Anti-Angiogenic Effects of Diltiazem, Imatinib, and Bevacizumab in the CAM Assay

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Abstract- Angiogenesis that plays important roles in a variety of physiological processes is strictly delimited and finely tuned by a balance of pro-angiogenic and anti-angiogenic factors. Angiogenesis is essential for viability, growth, invasion and metastasis of tumors. The chorioallantoic membrane model is considered to be a valuable method for investigation of anti-angiogenic effects of drugs and other substances. The aim of the present study is to investigate the anti-angiogenic effects of a calcium channel blocker (diltiazem), a tyrosine kinase inhibitor (imatinib), and a vascular endothelial growth factor inhibitor (bevacizumab) with the means of the chicken chorioallantoic membrane (CAM) model. Each drug was administered onto the chorioallantoic membrane at the concentrations of 10^{-4}M, 10^{-5}M, and 10^{-6}M on 8th day of incubation. After 24 h, development of the blood vessels under a stereo-microscope was investigated. The evaluations were performed using a scoring system. We evaluated anti-angiogenic effects of the drugs in two ways according to their average anti-angiogenic effect and comparison of median anti-angiogenic score. These two angiogenesis evaluation techniques provided similar results to measure anti-angiogenic effects of the study drugs. Bevacizumab has the strongest anti-angiogenic effect compared to the diltiazem and imatinib, and althought not reaching statistical significance imatinib has a strong anti-angiogenic effect than that of the diltiazem.

Index Terms- Angiogenesis, diltiazem, imatinib, bevacizumab, chorioallantoic membrane, chicken egg

I. INTRODUCTION

Angiogenesis as a physiological process involves the growth of new blood vessels from pre-existing vessels and plays a central role in embryonic and normal developments and wound healing. It has also important roles in the etiology of many diseases such as chronic inflammatory disorders, cancer, and some pregnancy related diseases such as intrauterine growth restriction and preeclampsia (1-3). The remarkable diversity in angiogenic signaling pathways provides many options for therapeutic intervention, and since angiogenesis plays an essential role in tumor growth and invasion, anti-angiogenesis is currently a major area of oncologic research. A tumor is unable to grow more than 2 mm in diameter unless there is the development of new vessels by angiogenesis (4). Some approved anti-angiogenic agents such as bevacizumab, sorafenib, sunitinib, and thalidomide are used clinically as effective drugs for several types of cancer, and many new agents are in phase II trials. The anti-angiogenic agents can act synergistically with conventional chemotherapy drugs and tend to have non-overlapping toxicities (5).

Molecular processes related to angiogenesis include stimulation of endothelial growth to develop new vessels by cytokine production (i.e. vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2), degradation of extracellular matrix proteins by matrix metalloproteinases, and migration of endothelial cells mediated by cell membrane adhesion molecules like integrins. Drugs targeting pathologic angiogenesis have been designed to interfere with any of these steps and some of them are currently undergoing evaluation in clinical studies (6).

Diltiazem is a potent vasodilator and belongs to a non-dihydropyridine group of drugs that are a class of calcium channel blockers, used in the treatment of hypertension, angina pectoris, and some types of arrhythmia. Sartippour et al. (7) investigated the effect of some drugs, such as diltiazem, enalapril, and omeprazole as generally administered agents in cancer patients, on in vitro angiogenesis. They concluded that these drugs in massive doses had a potential to inhibit endothelial proliferation but they had no effect at human therapeutic ranges. Higgins et al. (8) examined the effect of diltiazem on oxygen-induced retinopathy in a mouse model with evaluation techniques for neovascularization. They concluded that diltiazem decreases retinal neovascularization and improved retinopathy. Except that investigation, there is no study included diltiazem to evaluate its angiogenic effect in the chorioallantoic membrane (CAM) and similar assays.

Imatinib mesylate is a member of a new class of anticancer agents, the so-called small molecules. It is a phenylaminopyrimidine analogue that competes with ATP for its specific binding site in the kinase domain of specific tyrosine kinase receptors (9). It is a signal transduction inhibitor that specifically targets several protein tyrosine kinases, c-abl, c-kit, and the platelet derived growth factor (PDPG) receptor (10-12). PDGF is a crucial angiogenic factor involved in maturation of the microvasculature (13). Balke et al. (14) investigated eight osteosarcoma cell lines for their ability to form vascularized tumors on the CAM with or without imatinib. They concluded that treatment with imatinib potently inhibited tumor angiogenesis and growth in their model. Rocha et al. (12) examine the effects of imatinib on Human Aortic Smooth Muscle Cells and Human Umbilical Vein Endothelial Cells after

incubation with progesterone. They suggested that imatinib had anti-angiogenic effects related to smooth muscle cells but not endothelial cells, and it was probably preventing vessel stabilization.

