. Each time point represents data from autopsies of three animals.*

$p<0.03$; FGFR - day 14: $p<0.04$; day 17: $p<0.02$; day 20: $p<0.007$; day 23: $p<0.03$; day 26: $p<0.005$) (Figure 2A). By contrast, *VEGF* expression increased significantly, with a well-defined maximum on day 20 (day 14: $p<0.03$; day 17: $p<0.03$; day 20: $p<0.02$; day 26: $p<0.03$) while the expression of the corresponding receptor gene *VEGFR* significantly decreased in lymphoma cells during the lymphoma tissue progression (day 17: $p<0.03$; day 20: $p<0.03$; day 26: $p<0.002$) (Figure 2B).

On the other hand, relative expression of *PDGF* gene persisted at normal values compared with *PDGF* expression in control tissue. However, the expression of *PDGFR* gene significantly increased during the growth of the lymphoma (day 20: $p<0.05$; day 23 $p<0.04$; day 26: $p<0.0006$) (Figure 2C). On the contrary, the expression of both *EGF* and *EGFR* genes did not change in the course of the 26 days when the expression of these genes was followed (data not shown).

Very significant *VEGF* and *VEGFR* gene expression enhancement observed in SD-lymphoma conforms to the findings in many different types of tumors. Both genes are considered to initiate neovascularization in tumor tissue (19, 21, 22).

In the second experiment, the relative expression of previously mentioned angiogenesis-associated genes were followed in the PMEDAP and PMEG treated SD-lymphoma bearing animals with significantly lower lymphoma weights compared to untreated SD lymphoma rats (Table I). The results show (Figure 3A) that 24 h after the last injection of the drug, the PMEDAP treatment induced non-significant changes in the expression of *EGFR, EGF, FGFR, FGF, PDGFR* and *PDGF* genes, while that of *VEGFR* was significantly down-regulated ($p<0.0006$) and that of *VEGF* was still up-regulated ($p<0.02$) when compared with the lymph node of healthy animals. Forty-eight hours after the last PMEDAP administration, the regulation of *VEGF* showed an entirely reverse course and its expression returned towards a normal level ($p<0.01$) while by that time the expression of *PDGFR* had risen ($p<0.03$) (Figure 3B).

Table I. *Antitumor potency of PMEDAP and PMEG.*

Compound	Mean lymphoma weight					
	24 h after the last dose			48 h after the last dose		
	No. of rats	g ± s.d.	*t*-test	No. of rats	g ± s.d.	*t*-test
Control	3	16.9±3.6	-	3	21.3±2.4	-
PMEDAP	3	11.2±1.8	0.08	3	16.7±1.2	0.05
Control	3	11.4±1.8	-	3	14.1±1.6	-
PMEG	3	9.5±2.0	0.2	3	9.8±0.6	0.02

