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A Review on Angiogenesis Assays

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ABSTRACT
The angiogenesis activity was involved in many physiological and pathological conditions. The research on angiogenesis activity was increased and many new methods are come for the evaluation of the angiogenesis activity. Previously Robert Auerbach *et al*., described the methods used for the evaluation of angiogenesis activity. We added some more techniques to that which are currently used for the evaluation of angiogenesis activity. Some of ischemic models are also used for the evaluation of angiogenesis like hindlimb ischemia model, coronary artery ligation model and Langendorff isolated model. Some of the new methods are introduced for the evaluation of inflammatory angiogenesis i.e. sponge granuloma angiogenesis assay. The CAM assay, corneal angiogenesis assay and matrigel plug assays are used for the evaluation of both angiogenic and anti-angiogenic agents. We also described some of the quantification techniques which are easily performed in laboratory like capillary density and estimation of hemoglobin in Matrigels.

Keywords: Angiogenesis, Angiogenesis assays, Hindlimb ischemia model, LAD model, CAM assay.

INTRODUCTION

Over the last few years there has been a logarithmic increase in the number of reports dealing with angiogenesis. At the year of 2003 there was 14,851 references indexed for angiogenesis. [1] In the year of 2009 it became 2,910,000 references indexed in angiogenesis study. So number of new methods was introduced and quantification methods also increased. This is the important background to make a review on angiogenesis assays.

Neovascularization is the growth of the vascular system which plays a major role in both health and diseases. In physiological it plays a role in embryogenesis and development of the female reproductive system and wound healing.

Furthermore it contributes to the pathogenesis of many disorders either by excessive vessel growth for example in cancer, diabetic retinopathy, psoriasis and arthritis (or) by insufficient vessel growth for example in ischemic diseases of heart, limb (or) brain, neurodegeneration, pre-eclampsia and osteoporosis. [2] Thus every organ system may involve diseases in which angiogenesis is an important component. Three processes are responsible for the formation of new blood vessels. Those are

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a. **Angiogenesis** involve the sprouting of new capillary like structures from existing vasculature and is regulated by pro and anti-angiogenic factors. [3]
b. **Vasculogenesis** was originally defined by Risau as the formation of a capillary plexus from blood islands and is presently commonly used for the intussusceptions of bone marrow derived progenitor cells into the expanding vasculature.
c. **Arteriogenesis** was defined by Wolfgang Schaper as the development of adult collateral arteries from a pre-existing arteriole network via arteriogenesis a natural bypass is developed around an occluded main artery. [4]

MECHANISM OF ANGIOGENESIS

Angiogenesis is a complex process involving extensive interplay between cell soluble factors and extracellular matrix (ECM) components. The angiogenesis involve different sequential steps including: The release of proteases from activated endothelial cells, degradation of the basement membrane surrounding the existing vessel, migration of the endothelial cells into the interstitial space, endothelial cell proliferation, lumen formation, generation of new basement membrane with the recruitment of pericytes, fusion of the newly formed vessels, Initiation of blood flow. [5]

Angiogenesis activity can be evaluated by using *in-vivo, in-vitro, in-ovo* and *organ culture assay systems*.

IN-VIVO ASSAYS

Hind Limb Ischemia Model

This method is mostly used for the evaluation of angiogenesis substances. The mechanism involved in this model is haemodynamic changes leads to the formation of new blood vessels i.e. while large vessels with low flow tend to augmentation of blood flow which leads to the stimulation of vascular sprouting and maintain the potency of the newly formed collateral vessels thereby providing blood flow to the ischemic tissue. [6]

So far the animal model of persistent ischemia has been tried in a cat, canine, rabbit and rat by ligating the vessels. The rabbit is mostly used animal model for this study. The reasons that most studies choose the rabbit for the experimental animals are adequate cost, good management, easy maintenance and less complete formation of collaterals than the dog. [7] In this method the animals are anaesthetized and insion is making in the skin overlying the middle portion of the hindlimb. Then the proximal end of the femoral artery is ligating and distal portion of saphenous artery is ligating and artery and their side branches were dissected free. The femoral artery and attached side branched are excised and overlying skin is then closed. [8]

Left Coronary Artery Ligation Model

This model is mostly used for the myocardial ischemic studies and substance which have myocardial angiogenesis activity. The mechanism involved in this model is shear stress and stretch which leads to myocardium to up-regulate the adhesion molecules in the endothelium attraction of inflammatory cells and stimulation of endothelial cells to produce growth factors which causes angiogenesis. [6]