Overexpression of VEGF, the key mediator of angiogenesis in physiologic and pathophysiologic conditions, is accepted as related to poor prognosis in malign tumors. Bevacizumab, a recombinant humanized anti-VEGF monoclonal antibody, is the most clinically advanced anti-angiogenic agent. Although bevacizumab has been gained a place in the first-line treatment of advanced colorectal and non-small-cell lung cancer, there is a rapidly growing body of evidence for its efficacy in treatment of a number of other solid tumors (15).

To date, a variety of in vivo angiogenic assays have been developed to investigate angiogenesis in physiological and pathological circumstances and pro- and anti-angiogenic effects of compounds. The chick embryo CAM developed as a successful, feasible, and sensitive model for in vivo research on angiogenesis and anti-angiogenesis (16, 17). There was no study conducted to examine and compare the anti-angiogenic effects of diltiazem, imatinib and bevacizumab in the same setting of CAM assay. In the management of cancer patients with cardiovascular diseases, many drugs belonging to these drug groups may need to be used together in clinical practice.

In this study, we sought to determine the effects of diltiazem, imatinib and bevacizumab on angiogenesis in the CAM assay.

II. Materials and Methods

Drugs and preparation of the pellets

In this study, the effects of diltiazem (Diltizem® 25 mg flakon, Mustafa Nevzat İlac Sanayi Anonim Sirketi®, Istanbul), imatinib (Novartis Institutes for BioMedical Research, Basel, Switzerland), and bevacizumab (Altuzan 400 mg/16 mL vial, Roche Mustahzarlari Sanayi Anonim Sirketi, Istanbul) were investigated. While diltiazem and imatinib were prepared by solving in sterile distilled water, bevacizumab was in the form of soluble infusion. Suramin, a prototype of a pharmacological antagonist of growth factors, has a potential to inhibit multiple control points of angiogenesis (18). Thalidomide inhibits the activity of basic fibroblast growth factor-2 (5). Suramin and thalidomide (Sigma-Aldrich, St. Louis, MO, USA) was dissolved or emulsified immediately prior to use in positive controls (19, 20).

Three concentrations (10^{-4}M, 10^{-5}M and 10^{-6}M) of the study drugs and a 10^{-4}M concentration of suramin and thalidomide were used. The choice of these concentrations were based on the results of the previous studies which have shown that the concentration of 10^{-4} M provides submaximal efficacy of the drugs, and the observations regarding the efficacy of 10^{-6} M concentration of the drugs in human body. First of all, the concentrations of 10^{-4} M of the drugs were prepared; the more diluted concentrations were prepared by diluting these solutions. The classical molarity formula (M=m/V) was used to find out the required drug amount to provide 10^{-4} M concentration. The study drugs and positive controls were administered in a 2.5% (w/v) solution of agarose (Merck, Darmstadt, Germany). For easy administration, the pellets were prepared as 10 µl drops on circular stainless steel surface which is 5 mm in diameter. The pellets were then solidified at room temperature, and applied onto the CAM within 1 h. In first negative controls, no pellet was administered. The pellets with no drugs including only 10 µl agarose solution were used in second negative controls.

III. Chorioallantoic Membrane Assay

Ross 308 impregnated chick eggs were obtained from Yemsel Tavukculuk Hayvancilik Yem Hammaddeleri Sanayi ve Ticaret Anonim Sirketi (Kayseri). The impregnated chick eggs were incubated in horizontal position in 37.5°C and 80% relative humidified environment. On the day 5 of incubation period, 5 ml of albumin was taken from the solid side of the eggs by a syringe allowing detachment of the embryo from the eggshell and a shell piece of 2-3 cm in diameter was removed to open a window on the other side of the eggs. Normal development was verified and embryos with malformations or dead embryos were excluded. The window was sealed with gelatin and thereafter, the eggs were returned to the incubator for 72 hours to have CAM reached approximately 2 cm in diameter, and then on day 8 the pellets were placed on chorioallantoic membranes of each egg. The eggs were then incubated for 24 hours and after this period angiogenesis was evaluated.

