The Effects of TNF-α and bFGF on proliferation of the Smooth Muscle cells of Lymphatics

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Abstract **Objective** To study the regulatory effects of cytokines on smooth muscle (SM) cells of lymphatics in guinea pig´s mesentery. **Methods** For determination of the cytokine effects on lymphatic SM cells proliferation, the fifth passage of lymphatic SM was seeded in a 96-multiwell plate with a density of 2000 cell/well. One day later, cells were incubated with fresh medium containing cytokine or with medium alone, 6 well for each concentration were treated identically. The medium was renewed after two days. One and half days later each well was supplemented with 0.5μci^{3}H-TDR and incubated for another half day. Then the cells on glass fiber paper were counted in a liquid scintillation counter. **Results** Treatment with bFGF induced a dose-dependent stimulation of lymphatic SM proliferation as measured by ^{3}H-TDR incorporation, the minimal effective concentration was 5u/ml, and the highest stimulation of cell proliferation was observed at about 50u/ml. The stimulation come down when the concentration of bFGF was higher than 50u/ml. TNF-α has inhibition to cell´s proliferation of SM cells with higher effect as the increase of the concentration. **Conclusion** The results of the study indicates that bFGFcan promote the growth of lymphatic SM cells in a dose-dependent form, whereas, TNF-α can suppress the cells´ growth in a dose-dependent form.

Key Words lymphatics; smooth muscle cells; TNF-α; bFGF; guinea pig

In the past years, many experiments had been done for the effects of cytokines on the angiogenesis and heal, and many cytokines have been used in clinic recently. TNF-α is a cytokine secreted by macrophages and the name comes from the functions of anti-tumor and killing tumor cells. In vitro, they could restrain the proliferation of endotheliocytes of blood vessel [1] and smooth muscle cells and they also could restrain the proliferation of endotheliocytes of lymphatic[2] . bFGF come from macrophages/mononuclears, endotheliocytes, osteocyte, fibroblast et al. All the cells above could accelerate karyokinesis of many kinds of cells such as endotheliocytes of blood vessel, SM cells [3] , which could induce the growth of blood vessels in vivo. The TNF-α could also accelerate the proliferation of endotheliocytes of lymphatic that incubated in vitro[2]. In order to determine the effects of cytokines on lymphatic endotheliocytes and the change of the pathology about lymphatic vessels in tissue of inflammation or edema, isotopes had been utilized in the first study about the effects of TNF-α and bFGF on lymphatic SM cells´ proliferation.

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MATERIALS AND METHODS

Reagent

DMEM: GIBCO Company. Serum of newborn cattle (NCS): Biologic Preparation of Dalian. Trypsin (1:25): SIGMA Company. TNF-α (tumor necrosis factor): The Biomedicine Company of Bangding. bFGF (basic fibroblastgrowth fcator): The Biotechnology Company of BaiLuYuan in Beijing. ^{3}H-TDR (^{3}H-metmine): The atomic-energy Graduat institute of China. The liquid of scintillant, PPO 5g, POPO 0.2g, Add xylene up to 1000ml.

Methods

The fifth passage of lymphatic SM cells had been proofed that its´ survival probability was more than 98% and the purity was higher than 95%, then seed it in a 96 multiwell plate with a density of 2000 cells/well and 0.1ml/well. One day later, aspirated the foster liquid and incubated with fresh medium containing cytokine or with medium alone for contrast, renewed the medium after 2 days. After one and half days, each well was supplemented

with 0.5 μci3H-TDR and incubated for another half day, then syringed twice by PBS and digested it by trypsin, filled with the liquid of Hanks to stop the digest, transfer the cells on glass fiber paper of model 39 by cell harvester. Drying for half an hour at 80℃, cut and added it into 20ml scintillation bottle with 5ml scintillation liquid then counted it by a liquid scintillation counter.

