Purinergic regulation of vascular endothelial growth factor signaling in angiogenesis

SM Rumjahn1, N Yokdang1, KA Baldwin1, J Thai1 and ILO Buxton*,1
1Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557, USA

P2Y purine nucleotide receptors (P2YRs) promote endothelial cell tubulogenesis through breast cancer cell-secreted nucleoside diphosphate kinase (NDPK). We tested the hypothesis that activated P2Y1 receptors transactivate vascular endothelial growth factor receptor (VEGFR-2) in angiogenic signaling. P2Y1R stimulation (10 µM 2-methyl-thio-ATP (2MS-ATP)) of angiogenesis is suppressed by the VEGFR-2 tyrosine kinase inhibitor, SU1498 (1 µM). Phosphorylation of VEGFR-2 by 0.0262 or 2.62 nM VEGF was comparable with 0.01 or 10 µM 2MS-ATP stimulation of the P2Y1R. 2MS-ATP, and VEGF stimulation increased tyrosine phosphorylation at tyr1175. 2MS-ATP (0.1 – 10 µM) also stimulated EC tubulogenesis in a dose-dependent manner. The addition of sub-maximal VEGF (70 pM) in the presence of increasing concentrations of 2MS-ATP yielded additive effects at 2MS-ATP concentrations < 3 µM, whereas producing saturated and less than additive effects at ≥ 3 µM. We propose that the VEGF receptor can be activated in the absence of VEGF, and that the P2YR–VEGFR2 interaction and resulting signal transduction is a critical determinant of vascular homoeostasis and tumour-mediated angiogenesis.

Keywords: breast cancer; angiogenesis; purinergic receptor; P2Y; VEGF; VEGFR2; phosphotyrosine

The secretion of nucleoside diphosphate kinase (NDPK; EC 2.7.4.6) orthologues by intracellular parasites (Gounaris et al, 2001; Chopra et al, 2003), NDPK secretion by various carcinomas (Anzinger et al, 2001; Okabe-Kado and Kasukabe, 2003), and NDPK’s extracellular role in blood flow regulation (Buxton et al, 2001) first lead us to propose a pathological role for secreted NDPK in cancer and tumour angiogenesis. We have recently provided evidence for a nucleotide-dependent regulation of angiogenesis by breast cancer-secreted NDPK (Rumjahn et al, 2007). We observed that extracellular NDPK by regeneration of extracellular nucleotides can use endothelial P2 (Y) nucleotide receptors to stimulate angiogenesis. Supporting our findings, the disruption of CD39 (ecto-apyrase EC 3.6.1.5) activity, the dominant vascular ecto-nucleotidase and its regulation of nucleotide signaling, has been observed to inhibit tumour angiogenesis and metastasis (Goepfert et al, 2001; Jackson et al, 2007). The regulation of extracellular ATP and ADP levels by ecto-apyrase is also known to play important roles in cardiovascular physiology and pathophysiology by activation of purinergic type-2 (P2) nucleotide receptors (Erlinge and Burnstock, 2008).

P2 nucleotide receptors activated by ATP include both ligand-gated ion channels (P2X) and heterotrimeric G protein-coupled receptors (P2Y). P2Y receptors have become recognised as the important regulators of carcinogenesis, endothelial regulation, and blood flow regulation (Buxton et al, 2001; Burnstock, 2006; White and Burnstock, 2006). Little is known about the role of P2Y receptors (P2YRs) in angiogenesis with only a handful of reports providing evidence supporting this notion. We have shown earlier that P2YR signaling promotes angiogenic properties such as endothelial cell tubulogenesis (Rumjahn et al, 2007), whereas others have reported P2YR-mediated migration (Satterwhite et al, 1999; Kaczmarek et al, 2005) and permeability (Tanaka et al, 2004, 2006). Activated P2Y2 receptors can transactivate vascular endothelial growth factor receptor-2 (VEGFR-2), suggesting a direct link between extracellular nucleotides and established tumour angiogenesis signaling (Seye et al, 2004). VEGFR-2 mediates the majority of the angiogenic and permeability-enhancing effects of VEGF (Shibuya, 2006). Given this evidence, we hypothesised that endothelial P2YR signaling interacts to regulate VEGFR-2 signaling. Here, we provide evidence that P2Y1R stimulation of human endothelial cells activates VEGFR-2 intracellular signaling to stimulate endothelial cell tubulogenesis, a direct in vitro measure of angiogenesis. These data suggest that tumour-mediated angiogenesis signaling may be, in part, mediated by nucleotide receptor activation of the VEGFR-2 pathway and may effectively lower the local requirement for VEGF.

