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Angiopoietins differentially influence in vitro angiogenesis by endothelial cells of different origin

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Abstract:

Angiopoietins are important growth factors for vascular development and quiescence. They are promising targets for pro- or anti-angiogenic therapies in diverse pathologies, but the mechanisms of the ANGPT/TIE2 system are complex and not well understood. The function of the angiopoietins seems to depend on the specific tissue and on its environment. However, most in vitro experiments with angiopoietins have been performed with human umbilical vein endothelial cells only.

In the present study, the influence of the angiopoietins was studied with microvascular endothelial cells (EC's) originating from different vascular beds, i.e. dermal EC's and cardiac EC's, using a recently developed in vitro angiogenesis model that allows both a quantitative and qualitative evaluation of the angiogenic cascade. In addition, the expression of the angiopoietins and the receptors, TIE1 and TIE2 was analyzed with RT-qPCR.

This study revealed that the angiopoietins provoked a differential response in these distinct endothelial cultures. Both angiopoietin-1 as well as angiopoietin-2 elicited an angiogenic cascade in the dermal EC's but not in the cardiac EC's. In addition, the RT-qPCR data revealed marked differences in the endogenous expression pattern of these factors, indicating that the origin of endothelial cells might have an important impact on their angiogenic potential.

Keywords: Angiopoietin, TEK, real time RT-PCR, endothelial cells

Introduction:

Angiogenesis, the remodelling and expansion of a pre-existing vascular network is crucial for development. In the adult it is confined to the cyclic ovary, the mammary gland and to the placenta, but it is activated during wound healing or in diverse pathological situations including tumorigenesis, ischemic disease, chronic inflammation and other cardiovascular diseases [1, 10, 17]. As a consequence, both pro- and anti- angiogenic treatments are promising therapeutic strategies for a range of pathologies. However, the process of angiogenesis is highly complex, and a better knowledge of the molecular mechanisms of angiogenesis will aid the development of future therapies that aim to enhance or inhibit angiogenesis [9].

A range of growth factors have already been described to influence the process of angiogenesis. One of the most important and best known amongst them is the vascular endothelial growth factors (VEGF). VEGF induces capillary sprouting by stimulating endothelial migration and proliferation. Due to its importance in both development and in various pathological situations, VEGF has been extensively studied in the past decades [23]. The angiopoietins are another family of endothelial specific growth factors. Their role during angiogenesis is still elusive, they are expressed in a range of developing and adult tissues [11, 12, 16], and it is currently hypothesised that the angiopoietins play a major role during the maturation of the vasculature following VEGF induced angiogenesis [2, 25]. Angiopoietin 1 (ANGPT1) and angiopoietin 2 (ANGPT2) are the most common and best described members of the angiopoietin family. These ligands both bind the endothelial specific TIE2 receptor [27]. ANGPT1 is described as a vascular maturation factor; it induces lumen formation and vascular quiescence by activating the TIE2 receptor. ANGPT2 is an antagonist of ANGPT1, as it binds the TIE2 receptor with similar affinity as ANGPT1, but it has a weak ability to activate TIE2 [7, 22]. In this way, ANGPT2 induces vascular destabilisation, which can either lead to the onset of angiogenesis or endothelial regression, depending on the presence or absence of VEGF respectively. However, the antagonistic action of ANGPT2 seems to be context and tissue dependent [25]. Recent data indicates

that ANGPT-TIE2 activation is regulated by the TIE1 receptor [27]. This TIE1 receptor is another endothelial specific receptor that is structurally highly similar to TIE2, but does not interact directly with the angiopoietins [22].