Table 1 presents the ratio of angiogenesis evaluation/test compound administration. For each concentration of the study and positive control drugs, fifteen eggs were used in each egg set. All of the tests were duplicated. For no pellet and agar only negative controls, one and two egg sets, respectively, were used. We excluded the eggs with inflammation and embryo toxicity caused by the pellets. When used in 10^{-4}M and 10^{-5}M concentrations, test drugs caused embryo toxicity in 28 eggs; therefore, data obtained from the 343 eggs that were suitable for evaluation were used. There was no exclusion related to inflammation.

Table 1. The number of angiogenesis evaluation/study compound administration in the study and control groups.

	10-4 M	10-5 M	10-6 M
Diltiazem	14/30	28/30	30/30
Imatinib	28/30	29/30	30/30
Bevacizumab	24/30	29/30	30/30
Suramin	15/15		
Thalidomide	15/15		
No pellet	15/15		
Agar	30/30		

Angiogenesis scoring

The vascular composition of the chorioallantoic membranes where the pellets were administered was evaluated under stereoscopic microscope by using the scoring system of Burgermeister et al. on day 9 (21) (Table 2).

Table 2. The scoring system of angiogenic effect of the compounds on chorioallantoic membrane.

Score	Effect	Definition
0	Absent	Normal embryo, no difference with respect to surrounding capillaries
0.5	Weak	There is no area lacking capillary vessels. The density of the capillaries is decreased but not larger than the pellet
1	Moderate	The area lacking capillaries is small or capillary density is decreased in a certain area. The effects are not more than twice of the pellet area
2	Strong	The area lacking capillaries is at least as twice as pellet area

After the scoring, the equation developed by Krenn & Paper (20) was used for the determination of the average mean score for each drug concentration:

Average score = [Egg number (Score 2) X 2 + Egg number (Score 1) X 1] / [Total egg numbers (Score 0, 1, 2)]

Average score <0.5 = no anti-angiogenic effect. Average score 0.5 to 0.75 = weak anti-angiogenic effect, score >0.75 to 1 = good anti-angiogenic effect, and score >1 = very good anti-angiogenic effect.

3.3. Statistical Analysis

The data were expressed as median (min-max) in dot-plot graphs. Anti-angiogenic scores of study drugs and positive controls were analyzed by Kruskal-Wallis with post hoc Dunn's multiple comparison test tests and expressed as median (min-max). A p value of less than 0.05 was considered as statistically significant.

IV. RESULTS

In the first negative controls, angiogenesis of chorioallantoic membranes was normal. The pellets with no drugs including only 10 µl agarose solution as the second negative controls did not cause any significant anti-angiogenic effect (average anti-angiogenic score = 0.2).

The average anti-angiogenic scores of suramin and thalidomide were 0.5 (weak anti-angiogenic effect) and 1 (good anti-angiogenic effect), respectively. The average anti-angiogenic scores of diltiazem 10^{-4}M, 10^{-5}M and 10^{-6}M were 0.9 (good anti-angiogenic effect), 0.6 (weak anti-angiogenic effect), and 0.4 (no anti-angiogenic effect), respectively. The average anti-angiogenic scores of imatinib were 1.0 (very good anti-angiogenic effect), 0.7 (very good anti-angiogenic effect), and 0.5 (good anti-angiogenic effect) for 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations, respectively. The average anti-angiogenic score of bevacizumab was 1.58 (very good anti-angiogenic effect), 1.55 (very good anti-angiogenic effect), and 1.16 (very good anti-angiogenic effect) for 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations, respectively.