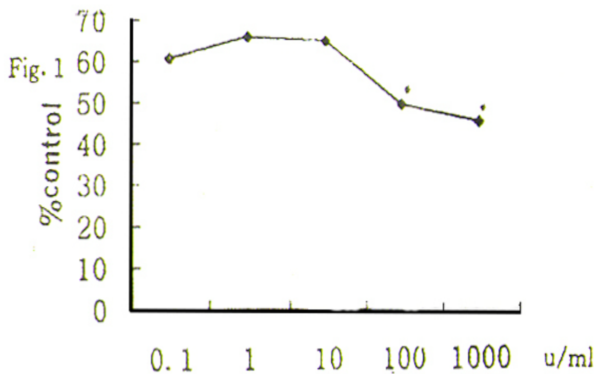
Each concentration of the bFGF is 0.1, 1, 10, 50, 100u/ml, and the TNF-α is 0.1, 1, 10, 100, 1000u/ml, every concentration has 6 wells which were treated identically. o n the other hand, 6 wells were made for contrast without cytokine . All the concentrations of NCS are 10%.

Statistic methods

All the measurement data of cpm was represented with $\bar{x} \pm s$, each group with cytokine and group of contrast undergone the Dunnett´s test.

Table.1 Effect of TNF-α on lymphatic SM cells´ DNA synthesis

Doses of TNF-α (u/ml)	n	cmp($\bar{x} \pm s$)	% control	P
. 0.1	6	559 ±38.8	60.4	
1	6	508 ±48.6	65.7	
10	6	595 ±46.4	64.3	
100	6	452 ±29.2	48.9	<0.05
1000	6	411.25±37.6	44.5	<0.05
control	6	925 ±32.1		



Fifth passage of Lymphatic SM cells, cell growth as determined by 3H-TDR incorporation four days after treatment with TNF-α. The concentration is 0.1, 1, 10, 100, 1000u/ml respectively. The changes are expressed as percent variation from control
* $p<0.05$

RESULTS

The effects of TNF-α on proliferation of the SM cells

Different concentration of TNF-α´s cpm was lower than contrast group, and with the ascent of the concentration, the data of the cpm was decreased obviously. (Table. 1 and fig. 1)

The effects of bFGF on proliferation of the SM cells

With the ascent of the concentration, the bFGF ´s cpm rose at first and reached the highest data at about 50u/ml, it was four times than contrast group, over 50u/ml, the data of cpm decreased rapidly. And all the cells came to death at the concentration of 1000u/ml in a test. (Table. 2)

Table.2 Effect of bFGF on Lymphatic SM cells´ DNA synthesis

Doses of bFGF (u/ml)	n	cmp($\bar{x} \pm s$)	% control	P
1	6	1501.50±78.3	126.6	
5	6	3267.80±81.1	275.5	<0.01
10	6	3512 ±53.8	296.1	<0.01
50	6	4692.30±47.7	395.6	<0.01
100	6	1762.25±28.8	148.6	
control	6	1186.30±59.1		

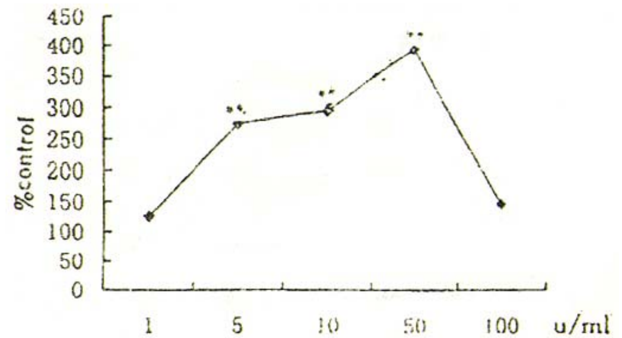


Fig.2 Fifth passage of Lymphatic SM cells, cell growth as determined by ^{3}H-TDR incorporation four days after treatment with bFGF. the concentration was 0.1,1,10,50,100u/mll respectively. The changes are expressed as percent variation from control. *$p<0.05$

