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Abstracts of the conference: Endothelial Cell and Tumor Angiogenesis

1. ENDOTHELIAL CELL: FUNCTION AND DYSFUNCTION

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ABSTRACT

A cell with a simple structure and complex functions. A squamous type of epithelium considered initially a gratuitous cellophane-like sheet, the endothelial cells (EC) have earned in time, the respect of biologists and pathologists. *Biologists discovered* that the EC are endowed with all common cellular organelles, coated pits and vesicles, a high number of vesicles (caveolae), transendothelial channels, specific Weibel-Palade bodies, and differentiated microdomains of the plasmalemma. They are social cells establishing homotypic and heterotypic intercellular junctions. ECs monitor transcytosis of plasma molecules, function in endocytosis, synthesis and secretion of matrix components, guard the vascular tone, administer hemostasis, immunity, angiogenesis and have other functions that insure the body homeostasis. Furthermore, the EC possess an innate heterogeneity, expressed by differences in their structure and function according to the tissues in which they reside.

Aggressive factors induce EC dysfunction. *Pathologists discovered* that the heterogeneity of EC concurs to a blood vessel-specific pathology: i.e. atherosclerotic plaques develop in arteries, thrombosis in veins and vascular leakage occurs in venules. To insults EC respond gradually, initially by modulation of constitutive functions, which is followed by EC *dysfunction*, and only ultimately by *injury* and apoptosis. As example, hyperlipemia with or without hyperglycemia leads to enhanced transcytosis of plasma LDL, increases EC biosynthetic activity expressed by augmented synthesis of matrix components, MCP-1 and new cell adhesion molecules which function in recruitment and diapedesis into the intima of inflammatory circulating cells. Ultimately, lipid accrual leads to the formation of EC-derived foam cells and eventually to EC death.

Endothelial Cells: a therapeutic target and therapeutic tool. Modern approaches for the treatment of cardiovascular disease (CVD) include specific targeting of EC altered mechanisms and molecules such as MCP-1, chemokine receptor antagonists, drugs designed to act on specific enzymes involved in the intracellular signaling cascade or inhibitors of vascular specific NAD(P)H oxidase. As tools, endothelial progenitor cells derived from bone marrow or vessel wall stem cells are a novel option for replacement of damaged EC (re-endothelialization) as well as for neovascularization of ischemic tissues.

Instead of conclusion: the wealth of data on the vital role of EC in health and diseases generated a complex discipline that theoretically includes: **endotheliology,** the investigation of normal EC functions; **endotheliopathy,** the search for the altered mechanisms in vascular diseases and **endotheliotherapy,** the quest for novel drugs targeted specifically to dysfunctional EC, a promising venue for the reversal of CVD.

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2. THE ROLE OF MAST CELLS IN TUMOR ANGIOGENESIS

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ABSTRACT

The current wisdom is that tumors are endowed with an angiogenic capability and that their growth, invasion and metastasis are angiogenesis-dependent. It is now well documented that neoplastic cells are influenced by the their microenvironment and vice versa. The specific organ microenvironment

determines the extent of cancer cell proliferation, angiogenesis, invasion and survival. Tumor cells are surrounded by an infiltrate of inflammatory cells, namely lymphocytes, neutrophils, macrophages and mast cells (MC), which communicate via a complex network of intercellular signaling pathways, mediated by surface adhesion molecules, cytokines and their receptors.
In my presentation, I will summarize:

i) the MC mediators involved in angiogenesis;
ii) the experimental evidence concerning the role played by MC in angiogenesis;
iii) the list of solid and haematological tumors in which a close relationship between angiogenesis, tumor progression and MC has been demonstrated;

the circumstances in which MC are a critical source of angiogenic factors *in vivo*, and in such cases, the signals that regulate their production and secretion that need to be determined as a prelude to the elaboration of new therapeutic strategies associated with MC presence and activation.

