CHAPTER 7

IN-VIVO TUMOR MICROVESSEL DENSITY EVALUATION

7.1 ANGIOGENESIS: CONCEPT & THERAPEUTICS

7.1.1

Aneiogenesis, the formation of new blood vessels out of preexisting capillaries, is a sequence of events having key importance in many physiologic (embryonic development, wound healing, menstrual cycle) & pathological (cancer, cardiovascular diseases, inflammation, diabetic retinopathy) conditions. The initial recognition of angiogenesis as therapeutically important process began in the area of oncology in 1970s. To date, anti-angiogenesis therapy is recognized globally, a promising approach, supposedly leading to desperately needs d breakthrough in cancer therapy. [1]

7.1.2 Tumor Angiogenesis

Angiogenic sprouting of blood vessels occurs in a series of steps, which can roughly be divided into a destabilization phase, a proliferation and migration phase and a maturation phase. All these steps offer potential points of pro-or anti-angiogenic clinical intervention. [2]

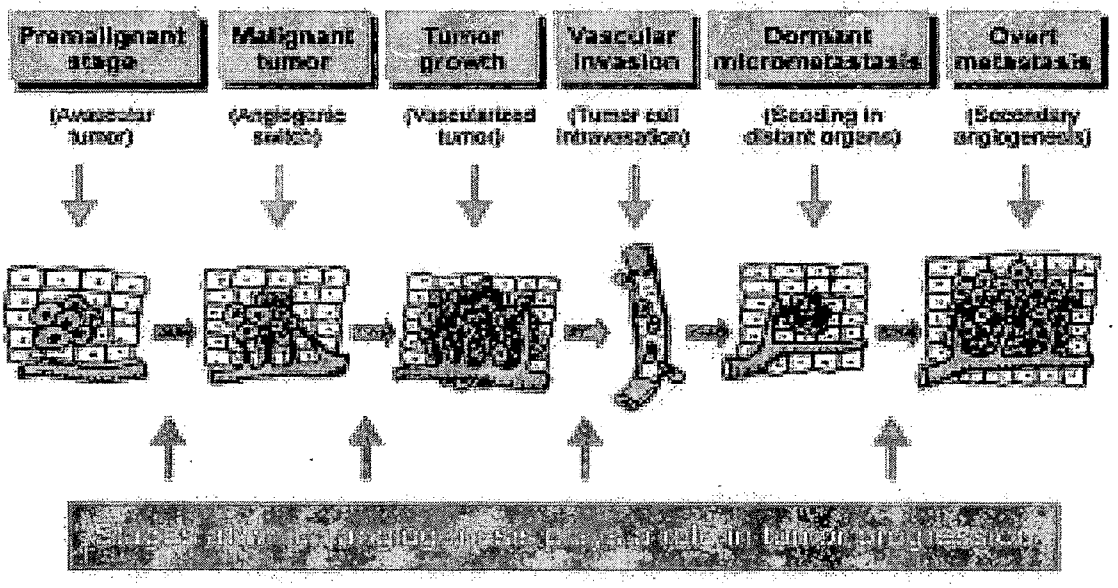


Figure 7.1.2: Angiogenesis in cancer development, progression and metastasis.

Clinical applications will therefore include the administration of an angiogenesis specific for the vascular endothelium in the tumor bed, optimization of the dose schedule of conventional cytotoxic chemotherapy for the vascular endothelium, the targeting of low-dose cytotoxic chemotherapy only to the vascular interstium in the tumor bed. It has been shown that cells in pre-cancerous tissue antigiogenic capacity on their way to becoming cancerous, and it have been that this concept be used to design strategies to prevent cancer. [3] [4]

VEGF-A, expressed, by tumor cells, up-regulates expression of the antiapoptotic protein Bcl-2 in endo' nelial cells in vitro and overexpression of Bcl-2 is sufficient to enhance endothelial cell survival and protect against apoptosis induced by growth factor deprivation.[5] Survivin, a member of inhibitor of apoptosis protein family is also upregulated by VEGF-A, and inhibit endothelial cell apoptosis. Since both Bcl-2 and survivin are upregulated by VEGF-A, inhibition of endothelial cell apoptosis during angiogenesis may occur simultaneously through parallel and non-overlapping pathways. [6]

A defective host antitumor immune response is an important mechanism allowing tumors to evade control by the immune system. VEGF-A via the VEGFR-1 receptor indirectly leads to defective functional maturation of the dendritic cells (important for antitumor immunity) and thus contributes to suboptimal induction of immunity in cancer patients. [7]

7.1.3 VEGF as A Prognostic Factor or an Indicator of Angiogenesis

Researchers have attempted to quantify angiogenesis and provide prognostic information by the measurement of tumor microvessel density (MVD), VEGF determination or monitoring circulating VEGF.