MATERIALS AND METHODS

Cell culture

Human cardiac endothelial cells (HCECs) were earlier isolated by Fluorescence Activated Cell Sorting for CD31 (PECAM) and immortalised by human telomerase reverse transcriptase (hTERT). Cloned human cord blood endothelial colony forming cells (ECFCs) were purchased from Dynacell Life Sciences (Spring
In vitro angiogenesis scoring technique

A representative endothelial cell tubulogenesis (angiogenesis) score for each condition was obtained by analysing digital images (∼100) collected from the central pointing corners of quadrants 1–IV in each culture well and averaging the four scores. As described earlier (Rumjahn et al., 2007), an angiogenesis score (s) represents the product of mean number of branch points (bp) multiplied by mean pixel length (l) multiplied by mean pixel cell surface area (a). Thus, s = bp × l × a.

Effect of disrupting VEGF signaling in P2Y receptor-mediated angiogenesis

Human cardiac endothelial cells (3 × 10⁴) on collagen-coated plates were incubated for 24 h with P2Y receptor agonists ATP (P2Y₁/₂R; 100 μM) and 2-methyl-thio-ATP (2MS-ATP) (P2Y₁/₂; 10 μM). EC tubulogenesis was also observed in the presence of 1 μM SU1498 (~IC₅₀ of VEGFR-2 tyrosine kinase inhibitor (Strawn et al., 1996); Sigma, St Louis, MO, USA) with either 100 μM ATP or 10 μM 2MS-ATP. EGM-2 was employed as a positive control, whereas non-treatment controls were performed for normalisation and comparison. EGM-2 stimulated growth was also observed in the presence of 100 μM M2MS-ATP. EGM-2 was employed as a positive control, whereas non-treatment controls were performed for normalisation and comparison.

Effect of 2-Methyl-Thio-ATP signaling on angiogenesis

Endothelial colony forming cells (5 × 10⁴) on GFR Matrigel-coated plates were incubated for 24 h with various concentrations of 2MS-ATP (0.1–10 μM in half-log increments). VEGF (262 pM) was used as a positive control, whereas non-treatment controls were performed for normalisation and comparison.

Statistical analyses

All graphs were prepared using Prism Graphing Software (V5.01; GraphPad Software, San Diego, CA, USA) and statistical analyses were carried out using InStat Statistical Software (V3.06; GraphPad Software), with P ≤ 0.05 considered significant. Significance was determined using non-parametric analysis of variance (Kruskal–Wallis) with Dunn’s multiple comparisons post-test or non-parametric t-test (Mann–Whitney). Data points and error bars represent means ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001 (vs negative control, unless otherwise indicated); †P < 0.05 (2MS-ATP alone vs 2MS-ATP + VEGF combination).

RESULTS

Endothelial P2Y₁/₂ receptors mediate in vitro angiogenesis through VEGF signaling

As P2Y receptors have earlier been reported to interact with VEGFR-2 (Seye et al., 2004), we investigated whether nucleotides and VEGF-induced angiogenesis involved a common signaling pathway. HCECs incubated with P2Y₁/₂ receptor agonists (100 μM ATP or 10 μM 2MS-ATP) for 24 h showed a 2.0-fold angiogenic stimulation (P ≤ 0.01; Figure 1A) that was suppressed back to ~1.3-fold control by 1 μM SU1498. The ~IC₅₀ of SU1498, a VEGF-2 tyrosine kinase inhibitor, was used to limit non-specific
inhibition of other RTKs (e.g., platelet-derived growth factor receptor and epidermal growth factor receptor) as reported earlier with higher concentrations of this agent (Strawn et al., 1996). The EGM-2 control produced a ~2.5-fold increase over control (P = 0.001; Figure 2), which, interestingly, was not affected by the addition of 1 μM SU1498. EGM-2 (a proprietary cocktail) includes a mix of various angiogenic factors, including VEGF.