3. CLINICAL SIGNIFICANCE OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IMMUNOHISTOCHEMICAL EXPRESSION IN SOLID TUMORS

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ABSTRACT
Angiogenesis is the process of blood vessels formation from preexisting vessels and has been demonstrated to play a crucial role in growth and differentiation. As Folkman pointed out three decades ago, solid tumors without neovascularization do not grow over 2-3 mm in diameter. The angiogenic phenotype of a malignant tumor is mainly but not exclusively, characterized by the ability of tumor cells to secrete growth factors. From them, vascular endothelial growth factor (VEGF) seems to be the most powerful in terms of proliferation and migration of endothelial cells.
The immunohistochemical demonstration of VEGF in normal tissues and malignant tumors became a usual investigation of the angiogenic switch and angiogenic phenotype. The final reaction product is cytoplasmic with granular pattern. VEGF is secreted by a large variety of normal cells, as others and we demonstrated in the normal human kidney, stomach, thymus, epidermis, and urothelium. A strong reaction is constantly noticed in the epithelial cells of the kidney tubules that probably represent the best external control of the reaction.
Many solid human tumors express VEGF, but the incidence of positive cases depends on the site of the primary. VEGF scoring includes the intensity of the final reaction product and number of positive cells. In renal cell carcinoma VEGF expression was found in 75.5% of cases and results does not correlate with microvessel density, histopathologic form and Fuhrman's nuclear grade. In these cases, tumor cells also express VEGF-1 and –2. In gastric carcinoma a positive reaction was found in 70% of cases; a positive reaction was noticed in the associated intestinal metaplasia, and in mucus-secreting glands of the normal gastric antrum. The colorectal carcinoma became a favorite target for anti-VEGF humanized monoclonal antibody, based on the expression of VEGF by tumor cells in over 50% of cases. In thymoma, the VEGF expression correlates with the pathological form and has the strongest intensity in thymoma B3. A correlation was found between VEGF expression, microvessel density and the invasive character of thymoma. Of particular interest is the breast cancer, which express VEGF in early stages, but just rarely in advanced stage; in this tumor, the initiation of tumor angiogenesis is induced only by VEGF, and as the tumor progresses, other angiogenic factors are secreted by both tumor cells and normal cells of the stroma. This could be an explanation for the reduced number of cases with advanced-stage breast cancer that express VEGF. Besides its prognostic value, as demonstrated by others for many solid tumors, VEGF expression (together with specific phosphorilated receptors) is a marker of the angiogenic phenotype and a target for therapy.

4. HUMAN ENDOTHELIAL CELL CULTURE: A USEFUL TOOL FOR ENDOTHELIAL RESEARCH

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ABSTRACT
Blood vessels consist of endothelial cells (EC) that are in direct contact with the blood, and pericytes, smooth muscle cells, fibroblasts, basement membrane and extracellular matrix, which are subendotelially located. Depending on body region, the cellular and non-cellular constituents of the vasculature differ in composition, phenotype and function.
The endothelium is a confluent monolayer of thin, flattened, rhomboid-shaped cells lining the intimal surface of all blood vessels, including veins. In the adult human, the endothelium is composed of approximately 1 to 6 x 10^{13} cells. The net mass of the endothelium is 1 kg and has a surface area of approximately 5,000 m^2.
The endothelium plays a key role in cardiovascular homeostasis through its diverse influences on blood vessel structure and function. Regulation of vascular smooth muscle tone, hemostasis and fibrinolysis control, participation to immune surveillance, inflammation, and atheroma formation, as well as metastasis development are the most important phenomena in which endothelial cell are involved.
The following human endothelial cell cultures are available:
- umbilical vein endothelial cells - represent the most used and studied human endothelial cells, because they are easily available, are normal cells, and probably physiologically more relevant than many established cell lines.
- arterial endothelial cells (aortic EC, pulmonary artery EC, coronary artery EC, iliac artery EC).
- venous endothelial cells (saphenous vein EC, iliac vein EC).
- microvascular endothelial cells; microvascular EC have been successfully isolated from many human tissues (adipose, brain, dermal, endometrial, gastric, heart, intestinal, liver, lung, placenta, renal, synovial, and tonsil tissue) using paramagnetic dynabeads coated with a specific ligand (such as CD31/PECAM).

