INTRODUCTION

Angiogenesis, the formation of new capillary network from preexisting vasculature, is regulated by different growth factors and dependent on local physico-chemical mechanisms (1-3). Vascular endothelial growth factor (VEGF) affect the endothelial vascular cells by stimulation of the VEGFR-2 receptor (KDR), mitogen-activated kinase (MAPK) and phospholipase C activation and induction of the PI3K/Akt pathway (4, 5). It has been demonstrated that the p38 MAPK and PI3K/Akt activation results in actin filament reorganization and modulation of migration of endothelial cells (EC) (6). The basic fibroblast growth factor bFGF (FGF2) binds to its receptor (FGFR), causing MAPK activation, promotes cell proliferation and migration during development and similarly to VEGF inhibits the apoptosis of EC (1, 7). It has been shown, that inhibition of p38 MAPK activity enhances VEGF and bFGF-induced angiogenesis in vitro and in vivo. That is accompanied by prolonged p42/44 MAPK activation and increased endothelial survival (7, 8). Similarly treatment with the p38 inhibitor, SB202190, also enhances the bFGF-induced tubular morphogenesis by decrease of apoptosis, increase of DNA synthesis and cell proliferation and enhancement of kinetics of cell differentiation, which includes regulation of the intracellular Notch ligand - the Jagged –1 protein expression (9, 10).

The Notch/Jagged signalling, plays a crucial role in cell differentiation. Angiogenesis, is regulated by VEGF, bFGF as well as by the free fatty acid metabolites, which are regulators of transcription factors such as peroxisome proliferation activating receptors (PPARs). The study analyzed the signalling pathways involved in the regulation of Jagged-1/Notch-4 expression in endothelial cells (HUVECs) in response to VEGF, bFGF and PPAR-γ exogenous activator - ciglitazone. HUVECs were incubated with investigated substances for 24 hours, with or without the presence of the MAP-kinases inhibitors were used. Jagged-1 and Notch-4 gene expression was determined using quantitative Real-Time PCR. The Jagged-1/Notch-4 protein expression was compared by flow cytometry, when the phosphorylation-dependent activation of HUVEC was estimated by Western-blot method. The opposite effect of VEGF, bFGF, or ciglitazone on the Jagged-1/Notch-4 expression on HUVEC was connected with the different activation of MAPKs. Cigtizone, activated p38 MAPK pathway and simultaneously inhibited phosphorylation of p42/44 MAPK. The pro-angiogenic: bFGF and VEGF, also activated the p38 MAPK, but they did not attenuate the p42/44 MAPK phosphorylation. Maintaining of the Jagged/Notch interactions by VEGF, when down-regulation by bFGF and ciglitazone, seems to be dependent on the different effect on p38 MAPK and p42/44 MAPK pathway regulation.

Key words: angiogenesis, growth factors, lipids signalling, mitogen-activated kinase, signal transduction, peroxisome proliferation activating receptors, vascular endothelial growth factor
ciglitazone was found to inhibit angiogenesis and branching of capillaries in a 3D model of angiogenesis in vitro (21). PPAR\(\gamma\) activators have been shown to significantly inhibit both VEGF and leptin-induced migration of endothelial cells by targeting Akt (21, 22).

The objective of the presented study was to follow the intracellular signalling involved in the Jagged-1 and Notch-4 expression in proliferating and differentiating endothelial cells in the presence of angiogenic VEGF, bFGF as well as anti-angiogenic ciglitazone.

**MATERIALS AND METHODS**

**Cell culture**

The primary human umbilical vein endothelial cells (HUVECs) were isolated from umbilical vein. HUVECs were incubated with bFGF 0.5 nM (Sigma-Aldrich, Poznan Poland), VEGF-0.2 nM (Sigma-Aldrich), or ciglitazone (the PPAR\(\gamma\) agonists) (Sigma-Aldrich) at concentrations: 1 \(\mu\)M, 3 \(\mu\)M, and 10 \(\mu\)M for 15 min (for the kinases regulation assay); or for 24 hour for the estimation of proliferation, tubulogenesis and the Jagged-1/Notch-4 gene expression. For the kinase inhibition assay the inhibitors of the p42/44 MAPK: UO126 (10 \(\mu\)M) (Promega,); PI3Kinase (Akt pathway): LY294002 (10 \(\mu\)M) (Calbiochem), and p38 MAPK: SB202190 (20 \(\mu\)M) (Calbiochem) were used. HUVECs were incubated with or without growth factors, ciglitazone and kinases’ pathways inhibitors for 15 min before the measurement of protein phosphorylation by the Western blot method.