DISCUSSION

In the experiment, the TNF-α has a dose-dependent inhibition to lymphatic SM cells´ proliferation. TNF-α was mainly secreted by kinds of

macrophages/mononuclears[4], it bring many physiological reactions on endotheliocytes of blood vessel such as accelerating hemopexis, expressing some surface antigens, inducing the change of morphologic, stimulate adhering of leukocytes and lymphocyte. In vitro, TNF-α could inhibit the proliferation of cattle´s endotheliocytes in mesentery lymphatic at low concentration with dose-dependent, and similar to endotheliocytes of blood vessel[5-7]. In spite of that, in vivo, TNF-α is an indirect activator on angiogenesis[8]. In inflammation or edema tissue the macrophages were stimulated to secrete TNF-α [9] which could lead leukocytes adhering to the endotheliocytes of blood vessel, first is the adhesion of neutrophils, then mononuclears and lymphocytes, in the end, the leukocytes assemble at inflammatory tissue. The mononuclears secreted EGF[10] FGF[11] and so on, these cytokines could stimulate the proliferation of endotheliocytes at inflammatory tissue, especially the SM cells in lymphatic, so the SM cells could be seen under endotheliocytes[12]. TNF-α could accelerate the differentiation of endotheliocytes and connecting with each other to form a intact blood vessel[13], so in chronic lymphedema, we will find that the number of blood vessels and lymphatics increase obviously[14]. Accelerating the proliferation of endotheliocytes and SM cells is the indirect function of TNF-α, and its direct function is accelerating endotheliocytes differentiation, and weather it could accelerate SM differentiation (such as the growth of muscle fibril) is unknown. The pathological mechanism of lymphatic´s regeneration which is effected by these cytokines is unknown now.

The functions of bFGF on SM cells of lymphatic are dualism, accelerating the proliferation within the limits and inhibiting proliferation out of the limits. In many pathologic models of animals, FGF could induce capillary vessels regeneration. On the endotheliocytes, which were cultured in vitro, FGF could stimulate their proliferation besides keeping them in differentiation. In vitro, bFGF might make endotheliocytes form tube, constitute capillary vessels and stimulate the proliferation of SM cells and pericytes, the relation of these cells and the growth of regenerated blood vessels is tightly. bFGF might accelerate proliferation of lymphatic endotheliocytes and SM cells in vitro, and the further study in vivo has not been done recently.

Proliferation was determined by mitosis, cytokines could control mitosis, which is related with the periods of G0 and G1. In the period of G1, there could be a cell division´ restriction point. The cells of G1 within the restriction point is in a state of inactivity, and will enter the state of proliferation once get out of the restriction point. Weather the cells could get out of restriction point was affected by a series of specific or non-specific environment signals, and the most important factor is cytokines. Many cytokines including PDGF, FGF, EGF, trypsin, and so on, could induce the cells in the period of G0 or G1 to get out of the restriction point and enter cell division and proliferation. These functions often divided into two kinds: the first is called competence factor, such as PDGF and FGF; they can induce effector cells in G0 or G1 come into competence before cell division; the second is called promote progression factor such as EGF, trypsin, they can induce competence cells get into the period of S and synthesize DNA. After these, cells division and proliferation do not depend on growth factor, they pass through the periods of S, G2, M and get into G1. How does growth factor induce cells get out of restriction point? In the study, PDGF, FGF and so on, may bind to cell membrane´s receptor and activate tyrosine kinase of receptor in cytoplast, to phosphorylate tyrosine in some important molecule, then activate PI, enhance pH in cytoplast, increase Ca^{2+}, oncogene such as myc,fos,ras and so on express temporarily and the most important thing is to accelerate some proteins synthesis rapidly, these proteins could trigger the synthesis of DNA. On the base of the competence factors, EGF, TGF may further enhance the effect, and increase concentration of protein in restriction point to a threshold, so 2~3 hours later, the cells get out of restriction point and come into the period of S.

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