This presentation reviews human endothelial cells types available for research, the protocol for isolation and culture of human umbilical vein endothelial cells, as well as human endothelial cells derived from hematopoietic stem cells. Finally, aspects concerning endothelial seeding of vascular grafts, and circulating endothelial cells are detailed.
Key words: human endothelial cells, culture, stem cells, endotheliazation of vascular grafts

5. TUMOR ANGIOGENESIS: REGULATION BY HYPOXIA AND MACROPHAGES

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ABSTRACT
Aims: We have investigated the molecular mechanisms of angiogenesis in stroke, breast cancer, and two distinct types of brain tumors, namely glioblastoma and hemangioblastoma.
Methods: Northern blotting, Western blotting, Luciferase assays, in situ hybridization and immunohistochemistry was performed. Transgenic and knock-out mice were analyzed in addition.
Results: In all pathologies examined, we observed upregulation of VEGF and of VEGF receptors –1 and –2 in a cell type scecific fashion. VEGF was predominantly expressed around necrotic areas, suggesting a hypoxia-dependent upregulation of the VEGF gene in vivo. Whereas Angiopoietin-1 and tie2 expression was not significantly altered, we observed a cell-type specific upregulation of Angiopoietin-2 in vascular cells, suggesting a modulation of tie2 function mainly via Ang-2 expression levels. The expression of VEGF, VEGFR-2 and Ang-2 witin the same tumor microenvironment (e.g. in perinecrotic regions) suggests a connection between VEGFR-2 and Tie-2

signaling pathways. The hypoxia-inducible transcription factors HIF-1a and HIF-2 a were also up-regulated in stroke and tumor specimens, suggesting that hypoxic induction of several genes underlies the observed vascular phenotypes.
Conclusions: Our results thus suggest a coordinate upregulation of genes (synexpression groups) mediated by hypoxia or by hypoxic mimicry (e.g. von Hippel Lindau Tumor Suppressor gene loss of function) which mediate the vascular and metabolic responses to hypoxic/ischemic and neoplastic brain injury.

6. PRO-ANGIOGENIC MYELOID PROGENITORS CIRCULATE IN BLOOD AND TARGET TUMORS

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ABSTRACT
Objective: Angiogenesis has been shown to be a prerequisite for tumor growth and metastasis, and a number of anti-angiogenic molecular therapies have been developed to suppress tumor growth. Pro-angiogenic bone marrow-derived progenitor cell populations (BM-PCs) which home to tumors have been shown to be suitable cellular vectors to efficiently eradicate tumors by suicide gene therapy (De Palma, Nat Med 14:1193, 2003; De Palma, Cancer Cell 8:211, 2005). We therefore asked which type of HPCs may be mobilized into blood and whether they have the capacity to form pro-angiogenic cells in solid tumors or to metastases. **Methods:** The Lewis Lung Carcinoma (LLC) and the highly metastatic melanoma B16 (B16-F10) cell lines were established in C57/BL6 mice. Transplantation of CD45.1 and CD45.2 mouse strains and of donor bone marrow cells from beta-actin/GFP C57/BL6 mice was used to follow bone marrow origin of the BM-PCs. Flow cytometric analysis and cell sorting were used to enumerate and to purify BM-PCs, and immunohistochemistry to follow the intra-tissue localisation of bone marrow-derived cells with proangiogenic phenotypes. Results: We found incorporation of bone marrow-derived cells into both LLC tumors and B16 metastases in C57/BL6 mice within a period of ~ 9-20 days. Tissue localisation of migrated BM-PCs in the tumors was confirmed using immunohistochemistry of CD45 (BM-PCs) and CD31 (endothelial cells) antigens. Flow-cytometric analysis demonstrated the presence of the pro-angiogenic Tie 2-expressing monocytes (TEMs, CD45+Tie2+) and endothelial progenitor cells (EPCs), which were CD45+VEGFR2+. Both populations co-expressed CD11b/Mac1. In their blood, tumor-bearing mice showed increased numbers of myelopoietic colony-forming cells (CFCs) and lineage marker (lin)-Stem Cell Antigen (Sca 1) + progenitor cells.
Flow cytometric cell sorting identified a population of lineage marker (CD4, CD8, CD45R0, TER119)-negative, CD11b+VEGFR2- cells from bone marrow as a precursor for EPCs in clonogenic assays, which during culture generate CD45+VEGFR2+CD11b+ EPCs. In contrast to the lin- CD11b+ VEGFR2- HPCs, both CD45+VEGFR2+CD11b+ EPCs and CD45+tie2+CD11b+ TEMs isolated from the tumors were found to be non-clonogenic in these progenitor assays. **Conclusion:** Myeloid lin- progenitors circulate in the blood of tumor-bearing mice and are a potent source for tumor-infiltrating pro-angiogenic cells. Tumor-bearing mice show enhanced mobilisation of these myeloid progenitors, resulting in accumulation of pro-angiogenic TEMs and EPCs in tumors. BM-PCs generated from lin- progenitors could thus be an attractive cellular therapeutic to target the tumor vasculature.