**Biological effect of investigated substances**

The absence of possible cytotoxic effect of used concentrations of investigated substances (VEGF (0.2 nM) and bFGF (0.5 nM), ciglitazone (1-10 \(\mu\)M) as well as kinases’ inhibitors: UO1226 (10 \(\mu\)M), LY294002 (10 \(\mu\)M), SB202190 (20 \(\mu\)M)) was confirmed by measurement of lactate dehydrogenase (LDH) activity in the medium (Cytotox 96 kit, Roche).

To determine the effect on cell proliferation, the rate of DNA synthesis was measured by the bromo-deoxyuridine (BrdU) incorporation to DNA using the anti-BrdU kit (Roche).

For pro-angiogenic activity the matrigel tubulogenesis assay was performed according to manufacturer protocol (Calbiochem), after the incubation of HUVEC for 24 hours with...
investigated factors at given above concentrations. Tubules formation was then investigated under the optical Olympus microscope (magnitude 50 x) and photographed in the presence of the 1 mm length scale. Three images per well were then captured and analysed for the tubular length. The results are given as an average of the total tubule length presented on pictures.

Measurement of the intracellular signalling pathways (Western blot)

Protein content, isolated from harvested using Cells Harvesting Buffer (Sigma), HUVEC samples, was estimated using Bradford method (23). Immunoblot analysis were performed according to Laemmli (24). Detection of kinases activation was performed using commercial Western-blot kits using NEB antibody: for phosphorylated p38 MAPK, phosphorylated Akt, total p38 MAPK, total Akt, and for p42/44 MAPK as well as total MAPK. As the secondary, the horseradish peroxidase-conjugated sheep anti-mouse, or anti-rabbit antibody were applied. Electrochemiluminescence reagent (ECL) was used for detection. Enhanced chemiluminescence, performed according to manufacturer’s instructions (Amersham) was used to demonstrate positive bands, that were visualized after exposure on a transparent medical X-Ray film.

Regulation of Jagged-1 and Notch-4 gene expression (quantitative real-time PCR assay)

Total RNA was isolated from HUVECs after 24 hours of stimulation with investigated factors. The amplification was performed using the continuous fluorescence detection system DNA Engine Opticon (MJ research Inc., Waltham, USA). The quantitative Real-Time PCR assay (qRT-PCR) was performed with the QuantiTect SYBR Green PCR Kit (Qiagen) using the following sets of primers: Jagged-1 sense: 5’ACCAAGCAACAGATCAAAGC3’, antisense: 5’GAAACAGCTCGTGATGTGCT3’; Notch-4 sense: 5’ACAGATGCAACCTCTAATTCC3’, antisense 5’CGTGGATTCAAATACAGCA3’; GAPDH sense: 5’CATCCATGACAACTTTGGTATCGT3’, antisense 5’CAGTTTCTGGGTTGCAGTGA3’. Calculation was performed using the program Calculation Matrix for PCR Efficiency REST-XL (gene.quantification@wzw.tum.de). The expression rates were calculated as the normalized C\textsubscript{T} difference between the control probe and sample with the adjustment for