The rabbit is the mostly used animal model for this study. In this model animals are anaesthetized. Under sterilization and artificial respiration the left thoracotomy in the 5th intercoastal space was done and heart is exposed. Then the left anterior descending coronary artery distal to its diagonal branch is ligating with a suture which produces myocardial ischemia, after haemodynamic stability the pericardium and chest is closed. [9]

Matrigel Plug Assay

This model is used for the evaluation of both angiogenic and anti-angiogenic agents. The mechanism involved in this model is injection of foreign substances in to the animal leads to the stimulation of the inflammatory cells including macrophages and neutrophils leads to the stimulation of angiogenesis. [6]

The mostly used animal model is mice. Matrigel is a gelatinous material derived from mouse tumor cells that is commonly used *in-vitro* and *in-vivo* as a substrate for cells**.** When pro-angiogenic and anti-angiogenic agents are also added to the matrigel and it is injected into the subcutaneous space of an animal, which forms the single solid gel plug will stimulate the new blood vessels invade the matrigel. This is the basis of an assay referred to as the "matrigel plug" assay.

The matrigel can be harvested and the new vessel formation in the plug can be assessed. The difficulty with the traditional matrigel plug assay is that the matrigel disperses easily in the subcutaneous tissue and does not form a tight solid mass. [10]

Sponge Implantation Method

This model is used for the evaluation of angiogenesis and anti- angiogenic agents. The mechanism involved in this is stimulation of inflammation by foreign substance leads to the angiogenesis. In this method the sponge can be prepared by using sterile absorbable gel foam. The gel foam is cut and strengthened with sterile agarose and that is used for angiogenesis study. The animals are anaesthetized and an incision is given at midline and gel piece is inserted in to subcutaneously. Animals are allowed to recover and at 14th day the animals are sacrificed and gel foams are harvested and quantification can be done for angiogenesis activity. Mostly used animal models are mice and rat. [11] The major disadvantage is implantation of the sponge materials is associated with non-specific immune response which may cause a significant angiogenic response even in the absence of exogenous growth factors in the sponge.

Corneal Angiogenesis Assay

This is the "gold standard" method for the following the effect of defined substances to promote neovascularization of the normally avascular cornea. Naturally the eye does not contain any blood vessel. So when applying test substances in the animal eye leads to the stimulation of angiogenesis which can be easily identified.

Several corneal angiogenesis models in the rabbit eye have been described including direct intrasomal injections of substances, chemical (or) thermal injury, intrasomal tumor implantation and sustained release sucralfate assay. Among these models the sustained release sucralfate assay is unique because it gives a predictable, persistent and aggressive neovascular response which is dependent on direct stimulation of blood vessels rather than on indirect stimulation by induction of inflammation.

In this method a pocket is making in the cornea and the test substance when introduced into this pocket will stimulate the formation of new vessels from the peripheral limbal vasculature. Slow release materials such as ELVAX (ethylene vinyl acetate copolymer) or Hydron have been used for the introduce test substance into the corneal pocket. The sponge material to hold test cell suspensions or test substances to induce angiogenesis can also be used because the slow releasing formulations may cause toxic. The original method was developed for rabbit eyes but now mostly used animal model is mice. [12]

The advantages of this method is visibility, accessibility and avascularity of the cornea are highly advantageous and facilitate the biomicroscopic grading of the neovascular response and the topical application of test drugs are the advantages of this method. The disadvantage of this method is it needs technically more demanding and more expensive than the CAM assay which makes it is not a potential screening assay.

Sponge Granuloma Angiogenesis Assay

This model is used for the evaluation of inflammatory angiogenesis which was described by Fajardo and colleagues. The sponge discs are prepared by cutting of sterile polyvinyl alcohol foam sponges. A hole is cut into the disc center to serve as a depot for administration of test substances and the back of the disc is close with the cotton plug. After adding the stimulants to the center hole the sponge discs are coat with an inert slow release ethylene vinyl acetate copolymer (ELVAX) and both the disc surfaces are sealed with filter paper.

The sterile discs are inserted into the subcutaneous layer at a site 2 cm distant from the incision which is then sutured to prevent disc dispersion. After 9-12 days the animals are sacrifice and the sponge discs are harvested. The discs are quantified for angiogenesis activity. [13]

IN-VITRO ASSAYS

In-vitro assays are performed by using different types of endothelial cells isolated from either capillaries or large vessels. Mostly used cell lines are Bovine aortic endothelial cells (BAECs), Chicken endothelial cells (CECs), Human microvascular endothelial cells (HMVECs) and Human umbilical vein endothelial cells (HUVECs).