Using primary human endothelial cells, we also observed that P2Y1 receptor activation stimulates VEGFR-2-mediated EC tubulogenesis. ECFCs incubated with 10 μM 2MS-ATP (P2Y1 receptor agonist) for 24 h exhibited a ~2.6-fold angiogenic stimulation (P = 0.001; Figure 1B) that was suppressed back to ~1.9-fold control by 1 μM SU1498. The VEGF control (262 pm) observed a ~2.4-fold increase over control, which was suppressed back to ~1.5-fold control by 1 μM SU1498. The SU1498 control showed minimal angiogenic effect alone.

Activated endothelial P2Y1 receptors transphosphorylate VEGFR-2

It is well known that ligand binding of a RTK (e.g., VEGFR-2) induces dimerisation and subsequent activation of the receptor, resulting in the autophosphorylation of tyrosine residues in its cytoplasmic domain (Arora and Schollar, 2005). Knowing that P2Y1R-mediated EC tubulogenesis uses VEGF-2 intracellular signaling, we further asked if P2Y1R activation by 2MS-ATP would transphosphorylate (i.e., transactivate) VEGFR-2. ECFCs stimulated with VEGF (0.0262 or 2.62 nm) showed phosphorylation of VEGF-2 in a dose-dependent manner (Figure 1C; lanes 1 and 2), consistent with the known activation of this receptor by its natural ligand. ECFCs stimulated with 2MS-ATP (0.01 and 10 μM) also exhibited a similar dose-dependent phosphorylation of VEGF-2 (Figure 1C; lanes 3 and 4). The non-stimulated (negative) control showed a minimal level of basal VEGF-2 tyrosine phosphorylation (Figure 1C; lane 5).

Phosphorylated tyrosine 1175 of VEGF-2 is a binding site for the SH2 domain of phospholipase C, which is an important mediator of VEGFR-2-induced angiogenesis (Shibuya, 2006). We further observed that ECFCs stimulated with either VEGF or 2MS-ATP phosphorylated VEGF-2 Tyr1175 in a similar dose-dependent fashion (Figure 1D and E). Human umbilical vein endothelial cells also showed similar levels of VEGF-2 tyrosine phosphorylation by VEGF and 2MS-ATP (data not shown).

Endothelial P2Y1 receptor activation stimulates EC tubulogenesis in a dose-dependent manner

We observed that 2MS-ATP promotes a dose-dependent angiogenic response with significant stimulation seen at higher concentrations of ≥3 μM 2MS-ATP, ~2.5-fold increase over control (P ≤ 0.05; Figure 2A). We observed earlier that ≥10 μM 2MS-ATP does not provide additional angiogenic stimulation (Rumjahn et al., 2007), therefore an apparent tubulogenesis EC50 would be ~3 μM. The VEGF control (262 pm) stimulated angiogenesis similar to that seen with 10 μM 2MS-ATP.
Figure 2  2-methyl-thio-ATP (2MS-ATP) and vascular endothelial growth factor (VEGF) cooperatively promote in vitro angiogenesis. P2Y1 or VEGF signaling alone, as well as together stimulated EC tubulogenesis over a 24 h duration. (A) ECFCs treated with varying amounts of 2MS-ATP produced a dose-dependent stimulation of tubulogenesis. Control mean = 1292.8 ± 65.1 angiogenesis units. Negative control A, ECFCs incubated in EBM-2 supplemented with 2% FBS. The angiogenic stimulation control used was EBM-2 containing VEGF. (B) VEGF (natural VEGFR-2 agonist) produced a dose-dependent stimulation of tubulogenesis. Angiogenic responses varied between 1.25- and 3.00-fold control (defined as 0 and 100% stimulation). Negative control B, ECFCs incubated in EBM-2 supplemented with 2% FBS. Curve trace was calculated using a non-linear fit of the data employing an equation describing a sigmoidal curve. (C) ECFCs incubated with varying concentrations of 2MS-ATP combined with a constant sub-maximal level of VEGF (apparent tubulogenesis EC50 of 70 pM) produced additive stimulation of EC tubulogenesis only at lower concentrations of 2MS-ATP. Control mean = 655.7 ± 81.8 angiogenesis units. Negative control C, ECFCs incubated in EBM-2 supplemented with 2% FBS. Fold control – 1.00 equals non-stimulated (negative) control. (D) Hypothetical curve illustrating two molecules promoting a biological response (e.g., angiogenesis) via convergent signaling pathways, which limits the potential of a larger response at higher concentrations. *P<0.05; **P≤0.01; ***P≤0.001.

