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Tumor necrosis factor mediated release of platelet-derived growth factor from bovine cerebral microvascular endothelial cells

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KEY WORDS tumor necrosis factor; platelet-derived growth factor; cultured cells; cell division; cerebral arteries; vascular endothelium; opyridazines; verapamil

AIM: To study the effect of tumor necrosis factor (TNF) on platelet-derived growth factor (PDGF) release from bovine cerebral microvascular endothelial cells (BCMEC), and the protection of drugs on proliferation of bovine cerebral microvascular smooth muscle cells (BCMSMC). **METHODS:** BCMEC and BCMSMC were cultured *in vitro*. Effects of TNF on PDGF release from BCMEC were investigated by crystal violet assay. **RESULTS:** TNF did not promote the proliferation of BCMSMC directly. But the postculture medium of BCMEC stimulated by TNF promoted the proliferation of BCMSMC in a concentration ($5-20$ $\mu g \cdot L^{-1}$)-dependent manner, and the mitogenic activity was neutralized by antibody to PDGF. The BCMSMC proliferation was increased 34 ± 4 % over control by TNF (20 $\mu g \cdot L^{-1}$). Imperatorin (Imp), iso-imperatorin (iso-Imp), and 6 - (α, α-phenylacetylpiperazinyl) phenyl-5-methyl-4, 5-dihydro-3 (2*H*)-pyridazinone (PMDP) $1-100$ $\mu mol \cdot L^{-1}$ did not affect the PDGF release from BCMEC stimulated by TNF, but inhibited the BCMSMC proliferation promoted by PDGF concentration-dependently. **CONCLUSION:** TNF promoted PDGF release from BCMEC. The proliferation of BCMSMC stimulated by TNF must be mediated by growth factor, such as PDGF. Imp, iso-Imp, PMDP inhibited proliferation of BCMSMC stimulated by PDGF.

Tumor necrosis factor (TNF), a polypeptide with high biological activity[1], affected proliferation of cultured bovine cerebral microvascular smooth muscle cells (BCMSMC)[2]. TNF promoted cultured endothelial cells (EC) production of platelet-derived growth factor (PDGF) that stimulated growth of bovine aortic smooth muscle cells (SMC)[3]. The present study was designed to examine the effect of TNF on mitogenic activity production of BCMEC. And the protection of drugs on the proliferation of BCMSMC mediated by PDGF was also studied.

MATERIALS AND METHODS

Reagents and drugs Recombinant human TNF was from Dainippon Pharmaceutical Co. PDGF (two isoforms AA and BB) and anti-PDGF were purchased from Promega. Imperatorin (Imp), iso-imperatorin (iso-Imp), and PMDP were synthesized (purified by crystallization) by Department of Medicinal Chemistry in our College.

BCMSMC culture[4] Early passages ($P_2 - P_5$) were grown to confluence on culture plates (10^5 cells per well in 24-well culture plate). Cells reached confluence by $24-48$ h, and were used within $3-5$ d of plating. Culture medium consisted of modified Eagle's medium (MEM) containing benzylpenicillin 50 $kU \cdot L^{-1}$, streptomycin 50 $mg \cdot L^{-1}$, and 20 % fetal calf serum (FCS). Cells were grown at 37 ℃ in a humidified atmosphere with flowing 5 % CO_2/95 % air.

Treatment of BCMEC with TNF Confluent BCMEC were washed twice with D-Hanks' solution, and treated for $2-48$ h with MEM-free serum containing various concentrations of TNF. The postculture medium was harvested and added immediately to semiconfluent BCMSMC (10^4 cells per well in 96-well culture plate) previously washed twice with D-Hanks' solution. BCMSMC proliferation was determined after a 24-h incubation.

Mitogenesis assay The proliferation of BCMSMC was measured as described before[5].