7. HETEROGENEITY OF TUMOR BLOOD VESSELS: CLINICAL AND THERAPEUTIC ASPECTS

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ABSTRACT

Tumor vasculature is irregular, abnormal, and essential for tumor growth. Heterogeneity of blood vessels distribution is well documented in surgical specimens. Each tissue has a specific vascular network adapted to its function. The heterogeneity of tumor blood vessels is closely linked to the tumor microenvironment. By the time of diagnosis, malignant neoplasm is biologically heterogeneous and consists of cells with different properties, morphology, growth rate and ability to induce expression of specific receptors. Some angiogenic factors are overexpressed at the beginning of the process and other in the late stage of tumor blood vessels development. Also, the distribution of angiogenic factors is not similar for various parts of a tumor. The density of tumor blood vessels is not the same for the whole tumor; the tumor exhibits intralesional or zonal heterogeneity of MVD. Tumor vasculature has a chaotic pattern of development. It is composed of vessels with different size, irregular thickness of the wall that is tortuous and leaky. The wall of the tumor blood vessels consists of various types of cells: endothelial cells, endothelial progenitors cells, tumor cells. Basal membrane has different features compared with normal one. Pericyte coverage seems to be an important step in the vascular stabilization but the origin and structure of perivascular cells is subject of debate. Also the extracellular matrix shows structural and molecular differences between tumors Genetic instability of activated endothelial cells could interfere with the efficiency of the antiangiogenic therapy. No model for study of angiogenesis is perfect. None of them characterize properly tumor angiogenesis. Linked with tumor angiogenesis heterogeneity the proper therapy might cover all steps of it. Diversity of tumor vessels formation as multistep process makes us to think that antivascular therapy could be better than antiangiogenic one.

8. EXPERIMENTAL MODEL FOR *IN VITRO* STUDY AND QUANTIFICATION OF NEOANGIOGENESIS

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ABSTRACT
Introduction: Knowledge of angiogenesis molecular mediators is important for understanding its control mechanisms and for the therapeutic approach. Anti-angiogenic tumor therapies require complementary reproductible tools and techniques to assess the individual pattern of tumor neoangiogenesis in order to define the optimal therapeutic approach and to monitor its efficacy for each patient.
Purpose: 1. *In vitro* quantification of neoangiogenesis 2. The study of endothelial cells response to various growth factors (VEGF, FGF-basic, IGF, BMP2, BMP4) and angiogenic inhibitors (TGFβ1, heparin, chemotherapeutic drugs).
Material and method: HUVEC cells were seeded in serum-free medium supplemented with endothelial growth factors, on a fibrin gel, at a concentration of 5 x 10^4 cells/ml. After 24 h another fibrin gel was added on the cell monolayer surface. The probes were visualized with an inverted microscope with X100 and X200 magnitude.
Results: Capillary tube formation was tracked at 12h intervals. An angiogenic response was already observed within the first 24h, consisting in the formation of tubular structures. In the next time intervals, the elongation of the existing structures and appearance of new, vasculary-like, tubular structures was noticed. A neoangiogenesis evaluation score was elaborated based on the following criteria: cell migration and alignment, formation of capillary tubes and their ramification, formation of polygonal structures and capillary network.
Conclusions: Our method of obtaining the fibrin gel is adequate for the *in vitro* formation of capillary tubes from HUVEC cells and probably for the study of angiogenesis in tumoral fragments. This experimental model will allow the evaluation of the angiogenic potential of individual tumors as well as the answer to different antiangiogenic agents.