The median anti-angiogenic score of thalidomide 10^{-4}M was significantly higher than that of the suramin 10^{-4}M [1 (0-2) versus 0 (0-1); $p<0.05$]. Figure 1 shows the median anti-angiogenic scores of diltiazem in 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations. As shown in the scatter-dot graph, the following significant differences were found. The median anti-angiogenic score of diltiazem 10^{-4}M was significantly higher than those of the diltiazem 10^{-6}M and suramin 10^{-4}M [1 (0.5-1) versus 0.5 (0.5-1) and 0 (0-1), respectively; $p<0.05$]. The median anti-angiogenic score of diltiazem 10^{-5}M was significantly higher than that of the suramin 10^{-4}M [1 (0.5-1) versus 0 (0-1); $p<0.05$].
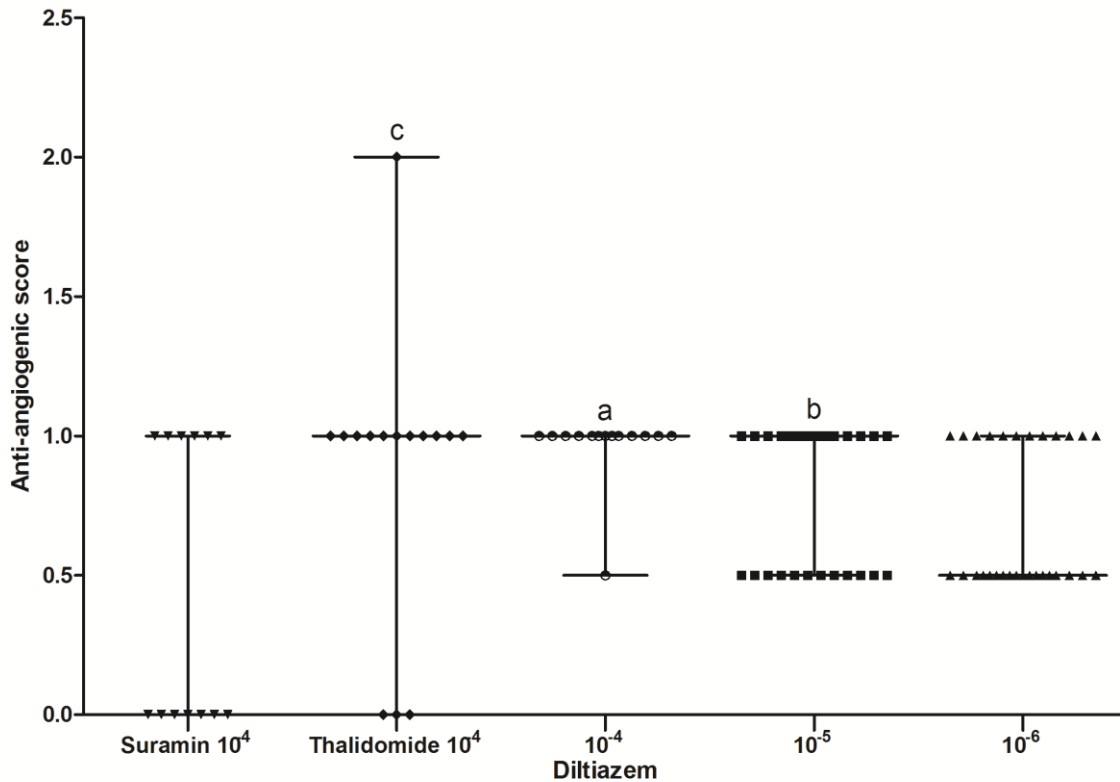
Figure 1. Anti-angiogenic scores of diltiazem in 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations. The data were expressed as median (min-max) on scatter-dot plots. [a]$P<0.05$ versus diltiazem 10^{-6}M and suramin 10^{-4}M. [b]$P<0.05$ versus suramin 10^{-4}M. [c]$P<0.05$ versus thalidomide 10^{-4}M.