VEGF stimulates in vitro angiogenesis in a dose-dependent manner

Endothelial colony forming cells incubated with varying concentrations of VEGF (0.00131 – 0.524 nM) over 24 h also exhibited a dose-dependent increase in EC tubulogenesis with maximal response at levels ≥ ~0.262 nM (Figure 2B). On the basis of these observations, an apparent VEGF EC50 of ~70 pM was used as a sub-optimal level of VEGF stimulation of tubulogenesis.

2MS-ATP and VEGF use a common angiogenic pathway

To further investigate the notion of P2Y and VEGF cooperative signaling, we incubated ECFCs with varying concentrations of 2MS-ATP (0.1 – 10 μM) in the presence of constant tubulogenesis EC50 concentration for VEGF (70 pM). We observed that the combination of sub-optimal levels of 2MS-ATP and VEGF do indeed promote a significant dose-dependent angiogenic response when compared with 2MS-ATP alone and control (P≤0.05; Figure 2C). Lower concentrations of 2MS-ATP (< 3 μM) in combination with VEGF (70 pM) exhibited additive-like angiogenic stimulation, whereas higher concentrations (≥ 3 μM 2MS-ATP) produced a more saturated and less than additive promotion of EC tubulogenesis. VEGF at 70 pM provided ~ 51% of maximal VEGF stimulated EC tubulogenesis (compared with 262 pM), consistent with our preliminary determination of the EC50.

DISCUSSION

We observed earlier that pathologically secreted NDPK stimulates angiogenesis in a nucleotide-dependent manner principally by P2Y1R (Rumjahn et al, 2007). Supporting this notion of P2Y-mediated angiogenesis, it has been reported that endothelial P2Y1R-mediated VEGFR-2 activation stimulates the expression of pro-inflammatory vascular cell adhesion molecule 1 (VCAM-1) (Seye et al, 2003, 2004). Inflammation and angiogenesis share common mechanisms and are often seen concurrently, especially in carcinogenesis. An appropriate example would be the use of VCAM-1 in the recruitment of monocytes (undifferentiated macrophages) and the differentiation of these cells into tumour-associated macrophages, which can provide a microenvironment conducive to tumour growth, metastasis, and of course angiogenesis, as especially prevalent in breast and prostate cancers (Pollard, 2004).