9. THE EFFECT OF PBLAST49-HVEGF ON VASCULATURE OF CHICK CHORIOALLANTOIC MEMBRANE

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ABSTRACT
Aim. There are few studies with pBLAST49-hVEGF gene in wound healing and treatment of pulmonary hypertension on animal model. The aim of the present study was to describe the dinamic changes of morphology and growth pattern of chick CAM blood vessels after applying several doses of pBLAST49-hVEGF.
Material and methods. Fertilized chicken eggs were prepared following standard protocol. On day three of incubation 2 ml of albumen were removed and we made an window in the shell egg. We started to apply 40 µL diluted pBLAST49-hVEGF on day 7 after initial incubation for the next five days. We monitorized the macroscopic features of the blood vessels growth and also a microscopic changes during the procedure. The fresh CAM was examined by microscopy. An alcian blue saphranin stain was applied to detect mast cells.
Results. The growth of the vessels was rapidly increased after the second application of pBLAST49-hVEGF. There were significantly differences in the number of vessels day by day. We observed a dense network of perfused small vessels with narrow lumen, which had a different growth pattern compared with control egg. The number of mast cells increased after the fourth day of treatment. Ghost vessels phenomenon was observed in few cases. No bleeding, spot hemorrhages or inflammatory events were observed.
Conclusions. The pBLAST49-hVEGF stimulates blood vessels formation in a dose dependent manner and promotes the maturation of functional vascular network. This might be a therapeutic alternative for patients with myocardial or lower limb ischemia.

10. ENDOTHELIAL CELL PROLIFERATION RATE DURING ANGIOGENESIS IN SOFT TISSUE TUMOURS

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ABSTRACT
Introduction. The pathologist finds it difficult to diagnose soft tissue tumours (STT), as they have an unpredictable evolution, and the prognostic tissue factors are less known. The present data doesn't include STT on the list of potential targets for antiangiogenic therapy. The results on the angiogenic phenotype of the STT are controversial, with regard not only to the microvascular density, but also to the angiogenic factors expression. At present, as there are no references on the proliferative potential of endothelial cells from the vessels of these tumors, we have set out to focus on that subject in the present paper.
Material and methods. 54 patients with soft tissue tumours, of which 43 malignant, and 11 benign, were examined. The biopsy samples obtained surgically were processed using the traditional techniques, the sections having been stained, using conventional methods of histopathological diagnosis and grading. The immunohistochemical profile contributed to establishing the final diagnosis of liposarcoma (n=15), malignant fibrous hystiocytoma (MFH) (n=14), leiomyosarcoma (n=9), rhabdomyosarcoma (n=3), fibrosarcoma (n=2) and benign tumors (n=11). The methods for PCNA and Ki67 (clone MIB-1) were used for assessing the proliferation rate. The observations focused on the positive reaction within the nuclei of endothelial and perivascular cells, a semiquantitative type of estimations.
Results. The tumoral blood vessels express Ki67 and PCNA, most endothelial cells being positive at nuclear level, even though the vessel was very big. The reaction for the proliferation marker in the

perivascular cells was more often than not negative, and very rarely positive. However, we noticed a constant correlation between the positivation of tumoral cells and the positive reaction for the proliferation marker at the level of endothelial cells. For most intratumoral blood vessels, the positivation rate for MIB-1 and PCNA was between 48 and 62%. The highest values were obtained for the vessels of the immature and intermediate type. In tumoral cells the index of postive nuclei was significantly higher for PCNA then for MIB-1, with no significant differences for endothelial cells. Perivascular cells are positive only when the endothelial ones are negative, marker of the blood vessel maturation. The endothelial cells proliferation index is correlated with the differentiation degree ($p<0.0001$). Very rarely did we notice a positive reaction in endothelial cells from benign tumours, with a proliferation index below 2%.

Conclusions. The study of the proliferation rate of endothelial and perivascular cells of STT highlights the steps of positivation of Ki67 and PCNA. The endothelial cells within tumour area have high proliferative rate and the reactions are negative in the vessels from the periphery of the tumour. Our results suggest that the proliferation index of endothelial cells may represent useful criteria for the characterisation of the angiogenic phenotype of sarcomas.

