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Anti-Plasminogen Monoclonal Antibody (MC2B8) Inhibites Angiogenesis

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Abstract: Angiogenesis is a complex process during which of new blood vessels are produced from the pre-existing blood vessels. Formation and growth of new vessels play an important role in the physiologic process (embryonic growth, tissue repair) and pathologic process (tumor growth, inflammation) for surviving of the tissues. In fact, the development of tumors is depended upon new vessel formation through which the tumor is provided with nutrient and oxygen. In this research, the role of plasminogen conformation with MC2B8 mAb (an antibody directed against C-terminal part of plasminogen) in clot lysis and angiogenesis is observed. In experimental model of angiogenesis, beads, covered with endothelial cells of bone marrow capillaries, are the source of endothelial cells. It coated in three-dimensional structure to be provided through fibrin gel. Different titers of monoclonal antibody (30-480 μg mL^{-1}) MC2B8 were added in fibrin gel. 3-5 days after culturing of endothelial cells, growth and migration was seen as the result of capillary formation MC2B8 mAb delayed clot lysis and inhibited angiogenesis at the concentration of 240 μg mL^{-1}. Present findings suggest that these effects on capillary tube formation and clot lysis caused blockage or conformational changes in plasminogen epitopes involved in angiogenesis and fibrinolysis.

Key words: Plasminogen, fibrinolysis, angiogenesis

INTRODUCTION

Angiogenesis is a complex process through which blood vessels are produced from pre-existing blood vessels. New vessels formation and growth play an important role in physiologic (emberyo growth, tissue healing and etc.) and pathologic (diabetic arthritis retinopathia, tumor growth and metastasis) processes, for surviving of the tissues. In fact, the development of tumor is depends upon new vessel formation through which the tumor is provided with nutrient and oxygen (Folkman, 1992; Yan Copolulos, 1998; Folkman, 19762.

The angiogenesis process includes endothelial cells migration, cell division and maturation which leads to vessel formation. Enzyme production involved in extracellular matrix modifying, is a key process enabling endothelial cells to bud from extracellular matrix (Taipale, 1997; Voldasky, 1998). The vessels development is regulated by several factors involved in coagulation control and capillary formation. Endothelial cells participation is regulated by extracellular matrix and requires integrine mediated cell-matrix complex formation and it's subsequent dissociation. Separation, the repeated cycle of reversible matrix-integrine bounding and dissociation of cytoskeleton components accompanied by matrix degradation occur as a results of moving cells margin progression.

Therefor, migration of endothelial cells is caused due to the cooperation between the molecular active complexes such as integrine ligands interaction with methaloproteinases and plasminogen activators as well temporary cell-matrix inhibitors. Endothelial cells, infact, release angiogenic stimulators, including plasminogen activator, resulting in plasmina formation through bonding to plasminogen. The catalytic active site of plasminogen is in c-terminal part of the molecule and serin protease part is activated by Arg561-val562 bound cleavage. As a results, plasminogen molecule is converts into plasmin. Plasmin activates promethaloproteases which not only facilitate cell migration by non-collagenous matrix components degradation, but also, release peptides which can be either stimulator or inhibitor

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of angiogenesis (Folkman, 1992; Collen, 1999). Any scientists, have so far tried to produce antibodies against plasminogen. Their significant studies have shed light on our knowledge about structure and mechanism of plasminogen activation, physiologic condition and fibrinolysis.

Considering, the diagnostic site, monoclonal antibodies against plasminogen can be divided into 4 categories:

- Antibodies against plasminogen PAP domain (MPW1 PG., LMP1, A1D12).
- Antibodies against one or more plasminogen kringle (10-F1, TPM1, F1P6, F3P2, MA-HAL, ...)
- Antibodies against c-terminal plasminogen domain (V-ICI, MC2 B8).
- Antibody against a complexity of domains (aPg-28, apg-96).

Functionally, these antibodies can also be divided into three major categories: One of them increases the rate of plasminogen activation in the presence of all plasminogen activators. These antibodies include: MPW2PG, MPW1PG, F12P18, F11P6, F111P5, F3P2 and A1D12. Second category of antibodies (e.g., Apg-96) inhibit plasminogen activation in the presence of these activators. Finally the third group are those antibodies which in the presence of some plasminogen activators have activatory or inhibitory effect and in the presence of other plasminogen activators show no effect or adverse effect. The researches carried out on the synthetic of plasminogen activation had shown a direct relation between rate of activation of this molecule and it's conformational status (Hatty *et al*., 1987; Ehrenreich *et al*., 1998; Sim *et al*., 1986). The α form of this molecule has less tendency for activation due to it's closed conformation, whereas the β form has a semiopen structure and is activated more rapidly. Semiopen (β) or open (γ) structural forms of plasminogen molecule have higher affinity for fibrinogen (fibrin) rather than it's closed structural form (α form). This is due to being more accessible of lysine hinding site (LBS) of plasminogen for binding to fibrinogen lysin reminants (Hatty *et al*., 1987; Ehrenreich *et al*., 1998; Sim *et al*., 1986; William *et al*., 1991; Slominskii, 1999; Makohoneko *et al*., 2000). Use of laboratory angiogenesis model allows us to study the rule of monoclonal antibody against plasminogen c-terminal part in angiogenesis.