Treatment of cells with drugs Cells were preincubated with various concentrations ($1-100$ $\mu mol \cdot L^{-1}$) of Imp, iso-Imp, PMDP, and verapamil for 6 h, and then washed 3 times with D-Hanks' solution before treatment with stimulants and drugs.

Immunoinhibition studies Postculture medium collected from TNF-stimulated BCMEC was preincubated with anti-PDGF antibody for 2 h, then the medium was transferred to cultured BCMSMC for proliferation assay.

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Statistics method Statistical analyses were made using t tests. Data are expressed as $\bar{x} \pm s$, n was number of independent experiments.

RESULTS

Effect of standard PDGF (AA, BB) on BCMSMC proliferation Standard PDGF promoted BCMSMC proliferation in a dose-dependent manner (Tab 1).

Tab 1. Effect of standard PDGF on BCMSMC proliferation ($n=6$). $^cP<0.01$ vs control.

PDGF/ $\mu g \cdot L^{-1}$	Absorbance at 595 nm AA	BB
0	0.253 ± 0.011	0.253 ± 0.011
5	0.401 ± 0.022^c	0.414 ± 0.034^c
10	0.412 ± 0.013^c	0.441 ± 0.022^c
20	0.434 ± 0.033^c	0.461 ± 0.023^c

Direct effect of TNF on proliferation of BCMSMC The absorbance at 595 nm of the crystal violet fixed in the BCMSMC stimulated by TNF was 0.158 ± 0.009 ($n=6$), Control 0.157 ± 0.027 ($n=6$, $P>0.05$). TNF in high concentration (20 $\mu g \cdot L^{-1}$) did not affect the proliferation of BCMSMC cultured with MEM free FCS.

Mitogenic activity of postculture medium collected from BCMEC stimulated by TNF

The time course of BCMEC production of mitogenic activity stimulated by TNF 10 $\mu g \cdot L^{-1}$ showed a slight increase in mitogenic activity after 2 −6 h, and the peak appeared at 24 h (Tab 2).

Tab 2. Mitogenic activity in postculture medium from TNF (10 $\mu g \cdot L^{-1}$)-stimulated BCMEC ($n=6$). $^aP>0.05$, $^bP<0.05$ vs control.

Time/ h	Absorbance at 595 nm Control	TNF	% Increase of proliferation
2	0.302 ± 0.015	0.317 ± 0.023^a	4.9
4	0.301 ± 0.014	0.330 ± 0.022^b	9.7
6	0.297 ± 0.015	0.323 ± 0.032^a	9.0
12	0.450 ± 0.033	0.495 ± 0.019^b	10.0
24	0.267 ± 0.055	0.326 ± 0.021^b	21.9
48	0.642 ± 0.037	0.706 ± 0.044^b	9.1

Incubation of BCMSMC with postculture medium from BCMEC stimulated with TNF led to a concentration-dependent increase in proliferation of BCMSMC (Tab 3).

Tab 3. Effect of TNF on release of mitogenic activity by BCMEC ($n=6$). $^bP<0.05$ vs control.

TNF/ $\mu g \cdot L^{-1}$	Absorbance at 595 nm
0	0.267 ± 0.035
5	0.309 ± 0.012^b
10	0.326 ± 0.021^b
20	0.357 ± 0.047^b

Immunoinhibition study Anti-PDGF antibody inhibited significantly the mitogenic activity of postculture medium from BCMEC stimulated by TNF. The inhibitory rate was 24 ± 5 % ($n=4$).

Protection of drugs Incubation of BCMSMC with Imp, iso-Imp, PMDP, and verapamil in high concentration (100 $\mu mol \cdot L^{-1}$) did not affect the proliferation of BCMSMC directly, and did not inhibit the release of PDGF from BCMEC simulated by TNF, but inhibited the proliferation of BCMSMC promoted by postculture medium collected from BCMEC in a concentration-dependent manner. This kind of antagonism was also found in the proliferation of BCMSMC stimulated by standard PDGF (Tab 4).