Fig. 2. HUVEC proliferation (BrdU incorporation). Influence of VEGF (0.2 nM), bFGF (0.5 nM), or ciglitazone (1-10 µM) (A). Evaluation of inhibitory effect of the p42/44 MAPK inhibitor U0126 (10 µM) (B); the Akt pathway inhibitor LY29002 (10 µM) (C); and the p38 MAPK inhibitor SB202190 (20 µM) (D). Results are shown as mean values, derived from three-five separate experiments, +/-SD. Significance: *p< 0.05 vs. control; **p<0.05 vs. VEGF, *p<0.05 vs. bFGF. BrdU- bromo-deoxyuridine; IP3Kinhibitor- IP3Kinase inhibitor (the Akt pathway inhibitor); ciglit (1 µM) - ciglitazone 1 µM, ciglit (3µM) - ciglitazone 3 µM, ciglit (10 µM) - ciglitazone 10 µM; VEGF (0.2 nM) + ciglit (1-10 µM)- coinubcation of VEGF with ciglitazone; bFGF (0.5 nM) + ciglit (1-10 µM) coinubcation of bFGF with ciglitazone.
the amplification efficiency relative to the expression level of the housekeeping gene GAPDH.

**Analysis of Jagged-1 and Notch-4 protein expression by flow cytometry**

After incubation with investigated factors HUVECs were harvested using Cells Harvesting Buffer (Sigma), centrifuged and then blocked in PBS with 10% BSA (Sigma). After that, Jagged 1 (goat) or Notch4 (mouse) (Santa-Cruz Biotechnology) primary monoclonal antibodies were added for 30 min. Then, cells were incubated with secondary antibody anti-mouse PE, or anti-goat FITC (Becton Dickinson). After labelling with the antibodies, the suspensions were diluted with 20 volumes of PBS, washed and directly measured by FACS Calibur Flow Cytometer (Becton Dickinson).

**Statistical analysis**

Descriptive results of continuous variables were expressed as mean values±SD from 3-5 experiments. The t-test for comparisons of quantitative variables were used. Levels of statistical significance were set at p<0.05. The statistical analysis were performed with the Statistica for Windows from Statsoft.

**RESULTS**

Any of investigated effects was related to the toxic effect of the used concentration of compounds as measured by the cell membrane LDH leakage (Cytotox 96 kit, Roche).

**Regulation of MAP kinases by used compounds (Western blot)**

The pre-treatment of HUVEC with VEGF resulted in activation of all investigated MAPK pathways (Fig. 1A,B,C). Whereas in the presence of bFGF the activation p42/44 MAPK (Fig. 1A) and slight activation of p38 (Fig. 1C), but not Akt (Fig. 1B) was observed. Incubation of HUVECs with ciglitazone increased p38 (concentration-dependently) (Fig. 1C), and decreased MAPK activity (Fig. 1A), whereas Akt phosphorylation remained unchanged (Fig. 1B).

**Influence of VEGF, bFGF, ciglitazone and the MAPK inhibitors on proliferation of HUVEC**

Incubation with VEGF (0.2 nM) and bFGF (0.5 nM) for 24 hours resulted in the significant increase of HUVEC proliferation (Fig. 2A). This effect was potently inhibited by ciglitazone, added simultaneously with the growth factors (Fig.
PPARγ activation also affected the spontaneous proliferation of HUVEC (Fig. 2A). This effect was not connected with the cell toxicity, since at the all used concentrations of ciglitazone we did not observe the cytotoxic effects investigated as described previously. Inhibition of p42/44 MAPK pathway by U0126 (Fig. 2B) and Akt pathway by LY294002 (Fig. 2C), but not inhibition of p38 MAPK by SB202190 (Fig. 2D) decreased the rate of HUVEC proliferation after VEGF and bFGF treatment. This effect was not connected with the cell toxicity, since at the all used concentrations of all MAPK pathways inhibitors had no cytotoxic effects; investigated as described previously. The inhibition of p42/44 pathway leads to statistical significant decrease of VEGF-induced proliferation (Fig. 2B). Pre-treatment of HUVECs with the Akt pathway (IP3K) inhibitor exerted even more potent retardation effect on the VEGF and bFGF- induced cell proliferation (Fig. 2C). While pre-treatment with the p38 MAPK inhibitor significantly attenuated the inhibitory effect of ciglitazone on cell proliferation (Fig. 2D).