1. Microvessel density. MVD determined immunohistochemically, is thought to be a marker representing the effect of angiogenesis and often correlates with intratumoral VEGF mRNA levels. It is not only important for oxygen and nutrient supply of proliferating tumor cells, but it also reflects the potential for invasion and metastasis.
2. Intratumoral VEGF determination. Intratumoral VEGF content can be measured using several methods, such as immunohistochemistry, in situ hybridization, quantitative immunoassays, Western blotting, or the reverse-transcriptase polymerase chain reaction. Although it is clear that the amount of VEGF-A expressed by tumors affects clinical outcome, none of the techniques used to quantitate tumor VEGF-A expression in solid tumors are routine, and at present it is uncertain if such studies were widely undertaken that they would be clinically useful or cost-effective for predicting outcomes in individual patients. [6]

7.1.4 Cyclooxygenase-2 (COX-2) Expression and Angiogenesis

Inflammatory mediators are also thought to contribute to the process of angiogenesis. One of the earliest observations regarding COX-2 and angiogenesis was made while studying the anti-tumor effect of existing COX inhibitors. It was noticed that a non-selective COX inhibitor, diclofenac suppressed the growth of COX-2 positive eolon-26 cells in nude mice through blocking angiogenesis. [8] Subsequently, studies on corneal models indicated that COX-2 specific inhibitors block new vessel formation. [9] Numerous studies showed co-localization of angiogenesis factors, such as VEGF, bFGF and tumor growth factor-P (TGF-P) with COX-2 by immunohistochemical staining in different cancer types. In breast and cervical cancers, enhanced COX-2 expression has been further associated with increased micro-vascular density (MVD) and with poor prognosis. [10]

bFGF can induce COX-2 messenger RNA expression and protein in several cell lines. In tumor cells. COX-2-derived prostaglandins up regulate the production of growth factors, including VEGF, which can act directly on endothelial cells, and bFGF, which stimulates COX-2 upregulation in fibroblasts. COX-2-derived prostaglandins in fibroblasts stimulate VEGF production, which acts on endothelial cells to again upregulate COX-2 and facilitate vascular permeability and angiogenesis. Prostaglandin E2 by itself has been shown to stimulate angiogenesis in vivo. [11]

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer related mortality worldwide. Recent evidence suggests that COX-2 signaling is implicated in hepatocarcinogenesis and COX-2 inhibitors prevent HCC cell growth in vitro and in animal models. [12]

Several studies showed that regularly taking aspirin or other conventional non steroidal anti-inflammatory drugs (NSAIDs) provides a 40-50% reduction in relative mortality by colon cancer, indicating the inhibition of COX in humans has a chemopreventive effect. However, the ability of NSAIDs to induce apoptosis does not always correlate with their ability to inhibit the COX enzymes. Therefore several COX independent mechanisms have been proposed. The first indication carne from the study of sulindac metabolites. Sulindac is metabolized to sulindac sulfide (an active COX inhibitor) and sulindac sulfone (not an nhibitor of COX). But both metabolites induced apoptosis with similar efficiency in cell culture models.[13] From another study carried out with PC3 prostate cancer cells with varying levels of COX-2 protein expression, it was found that apoptosis induced by both celecoxib and its non-COX-2 inhibiting derivatives was similar regardless of the levels of COX-2 protein, which support the COX independent function of NSAIDs. [14]

7.1.5 In vitro and In vivo Models to Study Angiogenesis

In vitro research has mainly focused on proliferation and migration of endothelial cells. For human research most laboratories make use of Human Umbilical Vein Endothelial Cells (HUVECs). But the major drawback of these cells is their macrovascular origin, which makes them less suitable for studies on angiogenesis which is a microvascular process. Assays to study proliferation of endothelial cells are based on cell counting or radiolabeled thymidine incorporation, or on colorimetric systems for measurement of mitochondrial activity. Another most common assay system to measure migration of endothelial cells is the growth factor induced sprouting or capillary-like structures from endothelial cells grown on a thick gel matrix of either matrigel or collagen. Assays based on the sprouting of capillaries out of fresh tissue embedded in matrix gels more closely reflect the in vivo condition. This makes use of rat aortic rings or human placental tissue.

Advantages of these in vitro systems include the possibility to control the different parameters (i.e. the spatial and temporal concentration of angiogenic mediators) involved, the ability to study individual steps in the angiogenic process, and the lower costs and efforts, as con-oared to in vivo experiments. However, the great diversity in the reagents (endothelial cell origin and passage number, content of matrigel substrate, growth media, etc) that are being used makes comparison between different studies rather difficult. [15]

To study angiogenesis in vivo, the most frequently used assay systems are the chicken chorioallantoic membrane (CAM) assay, [16] the corneal pocket, transparent chamber preparations such as the dorsal skin-fold chamber [17] and the polymer matrix implants. [18] However, in vivo assays also have several disadvantages: the pharmacokinetic properties of the compounds tested, necessary for proper interpretation of results, are often not known and the host will respond nonspecifically to the implantation.

