Published in final edited form as:
Chin J Integr Med. 2012 July ; 18(7): 502–506. doi:10.1007/s11655-012-1143-6.

A Microarray Analysis of Angiogenesis Modulation Effect of Xuefu Zhuyu Decoction(血府逐瘀汤) on Endothelial Cell*

Jun SONG[1], **Wen-yuan CHEN**[2], **Li-ya WU**[2], **Liang-pu ZHENG**[3], **Wei LIN**[3], **Dong GAO**[2], **Ted J. Kaptchuk**[4], and **Ke-ji CHEN**[5]

[1]Experimental Research Center, China Academy of Chinese Medical Sciences, Beijing (100700), China

[2]Department of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou (350108), China

[3]Fujian Academy of Integrative Medicine, Fuzhou (350108), China

[4]Harvard Medical School, Boston, MA02215, USA

[5]Xiyuan Hospital, China Academy of Chinese medical Sciences, Beijing (100091), China

Abstract

Objective—To study the angiogenesis modulation mechanism of Xuefu Zhuyu Decoction(血府逐瘀汤) on endothelial cell ECV304.

Methods—ECV304 cells were treated by 2.5% Xuefu Zhuyu Decoction-containing serum (XFZYD-CS) for 24h, 48h and 72h respectively. MTT, FACS, migration, adhesion and in vitro tube formation assay confirmed an angiogenesis effect of XFZYD at 3 time points. Then an analysis of angiogenesis regulator profiles at 3 times with Real-time PCR Supperarray was performed.

Results—At 48h, XFZYD-CS induced ECV304 significantly improved cell vigor, number in S phase, migration, adhesion and tube formation. At 24h and 72h, only cell migration was elevated. Microarray results showed that 18,14 and 16 genes changed expressions at 24h, 48h and 72h respectively. 6 genes changed consistently and 7 genes varied between 48h and the other two times.

Conclusion—The changes of ECV304 induced by XFZYD-CS at 48h. The regulation mode at 48h had less genetic change but had greater cellular effect.

Keywords

Xuefu Zhuyu Decoction; angiogenesis; Microarray; Chinese herbs

Angiogenesis is important for the treatment and prognosis of ischemic diseases. Xuefu Zhuyu Decoction (Drive Out Stasis from the Mansion of Blood Decoction) was first recorded in 1830 in Wang Qing-ren's *Yi-lin Gaicuo* (医林改错). It is a classical formula for activating blood and removing stasis, composed of the following ingredients: Angelica sinensis (Oliv.) Diels 9 g, Rehmannia glutinosa Libosch. 9 g, Prunus persica 12 g, Carthamus tinctorius L. 9 g, Citrus aurantium L. 6 g, Paeonia lactiflora Pall. 6 g, Bupleurum

*Supported by the National Natural Science Foundation of China (NO. 81072933); Fujian Natural Science Foundation (No. 2007J0101); Fujian Academy of Integrative medicine Foundation (No. CKJ2008001), NIH-NCCAM, US (No. K24 AT004095).

Correspondence to: Prof. GAO Dong, Tel: 86-59122861151, gd1026@yahoo.com.cn.

chinese DC. 3 g, Glycyrrhiza uralensis Fisch. 6 g, Platycodon grandiflorum 4.5 g, Ligusticum Chuanxiong Hort. 4.5 g, and Cyathula officinalis Kuan 9 g,

Previous work by our team demonstrated that Xuefu Zhuyu Decoction (XFZYD) has angiogenesis effects that not only increase the vessel number in chicken embryo chorioallantoic membrane (CAM) model[1], but also mobilize marrow endothelial progenitor cell(EPC)[2,3], promoting EPC differentiation[4] and tube formation[5]. The mechanism of its pro-angiogenesis is unclear. In this study of angiogenesis modulation function and the regulation mechanism of XFZYD, we first examined relevant endothelial cell migration, proliferation, adhesion and tube formation with endothelial cell line ECV304 to demonstrate the angiogenesis effect of XFZYD. We then used microarray technique analyze gene expression profiles.

MATERIAL AND METHODS

Preparation of XFZYD-containing Serum

XFZYD-containing serum were made according to the protocol adopted in our previous study[3].