Figure 2 shows the median anti-angiogenic scores of imatinib in 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations. As shown in the scatter-dot graph, the following significant differences were found. The median anti-angiogenic score of imatinib 10^{-4}M was significantly higher than those of the imatinib 10^{-6}M and suramin 10^{-4}M [1 (0.5-2) versus 1 (0-1) and 0 (0-1.0), respectively; $p<0.05$]. The median anti-angiogenic score of imatinib 10^{-5}M was significantly higher than that of the suramin 10^{-4}M [1 (0.5-2) versus 0 (0-1); $p<0.05$]. Overall, these results suggest that, being more significant in higher concentrations, imatinib has anti-angiogenic effect on chick CAM model.
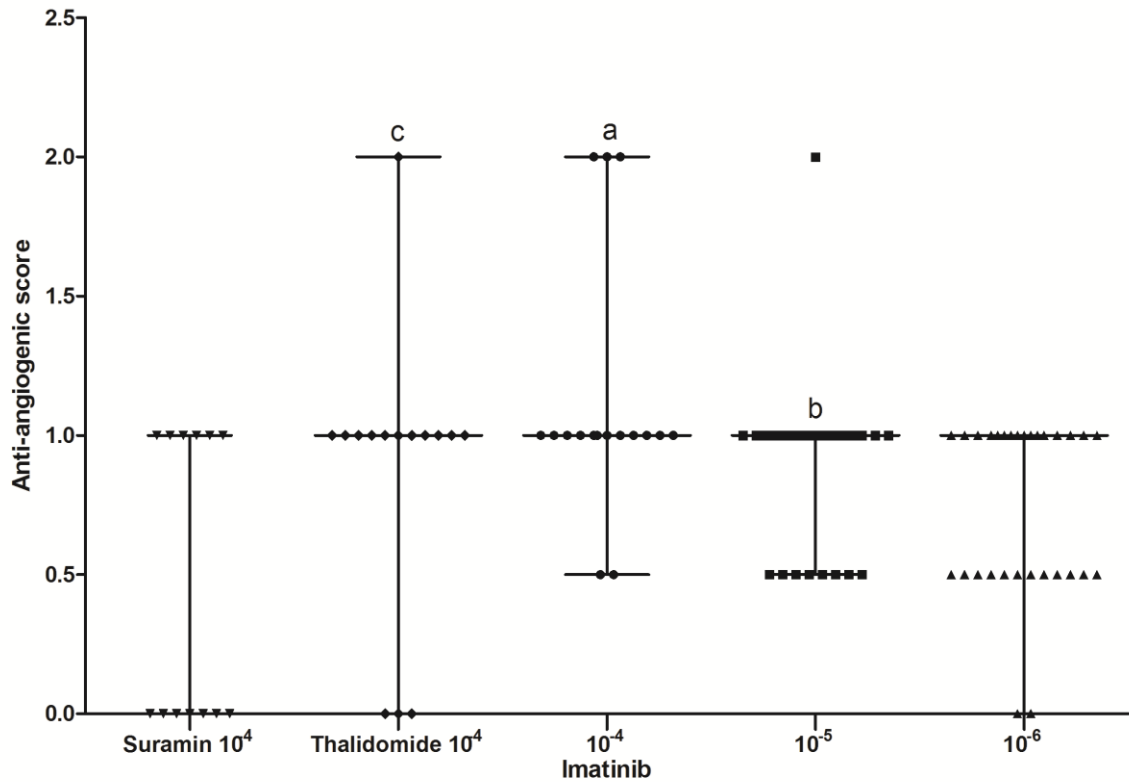
Figure 2. Anti-angiogenic scores of imatinib in 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations. The data were expressed as median (min-max) on scatter-dot plots. [a]$P<0.05$ versus imatinib 10^{-6}M and suramin 10^{-4}M. [b]$P<0.05$ versus suramin 10^{-4}M. [c]$P<0.05$ versus thalidomide 10^{-4}M.

Figure 3 shows the median anti-angiogenic scores of bevacizumab in 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations. As shown in the scatter-dot graph, the following significant differences were found. The median anti-angiogenic score of bevacizumab 10^{-4}M was significantly higher than those of the bevacizumab 10^{-6}M, suramin 10^{-4}M, and thalidomide 10^{-4}M [2 (1-2) versus 1 (0.5-2), 0 (0.5-1) and 1 (0-2), respectively; p<0.05]. The median anti-angiogenic score of bevacizumab 10^{-5}M was significantly higher than those of the bevacizumab 10^{-6}M, suramin 10^{-4}M, and thalidomide 10^{-4}M [2 (1-2) versus 1 (0.5-2), 0 (0-1), and 1 (0-2), respectively; p<0.05]. The median anti-angiogenic score of bevacizumab 10^{-6}M was significantly higher than that of the suramin 10^{-4}M [1 (0.5-2) versus 0.5 (0-1.0); p<0.05]. Overall, these results suggest that, being more significant in higher concentrations, diltiazem has anti-angiogenic effect on chick CAM model.
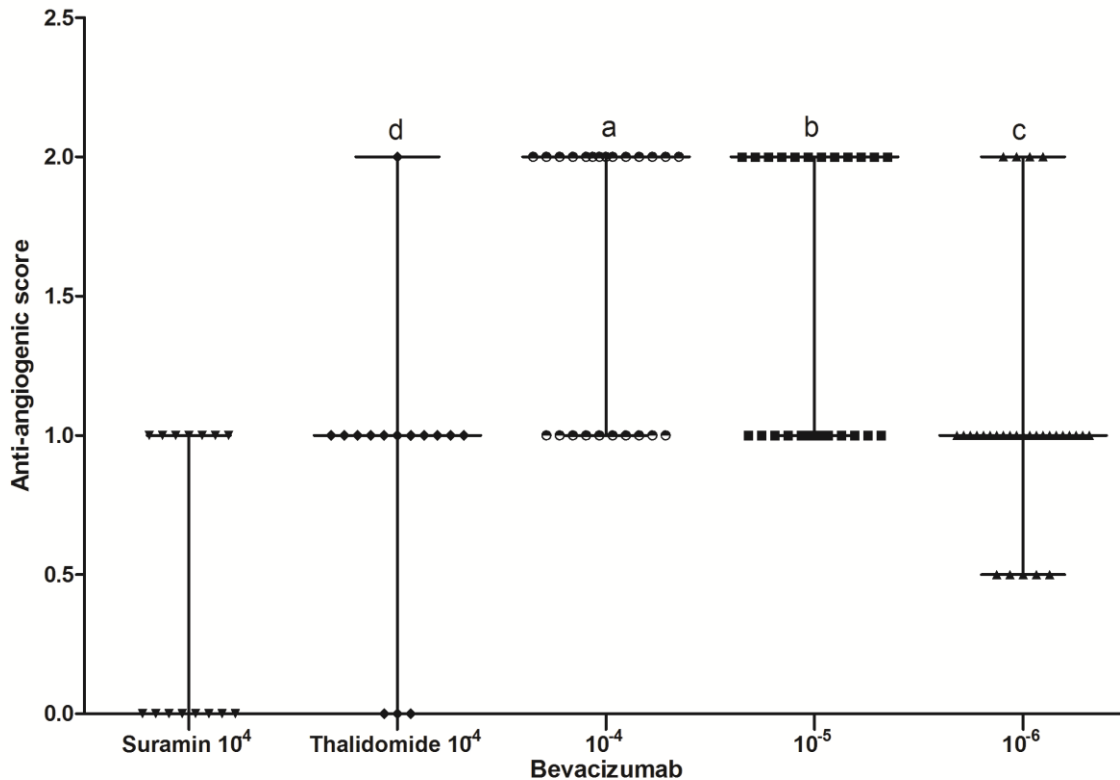
Figure 3. Anti-angiogenic scores of bevacizumab in 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations. The data were expressed as median (min-max) on scatter-dot plots. [a,b]P<0.05 versus imatinib 10^{-6}M, suramin 10^{-4}M, and thalidomide 10^{-4}M. [c]P<0.05 versus suramin 10^{-4}M. [d]P<0.05 versus thalidomide 10^{-4}M.