The advantages of these in-vitro systems include the possibility to control the different parameters i.e. the spatial and temporal concentration angiogenic mediators can be possible. These models have the ability to study individual steps in the angiogenic process. It needs lowest cost and efforts as compared to the *in-vivo* systems. In this the target tissue-human tissue specific endothelial cells is possible to use for the study. The major disadvantage of *in-vitro* system is any compound has the effect on the endothelial cell proliferation and migration *in vitro* that compound might not have the same effect *in vivo* due to its metabolic changes in the body leads to its inactivation. [5]

Cell Cord Formation Assay

In this method the growth factor reduced matrigel is pipetted into a well of a 48-well plate and polymerized for 30 min at 37°C. Then the endothelial cells are incubated in 1% FBS-containing growth medium for 12 h respectively. Then they were trypsinized and resuspended in the same medium and dispersed onto the matrigel. Then the cells were treated with the test substances. After 18 h cord formation in each well is monitored and photographed using an inverted microscope. The tubular lengths of the cells are measured using software. [14]

Cell Migration Assay

A substantial number of published reports emphasize the predictable value that assays of endothelial cell migration have for selecting biologically active assay for the evaluation of stimulants and inhibitors of angiogenesis. This assay was carried out in a 48-well microchemotaxis chamber. The polycarbonate membrane with 12-µm pore is coated with gelatin endothelial cells are resuspended in cell culture medium. The bottom chamber is loaded with endothelial cells and the membrane is laid over the cells. Invertation and incubation of the chamber is carried out in sequence. After 2 h incubation the upper wells are loaded with cell culture medium and test samples. Then the chamber is reincubated for 2 h and membrane filter is fixed and staining Diff-Quick reagent. The number of cells that migrated through the filter is counted under a microscope. [15] The advantage of this assay is it is reproducible in practice to very format screening for angiogenesis modifiers.

Cell Proliferation Assay

The proliferation studies are based on cell counting, thymidine incorporation (or) Immunohistochemical staining for proliferation (or) cell death. In this method the endothelial cells are isolated and cultured in medium at 37°C in a humidified atmosphere containing 5% CO_2. Cell proliferation is determined using a 5-bromo-2'-deoxyuridine (BrdU) colorimetric assay kit. Then the endothelial cells are seeded onto gelatin coated well plates in the presence and absence of test samples and incubated for 48 h at 37°C.

Then 10 ml of BrdU is added to each well and the cells are further incubated for 6 h at 37°C. Then the cells are fixed and incubated with anti-BrdU and then detected by the substrate reaction. The reaction is stopped by the addition of 1 M H_2SO_4 and the absorbance is measured by using micro plate reader at 450 nm with 690 nm correction. [16]

Tube Formation Assay

The endothelial cells are isolated and cultured in medium in gelatin coated flasks. The cells from passages 4 to 7 are using for the angiogenesis study. Three dimensional collagen gels containing endothelial cells are prepared. After gelation at 37°C for 30 min the gels are overlaid with basal medium supplemented with test substances at indicated concentrations. Gels are examined and the tube length is determined for each well followed by determination of each group by using software. All experiments are terminating at 48 h. [17]

Gelatin Zymography

This assay can also be called as Matrix Metalloproteinase (MMP) assay. In this the matrix metalloproteinase activities of the myocardial tissue is measured by using sodium dodecyl sulphate (SDS)-polyacrylamide gels. Gelatin is used as a substrate because connective tissue degrading enzymes such as gelatinase rapidly cleave it and it is easily incorporated into polyacrylamide gels. Test samples are diluted to a final protein concentration with distilled water and mixed with SDS sample buffer. The complexes are loaded onto the gel and electrophoresed at 200 V for approximately 45 min at room temperature. After electrophoresed the gels are cut into pieces and one half of the gels are incubated for 18 h at 37°C analyzed by densitograph. [18]