Incubation and Grouping of ECV304

Endothelial cell line ECV304 (China Center of Type Culture Collection, Wuhan University, China) was grown in M199 containing 5%FBS(v/v) at 37°C in a 5%CO_2 atmosphere. Once confluenced, cells were detached with trypsin-EDTA solution, synchronized by incubation for 24h in serum-free M199, then harvested and plated in 96-well plates (for proliferation assay) or 25cm^2 flask at a concentration of 2.5×10^3 cells/well or 2.5×10^5 cells/flask in 5%FBS. After 4h, the medium was discarded and the cells were exposed to 2.5%XFZYD-CS or control serum for 24h, 48h and 72h.

Cell vigor assay

The effect of XFZYD-CS in inducing ECV304 proliferation was estimated by methyl thiazolyl tetrazolium(MTT) assay. MTT(5mg/ml) was added to each well and incubated for 4h. After the MTT solution was discarded and replaced by 200μl DMSO, the plates were shaken for 10min. The optical density (OD) was assessed at 570nm (reference wave, 630nm) using a 96-well microplate reader(BioTek Co., USA).

Cell proliferation assay

Cell proliferation assay was tested by FACS as protocol described in the instruction book of Cycle TEST™ plus DNA REAGENT KIT. Cell Quest software was used to obtain data and ModiFit Version 3.0 was used for analysis.

Cell migration assay

Cell migration was evaluated by Boyden chamber assay. The upper chamber was covered by a 8μm polycarbonate membrane. All groups ECV304 (2×10^4 cells) were suspended in 100μl corresponding serum and added on the membrane. The lower chamber was loaded with 100μl corresponding cell culture supernatant. After incubatation at 37°C for 1h, the residual cells on the upper side of the membrane were removed with cotton swabs, and the membrane fixed with 4% neutral formalin for 10 min, and stained with hematoxylin. The stained cells from 6 high power (×400) fields (HPF) were counted. Photographs were taken by an inverted phase contrast microscope (IX70, Olympu Co., Japan).

Cell adhesion assay

96-well plates were coated with 1% gelatin for 1h. XFZYD-CS-treated or control serum-treated ECV304 (1×10^4 cells) were plated with 5%FBS for 30 min. The culture medium was subsequently removed and adherent cells from 6 random fields (×100) were counted.

In vitro tube formation assay

Matrigel assay was used to evaluate capillary tube formation activity as described in the protocol of In Vitro Angiogenesis Assay Kit (Millipore Co., USA). Briefly, ECMatrix™ solution was thawed on ice overnight, then mixed with diluent and placed in a 96-well plate at 37° for 2h to allow the matrix solution to solidify. Each group ECV304 was added on the polymerized matrigel at 10^4 cells per well. After incubation at 37° for 10h, capillary tubes were inspected at a magnification of 400× with an inverted phase contrast microscope. Capillary tubes were defined as endothelial cord formations that were connected at both ends. The number of tubes in six random fields per well was determined.

Real-time PCR microarray

Prepared RNA by Trizol, its yield and quality assessed by UV absorbance and denaturing agarose gel electrophoresis as common, synthetized first strand cDNA by Reverse Transcriptase (invitrogen Co., USA), diluted cDNA sample and loaded for the 96-Well Real-time PCR Arrays (SuperArray Co., USA). All assays were performed according to the manufacturer's protocols.

Data analysis and statistics

Data analysis of PCR array adopted the ΔΔCt Method. We calculate the ΔCt and ΔΔCt as the formula: ΔCt = average Ct − average of HK genes' Ct, ΔΔCt = ΔCt (XFZYD group) - ΔCt (control group), then calculated the fold-change for each gene as $2^{-\Delta\Delta Ct}$, Significance was set at fold difference≥2, negative meaned down-regulation. Except that, continuous variables were compared using the Student's t-test for two independent variables, or one-way ANOVA for comparisons of more than two means. Significance was set at $P < 0.05$.

RESULTS

Effect of XFZYD-CS on cell vigor

At 24 h, the MTT colorimetric assay revealed that compared to the control group, 2.5% XFZYD-CS treated ECV304 could elevate the mitochondria metabolism capacity to improve the cell vigor (Table1).

Effect of XFZYD-CS on cell proliferation

In time course experiments, only at 48h, did results of the Flow Cytometer show, XFZYD-CS treated ECV304 elevated cell proliferation significantly compared to the control group (Table1).