After comparison of the median anti-angiogenic scores of diltiazem, imatinib, and bevacizumab, overall, bevacizumab was the strongest anti-angiogenic agent compared to the other drugs (p<0.05). The median anti-angiogenic scores of diltiazem, imatinib, and bevacizumab were significantly higher than that of the suramin (p<0.05). The median anti-angiogenic score of bevacizumab was significantly higher than that of the thalidomide (p<0.05). Diltiazem, imatinib, and thalidomide were comparable with regard to their median anti-angiogenic scores (p<0.05).

V. DISCUSSION

We used chick CAM assay for the evaluation of the diltiazem, imatinib, and bevacizumab on angiogenesis. There was no CAM assay investigated their anti-angiogenic effects. This was the first study investigated the anti-angiogenic effects of these drugs in the same setting of CAM assay. Our findings demonstrated that diltiazem, imatinib, and bevacizumab have anti-angiogenic effects. Overall, according to their average anti-angiogenic scores, study drugs and positive controls ranked from very good to weak anti-angiogenic effect as the following: bevacizumab, imatinib and thalidomide, diltiazem, and suramin. Overall, these results suggest that, being more significant in higher concentrations, all the study drugs have anti-angiogenic effect on chick CAM model. After comparison of the median anti-angiogenic scores of study drugs and positive controls, they

ranked as the following: bevacizumab, diltiazem, imatinib, and thalidomide, and suramin. In this study, we evaluated anti-angiogenic effects of the drugs in two ways according to their average anti-angiogenic effect and comparison of median anti-angiogenic score. These two evaluation methods provided similar results to measure anti-angiogenic effects of the study drugs.

CAM is an extra-embryonic membrane formed on day 4 of incubation by fusion of the chorion and the allantois. Immature blood vessels grow very rapidly until day 8 and rive rise to a capillary plexus. Rapid capillary proliferation continues until day 11, and the vascular system attains it final form on day 18. CAM is a very angiogenic tissue until day 11 or 12 of chick embryo development. Between day 8 and day 10, CAM is highly responsive to pro- and anti-angiogenic effects of the drugs. In addition, 3 to 5 days are usually enough for the assays. There is no clear difference between in ovo or shell-less cultures. In this study, in ovo technique was used. Focal application of test substances is still the most used method (17).