**Tubular morphogenesis of HUVECs in the 3D matrigel model**

HUVECs cultured in matrigel with bFGF, or VEGF underwent tubulogenesis measured by the increase of the tubule length. Ciglitazone alone or added simultaneously with VEGF or bFGF inhibited tubulogenesis in concentration-dependent manner. The presence of MAPK inhibitors differentially influenced the tubulogenesis (Fig. 3). Inhibition of p42/44 MAPK pathway (by U0126), as well as IP3K (by LY294002) decreased the VEGF and bFGF induced tubulogenesis, but did not affect the influence of ciglitazone (Fig. 3A,B). Inhibition of p38 MAPK (by SB202190) enhanced the VEGF and bFGF-induced tubular length and statistical significant attenuated inhibitory effect of ciglitazone (Fig. 3C).

**Regulation of Jagged-1 and Notch-4 gene expression in HUVECs**

The quantitative RT-PCR method analysed the influence of investigated factors on the expression pattern of Jagged-1 (Fig. 4) and Notch-4 (Fig. 5). Incubation with VEGF slightly increased the Jagged-1 expression in HUVECs (ratio: 1.57), while the inhibition of Jagged-1 expression by bFGF (ratio: 1.98) and ciglitazone was observed (Fig. 4A-C). Inhibition of p38 MAPK pathway by SB202190, or Akt pathway (IP3K) by LY294002 attenuated inhibitory effect on Jagged-1 expression in both bFGF and ciglitazone treated cells (Fig. 4B, and C).
Incubation with VEGF non-significantly increased the Notch-4 expression in HUVECs, while bFGF and ciglitazone inhibited Notch-4 expression in HUVECs (Fig. 5A-C). Data presented as a ratio of relative gene expression. Results are shown as mean values, derived from three-five separate experiments, +/-SD. Significance: *p < 0.05. p42/44 inhibitor- U0126 inhibitor of p42/44 MAPK pathway; IP3K inhibitor- LY29002 inhibitor of Akt pathway; p38 inhibitor- SB202190 inhibitor of p38 MAPK pathway; ciglit (1 µM)- ciglitazone 1 µM; ciglit (3 µM)- ciglitazone 3 µM; ciglit (10 µM)- ciglitazone 10 µM.

**DISCUSSION**

Process of angiogenesis is regulated by different growth factors, hormones, epigenetic mechanisms and depends on local physico-chemical tissue status ie nutrient and oxygen supply (2, 25, 26).

The original finding of this paper is that the opposite effect of VEGF, bFGF, or ciglitazone on the Jagged-1/Notch-4 expression on HUVEC is connected with the different activation of MAPKs. We report that ciglitazone, PPARγ ligand activates, p38 MAPK pathway and simultaneously inhibits phosphorylation of p42/44 MAPK. The pro-angiogenic: bFGF and VEGF, also activate the p38 MAPK, but they did not attenuate the p42/44 MAPK phosphorylation. This might represent the principal distinction between angiogenic, differentiation and anti-angiogenic effect of investigated factors. Activation of the JNK/SAPK and p38 kinases has been shown to improve the EC motility and vascular permeability, but in consequence attenuate the VEGF and bFGF-induced differentiation and morphogenesis (27). Inhibition of p38 MAPK was reported to reduce an EC apoptosis and enhance phosphorylation of p42/44 MAPK. The pro-angiogenic: bFGF and VEGF, also activate the p38 MAPK, but they did not attenuate the p42/44 MAPK phosphorylation. This might represent the principal distinction between angiogenic, differentiation and anti-angiogenic effect of investigated factors. Activation of the JNK/SAPK and p38 kinases has been shown to improve the EC motility and vascular permeability, but in consequence attenuate the VEGF and bFGF-induced differentiation and morphogenesis (27). Inhibition of p38 MAPK was reported to reduce an EC apoptosis and enhance phosphorylation of p42/44 MAPK pathway (10). This has been suggested for explanation of the antagonistic effects of the p38 MAPK and p42/44 pathway influence on EC activity (10).
To confirm the role of p38 MAPK in ciglitazone-dependent inhibition of angiogenesis, the effect of inhibition of p38 MAPK on retardation of HUVEC proliferation and tubulogenesis was examined. In accordance with other authors (8,10) we observed the augmentation of VEGF or bFGF-induced tubulogenesis after pre-treatment with inhibitor of p38 MAPK pathway (SB202190). Moreover inhibition of this pathway led to the augmentation/restoration of differentiation and tubulogenesis in the ciglitazone treated cells. Inhibition of p38 MAPK increased the rate of HUVECs proliferation in the presence of ciglitazone, but did not affect the proliferation of the VEGF- and the bFGF-exposed cells, confirming results obtained by the others (10) that the p38 MAPK pathway is implicated in VEGF and Bfgf-dependent cell differentiation, but not proliferation. On the other hand inhibition of the p42/44 MAPK and Akt pathways resulted in the decrease of HUVEC proliferation rate what argue for the involvement and importance of p42/44 MAPK and Akt pathways in cell proliferation (5, 10). The effect was stronger for the Akt pathway (IP3K) inhibition, and was in agreement with the observed decrease of cell survival after inhibition of this pathway (28). It should be however stressed that the commonly used kinases inhibitors are not absolutely specific. In the elegant review concerning specificity and mechanism of action of the kinases inhibitors Davies et al. underlined the efficacy of used compounds to inhibit more then one protein kinase with similar potency (29). On the other hand in the same article the authors confirmed that the inhibitors used in our study (SB 203580 and U0126) were compounds with the most impressive selectivity (29).