Effect of XFZYD-CS on cell migration

At 24h, 48h and 72h, XFZYD-CS treated ECV304 significantly improved the number of the cell migrated to the underlayer of the membrane as compared to the control group (Table1). This demonstrated that XFZYD influenced cell migration ability.

Effect of XFZYD-CS on cell adhesion

At 48h, the cell adhesion assay indicated that XFZYD-CS treatment significantly elevated the number of cells adherent on gelatin compared to the control group. This demonstrated that XFZYD elevated cell adhesion ability. (Table1).

Effect of XFZYD-CS on tube formation

In vitro angiogenesis assay showed that only at 48h did XFZY-CS dramatically increase the tube number compared to controls. (Table1).

The results of Real-time PCR microarray

Microarray analysis illustrated that among the 84 angiogenesis regulator gene chosen, all 27 genes had been effected significantly at 3 times, and 18,14,16 genes changed expression at 24h, 48h and 72h respectively (Table1-3). The 48h was the optimal angiogenesis time demonstrated by the above assay but this time affected the least number of genes. There were 6 identical genes changed at 3 times as TGFβ2 and VEGFC upregulate; CXCL6, CXCL10, CDH5 and EGF downregulate (Table2). 7 genes changed expression differentially between 48h and the other two times (Table3) as EFNA1, CXCL5 and THBS2 downregulated at 48h; EFNB2, TYMP, FGF2 and NRP1 upregulated at 24h or 72h.

DISCUSSION

By comprehensive screening every aspect of endothelial cell in the process of angiogenesis including cell vigor, reproduction, migration, adhesion and tube formation, this experiment demonstrated that, at 48h, XFZYD-CS treated ECV304 had significant effects in all of the above stages. The 48h was the optimal time point for angiogenesis while the 24h and 72h only effected cell migration. There was not only a optimal time but also optimal concentration in angiogenesis induced by XFZYD that made the time and dose-response curve resemble an inverted U[6]. This was quite different from a pure angiogenesis factor such as VEGF etc. Previous clinical and laboratory research showed that XFZYD could promote angiogenesis while also reducing atherogenesis [1,7]. On the other hand, VEGF also promotes angiogenesis in atherosclerotic plaque by expanding the plaque and accelerate atherogenesis[7]. The different angiogenesis effects we observed between XFZYD and VEGF is perplexing and intriguing. Considering the inverted U curve of XFZYD pro-angiogenesis effect, we speculate that XFZYD does not entirely stimulate angiogenesis but rather primarily maintains vessel growth in physiological or repair range to avoid angiogenesis in the atheromatous plaque. This vessel appropriate growth phenomenon could be regulated by different aspect of angiogenesis and our experiment may provide us with a clue to understand the regulation mode. More research is warranted to test our hypothesis and fully develop angiogenesis in therapy of ischemic diseases.

Among the changed genes at the 3 time points in the 84 angiogenesis regulators, the changes of Eph super family interested us most. EphA1 and EphB2 were both pro-angiogenesis, but their expression changed in differentially at 48h and the other two times as EphA1 down-regulation in 48h and EphB2 up-regulation in 24h or 72h. In recent years it has become known that Eph receptor and ephrin interactions also regulate critical steps of angiogenesis, blood vessel formation and remodeling during vascular development[8-10]. EphrinA1 was the first to be isolated from cultured human umbilical vein endothelial cells in the whole family[10-12]. It has a major impact on endothelial cell behavior and can modulate capillary sprout formation in the rat cornea assay and promote capillary-tube formation in vitro angiogenesis assays[10]. Besides EphrinA1 function in angiogenesis, EphrinB2 is also a key factor in both vascular development[13,14] and postnatal angiogenesis[15,16], In our experiment, that EphA1 and EphB2 changed bidirectionally at different time points and

caused different angiogenesis effects may reflect the key work of Eph family in angiogenesis induced by XFZYD. Again, amore definitive understanding will require further research.