7.1.6 Modalities for Therapeutic Angiogenesis Inhibition

7.1.6.1 Naturally Occurring Angiogenesis Inhibitors

Endostatin (a 20-kDa C terminal fragment of collagen XVIII) specifically inhibits endothelial proliferation and potently inhibits angiogenesis and tumor growth. Systemic administration of human endostatin potently inhibits the growth of Lewis lung carcinoma and B16F10 melanoma in mice without detectable toxicity and resistance. [20] **Angiostatin** is a 30-kDa circulating endogenous, anti-angiogenic protein, which binds ATP synthase on the surface of human endothelial cells and induces apoptosis in endothelial and tumor cells. Remarkably, no drug resistance occurs with endostatin or angiostatin and when they are combined, the tumors do not recur once treatment is suspended. [20]

7.1.6.2 *Antagonists of Angiogenic Growth Factors*

Angiogenesis can be blocked by monoclonal antibodies (mobs) to VEGF or its receptor and by small molecule inhibitors of the tyrosine kinase activity of the VEGFRs. Small molecules inhibiting the tyrosine kinase domain of VEGFR as SU5416 (Sugen) have been shown to effectively and selectively inhibit angiogenesis in vitro and in vivo. [22] Inhibition of angiogenesis has beei shown with mAbs against bFGF and their derivatives, which bind to and complex bFGF as well as platelet derived growth factor (PDGF) and prevents them from binding to their receptors. [23] Small molecules binding to the intracellular kinase domain, inhibiting ATP binding such as SU6668 a broad spectrum receptor tyrosine kinase inhibitor have been shown to be anti-angiogenic and induce regression of established tumors and are in the process of clinical testing. [21]

Bevacizumab (AvastinTM, Genentech) is a humanized monoclonal antibody has been recently approved by the US FDA as a first-line therapy for widespread metastatic. colorectal cancer. [22]

Thalidomide is being used clinically as an anti-angiogenic molecule. It acts by blocking the activity of angiogenic growth factors such as bFGF and VEGF. It is effective orally and has been found useful in the treatment of malignant recurrent gliomas, especially when given in combination with carboplatin. An open label Phase II study of thalidomide at a dose of 100 mg daily in androgen-independent prostate cancer was conducted to evaluate its efficacy and tolerability. The results indicated that thalidomide could decrease prostate-specific antigen level in patients with androgen- independent prostate adenocarcinoma, suggesting the potential for improved disease control. [23]

It has already been reported that E7820, an aromatic sulfonamide derivative, is a novel angiogenesis inhibitor that inhibits both proliferation and tube formation of human HUVEC

induced by either bFGF or VEGF. Also it was found that E7820 has promising antitumor effects in a preclinical model through inhibition of angiogenesis and that monitoring the expression level of integrin α2 on platelets might be a valuable predictive marker for the biological effect of E7820. [24]

Inhibitors of platelet derived growth factor induced angiogenesis include specific as well as broad-spectrum RTK inhibitors of which imatinib mesylate (Gleevec) is approved for the treatment of myeloid leukemia and gastro-intestinal stroma tumors and is in the phase of clinical trial for other malignancies. Inhibitors of epidermal growth factor receptors (EGFR) include mAbs (as Erbicax), small molecule inhibitors (as Iressa) lave shown promising results in clinical trials. [25]

7.1.6.3 Gene Therapy in Angiogenesis Research

New targets in tumor endothelium have been identified by comparing libraries of genes [e.g. SAGE (Serial analysis of gene expression)] constructed from isolated endothelial cells from tumors and normal tissue. [26] Furthermore, the rapid development of tools for in silico analysis of gene expression, among others supported by the online availability of data via the Cancer Genome Anatomy Project (CGAP) offers additional opportunities to identify new genes of interest for vascular targeting. [26]

7.1.6.4 Miscellaneous Factors and Conditions

Anti-inflammatory drugs affecting the coagulation cascade, as the NSAIDs, aspirin and celecoxib showed anti-tumor, anti-angiogenic effects through decreased VEGF-production. The **antibiotic fumagillin-analogue TNP-470** inhibits endothelial cell (EC) migration and proliferation, tube formation and tumor growth and metastasis by inhibiting methionine aminopeptidase II. It proved to be effective in clinical trials alone and in combination with other agents. **Combrestatin A-4** is a tubule-polymerisation interfering compound specifically inhibiting endothelial migration as well as inducing apoptosis in proliferating EC and tumor cells, leading to extensive tumor necrosis in vivo (Bouis et al 2006). Angiogenesis inhibition by **Cephalotaxine alkaloids** (Cephalotaxus fortunie Hook and other related species), its derivatives and compositions were reported and granted a US patent [27].