MATERIALS AND METHODS

Drugs: Human fibrinogen, human thrombin cell culture medium MCDB131, Endothelial Growth Supplemented Factor (EGSF), cytodex-3 micrcarrier beads was collected from Sigma, USA. Aprotenin monoclonal was collected from Byer. Antibody MC2 B8 and Bone marrow endothelial cell was a gift from Dr. M. Mirshahi, Biochemistry Department, Tarbiat Modarres University.

Preparation of cytodex-3-microcarrier beads: We added cytodex-3-microcarrier beads in phosphate buffer for eight to ten h to be swelled, then, the swelled beads were autoclaved for 15 min at 15-20 p/in z pressure, then the understerile hood phosphate buffer was discarded and MCDB131 medium containing 5% EGSF was added.

Human bone marrow endothelial cell: The culture cells containing cryo were taken out from nitrogen tank and after melting of cryo at 37°C a complete culture medium was added instantly and the mixture was centrifuged for 5 min at 1000 rpm. Then, the supernatant was discarded and some complete culture medium were added to percipitate and centrifuged for 5 min at 1000 rpm. After centrifugation, the supernatant was discarded and cell perapitate was suspended in some MCD 131 medium and 5% EGSF. After cell count and determining cell viability percentage, the suspension was transferred into appropriate cell culture flask and then the culture medium was added to it. The culture containing container then was transferred to 37°C incubator with 5% CO_2 and the cells were observed for 3 to 4 days.

Designing and evaluating angiogenesis *in vitro*: HBMEC was cultured in 50 mL cell culture-flask and when the cell number reached up to more than one million, we exposed it to 0.5% Trypsin-EDTA to make a suspension. Then, cytodex-3-micro carrier beads was added to accumulate cells as a clone. For this purpose, we added the cells to the culture tube with a 30:1 ratio (30 cells to 1 beads) and incubated them at 37°C and 5% CO_2 for 8 h. Since the particles were covered with collagen, the cells bound to beads surface after 8 h. After checking the cell adhesion to the beads by microscope, the beads were mixed with fibrinogen solution (2.5 mg dL^{-1}), then thrombin was added (2 μ mL^{-1}) and distributed in the
24-well cell culture macroplate. After fibrinogen clotting and fibrin gel formation, complete culture medium (EGSF 5%, MCDB131) was added to each macroplate well in order to the inhibition of excessive fibrinolysis activity of the cells. 200 μ mL^{-1} aprotenin was then added to the fibrin gel. The model reproducibility was observed after 3 to 5 days of incubation of the macroplates at 37°C and 5% CO_2. In the next stage, IgG class MC2B8 antibody against plasminogen c-terminal part was added to the fibrinogen solution in dilutions of 30-48 μg mL^{-1} and it's effect on angiogenesis was studied in comparison with control sample after 72 h.

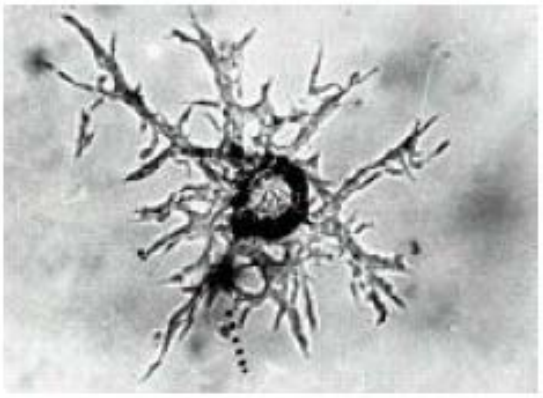

Fig. 1: Angiogenesis in control plate after 5 days, vessel spourtes aris from all carrier beads surface are seen