Langendorff Isolated Heart

This method is example for the in-vitro coronary artery ligation model. This can be performed by using the "isolated buffer perfused heart model". The heart is isolated by performing tracheotomy and the dominant branch of left circumflex coronary artery was sutured. Heart is excised and placed in iced buffer. Heart is hanging by using the aortic root on a Langendorff apparatus for retrograde non-recirculating buffer perfusion at a constant pressure of 85 mm of Hg. Heart is continuously oxygenated with 95% O_2 and 5% CO_2. The perfusate is warming to 37°C. The perfusate is modified Krebs-henselet solution. [19]

ORGAN CULTURE ASSAYS

Cultivation of Cardiac Myocytes in Agarose Medium

This method is used for the angiogenic drugs which have the action on cardiac myocytes. Cardiac myocytes are isolated from the left ventricular myocardium of the mice and placed in culture medium. Heart explants are incubating for 7 days. Angiogenic Stimulants are added every other day and after 7 days endothelial sprouts are photographed and sprout formation is calculated. [20]

The Aortic Ring Assay

The angiogenesis can also evaluating by culturing rings of mouse aorta in three dimensional collagen gels with some modification of the method originally reported for the rat aorta. Thoracic aortas are removed and transferred to a culture dish containing ice cold serum free medium. The peri-aortic fibro adipose tissue is carefully removed without damaging the aorta wall. After the aorta is cut into 1mm long rings and rinsing in minimum essential medium. Mouse aortic rings are place in the middle of a 24-well plate. The rings are overloaded with matrigel and leave to polymerize for 1 to 2 h at 37°C. The rings are exposed to hypoxia for 2 h

followed by reoxygenation for 5-7 days. The vessel sprouts are observed and areas of sprouts are measured. [21]

Rat Blood Vessel Culture Assay

The rat thoracic veins are isolated and fibro-adipose tissue is removed. The veins are washing with DMEM supplemented with 10% FBS. The veins are then cut into small fragments and cultured in fibrin gels which are forming by addition of thrombin to the same medium containing fibrinogen in 12-wellplate. On the following day the test substance in the same volume of medium is added to the fibrin gel in the wells. After 9th day tube formation and cell growth are examined using a microscope and graded. [22]

Chick Aortic Arch Assay

This method is the modification method of the rat aortic ring assay. It is rapid method and it takes 1-3 days with serum-free medium. The chick aortic arch assay can be performed by incubating chick aortic arch ring in culture medium contain test substances. The aortic arches are isolated from 12-14 chick embryos and cut into 1mm rings and cultured in well plate containing matrigel. Average sprouting is measured. [23]

***IN-OVO* ASSAY**

Chick Chorioallantoic Membrane (CAM) Assay

The cancer biologists, developmental biologists and ophthalmologists have described the chick chorioallantoic membrane (CAM) as a model system for studying development, cancer behavior, properties of biomaterials, angiogenesis and photodynamic therapy. This assay is the most widely used assay for screening of angiogenesis activity. [24]

This method is used for screening of both the angiogenesis and anti-angiogenesis substances. In this method the fertilized white leghorn chicken eggs on the second day of incubation is collect and incubated at 37°C and constant humidity. At the day of 3 small hole is drill at narrow end and the albumin is withdrawn. At the 7th day of incubation a small square window is open in the shell and test substances are implanted on the top of the membrane. The window was sealed and reincubated. Eggs are incubated up to appropriate incubation time and angiogenesis is quantified. [11]

By other method, the CAM vascular system is displayed in greater detail, except that the embryo and the extraembryonic membranes must be transferred to a petridish in the early stages of development, i.e. on the 3 to 4 incubation. There CAM develops at the top as a flat membrane, reaching the edge of the dish to provide a two-dimensional monolayer onto which grafts can be placed. Because the entire membrane can be seen, rather than just a small portion through the shell window, multiple grafts can be placed on each CAM and photographs can be taken periodically to document vascular changes over time.

The changes in the distribution and density of CAM vessels next to the implant which are evaluated in-vivo by means of a stereomicroscope at regular intervals following the graft procedure. The score is a 0 when no changes can be seen; it is +1 when the neovessel covers towards the implants and +2 when a considerable change in the number and distribution of converging neovessel is observed. [25] The CAM assay is relatively simple and inexpensive and thus suitable for large scale screening. The major disadvantage of this assay is that the CAM contains already a well developed vascular network which makes it difficult to discriminate between new capillaries and already existing ones. [5]

QUANTIFICATION OF ANGIOGENESIS ACTIVITY

Angiography Study

The angiography is used for the quantification of angiogenesis in coronary artery ligation model and rabbit hindlimb ischemia model. Under general anesthesia, the animals received a contrast agent, through a standard femoral puncture by using digital subtraction angiography. To precisely assess collateral filling of LAD territory, semi quantification of the collateral coronary circulation was performed with Image Pro plus 5.1 according to the manufacturer's instruction.