Hypericin, a perihydroxylated dianthraquinone was proved to be a highly potent inhibitor of angiogenesis in several ocular models examined in rat eyes. Extensive angiogenesis induced in the cornea and iris by intra-ocular administration of FGF-2 was selectively inhibited by hypericin administered via the intraperitoneal route. These findings identified hypericin as a potentially useful agent in the treatment of ophthalmic neovascularization pathogenesis. [28]

Novel substances designated as ICM0301A-H were isolated from the culture broth of Aspergillus sp. F- 1491. ICM0301s inhibited the growth of HUVECs induced by bFGF with IC50 values of 2.2-9.3 pg/ml. ICM0301A showed significant anti-angiogenic activity at lower than 10 mg/ml in the angiogenesis model using rat aorta cultured in fibrin gel. Furthermore, ICM0301A did not show any toxic symptom in mice by intraperitoneal injection at a dose of 100 mg/kg. [29]

Angiogenesis is a complex process involving an overabundance of activating and inhibiting factors. Nevertheless, the field of angiogenesis research is at an exciting stage, with important advances made in sorting out the regulatory pathways involved in the various steps that take place during angiogenesis. The cellular players, soluble factors, and environmental conditions that are able to affect one or more of the angiogenic steps have been identified in many studies. Therapeutic intervention inducing or enhancing angiogenesis with single angiogenic factors (VEGF, bFGF) has shown some success, yet the induced neovasculature might be immature, leaky and lacking periendothelial support. Therefore, the combination of angiogenic factors should be envisioned. As many angiogenic pathways are surplus, therapeutic inhibition of angiogenesis might need an even broader approach than might be required for angiogenesis induction. The elucidation of the human genome and subsequent gene function studies are likely to deliver a multitude of promising new factors for pro- and anti-angiogenic therapies. Angiogenesis inhibitors, when administered as protein therapeutics as well as in gene therapy studies, strongly affect tumor outgrowth. A prerequisite for these newer strategies is the specific delivery of the effector molecules to the tumor vasculature.

In the coming years, it will be a great challenge to untangle the 'underlying vs the significant' relation and the spatiotemporal misbalance in pro- and antiangiogenic entities in human disease in light of the development of better therapies. In this respect, the advent of novel molecular biological techniques such as the isolation of single cells from patient biopsies and subsequent profiling of gene expression with DNA array technology will be a helpful tool. However, it can be anticipated that the products approved to appear

on the market are approved/likely to be approved not as single agents, but as a part of a combination regimen with other traditional chemotherapeutic agents.

7.1.6.5 Gold Nanoparticles as an Anti-angiogenic property:

An amazing feature of gold nanoparticles is the antiangiogenic properties. [31, 32] Angiogenesis, the formation of new blood vessels from existing ones, plays an important role in the growth and spread of cancer. This process is also important for the promotion and maintenance of other diseases like neoplasia and rheumatoid arthritis. New blood vessels "feed" the cancer cells with oxygen and nutrients, allowing these cells to grow, invade nearby tissue, spread to other parts of the body, and form solid tumors. [33] Gold nanoparticles can interact selectively with heparin-binding glycoproteins, such as VEGF165, a critical cytokine for the induction of angiogenesis. Recently, Mukherjee et al. (2005) demonstrated that AuNPs inhibit the vascular endothelial growth factor 165-isoform (VEGF165). VEGF165 is an endothelial cell (EC) mitogen and a prime mediator of angiogenesis that plays an important role in pathological neovascularization including rheumatoid arthritis (RA), chronic inflammation, and neoplastic disorders.

This is the first example of an inorganic compound that is anti-angiogenic in nature and opens up a new area of research using inorganic nanoparticles as anti-angiogenic agent. [33]. Specifically, AuNP binds to the heparin-binding domain in VEGF165. [34] It is now recognized that angiogenesis plays a key role in the formation and maintenance of pannus in RA. The new blood capillaries promote synovial inflammation by transporting the inflammatory cells to the site of synovitis in RA. It is found that VEGF levels in serum, synovial fluid, and in inflamed synovium of RA patients are elevated. Therefore, AuNP therapy could be a potential treatment option for Rheumatoid arthritis.