11. TUMOR LYMPHANGIOGENESIS. A NEW ROLE OF TUMOR ASSOCIATED MACROPHAGES

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ABSTRACT

Metastasis of malignant tumors to regional lymph nodes is one of the early signs of cancer spread in patients, and it occurs at least as frequently as haematogenous metastasis.[1] However, little is known about the role of tumor lymphangiogenesis in metastasis and whether this process is important for spread via the lymphatics. During the last few years significant insight has been provided into the molecular mechanisms underlying the development of lymphatic vessels and the role of lymphangiogenesis in health and disease due to the discovery of the key lymphatic growth factors VEGF-C and –D and their corresponding receptor VEGFR-3, and, more recently, due to the identification of several specific molecular markers allowing to distinguish blood from the lymphatic endothelium.[2-4]

Breast lymphangiogenesis, as measured by staining with the lymphatic specific marker Podoplanin, is increased significantly during the development of cancer and is clearly associated with lymph node metastasis and worse clinical outcome. [5]

Lymphatic vessels generally arise within the peritumoral stroma, although the lymphangiopoietic vascular endothelial growth factors VEGF-C and –D are produced by tumor cells.[6] In squamous cell carcinoma of uterine cervix, a tumor with an active peritumoral inflammatory reaction, we showed a close association between the amount of peritumoral inflammatory reaction and the peritumoral lymphangiogenesis and lymphovascular invasion, providing evidence for a close relationship between tumor-associated inflammation and tumor-lymphangiogenesis. Further a subpopulation of CD14+, VEGFR-3-expressing monocytes recruited to, and activated at the site of tumor plays an important role in tumor-associated lymphangiogenesis producing large amounts of VEGF-C and inducing local sprouting of pre-existing lymphatic endothelial cells in breast cancer.[7],[8]

Understanding this novel mechanism for lymphangiogenesis and this new role of specialized TAMs may allow to open important ramifications for anti-lymphangiogenic therapies within the complexity of human cancer.

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12. MOLECULAR FEATURES OF THE LYMPHANGIOGENIC VEGFS

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ABSTRACT
In the last 10 years lymphangiogenesis has quickly evolved from an antiquated niche discipline into a mainstream research topic. The relevance of the lymphatic system for medical intervention, especially in the prevention of cancer metastasis, has led to the generation of a substantial amount of scientific data. Nevertheless, many of the interesting questions in lymphatic research remain unanswered. Much effort in clinical research has been spent trying to correlate the newly discovered lymphatic-specific markers with disease or disease outcome, while most of the experimental work has been devoted to various mouse models. Recently lymphangigenesis models have been described for experimental animals whose lymphatic system had been poorly described before, but it is still unknown which of the models is appropriate for which questions. At the molecular level, the multi-stage biosynthesis of the lymphangiogenic growth factors VEGF-C and VEGF-D has precluded a clear view on how the multiple forms of VEGF-C and VEGF-D differ from each other and which of the forms act as the immediate effectors of lymphangiogenesis in vivo. Despite using VEGF-D in human gene therapy trials, its physiological role remains entirely enigmatic, as it seems dispensable, at least for mice. In addition, fundamental questions of embryonic lymphatic development are still debated, for example the relative contributions of lymphangiogenesis versus lymphvasculogenesis. While more and more interactions between the molecular players in lymphangiogenesis are uncovered, the role of the "classical" VEGF receptors for lymphangiogenesis remains incompletely understood. Attempts to dissect the roles of the individual receptors for angiogenesis and lymphangiogenesis at a comprehensive level have so far not produced unequivocal results. It will be interesting to see whether the clinical applications of lymphangiogenesis and anti-lymphangiogenesis will live up to expectations despite our incomplete understanding of the biology underneath.

13. ENDOTHELIAL MOLECULAR MARKERS IN THE DIAGNOSIS OF ANGIOSARCOMA

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ABSTRACT
Molecular markers, including vascular types, may be useful tools in the management of malignancies, like breast cancer, facilitating positive and differential diagnosis, orientating