DISCUSSION

Early experiments showed that TNF promoted the proliferation of SMC[2]. But the SMC culture medium contained various kinds of serum which were full of growth factors that stimulated the proliferation of SMC, such as PDGF. To avoid the interference of growth factors, we used MEM free serum during the experiment. Under this condition TNF did not promote the proliferation of BCMSMC directly, but the postculture medium from BCMEC stimulated by TNF promoted the proliferation of BCMSMC and this kind of effect in postculture medium could be neutralized by antibody to PDGF. Thus, PDGF are apparently released by BCMEC in response to TNF challenge. We concluded that the promotion of TNF on BCMSMC proliferation must be mediated by growth factors, such as PDGF.

Time-course studies suggested that the major

Tab 4. Antagonism of drugs on proliferation of BCMSMC stimulated by standard PDGF (20 μg·L^{-1}) and postculture medium of TNF (20 μg·L^{-1}) stimulated BCMEC (n = 6). $^{b}P < 0.05$ *vs* control^{x}; $^{e}P < 0.05$ *vs* control^{y}.

Drug/μmol·L^{-1}		Absorbance at 595 nm PDGF AA	PDGF BB	TNF
Control^{x}		0.467 ± 0.012	0.450 ± 0.018	0.333 ± 0.021
Imp	100	0.372 ± 0.018^{b}	0.372 ± 0.018^{b}	0.153 ± 0.033^{b}
	10	0.395 ± 0.023^{b}	0.382 ± 0.019^{b}	0.210 ± 0.014^{b}
	1	0.407 ± 0.034^{b}	0.407 ± 0.023^{b}	0.230 ± 0.018^{b}
iso-Imp	100	0.372 ± 0.033^{b}	0.355 ± 0.031^{b}	0.175 ± 0.017^{b}
	10	0.398 ± 0.029^{b}	0.396 ± 0.027^{b}	0.195 ± 0.019^{b}
	1	0.420 ± 0.013^{b}	0.422 ± 0.020^{b}	0.182 ± 0.022^{b}
Control^{y}				0.738 ± 0.029
PMDP	100	0.352 ± 0.027^{e}	0.344 ± 0.013^{e}	0.463 ± 0.020^{e}
	10	0.387 ± 0.015^{e}	0.390 ± 0.028^{e}	0.548 ± 0.026^{e}
	1	0.424 ± 0.021^{e}	0.428 ± 0.013^{e}	0.582 ± 0.043^{e}
Verapamil	100	0.286 ± 0.024^{e}	0.235 ± 0.010^{e}	0.428 ± 0.026^{e}
	10	0.435 ± 0.013^{e}	0.361 ± 0.023^{e}	0.482 ± 0.022^{e}

release of mitogenic activity occurred at 24 h with only a slight increase in activity at 2 - 6 h. It appeared that a lag period may be required to produce new PDGF so that the stimulatory effect of TNF can be fully expressed.

Imp, iso-Imp, and PMDP inhibited the proliferation of BCMSMC stimulated by TNF[2]. The drugs did not inhibit the release of PDGF from BCMEC challenged by TNF, but antagonised the promotion of PDGF on the BCMSMC proliferation. Similarly, calcium channel blocker verapamil inhibited the mitogenic activity of PDGF in a concentration-dependent manner. This implied that at least, the exist of Ca^{2+} in cell is very important in the action of PDGF.

PDGF is a chief factor that is chemotactic and mitogenic for vascular SMC[6]. And the proliferation of SMC is a central factor in the etiology of heart and cerebrovascualr diseases[7,8]. Our data suggested that through a series of cell-cell interactions, the release of main factors such as TNF, PDGF play an important role in vessel wall biology. The further study about TNF and PDGF in the pathogenesis of heart and cerebrovascular diseases is being done in our Lab.