7.1.6.6 In vivo Anti-tumor Testing

Rodent models provide an important means of assessing anti-tumor activity vs toxicity for new developmental drugs as well as new formulations of already established drugs. Syngeneic mouse and rat models played important role in early cancer drug development but these models have largely been surpassed by the use of xenografted human solid tumors in athymic (nu/nu) mice, in severe combined immunodeficiency (SC1D) mice, or in rats. [33] Although the limitations of rodent models in predicting clinically active agents are well recognized, they still provide an important component of preclinical testing. Significant responses in multiple xenograft models increase the possibility of a new drug having clinical activity. [34]

Solid tumors are often grown subcutaneously on the flank or back of animals to allow an accurate serial determination of tumor volume with caliper by measuring the tumors in different dimensions . [35]

7.1.6.6.1 Establishing Subcutaneous Xenograft [35]:

1. Harvest tumor cells that are 75-80% confluent, highly viable and in logarithmic growth phase. It is preferred to grow cells in antibiotic-free medium to avoid masking microbial contamination.
2. Viable cell number is determined by hemocytometer counting using tryptan blue, and 5- 50 million tumor cells (depending on tumorigenicity and growth rate) are injected subcutaneously between the shoulder blades of athyhmic mice under strict aseptic conditions with a 1 cm^3 syringe and a 19 guage needle.
3. For injection, cells are suspended in culture medium without fetal bovine serum (FBS) such that a total of 200 µL are injected to deliver the desired cell dose.
4. Mice are observed biweekly and tumor growth monitored when tumors become palpable. The lag phase varies from 2 to 12 weeks depending on the amount of cells injected, the tumor type and the cell line employed.
5. When tumors reach about 1.5 cm^3, two to four mice are sacrificed, the skin over the tumor disinfected and the tumors removed under aseptic conditions.
6. The tumor cells are forced through a sterile 80-160 µm stainless steel mesh strainer, and mixed with cell culture medium to form slurry, such that injection of 200 µL subcutaneously between the shoulder blades of 20-25 mice delivers a tumor cell dose of approx 5-50 million cells, depending on how aggressive the tumor is?

7.1.6.6.2 *Assessing The Volume of Subcutaneous Tumors by Caliper Method* [36]

1. Tumors should be measured beginning when they are first palpable: the length (*L* = longest dimension/diameter), the width (*W* = the shortest diameter). The ellipsoid volume of the tumor (TV), calculated from ($L \times W^2$) x 0.5 provides the most accurate measure of tumor mass.
2. For multilobed tumors or those that grow in irregular shapes, the tumor should be divided visually into two, three or four lobes of similar dimension that are measured separately. The calculated volume for each lobe can be summed to obtain the volume of the entire mass.

7.1.6.6.3 *Determination of Therapeutic Effect in Murine Tumor Models* [37]:

Subcutaneous tumor models have the advantages of providing visual confirmation that 100% of the mice used in an experiment have tumors prior to therapy; and (2) providing a means of assessing tumor response or growth over a period of time.

Regardless of whether survival data or tumor measurements are the primary end points, mice should be weighed throughout the course of the experiments, as body weight provides another means of assessing toxicity, usually done in terms of percentage change of body weight from the weight at start of the experiment.

1. Subcutaneous tumor volumes should be measured as described earlier.
2. Data should be presented graphically [Tumor volume (mm^3) vs Time in days] in which the growth over time for averages of this data can be displayed or calculated.
3. For models in which treatment begins after tumor growth is documented, often when tumors are at a size of 30-100 mm, usually two measurements showing increasing tumor size prior to therapy are sufficient to establish tumor engraftment and progression.
4. Tumor volume should be measured twice weekly. The T/C ratio can be calculated as a T/C ratio = days to obtain a predefined tumor mass for the treated animals divided by the days to obtain the same size mass in the control animals.
5. Data can also be presented as a percent increase in lifespan (% ILS), which is calculated in days from initiation until a death for treated vs control as,
   % ILS=[(T- C) / C] x 100.

7.2 EXPERIMENTAL

7.2.1 Materials

- The cancer cell cultures were obtained from National Cancer Institute (NCI), USA. B16F10 (melanoma) cells
- RPMI-1640 medium and fetal bovine serum (J BS) was purchased from Gibco Corp. New Jersey.
- Reagents and solvents required for the preparation of cell culture medium were procured from S.D. Fine Chemicals and Qualigens Chemicals, respectively (Appendix - I).

7.2.2 Method

1. *In vivo* Tumor Growth Inhibition Studies

Animals: Female BDF1 mice (18-20g, 4-5 weeks of age) were obtained from 'Animal House' of Advanced Centre for Treatment, Research and Education, Navi Mumbai and kept under the controlled conditions (temperature: 22±2°C & relative humidity: 55±5%) with 12hr/12hr light/dark cycles. Standard rodent pellet diet (Amrut, Pranav Agro Ind. Ltd., Sangli, India) and tap water was provided ad libitum to the animals. The Institutional Ethics Committee for 'Animal Care and Use' of Advanced Centre for Treatment, Research and Education, Navi Mumbai, India had approved the animal study protocol. Animal care was in accordance with CPCSEA guidelines.