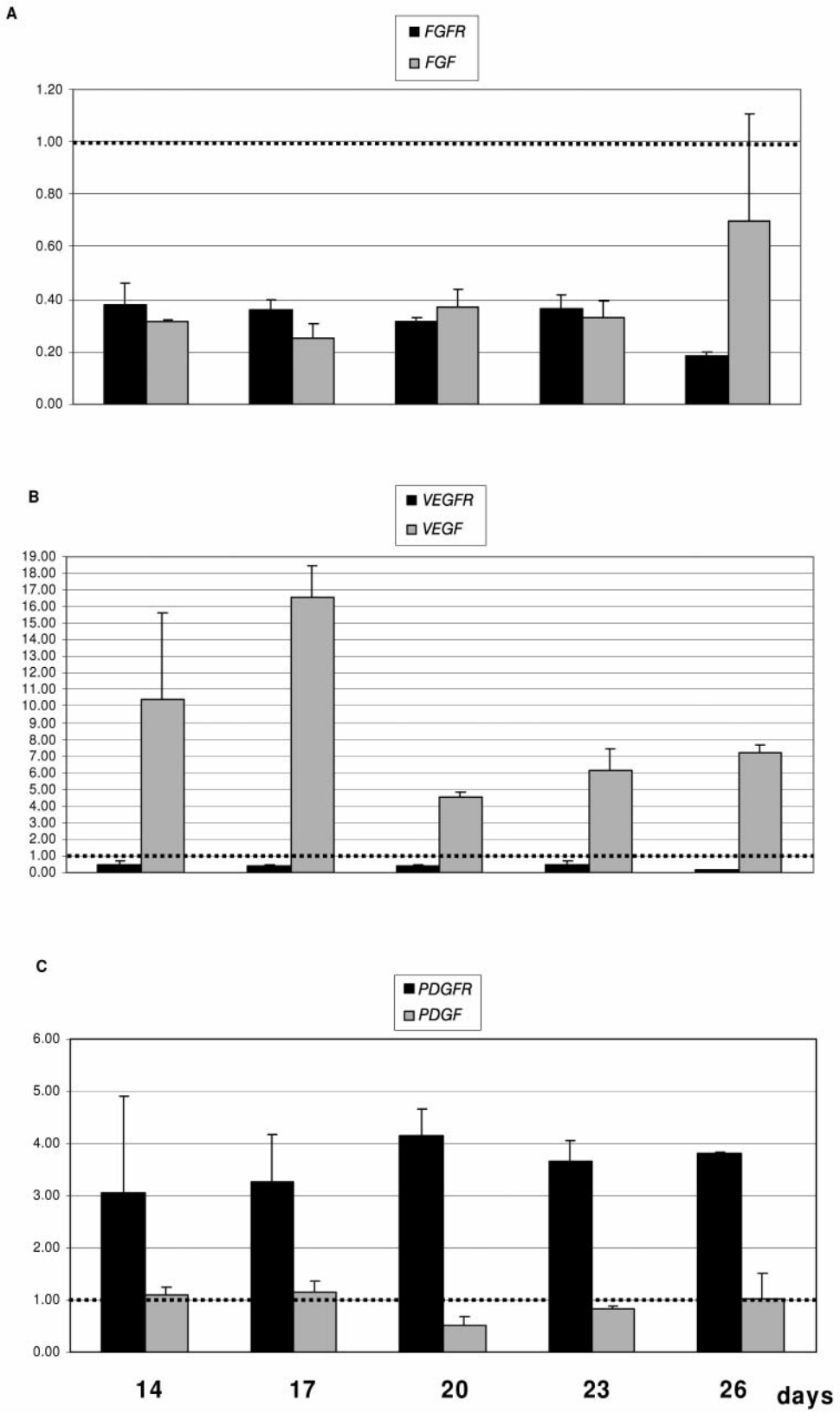

Figure 2. *Relative expression of genes associated with angiogenesis in tumor tissue as related to expression of the corresponding genes in lymph node cells of healthy SD/Cub rats (dotted line). A, FGF and its receptor FGFR. B, VEGF and its receptor VEGFR. C, PDGR and its receptor PDGFR.*