Most of the current knowledge on how the angiopoietins influence angiogenesis has been deduced from complex in vivo models with loss of function or gain of function mutations. These models are often lethal in the embryonic stage of development and hardly provide the possibility to investigate the mechanism in real time [5]. In vitro models of angiogenesis might provide an alternative, as these experiments are more standardized and can be monitored in real time. Various in vitro models of angiogenesis have been described. However, in most of these models, only a single stage of the angiogenic cascade is investigated, e.g. proliferation, migration or tube formation. Recently, an all-in-one angiogenesis assay has been developed that allows both the qualitative and quantitative assessment of the entire angiogenic cascade [3, 4, 6]. Until now, the majority of in vitro angiogenic models have been developed with human umbilical venous endothelial cells (HUVEC), because these cells are relatively easy to isolate and grow [29]. However, since in vivo angiogenesis mostly proceeds in the microvasculature, it would be more appropriate to investigate in vitro models with microvascular endothelial cells [29].

In the present study, the influence of exogenously administered ANGPT1 and ANGPT2 was investigated in the all-in-one angiogenesis model applied to microvascular endothelial cells derived from the human heart and neonatal foreskin. In addition, RT-qPCR was performed to map the endogenous expression of the angiopoietins and their receptors TIE1 and TIE2 at different stages during the angiogenic cascade.

Materials and methods:

Human endothelial cells and media

Human microvascular endothelial cells either derived from the heart muscle of a 54 year old female (HCMEC), or from the neonatal foreskin (HDMEC) were purchased from Lonza Biosciences (Verviers, Belgium; original catalogue names: HMVEC-C Cardiac MV Endo Cells and HMVEC-dBINEo, respectively). A basal medium (BM) was constituted by using the growth EBM-2 medium (Lonza Bioscience, Verviers, Belgium), to which 2% foetal bovine serum was added. This medium composition retains cell survival, but does not result in an angiogenic response of the cells. The growth medium (GM) consisted of the EBM-2 medium, supplemented with several growth factors as provided with the EGM-MV Bullet kit (Lonza Biosciences). This kit included Vascular Endothelial Growth Factor A (VEGF-A), Fibroblast Growth Factor 2 (FGF-2), Epidermal Growth Factor (EGF), Insulin-like Growth Factor 1 (IGF-1), hydrocortisone and some less specified components such as bovine brain extract and foetal bovine serum. Previous studies revealed that this medium stimulates endothelial cells to run chronologically through all phases of the angiogenic cascade in vitro within 30 - 40 days (Table 1) [6].

The growth factors ANGPT1 (CYT-413; Biotrend Chemikalien GmbH, Köln, Germany) and/or ANGPT2 (CYT-414; Biotrend Chemikalien GmbH, Köln, Germany) were added to the media at a concentration of 100 ng/ml, as previously determined [28]. In a preliminary study no angiogenic or antiangiogenic effect could be observed of ANGPT1 or ANGPT2 when these were administered at a concentration of 50 ng/ml, whereas application of both growth factors at concentrations of 100 or 200 ng/ml led to angiogenic effects (unpublished data).

In vitro angiogenesis assay

Endothelial cells were seeded in gelatine-coated 24-well plates (Iwaki, Tokyo, Japan) at a concentration of $4.1\text{--}4.5 \times 10^4$ cells per well. The angiogenic effects of the different experimental

treatments were investigated by staging the angiogenic cascade in vitro, according to a previously established method [2, 3]. On the first day of investigation, 4 visual fields were randomly allocated and defined by coordinates. Phase contrast micrographs were obtained from these visual fields, using an Axiovert 25 inverted microscope (Zeiss, Jena, Germany) with an Inteq 000610 video camera (Inteq, Berlin, Germany) and the image editing system Axiovision, version 3.0 (Zeiss). Based on the morphology of the endothelial cells, these micrographs were assigned to the respective stage of angiogenesis in vitro, as previously described (Table 1) [6].

Quantification of angiogenesis in vitro, induced by ANGPT1 and ANGPT2 was carried out as follows: For each cell culture, six experimental groups were cultivated, i.e. three in GM and three in BM. In the first group, only ANGPT1 was added, in the second group, only ANGPT2 was added and in the third group, both factors were added in combination to the medium. In addition, cells were cultured in BM as negative controls and in GM as positive controls. The documentation of the visual fields was performed twice a week over 60 days (17 investigation days). For each visual field, the sum of assigned stages at the 17 investigation days (S) was calculated. The angiogenic effects of ANGPT1, ANGPT2 or their combination were determined by calculating the ratio of the arithmetic mean of the sums of the visual fields of each treatment ($\overline{S^{growth\ factor}}$) [3]. Analysis of variance was performed with the Sigmaplot software (Systat, Erkrath, Germany).