Recent studies have shown that p38 MAPK plays essential role in cell differentiation of several cell types (8). Inhibition of this pathway was found to enhance expression of Jagged-1 during the bFGF-stimulated tubular morphogenesis (10). Therefore we put the question, whether the mechanism of anti-angiogenic effect of ciglitazone can be connected with the regulation of expression of the Jagged-1/Notch-4 genes. We observed the opposite effect on the Jagged-1 and the Notch-4 expression by used factors. VEGF increased expression, whereas bFGF, as well as ciglitazone, inhibited expression the Jagged-1 and the Notch-4. In parallel, the significant differences in regulation of the Jagged-1 and the Notch-4 expression by p38 MAPK was observed. Inhibition of p38 kinase increased the Jagged-1, whereas decreased the
Notch-4 expression in VEGF, bFGF and ciglitazone treated cells. On the other hand Akt pathway inhibition resulted in an increase of Jagged-1 expression in the ciglitazone treated cells, but IP3K inhibitor did not affect the Notch-4 expression. We also did not observe the influence of p42/44 MAPK on regulation of Jagged-1 expression, whereas inhibition of this pathway increased Notch-4 expression in the VEGF and ciglitazone stimulated HUVECs.

During development, the Notch-4 gene expression is restricted to the vasculature, while the Jagged-1 is widely expressed in different tissue (12). Notch receptors regulate the balance between cell differentiation and stem cell proliferation during the morphogenesis of numerous tissues (1, 12, 20, 21). It has been shown that HUVECs with dominant negative forms of Jagged-1 demonstrated significant inhibition of vascular network formation, when activation of Notch-4 was shown to inhibit the sprouting of human dermal microvascular endothelial cells (HMEC-1) in vitro and in vivo model (17, 18).

Isbrucker et al. demonstrated that inhibition of p38 MAPK enhances the VEGF- stimulated angiogenesis in vitro and in vivo, whereas blocks hyperpermeability (27). Also activation of PPARγ by rosiglitazone increases permeability of bronchial lung vasculature and promote oedema (30). Thus p38 MAPK activation promotes the permeability of EC.

Our findings provide an additional argument for understanding the regulatory mechanism(s) of endothelial biology by the involvement of p38 MAPK and p42/44 MAPK pathway in VEGF, bFGF and PPARγ regulated angiogenesis. The results might add to understanding how activation of p38 MAPK, leading to decrease of the Jagged/Notch system mediated cell-cell interaction, may lead to impairment of angiogenesis what may add to understanding of pathological angiogenesis associated atherosclerosis, diabetes or tumour growth.

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