Angiogenesis is essential for tumor progression and dissemination (22, 23). New blood vessel formation during tumor growth is regulated by pro- and anti-angiogenic factors (22, 24). Angiogenic switch occurs when the effects of anti-angiogenic factors become preponderant. This leads to formation of new blood vessels supplying tumor tissue (24). Anti-angiogenic approaches fell in two categories as agents that blocked the activity of pro-angiogenic molecules. Nowadays, over 300 anti-angiogenic molecules targeting different signaling pathways are being tested for their anticancer properties at preclinical and

clinical stages. Owing to its central role in promoting tumor growth, VEGF has become the most important target for blocking its action a different level of signaling pathways (17). The rabbit ear chamber, the mouse dorsal skin and air sac, the CAM, the iris and avascular cornea of the rodent eye, and the zebrafish are the classical assays for investigation of angiogenesis in in vivo methods. In vivo angiogenesis assays may be very sensitive to environmental factor, and the activation of inflammatory or other non-endothelial cell types can modify the development of new vessel formation. Because the CAM has many advantages related to cost, ease of use, reproducibility, and reliability, it was chosen for this experiment (17).

Calcium is an essential signal transduction element which plays significant roles in many eukaryotic cell functions including cell cycle progression (25). Control of intracellular level of calcium is of paramount importance for normal cell cycle progression and cell proliferation (26); therefore, calcium excess or disturbances of calcium signalization may cause cell death (27). Diltiazem is a calcium channel blocker which is more potent on L-type calcium channels. We found that diltiazem in 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations had poor anti-angiogenic effect. However, the drug in 10^{-4}M concentration was more potent inhibitor of angiogenesis. The findings show that, being more significant in high doses, diltiazem has poor anti-angiogenic effect.

Imatinib is a specific tyrosine kinase inhibitor that blocks the activity of the bcl-abl oncoprotein and the c-kit tyrosine kinase cell surface receptor. Imatinib is also an inhibitor of the PDGFR kinase (28). Protein kinases act as catalyzers in phosphorylation reactions in signaling cascades that affect cell growth and differentiation. These roles in cell progression make the kinases target agents for drug development in cancer. To the best of our knowledge, the effect of imatinib on angiogenesis on chorioallantoic membrane model has not been investigated to date. The results of our study indicate that imatinib in 10^{-4}M and 10^{-5}M concentrations has strong anti-angiogenic effect, whereas, 10^{-6}M concentration of the drug has poor anti-angiogenic effect. Imatinib is more potent inhibitor of angiogenesis when used in higher concentrations. Our findings suggest that imatinib as a specific tyrosine kinase inhibitor which also blocks PDGFR has anti-angiogenic properties. This effect of the drug may have been caused by the blockade of PDGFR of which expression significantly increases during angiogenesis, by imatinib.

Bevacizumab is a recombinant humanized monoclonal antibody directed against VEGF. The drug has been used as the first-line treatment of metastatic colorectal cancer in combination with 5-fluorouracil since 2004 (28). In this study, bevacizumab in 10^{-4}M, 10^{-5}M and 10^{-6}M concentrations was shown to have strong anti-angiogenic effects. However, the drug was more potent inhibitor of angiogenesis in 10^{-4}M and 10^{-5}M concentrations than 10^{-6}M concentration.

Like other in vivo angiogenesis assays, the CAM assay has some disadvantages. CAM tissue contains a well-developed vascular network and the vasodilation that invariably follows its manipulation may be hard to distinguish from the effects of the test agent. Another limitation is nonspecific inflammatory reactions caused by the implant containing the study substance. Non-specific inflammatory reactions are much less frequent when the implant is made very early in the CAM development and the chick immune system is relatively immature (29).

In conclusion, in the CAM assay, for the evaluation of anti-angiogenic effect of study drugs, the average anti-angiogenic effect and comparison of median anti-angiogenic score provides comparable results. Bevacizumab has the strongest anti-angiogenic effect compared to the diltiazem and imatinib, and althought not reaching statistical significance imatinib has a strong anti-angiogenic effect than that of the diltiazem.

REFERENCES

[1] Chung AS, Lee J, Ferrara N. Targeting the tumour vasculature: insights from physiological angiogenesis. Nat Rev Cancer. 2010 Jul;10(7):505-14.

[2] Khankin EV, Royle C, Karumanchi SA. Placental vasculature in health and disease. Semin Thromb Hemost. 2010 Apr;36(3):309-20.

[3] Barut F, Barut A, Gun BD, Kandemir NO, Harma MI, Harma M, Aktunc E, Ozdamar SO. Intrauterine growth restriction and placental angiogenesis. Diagn Pathol. 2010 Apr 22;5:24

[4] Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med. 2000 Apr;6(4):389-95.

[5] Eichholz A, Merchant S, Gaya AM. Anti-angiogenesis therapies: their potential in cancer management. Onco Targets Ther. 2010 Jun 24;3:69-82.