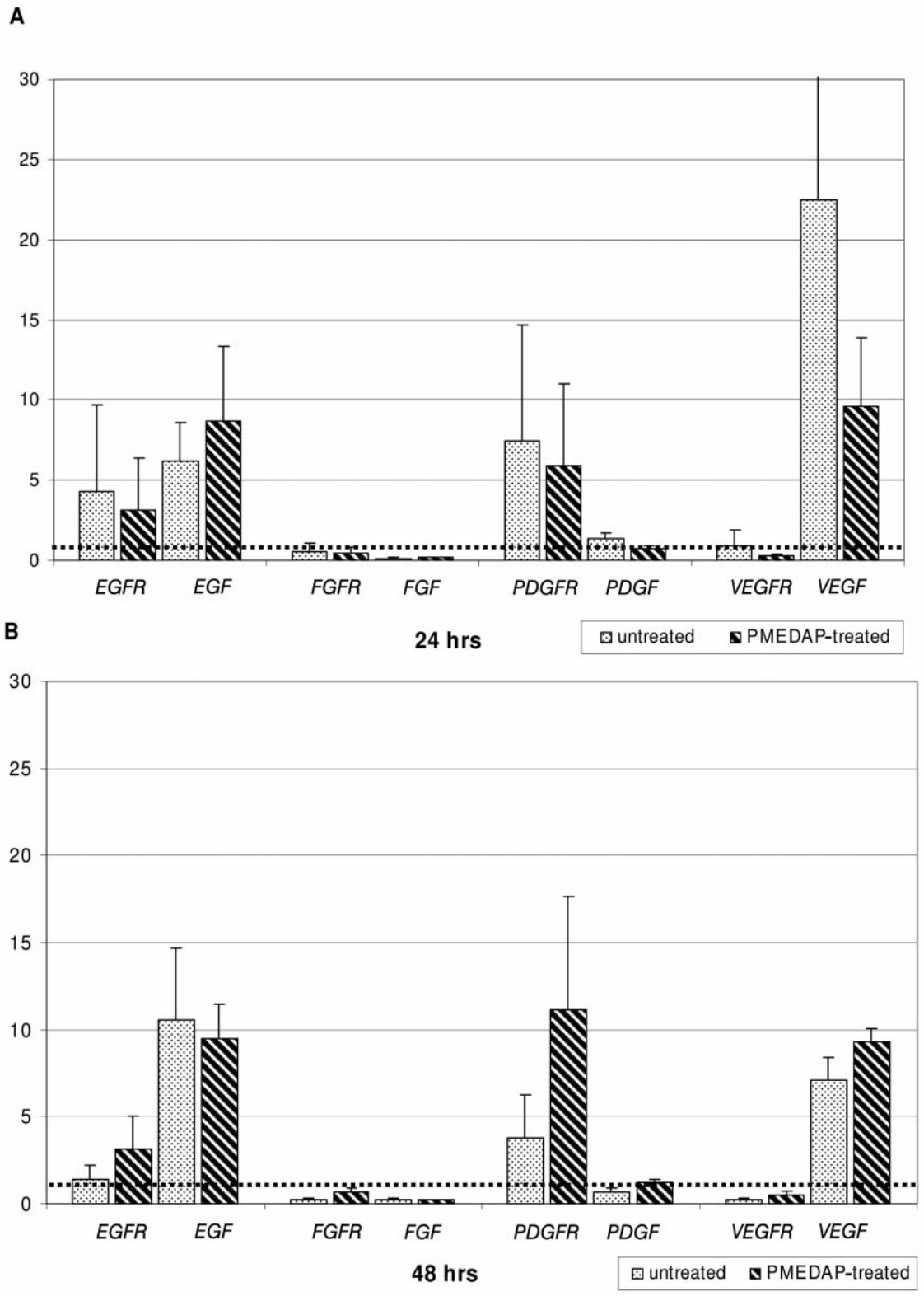

Figure 3. *Modulation of angiogenic gene expression by PMEDAP. Relative expression of EGF and its receptor EGFR; FGF and its receptor FGFR; PDGR and its receptor PDGFR and VEGF and its receptor VEGFR in tumor tissue as related to the corresponding gene expression in lymph node cells of healthy SD/Cub rats (dotted line). A, Relative expression of selected angiogenic genes 24 hours after last injection of PMEDAP. B, Relative expression of selected angiogenic genes 48 hours after last injection of PMEDAP.*