From our perspective, our most intriguing finding was that 48h was the time point having the optimal angiogenesis effect but it impacted the least number of gene: 14 genes of 84 selected regulation genes. As we know, angiogenesis is a highly complex process which is regulated by numerous positive and negative factors. Common positive factors include vascular endothelial growth factor, transforming growth factor, epithelial growth factor and fibroblast growth factor[17] and common negative factors include angiostatin, endostatin, thrombospondin etc[18,19]. Balance between angiogenesis promoting factors and inhibitors allows healthy angiogenesis to occur only in embryonic development, wound healing and the female reproductive cycle. Any imbalance will lead to proangiogenesis or antiangiogenesis. Proangiogenesis is caused by the inadequate production of angiogenesis inhibitors and/or excessive amounts of angiogenesis growth factors. Our experimental results may reflect not only the imbalance but also the complicated modulation of proangiogenesis. XFZYD did not elevate all positive factors, for example it upregulated VEGFC and TGFβ2 but downregulated EGF, the same effect as the inhibitors, and the general trend in the end is proangiogenesis.

In conclusion, our study showed that the angiogenesis mechanism of this classical Chinese herbal formula was multilevel, multi-targeted and had multiplepathways from endothelial progenitor cell to endothelial cell, from vascular development to adult angiogenesis. These findings provide a scientific rationale for the basis of adopting XFZYD in the treatment of ischemic disease. Further basic science and clinical research is necessary to gain a precise and full understanding of appropriate angiogenesis modulation mode.

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Table 1

Effects of XFZYD on Ecv304 vigor, proliferation, migration, adhesion and tube formation capacity ($\bar{x}\pm s$, n=6)

Time	Group	Vigor (570 nm light absorbance)	Cell cycle (S phase %)	Migration (migratory Cells×400)	Adhesion (adherent cells×100)	Tube formation (tubes ×400)
24h	XFZYD	0.193±0.005	38.00±1.41	36.67±3.50*	35.00±4.90	20.50±3.73
	Control	0.196±0.026	38.67±2.16	25.50±6.28	29.67±2.94	23.17±2.99
48h	XFZYD	0.253±0.014*	45.67±2.25#	39.83±3.71#	36.50±3.94*	36.50±4.85#
	Control	0.208±0.030	33.17±2.23	28.67±3.33	28.17±3.37	21.33±4.41
72h	XFZYD	0.387±0.018	32.00±1.79	35.50±4.93#	27.33±5.16	20.00±3.74
	Control	0.392±0.011	29.83±3.66	23.50±4.51	30.00±5.10	20.17±3.92

Compared to control group,

*P<0.05;

#P<0.01

Table 2

Same changed angiogenesis regulator genes detected at 3 times [a]

Symbol	Description	Fold difference		
		24h	48h	72h
TGFβ2	Transforming growth factor, beta 2	3.74	2.19	2.61
VEGFC	Vascular endothelial growth factor C	2.59	3.01	2.37
EGF	Epidermal growth factor (beta-urogastrone)	-2.23	-2.01	-2.50
CDH5	Cadherin 5, type 2, VE-cadherin (vascular epithelium)	-2.89	-6.13	-2.18
CXCL10	Chemokine (C-X-C motif) ligand 10	-5.82	-5.93	-4.07
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	-2.11	-2.39	-2.11

[a] Results shown are representative of three experiments.

Table 3

Differentially Changed expression genes between 48h and the other two times [a]

Symbol	Description	Fold difference		
		24h	48h	72h
EFNA1	Ephrin-A1	-1.15	-2.03	-1.12
EFNB2	Ephrin-B2	2.95	1.98	2.36
CXCL5	Chemokine (C-X-C motif) ligand 5	-1.58	-3.63	-1.23
THBS2	Thrombospondin 2	-1.40	-7.60	-1.13
TYMP	Thymidine phosphorylase	-2.27	-1.28	-3.55
FGF2	Fibroblast growth factor 2 (basic)	3.18	1.49	2.24
NRP1	Neuropilin 1	2.41	1.72	2.29

[a] Results shown are representative of three experiments.

Table 4

The other Changed expression significantly genes at 48h [a]

Symbol	Description	Gene Name	Fold difference		
			24h	48h	72h
ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	HEIR-1	2.54	2.89	1.81
IFNA1	Interferon, alpha 1	IFL/IFN	1.52	2.93	2.10
CCL11	Chemokine (C-C motif) ligand 11	SCYA11	-2.03	-2.09	-1.58
COL4A3	Collagen, type IV, alpha 3 (Goodpasture antigen)	TUMSTATIN	1.01	-2.35	-5.18
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	CD61/GP3A	1.04	-3.52	-2.22

[a]Results shown are representative of three experiments.

Chin J Integr Med. Author manuscript; available in PMC 2013 July 07.