We suggest that P2YR-mediated VEGFR-2 activation can promote tumour angiogenesis indirectly, as well as stimulate direct angiogenic effects on the endothelial cells where the original P2Y1/VEGFR signaling occurs. We investigated the latter notion and observed the inhibition of ATP and 2MS-ATP-mediated EC tubulogenesis by VEGFR-2 tyrosine kinase inhibitor SU1498. This showed that VEGFR-2 intracellular signaling is a substantial component of P2YR-mediated in vitro angiogenesis. The unaltered angiogenic potential of EGM-2 (includes angiogenic factors in addition to VEGF) by SU1498 is consistent with the presence of multiple angiogenic pathways that can compensate for impaired VEGF signaling. As this P2Y-mediated VEGFR-2 signaling was observed in immortalised endothelial cells (HCECs), we further showed that this signaling also exists in primary endothelial cells (ECFCs). In the presence of a more relevant VEGF angiogenic control, SU1498 (~IC50 tyrosine kinase activity) produced a ~60% reduction in stimulated EC tubulogenesis indicating a tight association between VEGFR-2 tyrosine kinase activity and our detection of EC tubulogenesis. VEGF (0.262 nM) exhibited maximal induction, whereas higher concentrations
The image contains a page from a scientific paper discussing the role of purinergic signaling in angiogenesis. The authors propose that dual inhibition of P2YR and VEGFR-2 signaling may provide an effective mode of combinational anti-angiogenic therapy. The paper includes references to experimental evidence supporting these findings.

**References**

Anzinger, J., Malmquist NA, Gould J, Buxton IL (2001) Secretion of a nucleoside diphosphate kinase (Ndpp2-H2) by cells from human breast, colon, pancreas and lung tumors. *Proc West Pharmacol Soc 44*: 61–63

Chopra P, Singh A, Koul A, Ramachandran S, Drlica K, Tyagi AK, Singh Y (2003) Cytotoxic activity of nucleoside diphosphate kinase secreted from *Mycobacterium tuberculosis*. *Eur J Biochem* 270(4): 625–634

Erlinge D, Burnstock G (2008) P2 receptors in cardiovascular regulation and disease. *Purinergic Signal* 4(1): 1–20

REFERENCES

Anzinger J, Malmquist NA, Gould J, Buxton IL (2001) Secretion of a nucleoside diphosphate kinase (Nm23-H2) by cells from human breast, colon, pancreas and lung tumors. *Proc West Pharmacol Soc* 44: 61–63

Arora A, Scholard EM (2005) Role of tyrosine kinase inhibitors in cancer therapy. *J Pharmacol Exp Ther* 315(3): 971–979

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E (1999) Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Pharmacol Exp Ther* 289(3): 1034–1041

Blustajn JK, Trinkaus-Randall V, Weisman GA, Robson SC (2005) The importance of purinergic signaling in angiogenesis. *Cancer Res* 65(18): 7438–7442

Ewan LG, Jopling HM, Jia H, Mittar S, Bagherzadeh A, Howell GJ, Walker JH, Zachary IC, Ponnambalam S (2006) Intrinsic tyrosine kinase activity is required for vascular endothelial growth factor receptor 2 ubiquitination, sorting and degradation in endothelial cells. *Traffic* 7(9): 1207–1218

Goepfert C, Sundberg G, Sevigny J, Enjyoji K, Hoshi T, Csizmadia E, Robson S (2001) Disordered cellular migration and angiogenesis in cd39-/- null mice. *Circulation* 104(25): 3109–3115

Gounaris K, Thomas S, Najarro P, Selkirk ME (2001) Secreted variant of nucleoside diphosphate kinase from the intracellular parasitic nematode *Trichinella spiralis*. *Infect Immun* 69(6): 3658–3662

Holmqvist K, Cross MJ, Rolny C, Hagerkvist R, Rahimi N, Matsumoto T, Claesson-Welsh L, Welsh M (2004) The adaptor protein Shb binds to tyrosine 1175 in vascular endothelial growth factor (VEGF) receptor-2 and regulates VEGF-dependent cellular migration. *J Biol Chem* 279(21): 22267–22275

Jackson SW, Sun X, Enjyoji K, Hoshi T, Csizmadia E, Sundberg C, Robson SC (2007) Disordered purinergic signaling inhibits pathological angiogenesis in cd39-/- null mice. *Am J Pathol* 171(4): 1395–1404

Kaczmarek E, Erb L, Kozik K, Jaryza R, Wink MR, Guckelberger O, Blustajn JK, Trinkaus-Randall V, Weisman GA, Robson SC (2005) The importance of purinergic signaling in angiogenesis. *Cancer Res* 65(18): 7438–7442

**Acknowledgements**

This work was supported by NIH HD053028 and CA09563.