postoperative therapy and prognosis. Primary epithelial tumors are dominant among breast malignant tumors, mesenchymal or mixed tumors presenting a relatively low incidence. Differential immunohistochemistry, using a panel of endothelial-specific markers versus a panel of epithelial-specific markers, certifies the histopathological diagnosis. The paper presents clinical, histopathological and immunohistochemical features of a rare breast tumor. Tumor had gross findings characterized by large size, the largest dimension being about 15 cm, imprecisely delimited margins, and spongy consistency. Cut surface showed extensive hemorrhagic areas, alternating with necrotic zones. Routine microscopy showed numerous interanastomosing vascular channels, of variable caliber, forming blood pools, micropapillae and solid areas composed of atypical spindle-shaped and polyhedrical cells, exhibiting frequent mitoses. Tumor limits were inflitrative into the glandular and adipose tissue. Final diagnosis required special stainings (van Gieson, Szekelly) and immunohistochemistry (using antibodies against Factor VIII, CD34, vimentin, desmin, citokeratin, and protein S100). Corroborating the results of the complex microscopical investigation, diagnosis of high grade breast angiosarcoma was established. Differential diagnosis with a carcinoma, imposed by the presence of solid epithelioid areas, was based on the negativity of epithelial-specific markers.

14. IMMUNOHISTOCHEMICAL EXPRESSION OF HIF-1A IN HUMAN ENCHONDROMAS

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ABSTRACT
Background: The aim of this study is to evaluate the expression of the transcription factor Hif-1α (hypoxia-inducible factor-1 alpha) in human enchondromas. Hif-1α plays a major role in the adaptative response of cells to hypoxia. In normoxia, Hif-1 is maintained at low and often undetectable levels in cytoplasmic compartment by continuous proteasome-dependent degradation. Under hypoxia, Hif-1 traslocates into the nucleus and activates transcription of several genes including VEGF and EPO, which stimulate angiogenesis and erythropoiesis, respectively. Hypoxic regions are a common feature of solid tumors and Hif-1 indirectly promotes neoangiogenesis, essential for tumor growth and progression.
Material/Method: Surgical specimens of femoral enchondromas were obtained from 3 pacients. Immunohistochemical staining was performed on 4 μm thick sections from formalin-fixed paraffin-embedded tissue. After deparaffinization / dehydration, antigen retrival through pressure cooking, Hif-1α expression was detected using a standard streptavidin-biotin immunoperoxidase ABC technique with DAB as chromogen, Methyl Green conterstaining and mounting. Anti-human Hif-1α MAb (Chemicon) was used as primary antibody (dilution 1:500).
Results: We found that in all the tumors of patients, there are chondrocytes showing cytoplasmic localization of Hif-1, whereas nuclear Hif-1 staining was detected in just a few cells.
Conclusions: The presence of some chondrocytes showing nuclear localization of Hif-1 could be responsible via VEGF for enhancement of angiogenesis in chondromas and increasing the risk for tumor growth and malignancy. Accumulation of Hif-1 in the nuclei of chondrocytes could be used as a marker in pre- and post-operative surgical assessment of enchondromas.

15. SIMULTANOUS ASSESSMENT OF LYMPHATIC VESSEL DENSITY AND MAST CELL DENSITY BY COMBINED IMMUNOHISTOCHEMICAL AND HISTOCHEMICAL METHODS IN LIP TUMOURS

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ABSTRACT

Introduction. Mast cells are ubicuous in the connective tissues, particularly in the loose connective tissue associated with mucosae and of the perineural sheaths. They were found to associate with lymph vessels in old studies, and they are accumulating peritumorally in several malignant tumours, where they have been found to secrete growth factors and cytokines, including vascular endothelial growth factor (VEGF – A). Several studies document the presence of VEGF – A and VEGF – C positive cells with morphology of mast cells / macrophages around different malignant tumours. A single study documents the role of mast cells in tumour lymphangiogenesis so far, referring to pancreatic cancer.

Aim, materials and methods. To study the correlation between mast cell density and lymphatic density in lip malignant tumours. Immunohistochemical identification of lymphatic vessels was made with podoplanin, whereas mast cells presence was assessed with alcian blue – safranin in 22 cases of lip malignant tumours. Hot spots of lymphatic vessels were identified first at 20x, then the lymphatic microvascular density was counted at 400x in the peritumoral area. Hot spots of mast cells were identified at 400x, and the ones in close vicinity of lymphatic vessels hot – spots were counted.