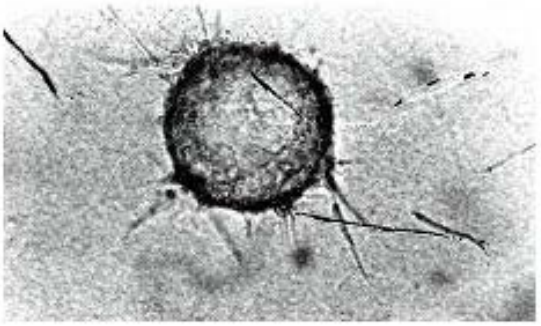

Fig. 2: MC2B8 Ab containing plate at 240 μg mL^{-1}. After 3-7 days angiogenesis was not seen and angiogenesis was inhibited at this concentration

RESULTS

Evaluation the angiogenesis of endothelial cell growth on cytodex-3-microcarrier beads: Following the designing of model, the results showed that angiogenesis is obviously visible from fifth day which was used as control in the present study (Fig. 1).

Evaluation the endothelial cell growth on cytodex-3-microcarrier beads: To evaluate the effect of MC2B8 monoclonal antibody on the angiogenesis, several doses of MC2B8 was added and the results in Fig. 2 showed that MC2B8 Ab inhibited angiogenesis process at concentration of 240 μg mL^{-1}.

DISCUSSION

Plasminogen is a single-chain glycoprotein with 791 amino acids and MW of 92 KD which has structurally three major parts. The first part of molecule with a amino acid sequence of 1 to 7 contains N-terminal domain called as preactivation peptid (PAP). Next part of the molecule includes five circular and identical structures (kringles), each containing 80 amino acids. The interaction of plasminogen with fibrin (which has many lysine residues) occurs via lysine binding sites in kringle structures. The c-terminal part of the molecule contains serine protease domain which is plasminogen catalytic site and respons for clot lysis. After the plasminogen binding to fibrin surface (via LBS sites) and it's conversion into plasmin, the serine protease domain of molecule is activated and fibrin lysis is occurred via it's catalytic site. After proteolytic cleavage of the plasminogen molecule by different proteases, plasminogen converts into active two-chain plasmin molecule with fibrinolytic activity (Ponting *et al*., 1992). Lysine binding site or LBS is the most important functional part in kringles. Plasminogen molecule can potentially bind to various molecules such as fibrin, fibronectin, collagen, Histidin rich glycoprotein, E-Amino caproic acid, thrombospondin, cell receptors and plasmin inhibitor or α 2-antitripsin (Malinoswki *et al*., 1984; Lijnen *et al*., 1980; Wiman *et al*., 1979).

Plasminogen concentration in adults is 200 mg dL^{-1} and in new born it is half of adults, while in children it is more than newborns and less than adults. It's concentration increases in third gestational period and plasma level of newborn plasminogen rapidly increases and reaches to adult's plasminogen level by 6 monthes. Plasminogen is mainly produced in liver, however, other cells including eosinophils and renal cells can also synthesis it (Benavent *et al*., 1984).

The primary stages of angiogenesis are the result of cooperation among active molecular complexes such as intergin complexes and metalloproteinases, plasminogen activators and their substrates and etc. In fact, plasminogen activation occurres via plasminogen and urokinase binding to receptor following the releasing of angiogenesis stimulators from tumoral cells (in pathologic condition) or endothelial cells (in physiologic condition) and results in plasmin formations which in turn activates metalloproteinases. These activated molecules not only facilitate cell migration via matrix degradation but, also, release peptides which can be either stimulator or inhibitor of angiogenesis. It is obvious, that proteolytic systems such as plasminogen, plasminogen activators and matrix metalloproteinases have play an important role in the invasion of tumoral cells and degradation of vessels construction during angiogenesis (Klagsbrun, 1991). Since tumor mass growth depends on angiogenesis and the possibility of tumor mass suppression and degradation by antiangiogenic agents, therefore, study of angiogenesis inhibitors have great importance. Monoclonal antibodies such as monoclonal antibody against Vascular Endothelial Growth Factor (VEGF) have been proposed as antiangiogenic drugs, among them is

Vitaxin. This drug is a monoclonal antibody against integrine α vβ 3 of endothelial cells which can decrease TGFα expression and increase apoptosis in these cells (Giavazzi *et al*., 2000).

In this study, we found that at *in vitro* condition, monoclonal antibody MC2B8 (directed against plasminogen c-terminal site) can act as dose-related inhibitor of angiogenesis process. Therefore, according to this finding we can hypotheses that either the inhibitory effect MC2B8 antibody can be the result of the blockage of some plasminogen epitopes involved in angiogenesis or conformational modification of plasminogen induced by MC2B8 may expose new epitopes of the molecule (for example, angiostatin epitopes containing kringle 1 to 4 of plasminogen) having antiangiogenic property.

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