**Figure 3** Putative role of extracellular nucleoside diphosphate kinase (NDPK) and P2Y receptor (P2YR)/vascular endothelial growth factor receptor (VEGFR-2) activation in angiogenesis. We have observed breast cancer-secreted NDPK-B to be a significant contributor in promoting angiogenesis. Extracellular NDPK would modulate nucleotides such as elevating ATP levels (Rumjahn et al., 2007). Our current data supports a scenario where P2Y purinergic receptor activation above an unknown threshold would produce conditions favourable to pathological angiogenesis. Moreover, this P2Y angiogenic signaling would cooperate with VEGF angiogenic signal. This posits the notion of dual inhibition of VEGF signaling through sequestering extracellular VEGF levels (i.e., Bevacizumab) as well as blocking P2YR-dependent activation of VEGFR-2.
Modulation of endothelial cell migration by extracellular nucleotides: involvement of focal adhesion kinase and phosphatidylinositol 3-kinase-mediated pathways. *Thromb Haemost* 93(4): 735–742
Lee S, Chen TT, Barber CI, Jordan MC, Murdock J, Desai S, Ferrara N, Nagy A, Roos KP, Iruela-Arispe ML (2007) Autocrine VEGF signaling is required for vascular homeostasis. *Cell* 130(4): 691–703
Okabe-Kado J, Kasukabe T (2003) Physiological and pathological relevance of extracellular NM23/NDP kinases. *J Bioenerg Biomembr* 35(1): 89–93
Pollard JW (2004) Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* 4(1): 71–78
Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* 50: 413–492
Rumjahn SM, Javed MA, Wong N, Law WE, Buxton IL (2007) Purinergic regulation of angiogenesis by human breast carcinoma-secreted nucleoside diphosphate kinase. *Br J Cancer* 97(10): 1372–1380
Sakurai Y, Ohgimoto K, Kataoka Y, Yoshida N, Shibuya M (2005) Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. *Proc Natl Acad Sci USA* 102(4): 1076–1081
Satterwhite CM, Farrelly AM, Bradley ME (1999) Chemotactic, mitogenic, and angiogenic actions of UTP on vascular endothelial cells. *Am J Physiol* 276(3 Part 2): H1091–H1097
Seye CI, Yu N, Gonzalez FA, Erb L, Weisman GA (2004) The P2Y2 nucleotide receptor mediates vascular cell adhesion molecule-1 expression through interaction with VEGF receptor-2 (KDR/Flk-1). *J Biol Chem* 279(34): 35679–35686
Seye CI, Yu N, Jain R, Kong Q, Minor T, Newton J, Erb L, Gonzalez FA, Weisman GA (2003) The P2Y2 nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells. *J Biol Chem* 278(27): 24960–24965
Shibuya M (2006) Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *J Biochem Mol Biol* 39(5): 469–478
Strawn LM, McMahon G, App H, Schreck R, Kuchler WR, Longhi MP, Hui TH, Tang C, Levitzki A, Gazit A, Chen I, Keri G, Orfi L, Risau W, Flamme I, Ulrich A, Hirth KP, Shawver LK (1996) Flk-1 as a target for tumor growth inhibition. *Cancer Res* 56(15): 3540–3545
Tanaka N, Kawasaki K, Nejime N, Kubota Y, Nakamura K, Kunitomo M, Takahashi K, Hashimoto M, Shinozuka K (2004) P2Y receptor-mediated Ca(2+) signaling increases human vascular endothelial cell permeability. *J Pharmacol Sci* 95(2): 174–180
Tanaka N, Nejime N, Kagota S, Kubota Y, Yudo K, Nakamura K, Kunitomo M, Takahashi K, Hashimoto M, Shinozuka K (2006) ATP participates in the regulation of microvessel permeability. *J Pharm Pharmacol* 58(4): 481–487
White N, Burnstock G (2006) P2 receptors and cancer. *Trends Pharmacol Sci* 27(4): 211–217