RT-qPCR

Total RNA was isolated with the Invisorb Spin Cell RNA Mini Kit (Invitex, Berlin, Germany) from cells, sampled in duplicate after 3, 10, 20 and 40 days in culture. RNA integrity was analyzed with the Experion™ Automated Gel Electrophoresis System (Bio-Rad, Nazareth, Belgium), using the Experion™ mRNA Std Sense chips (Bio-Rad) according to the manufacturers protocol. The Experion system uses an algorithm to analyze RNA-integrity on a scale from 1 to 10, with 1 being totally degraded RNA and 10 being totally intact RNA. The isolated RNA was reverse transcribed with the First Strand cDNA

Synthesis Kit (Fermentas, Leon-Rot, Germany) and qPCR was performed with the Rotor Gene 6000 (Corbett) using Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas). All samples were run in duplicate and each run included a serial 5-fold dilution of a pooled cDNA mix from all samples to calculate the standard curve. Gene specific primers (table 2 & 3) were either used as described by other researchers, or designed using RTprimerDB [21]. The technical details of these assays have been uploaded on RTprimerDB and are freely accesible [21].

Sample normalization was performed with the 4 most stable reference genes, i.e. GAPDH, UBC, TBP and HPRT, identified from a set of ten reference genes as described before [6]. Normalized relative quantities of mRNA coding for ANGPT1, ANGPT2, TIE2 and TIE1 (table 1) were calculated using the REST-software (Qiagen, Hilden, Germany) [13] and the qBase-software [15]. The REST software analyses if gene-expression is significantly different between two sample groups using bootstrap randomization [13]. The qBase software calculates normalized relative expression quantities which can then be further used for statistic analysis with Sigmaplot (Systat).

Results:

Angiogenesis assay

Endothelial cells, incubated in BM remained in stage 1 of angiogenesis in vitro. In contrast, incubation of the endothelial cells in GM, led to a run through all stages of angiogenesis in vitro. The response to the addition of angiopoietins was different between both cell types. Addition of the angiopoietins in HCMEC cultures led to a moderate angiogenic response (Fig. 1 and 2), in which the cultures did not go beyond stage 3 of angiogenesis in vitro, whereas addition of the angiopoietins in HDMEC cultures led to an angiogenic response (Fig. 3 and 4). After 20 days, the HDMEC's formed a network, but this network remained in a two dimensional state. Addition of GM led to higher cell densities in both HCMEC as well as in HDMEC cultures, but no additional effect on the angiogenic process was observed.

The sum of assigned stages are listed in table 4, for HDMEC's and for HCMEC's, cultured in GM was $\overline{S^{GM}} = 78 \pm 1.83$; $\overline{S^{GM}} = 68.50 \pm 2.83$, respectively. Addition of ANGPT1 in BM to HDMEC cultures, resulted in a $\overline{S^{ANGPT1}} = 59.75 \pm 0.50$, which was significantly lower than ANPT1 in GM $\overline{S^{ANGPT1}} = 63.25 \pm 1.71$ (P-value <0.05). Addition of ANGPT1 in HCMEC cultures lead to a significantly lower response in BM ($\overline{S^{ANGPT1}} = 31.25 \pm 1.71$) as compared to GM ($\overline{S^{ANGPT1}} = 34.00 \pm 0.00$, P-value < 0.05). Addition of ANGPT2 did not result in a significantly different response between BM and GM in either of the cell types, but it was significantly different between HDMEC's in GM ($\overline{S^{ANGPT2}} = 62.25 \pm 0.96$) and HCMEC's in both BM ($\overline{S^{ANGPT2}} = 29.00 \pm 10.30$; P-value < 0.05) as well as in GM ($\overline{S^{ANGPT2}} = 19.50 \pm 2.65$; P-value < 0.05). The addition of both ANGPT1 as well as ANGPT2 to the medium resulted in a significant difference between HDMEC's in GM ($\overline{S^{ANGPT1\&ANGPT2}} = 62.00 \pm 1.41$) and HCMEC's in GM ($\overline{S^{ANGPT1\&ANGPT2}} = 19.25 \pm 1.26$; P-value < 0.05), but not between the other treatments.