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肿瘤坏死因子介导牛脑微血管内皮细胞血小板-衍化生长因子的释放

R977

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关键词 肿瘤坏死因子; 血小板-衍化生长因子; 培养的细胞; 细胞分裂; 脑动脉; 血管内皮; 哒嗪类; 维拉帕米

目的：研究 TNF 介导的 BCMEC 释放 PDGF 及药物对 BCMSMC 增殖的保护作用. 方法：体外培养 BCMEC 和 BCMSMC，用结晶紫染色法观察 TNF 引起的 BCMEC 释放 PDGF 继而促进 BCMSMC 增殖作用. 结果：TNF 不能促进无血清培养的 BCMSMC 增殖，但 TNF (5 - 20 μg·L^{-1}) 剂量依赖地促进 BCMEC 释放 PDGF. TNF (20 μg·L^{-1}) 促进 BCMSMC 增殖的百分率为 34±4 %. 药物 Imperatorin，iso-imperatorin 和 PMDP 不影响 TNF 引起 BCMEC 释放 PDGF，但能剂量依赖地 (1 - 100 μmol·L^{-1}) 抑制 PDGF 促 BCMSMC 增殖的作用. 结论：TNF 促进 BCMEC 释放 PDGF 继而引起 BCMSMC 的增殖. 药物能抑制 PDGF 引起的 BCMSMC 增殖.

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Inhibitory effects of nifedipine on DNA and protein synthesis in cultured cardiac nonmyocytes of neonatal rats[1]

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KEY WORDS cultured cells; nifedipine; angiotensin Ⅱ; heart hypertrophy; heart

AIM: To observe the inhibitory effects of nifedipine (Nif) on cardiac nonmyocytes growth and proliferation. **METHODS**: Using nonmyocytes in culture as a model, [^{3}H]thymidine and [^{3}H]leucine incorporation were measured. **RESULTS**: There was a significant decrease in cell number and in total cellular protein after 72-h exposure to Nif 1 μmol·L^{-1} in the presence of angiotensin Ⅱ (Ang Ⅱ). A 48-h exposure to 1, 10, 100, 1000 nmol·L^{-1} of Ang Ⅱ caused a 19 %, 35 %, 46 %, 48 % increase in protein synthesis and 27 %, 46 %, 56 %, 57 % increase in DNA synthesis. Nif 1, 3, and 10 μmol·L^{-1} were able to reduce the Ang Ⅱ 100 nmol·L^{-1}-induced increase of protein synthesis and DNA synthesis. **CONCLUSION**: Nif had a direct inhibitory action on the growth of nonmyocytes, which was related to the regression of cardical hypertrophy.

The cardiac nonmyocytes are important in the patheophysiology of left ventricular hypertrophy (LVH)[1]. The renin-angiotensin system represents a cascade of biochemical events leading to the generation of angiotensin Ⅱ (Ang Ⅱ). Local generation of autocrine or paracrine actions of Ang Ⅱ have been shown in the heart[2]. It has been proposed that Ang Ⅱ participates in the mechanisms of structural cellular changes by enhancing cell growth in LVH[3]. Nifedipine (Nif), an dihydropyridine calcium antagonist, showed a conspicuous LVH regression[4]. Potential explanation for the mechanism of action of Nif in this situation included the effect of blood pressure reduction and direct effects on the myocardium. In this experiment, we observed the effects of Nif on the growth of cardiac nonmyocytes by using cardiac nonmyocytes in culture as a model, which may partly explain the benefical effects of Nif in LVH.

MATERIALS AND METHODS

Tissue culture Monolayer cultured cardiac nonmyocytes of neonatal rats were prepared as previously described[5]. Briefly, fragments of ventricles of 2 - 4 d old neonatal rats were aseptically removed and tissue fragments were placed in Hanks' solution. The cells were isolated with 0.06 % trypsin at 37 ℃. Cultures of cardiac nonmyocytes were prepared passing twice the cells adherent to the culture dish during the preplating procedure. Until the second passage,

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