B16F10 melanoma cells were used for tumor induction in animals. Mice were subcutaneously inoculated on day zero in abdominal region with 10^6 B16F10 melanoma cells each in sterile phosphate buffer saline (PBS). Animals were divided randomly into three groups and individual animal weights were recorded on day zero. Starting from day one, Group I was given injections via tail vein saline sterile phosphate buffer saline (PBS). Group II & Group III received treatment with 2 mg/kg plain doxorubicin drugs & gold nanoparticle complexed with doxorubicin. suspended/dissolved in PBS sterile phosphate buffer saline.

The mice were weighed every alternate day throughout the experiment. The day of tumor detection was recorded and the dimensions of the tumors were measured every 48 hr with calipers. Tumor volume (TV) was calculated in accordance with the equation, tumor volume (mm) =length (mm)xwidth^2 (mm^2)x0.5.

At the end of study the mice were sacrificed by cervical dislocation and the primary tumors and secondary tumors, if present were excised, appropriately photographed and fixed in 10% phosphate buffered formalin until further evaluation. The tumor development study lasted 21 days.

2. Tumor Microvessel Density (Tumor Angiogenesis) Evaluation

To evaluate tumor microvessel density, tumors were processed using an auto-technicon apparatus through increasing concentrations of ethanol and infiltrated in paraffin (melting point 58-60^{0}C). In order to evaluate microvessel density within the tumor mass, histological; sections of 5μm thick were obtained. Tumor microsections were stained with hematoxylin-eosin. The slides were observed by in independent observer under low-power microscopy and the angiogenesis response (tumor microvessel density) was scored as 4 (marked), 3 (moderate), 2 (mild), 1 (minimal) and 0 (complete absence).

7.2.3 Results & Discussion:

Mouse Melanoma tumor model was used in order to evaluate antitumor effects of in vivo. After the administration of total 7.2 mg/kg of doxorubicin-gold nanoparticles or doxorubicin alone. there was a significant difference in reduction of tumor growth and ascites formation between doxorubicin-gold nanoparticle and control groups. These results suggest to that although the low dose of Doxorubicin-gold nanoparticles treatment was used in this model, gold nanoparticles could facilitate the potency of doxorubicin. The low dose doxorubicin treatment, however, did not significantly reduce the tumor growth compared to Phosphate buffer control group. Inhibition of ascites formation was observed both in Doxorubicin-Gold nanoparticles, doxorubicin & gold nanoparticles groups that may attribute to the administration of chemotherapeutic agent and the antiangiogenic properties of gold nanoparticles.

7.2.4 In vivo Tumor Growth Inhibition in Mouse Melanoma Model:

7.2.4.1 Doxorubicin Gold Nanoparticles: From the Figure: it was evident that B16F10 melanoma cells injected subcutaneously into mice developed tumors reaching the size of approximately 3000 mm^3 in 21 days (control group). Treatment of animals with doxorubicin from the next day of tumor cell inoculation significantly retarded the growth of these primary tumors in a dose dependent manner. The tumor growth (expressed in terms of tumor volume) was found to be retarded to approximately 70 mm^3 and 176 mm^3 on day 21 with a multiple dose of 4,6 mg/kg, respectively in comparison to untreated control. Administration of doxorubicin as gold nanoparticle compelx containing equivalent dose of free doxorubicin improved the tumor growth retarding efficacy of plain doxorubicin. The dosing with doxorubicin-gold nanoparticle complex retarded the tumor growth to approximately 25 mm^3 and 86 mm^3 on day 21, respectively. Gold nanoparticle complex caused tumor growth delay in comparision to untreated control and plain drug treatment.
In all animals receiving doxorubicin-gold nanoparticle complex tumors were detectable only on day 11-13. In contrast, in control and plain drug treated animals, tumors were detectable from day 7-9.