RT-qPCR

The RNA quality indicator (RQI) varied between 9.5 and 8.5 for all samples, indicating that RNA degradation during processing was minimal [14]. The comparison of the combined gene expression between the different cell types with the REST software (Qiagen) revealed significant differences in gene expression between these cell types. ANGPT2 mRNA was significantly down-regulated in HCMEC's compared to HDMEC's, by a mean factor of 0.019 (S.E. range is 0.004 – 0.098; P-value < 0.05) whereas ANGPT1 mRNA was significantly up-regulated in HCMEC's compared to HDMEC's, by a mean factor of 3595.302 (S.E. range is 161.197 - 63881.461; P-value < 0.05) (Fig. 5). No significant difference was found for TIE2 mRNA expression between the two cell types. In contrast, TIE1 mRNA was less abundant in HCMEC's as compared to HDMEC's, by a mean factor of 0.308 (S.E. range is 0.085 – 1.231; P-value < 0.05) (Fig. 5).

Gene expression was also time dependent in the both cell cultures. Expression of ANGPT2 mRNA in HDMEC's was positively correlated with length of cultivation (P-value = 0.004; $R^2 = 0.771$). Expression of mRNA for ANGPT1 was not detected in de HDMEC's cells, after 3 and 10 days of culture, but it became expressed after 20 and 40 days of culture (figure 6). TIE2 expression in HCMEC's was negatively correlated with increasing days in culture (P-value = 0.004; $R^2 = 0.767$), and TIE1 expression was stably expressed in both cultures between days 3 to 20, but after 40 days of culture, it was downregulated in both cultures (Fig. 7).

Discussion:

The angiopoietins, i.e. ANGPT1 and ANGPT2 are important angiogenic growth factors. They are crucial for the normal development of the vasculature, but are also implicated in diverse cardiovascular pathologies [33]. The complex interactions between ANGPT1 and ANGPT2, and their exact function are still elusive, and seem to depend on the tissue environment [2]. In the present study, the effect of the angiopoietins was tested in a recently developed in vitro angiogenesis assay, which has previously been established to allow the quantitative assessment of the whole angiogenic cascade, without the use of an external extracellular matrix [3, 6].

ANGPT2 is frequently viewed as an antagonist to the function of ANGPT1 through competitive binding on the TIE2 receptor [26]. However, its function seems to be context dependent, and in certain situations, ANGPT2 has been observed to induce angiogenesis through the activation of TIE2 [25]. In the present study, the pro-angiogenic effects of ANGPT2 were similar to those of ANGPT1 and to those of the combination of ANGPT1 and ANGPT2, suggesting that ANGPT2 does not act as an antagonist to ANGPT1 in this in vitro setting. Conflicting data exists about the capacity of ANGPT2 to induce TIE2 activation in vitro [22, 32]. The action of the angiopoietins is thought to depend on interactions between TIE2 and TIE1 [18, 19], and these complex interactions are since long a subject of debate [2, 33]. Recent evidence has revealed that homodimerization and activation of TIE2 is