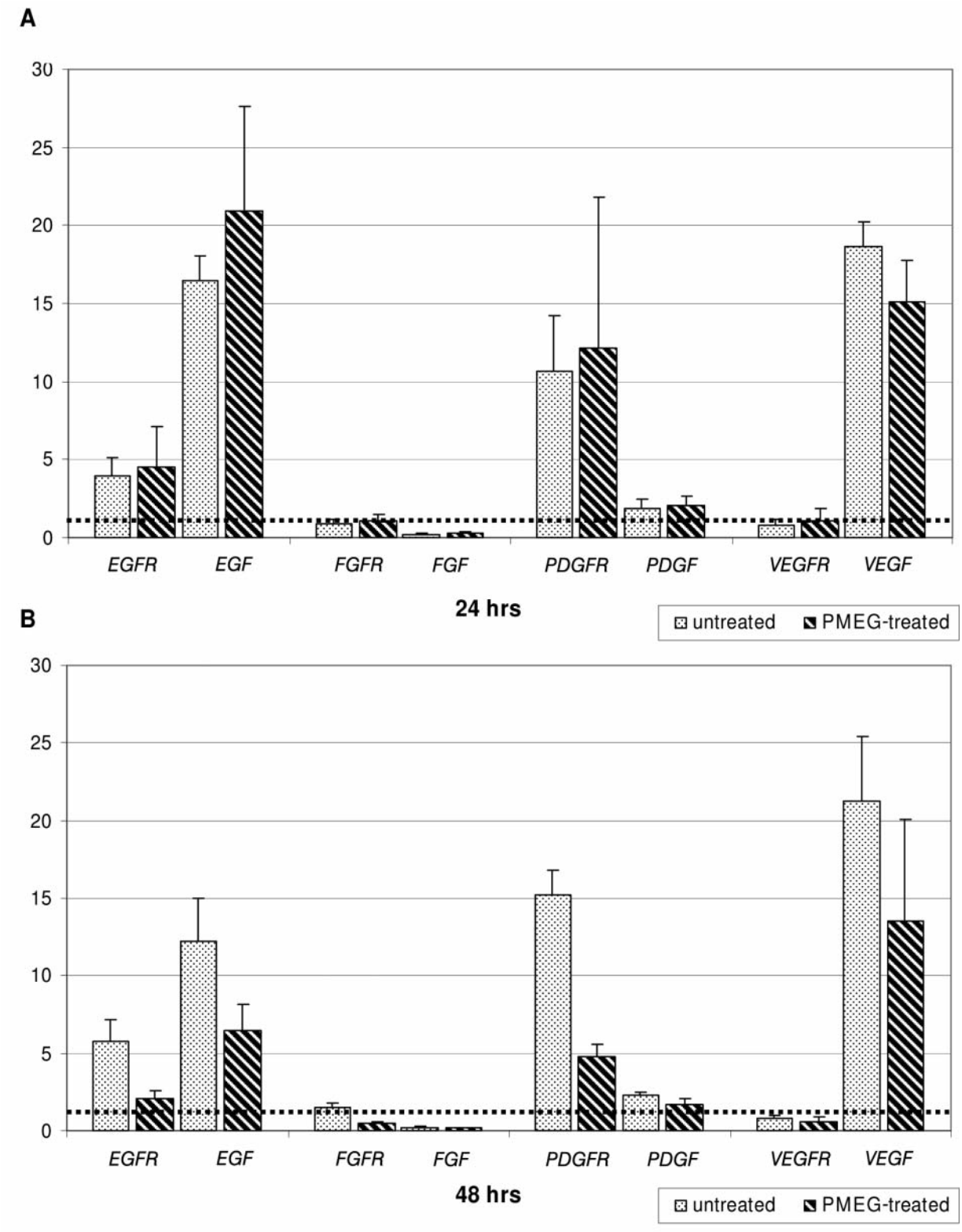

Figure 4. *Modulation of angiogenic genes expression by PMEG. Relative expression of EGF and its receptor EGFR, FGF and its receptor FGFR, PDGF and its receptor PDGFR, and VEGF and its receptor VEGFR in tumor tissue as related to expression of the corresponding genes in lymph node cells of healthy SD/Cub rats (dotted line). A, Relative expression of selected angiogenic genes 24 hours after last injection of PMEG. B, Relative expression of selected angiogenic genes 48 hours after last injection of PMEG.*

Surprisingly, after the treatment of SD-lymphoma suffering rats with PMEG, the selected angiogenesis-associated genes did not maintain the same profile as in the case of PMEDAP therapy. Twenty-four hours after the last PMEG injection, the *EGFR* and *EGF* genes were significantly up-regulated ($p<0.05$ and $p<0.007$ respectively) while *FGF* and *VEGF* genes were down-regulated ($p<0.0005$ and $p<0.001$ respectively, Figure 4A). Twenty-four hours later, all studied genes were significantly down-regulated compared to the lymph node of healthy animals and also when compared with untreated lymphomas, with the exception of a relative expression of *FGF* and *VEGFR* genes which persisted unchanged (Figure 4B).

VEGF, FGF, EGF and *PDGF* and their receptors are angiogenesis-modulating molecules. Their mode of action is mostly paracrine or autocrine. *VEGF* is one of the most important proangiogenic factors and acts as a mitogen for vascular endothelial cells. *VEGF* increases vascular permeability and contributes to endothelial cell survival in blood and lymphatic vessels. Linkage of *VEGF* with *VEGFR* initiates a cascade of intracellular signals through increased receptor tyrosine kinase activity, thus resulting in endothelial cell proliferation and formation of new blood vessels (21-23). Angiogenic growth factor *FGF* also induces endothelial cell growth and microvascular permeability (16, 24). *EGF* and its receptor *EGFR* are involved in pleiotropic signaling pathway in cancerogenesis. Activation of *EGFR* stimulates tumor growth, angiogenesis, invasion, metastasis and inhibition of apoptosis (25). Up-regulation of these genes is a marker of poor prognosis in malignant disease and hence advanced therapy of cancer is focused on an EGF blockade (26). The importance of *PDGF* consists in its direct tumor growth-promoting effect. On the one hand, it increases survival and proliferation of endothelial cells and moreover, it regulates vessel growth *via* pericyte recruitment and association with newly formed vessels. Pericytes in tumor vessels are critical for vessel integrity and function. Targeting pericytes actually provides additional benefits in anti-angiogenic treatment (27, 28).

The antiangiogenic efficacy of PMEDAP is relatively weak and is manifested mainly by down-regulation of *VEGF* and its receptor *VEGFR* detectable 24 hours after treatment. On the other hand, compound PMEG down-regulates selected proangiogenic genes much more efficiently. Its antiangiogenic potency persists and is more intensive 48 hours after treatment. It is highly possible that the novel prodrug of PMEG, compound GS-9219, which has a substantial antineoplastic activity in dogs with spontaneous non-Hodgkin's lymphoma (29) exerts antiangiogenic potential, as well.

In conclusion, we presented a study which shows that the *in vivo* antitumor efficacy of both ANPs PMEDAP and PMEG is not only caused by the inhibition of DNA synthesis but that these compounds consequently affect angiogenesis. This process plays an important role in tumor growth, since tumor tissue located distant from a blood vessel needs neovascular formation to ensure a sufficient supply of nutrients and oxygen.

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