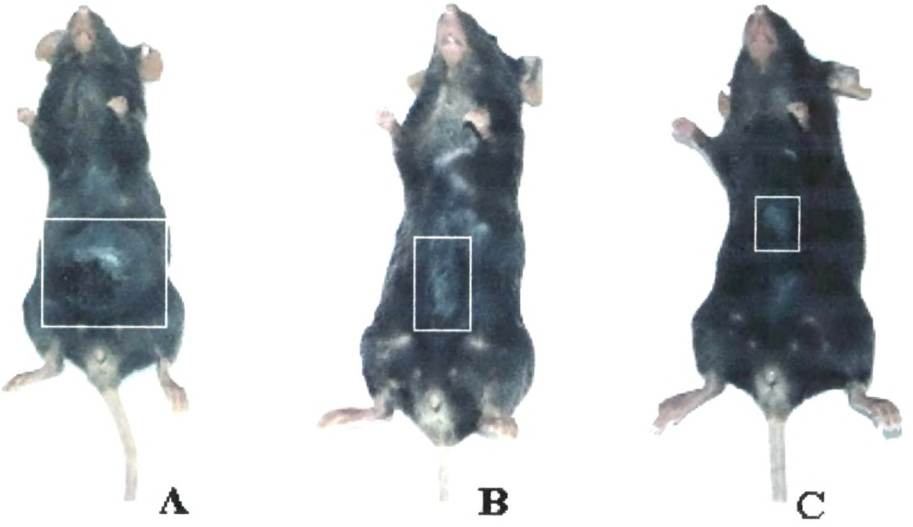


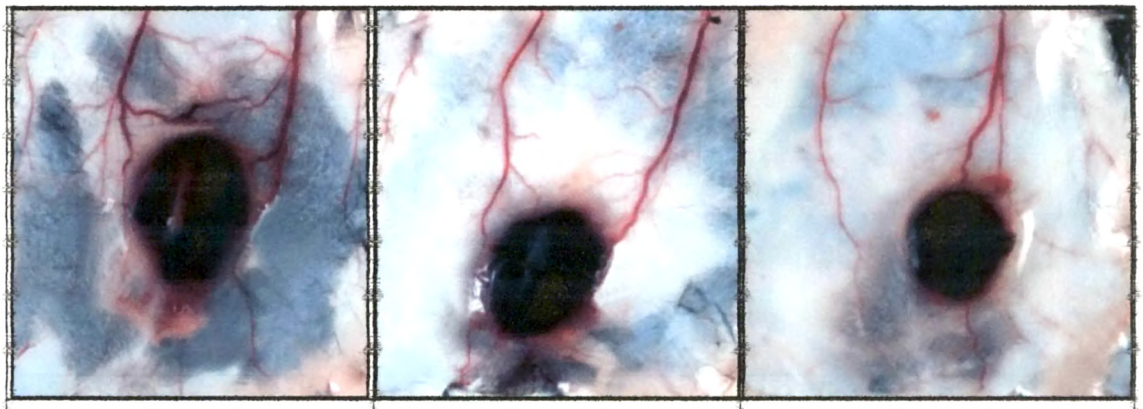
Figure 7.2.2: Tumor in BDF1 mice. At day 21 after tumor cell inoculation in control (A) tumors appeared as raised melantonic nodule. In contrast, with plain drug doxorubicin & its equivalent doxorubicin-gold nanoparticle complex treated appeared significantly smaller (B, C).

3. Tumor Angiogenesis Inhibition Study (Tumor Microvessel Density Evaluation)

Representative photomicrographs of hematoxylin-eosin stained tumor microsections (Figure:7.2.2) showed a dense vascularization in tumors of control group animals. Tumors

treated with plain drug doxorubicin had significantly fewer micro vessels (*Kruskal-Wallis test*; Dunnett s test, $\alpha = 0.05$) compared with the control. In fact, as evident from Table, the ant -angiogenesis effect was found to be m significantly improved (Tukev's HSD test and Dunnett's test, $\alpha = 0.05$) in the tumors of mice receiving drug-cyclodextrin complex in comparison to plain drag and control group (Tukey's HSD test and Dunnett's test, $\alpha = 0.05$).

7.2.4.2 Effect of combination drug treatment: Representative photomicrographs of stained tumor microsections (Figure: 7.2.3) showed that a marked/dense microvasculature was observed in the control tumors. Tumors treated with individual drugs had significantly fewer microvessels compared with the control (Kruskal-Wallis test; Dunnett's test, $\alpha = 0.05$). In fact, as summarized in Table 4, the anti-angiogenesis effect was found to be significantly improved (Tukey's HSD test and Dunnett's test, a = 0.05) in the tumors of mice receiving combination drug treatment in comparison to control and individual drug treated animal tumors.