inhibited through the formation of TIE1-TIE2 heterodimers, in which TIE1 might function as an inhibitory co-receptor [27]. ANGPT1 is able to break the association of the TIE2-TIE1 heterodimers, and to induce homodimerization of two TIE2's, resulting in receptor activation. In contrast, ANGPT2 is not able to break these heterodimers, and is unable to activate the TIE2 receptor when it is associated with TIE1 [27]. ANGPT2 is able to induce TIE2 activation in HUVEC's with experimentally downregulated TIE1 expression, suggesting that ANGPT2 is able to activate the TIE2 receptor and that it might have similar effect as ANGPT1 in the absence of TIE2-TIE1 heterodimers [19]. However, in endothelial progenitor cells, which both express TIE1 and TIE2, ANGPT2 is able to activate TIE2, because no heterodimers are formed in these cells [19]. In the present study, the expression of mRNA for both TIE2 and TIE1 was detected in HDMEC's and HCMEC's. The mRNA of TIE2 was expressed at similar levels in both cell types, whereas mRNA of TIE1 was more abundant in HDMEC's in comparison to the HCMEC's. This might suggest that a differential TIE1/TIE2 ratio in the cells might cause a different effect of Angiopoietins. In vivo, TIE1 is expressed at sites of active angiogenesis and at vascular branching points and bifurcations, whereas it is absent in stable vessels [20, 24]. This expression pattern indicates that the presence of TIE1 activates the vasculature by inhibiting TIE2 activation, whereas the absence of TIE1 might allow both ANGPT1 and ANGPT2 to activate TIE2 and to induce maturation and vascular quiescence [27]. In the present study, the higher expression pattern of TIE1 in the HDMEC's support this hypothesis, as these factors are angiogenically more active as compared to the HCMEC's that had a lowered expression of TIE1.

Although ANGPT1 is known as a vascular maturation factor, it has also been observed to be a pro-angiogenic compound in certain situations [8, 30]. In the present study, both the addition of ANGPT1, ANGPT2 and their combined addition stimulated an angiogenic cascade in the HDMEC cultures with basic medium. However, in the cultures with growth medium, it seemed to stabilize the ongoing angiogenesis as these cultures only attained stage four of the angiogenic cascade. These results indicate that the action of the angiopoietins might depend on the presence or absence of other

growth factors in the medium such as fibroblast growth factor, or endogenously expressed growth factors. In HCMEC's, both the addition of ANGPT1, ANGPT2 and the combination of both factors resulted in an anti-angiogenic response, suggesting that the action of the Angiopoietins might be dependent on the cell type.

The different angiogenic potential of these EC's of distinct origin may also be explained by a differential endogenous mRNA-expression pattern of the endothelial cells. ANGPT1 was highly expressed in the HCMEC, whereas this factor was not expressed in the early stages of angiogenesis of the HDMEC and became only expressed at low levels in the later stages of angiogenesis. In contrast, ANGPT2 was only expressed at basal levels in the HCMEC whilst being highly upregulated in the HDMEC's. This might indicate that the HDMEC are more prone to an angiogenic response due to a higher expression of endogenous ANGPT2, and a low expression of ANGPT1, resulting in an inhibition of TIE2 mediated stabilization of the endothelial cells. A previous study with the same cells has also revealed a significant difference in the expression pattern of mRNA for the VEGF-receptor VEGFR1 [6], indicating that endothelial cells of different origin might respond differently to a given stimulus, and that care should be taken when results of angiogenic tests on a specific vascular bed are extrapolated to another vascular bed.

In conclusion, the present study revealed that angiopoietins are able to provoke an angiogenic response in microvascular endothelial cell cultures. However, their effect might be highly dependent on the tissue environment and on the origin of the endothelial cells. The use of multiple endothelial cell lines with different origins should be supported, to aid the extrapolation of in vitro data to in vivo situations.

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327 **Tables:**

328 Table 1: stages of angiogenesis in vitro, and description of cell morphology within the different stages

Stage Nr.	Morphology of endothelial cells
Stage 1	<i>Confluent monolayer</i>
	Polygonal shaped cells
Stage 2	<i>Endothelial sprouting, late phase</i>
	> 50% elongated shaped cells
Stage 3	<i>Linear side by side arrangement, late phase</i>
	> 50% linearly arranged cells
Stage 4	<i>Networking</i>
	Network of linearly arranged cells
Stage 5	<i>Three-dimensional organisation, early phase</i>
	Appearance of capillary-like structures (linear structures of endothelial cells with a diameter of more than 28 µm; for these structures an internal lumen was shown by electron microscopy)
Stage 6	<i>Three-dimensional organisation, late phase</i>
	All linearly arranged cells form capillary-like structures; dissolution of the cell layer on the bottom