[6] Ziche M, Donnini S, Morbidelli L. Development of new drugs in angiogenesis.Curr Drug Targets. 2004 Jul;5(5):485-93.

[7] Sartippour MR, De Leon E, Rubio R, Brooks MN. The effect of commonly used drugs on angiogenesis. Anticancer Res. 2003 Jan-Feb;23(1A):231-4.

[8] Higgins RD, Yu K, Sanders RJ, Nandgaonkar BN, Rotschild T, Rifkin DB. Diltiazem reduces retinal neovascularization in a mouse model of oxygen induced retinopathy. Curr Eye Res. 1999 Jan;18(1):20-7.

[9] Buchdunger E, O'Reilly T, Wood J. Pharmacology of imatinib (STI571). Eur J Cancer. 2002 Sep;38 Suppl 5:S28-36.

[10] Barr RD. Imatinib mesylate in children and adolescents with cancer. Pediatr Blood Cancer. 2010 Jul 15;55(1):18-25.

[11] Waller CF. Imatinib mesylate. Recent Results Cancer Res. 2010;184:3-20.

[12] Rocha A, Azevedo I, Soares R. Anti-angiogenic effects of imatinib target smooth muscle cells but not endothelial cells. Angiogenesis. 2007;10(4):279-86. Epub 2007 Sep 18.

[13] Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. Angiogenic and angiostatic factors in the molecular control of angiogenesis. Q J Nucl Med. 2003 Sep;47(3):149-61.

[14] Balke M, Neumann A, Kersting C, Agelopoulos K, Gebert C, Gosheger G, Buerger H, Hagedorn M. Morphologic characterization of osteosarcoma growth on the chick chorioallantoic membrane. BMC Res Notes. 2010 Mar 4;3:58.

[15] Jenab-Wolcott J, Giantonio BJ. Bevacizumab: current indications and future development for management of solid tumors. Expert Opin Biol Ther. 2009 Apr;9(4):507-17.

[16] Paper, D.H., Vogl, H., Franz, G., Hoffmann, R., 1995. Macromol. Symp. 99, 219–225.

[17] Ribatti D. The chick embryo chorioallantoic membrane as an ın vivo assay to study antiangiogenesis. Pharmaceuticals 2010;3:482-513.

[18] Takano S, Gately S, Neville ME, Herblin WF, Gross JL, Engelhard H, Perricone M, Eidsvoog K, Brem S. Suramin, an anticancer and angiosuppressive agent, inhibits endothelial cell binding of basic fibroblast growth factor, migration, proliferation, and induction of urokinase-type plasminogen activator. Cancer Res. 1994 May 15;54(10):2654-60.

[19] Demirci F, Paper DH, Franz G, Başer KH. Investigation of the Origanum onites L. essential oil using the chorioallantoic membrane (CAM) assay. J Agric Food Chem. 2004 Jan 28;52(2):251-4.

[20] Krenn L, Paper DH. Inhibition of angiogenesis and inflammation by an extract of red clover (Trifolium pratense L.). Phytomedicine. 2009 Dec;16(12):1083-8.

[21] Bürgermeister J, Paper DH, Vogl H, Linhardt RJ, Franz G. LaPSvS1, a (1-->3)-beta-galactan sulfate and its effect on angiogenesis in vivo and in vitro. Carbohydr Res. 2002;337(16):1459-1466.

[22] Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2002;2:727–39.

[23] Fidler IJ. The pathogenesis of cancer metastasis: the ''seed and soil'' hypothesis revisited. Nat Rev Cancer 2003;3:453–8.

[24] Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3:401–10.

[25] Mariot P, Vanoverberghe K, Lalevee N, Rossier MF, Prevarskaya N. Overexpression of an alpha 1H (Cav3.2) T-type calcium channel during neuroendocrine differentiation of human prostate cancer cells. J Biol Chem. 2002;277(13):10824-10833.

[26] Ciapa B, Pesando D, Wilding M, Whitaker M. Cell-cycle calcium transients driven by cyclic changes in inositol trisphosphate levels. Nature 1994;368(6474):875-878.

[27] Choi DW. Ionic dependence of glutamate neurotoxicity. J Neurosci. 1987;7(2):369-379.

[28] Homsi J, Daud AI. Spectrum of activity and mechanism of action of VEGF/PDGF inhibitors. Cancer Control. 2007 Jul;14(3):285-94.

[29] Ribatti D. Chick embryo chorioallantoic membrane as a useful tool to study angiogenesis. Int Rev Cell Mol Biol. 2008;270:181-224.

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