Control	Treated	Treated

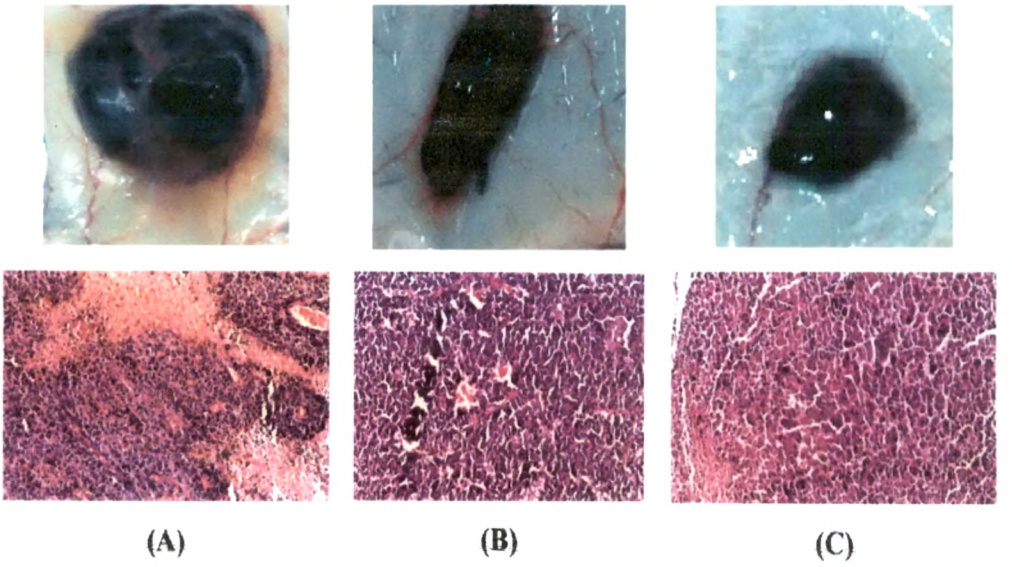


Figure 7.2.3: Peritumoral vascularization and microvessel density in B16F10 melanoma tumors at day 21 in BDF1 mice. A network of tumor feeding vessels was visible at the time of tumor excision in the subcutaneous tissue of control tumors (A) but has barely developed around tumors of drug treated mice (B, C). In control tumors (A), dense microvasculature (red colored patches) appeared throughout the tumor section whereas, tumor microvessel density was significantly reduced in plain doxorubicin treated animal tumors (B) and completely diminished in the tumors after treatment with Doxorubicin-gold nanoparticle complex (C).

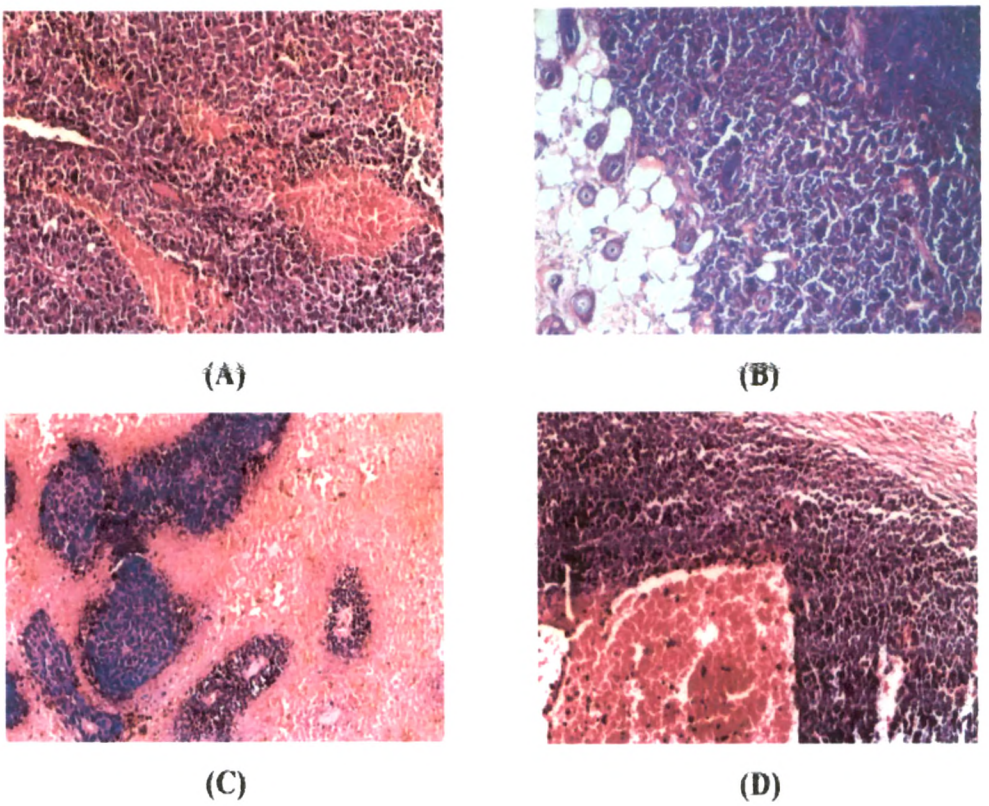


Figure 7.2.4: Tumor microvessel density in tumor microsections after hematoxylin-eosin staining. In control tumors (A), in plain Doxorubicin (2 mg/kg) treated animal tumors (B), Doxorubicin Gold nanoparticles treated animal tumors (C) and combination Doxorubicin-Gold complex Sophorolipid treatment animal tumors (D).

Groups	Degree of Angiogenesis
Control	3, 3, 3 ^{a,b,b,,b,,}
Plain Doxorubicin	2, 1, 1 ^{a,b,c,e}
Doxorubicin-Gold nanoparticle complex	2, 2, 2 ^{a,b,d,e}
Doxorubicin-Gold nanoparticles Sophorolipid treatment	0, 0, 0 ^{a,b,,c,d}
^{a} significant (Kruskal-Wallis test, α=0.05); $^{b,b',b''}$ significant (Tukey's HSD test, Dunnett's test, α=0.05); ^{c,d,e} significant (Tukey's HSD test, α=0.05).	

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