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331 Table 2: Primers, used for RT-qPCR

Gene		Primer sequence (5'-3')	amplicon length (bp)	Ta (°C)	Genbank accession nr or reference	RTprimerdB ID
ANGPT1	F	GGACAGCAGGAAAACAGAGC	129	60	NM_001146.3	8190
	R	CACAAGCATCAAACCAACCAT				
ANGPT2	F	ACTGTGTCCTCTCCACCAC	132	60	NM_001118888.1	8191
	R	GGATGTTAGGGTCTTGCTTT				
Tie2	F	GCTTGCTCCTTCTGGAAGTGT	112	60	[27]	2636
	R	CGCCACCCAGAGGCAAT				
Tie1	F	AGA ACCTAG CCT CCA AGATT	119	60	NM_005424	8212
	R	ACT GTA GTT CAG GGA CTC AA				

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334 Table 3: Full official gene names

official symbol	alias	name
ANGPT1	ANG1	Angiopoietin 1
ANGPT2	ANG2	Angiopoietin 2
Tie-1	TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TEK	TIE2	<i>TEK tyrosine kinase, endothelial</i>

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344 Table 4: sum of stages of the different treatments

Cell type	Visual field	$S^{\text{ANGPT1, BM}}$	$S^{\text{ANGPT2, BM}}$	$S^{\text{ANGPT1 \& ANGPT2, BM}}$	$S^{\text{ANPT1, GM}}$	$S^{\text{ANGPT2, GM}}$	$S^{\text{ANPT1 \& ANGPT2, GM}}$
HDMEC	1	60	59	55	61	62	60
	2	60	59	57	64	63	63
	3	59	59	60	63	61	63
	4	60	60	61	65	63	62
	mean	59.75 ± 0.50	59.25 ± 0.50	58.25 ± 2.75	63.25 ± 1.71	62.25 ± 0.96	62.00 ± 1.41
HCMEC	1	33	41	23	34	20	21
	2	32	34	29	34	23	18
	3	29	19	37	34	17	19
	4	31	22	19	34	18	19
	mean	31.25 ± 1.71	29.00 ± 10.30	27.00 ± 7.83	34.00 ± 0.00	19.50 ± 2.65	19.25 ± 1.26

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350 Figure 1: Micrographs of human cardiac microvascular cells (HCMEC) after 40 days in culture with
 351 either ANGPT1 in BM (A), ANGPT2 in BM (B), ANGPT1 & ANGPT2 in BM (C), ANGPT1 in GM (D),
 352 ANGPT2 in GM (E), ANGPT1 & ANGPT2 in GM (F). No clear network formation is evident in any of the
 353 treatments. HCMEC, grown in GM attain higher cell densities. Bars: 50µm.

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356 Figure 2: Stages of angiogenesis in vitro of HCMEC. Addition of ANGPT1, ANGPT2, or the combination
 357 of these factors did not lead to an angiogenic cascade.

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Figure 3: Micrographs of human dermal microvascular cells (HDMEC) after 40 days in culture with either ANGPT1 in BM (A), ANGPT2 in BM (B), ANGPT1 & ANGPT2 in BM (C), ANGPT1 in GM (D), ANGPT2 in GM (E), ANGPT1 & ANGPT2 in GM (F). Network formation is evident in all of the cell cultures. However, in the cultures with GM, this is less clear, because of an overall higher cell density in these cultures. Bars: 50µm.

Figure 4: Stages of angiogenesis in vitro of HDMEC. The addition of ANGPT1, ANGPT2 or the combination of these factors led to an angiogenic cascade that halted at stage four of angiogenesis in vitro.

Figure 5: Combined expression pattern of the angiopoietins and TIE2 in the HDMEC's and the HCMEC. Error bars: SEM.

Figure 6: Expression pattern of ANGPT1 and ANGPT2 at different culture days in the HDMEC's and in the HCMEC. Error bars: SEM.

Figure 7: Expression pattern of TIE2 at different culture days in the HDMEC's and in the HCMEC. Error bars: SEM.