Images with the best contrast were selected for analysis for each animal, and the angiographic index was calculated by using a standard protocol based on Rentrop's grading scales. The area of interest (AOI) was defined as a left ventricular region that included neocollaterals and a distal segment of the ligated LAD coronary artery. The profile of the LAD and collateral artery reperfusion were delineated carefully, and total collateral vascular areas were calculated. Each image was recorded three times. [26]

Assessment of Hind Limb Function

This method is used for the quantification of hindlimb ischemia model. The hindlimb function can be estimate by forced swimming test to determine the functional capacity of the ischemic hindlimb after treatment. In this method the animals are placed in a water filled tank to swim. Active strokes per minute of each limb are counted during 3 consecutive periods. Functional muscle activity is calculated as the ratio of number of strokes/min of the ischemic and healthy hind limbs. [27]

Capillary Density Estimation

This method is mostly used quantification method for the angiogenesis activity. In this method the test and control tissues are fixed in methanol and embedded in paraffin. The paraffin embedded tissues are cut into thick sections and the sections are stains with hematoxylin and eosin (H&E) and observe under microscope. The capillaries are counted and expressed as number of capillaries per (mm^2). [28]

This method is easy to perform in laboratory and it less cost. This method is used in most of the of angiogenesis evaluation techniques.

Enzyme Linked Immunosorbent Assay (ELISA)

This method of quantification can be used for the estimation of angiogenic growth factors. This assay is performing by using specific angiogenic growth factor ELISA kit according to the manufacture's protocol. The mostly 96-well micro liter plates are used for this study. [21]

Hemoglobin Determination in Matrigel Plugs

This method is particularly for the quantification of matrigel plug assay. In this method the Matrigels are dissected from the mice and weighed. Then they are homogenized for 5-10 min ice at 10,000 rpm on micro centrifuge for 6 min and supernatant is collected for hemoglobin measurements. The supernatants are mixed with Drabkin's reagent and hemoglobin in the samples is quantified calorimetrically at 540 nm in spectrophotometer. [13] This method was easy and less cost.

Laser Dropler Perfusion Image (LDPI)

This method is used for the quantification of coronary and hindlimb blood flow by using Laser Doppler Perfusion

Imager system. The LDPI uses a beam from a 2-mW helium-neon laser that sequentially scans a 12×12 cm tissue surface to a depth of 600 hundred microns. LDPI was used to record perfusion of both right and left limbs preoperatively and predetermined time points postoperatively. The animals are place on a heating plate at 37°C to minimize temperature variation. Consecutive measurements are obtained over the same region of interest. Colour photographs are record and analysis performed by calculating the average perfusion of the ischemic and non-ischemic tissues. [29-30]

Western Blot Analysis

The western blot analysis can be used for the quantification of angiogenic growth factors expression. In this the whole total proteins are extracted from the ischemic and non-ischemic tissue and proteins are separated by electro blotted on nitrocellulose membrane. The membrane is probe with polyclonal antibodies and complexes are visualized after 1 hr incubation. [30]

Reverse Transcriptase Polymerase Chain Reactions (RT-PCR) Assays

Reverse transcriptase polymerase chain reactions are performed on mRNA made from endothelial cells. This method is used for the estimation of what type of angiogenic growth factor involved in the angiogenic activity. In this method the total RNA was extracted using RNA extraction kit and RT-PCR reactions are carried out using reverse transcriptase systems and the results are given in relation to 18S rRNA molecule numbers. [31]

According to this elaborated review on various angiogenesis assays *in-vitro* models are easy than compared to the *in-vivo* angiogenesis assay models. In *in-vivo* angiogenesis models, the matrigel plug assay is the easiest model than compared to all other *in-vivo* angiogenesis assay models. Other than the pre-mentioned two models *in-ovo* angiogenesis assay model (CAM assay) is mostly used as a pilot method for most of the angiogenesis evaluation studies. CAM assay, corneal angiogenesis assay and matrigel plug assays are also used for the evaluation of anti-angiogenic agents. When compared to all other angiogenesis quantification methods capillary density estimation source will give better accuracy and precession. Hemoglobin estimation also easiest method when compared to all other angiogenesis quantification methods.

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