Results. Lymphatic vessels were identified accurately by this staining method, although positive postcapilary venules were identified also (they were excluded in counting by their erythrocytes content and by the presence of pericytes). Podoplanin expression was also noticed in tumor cells, Schwann cells, myoepithelial cells, ductal and mucus secretor cells of the minor labial salivary glands. Mast cells were all alcianophyllic. Statistic data regarding the series of different lip tumors are listed in the table below.

Series	**Statistic variable**	**Mast cells density**	**Lymphatic vessels density**
Squamous cells carcinoma	**Mean**	4.98 ± 2.86	5.642 ± 2.03723
	95% CI	3.32011; 6.49589	4.51118; 6.77292
	99% CI	2.70711; 7.10889	4.07449; 7.20951
Basal cells carcinoma	**Mean**	7.8325 ± 1.59965	3.665 ± 1.98065
	95% CI	5.28105; 10.38395	0.50587; 6.82413
	99% CI	3.15351; 12.51149	- 2.1284; 9.4584
Adenocarcinoma	**Mean**	6.66333 ± 3.17997	6.44 ± 2.16709
	95% CI	- 1.24865; 14.57632	1.04745; 11.83255
	99% CI	- 11.56744; 18.86415	- 5.98415; 18.86415

Correlation analysis by simple regression test showed that mast cells density correlates with lymphatic vessels density only in squamous cells carcinoma (p = 1,011), whereas in basal cells carcinoma and adenocarcinoma there is no correlation between these two variables (p=0,849 and 0,271, respectively).

Conclusions:

1. The combined method shown above identifies accurately lymphatic vessels and mast cells.
2. Hot spots of lymphatic vessels and mast cells were often in the same microscopic field.
3. Podoplanin expression was noticed also in tumour cells, Schwann cells, myoepithelial cells and both secretory and ductal cells of the minor labial salivary glands.
4. Lymphatic vessels density correlated significantly with mast cells density only in squamous cells carcinoma, whereas in basal cells and adenocarcinoma no signifficant correlations may be found.

16. INTRACORONARY INFUSION OF BONE MARROW-DERIVED PROGENITOR CELLS IN ACUTE MYOCARDIAL INFARCTION: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED MULTICENTER TRIAL (REPAIR - AMI)

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ABSTRACT
Background: Experimental studies indicated that mononuclear progenitor cells derived from the bone marrow may contribute to the functional regeneration of freshly infarcted myocardium. Meanwhile, a number of small clinical pilot trials using intracoronary infusion of bone marrow-derived progenitor cells indeed suggested that such a strategy appears to be feasible and safe in patients with acute myocardial infarction (AMI).
Methods: Therefore, we initiated the Reinfusion of Enriched Progenitor Cells And Infarct Remodeling in Acute Myocardial Infarction (REPAIR - AMI) trial, which is the first double-blind, randomized, placebo controlled, clinical multicenter trial assessing the effects of intracoronary progenitor cell therapy after AMI. In this investigators-initiated trial performed in 17 centers in Germany and Switzerland, a total of 204 patients with an AMI have been randomized to receive intracoronary infusion into the infarct artery of either progenitor cells (BMC group), isolated from 50 ml of bone marrow aspirates by Ficoll density gradient centrifugation, or placebo medium 3-6 days after AMI. Bone marrow aspirates were shipped via courier to a central cell processing lab and isolated cell suspension or placebo medium was sent back in a blinded fashion and infused into the infarct related artery by stop-flow technique within 24 hours thereafter. The primary endpoint is absolute improvement of cardiac pump function (global left ventricular ejection fraction, LVEF), measured by quantitative left ventricular angiography after 4 months, as assessed by a core lab.
Results: At the time of study therapy, LVEF was similar in the Placebo and the BMC group (47±1.1% vs. 48±1.5% [mean ±SEM], p=0.3). At 4 months, LVEF had significantly increased in both groups. However, the absolute increase in LVEF as the primary endpoint of the study was significantly (p = 0.014) greater in the BMC group (+ 5.5 ± 0.7%) compared to the Placebo group (+ 3.0 ± 0.7 %). At 4 months follow-up, LVEF was significantly (p=0.021) higher in the BMC group (54 ±1.1%) compared to the Placebo group (50 ±1.5%). Thus, the results of the trial confirm the hypothesis, that intracoronary infusion of BMC provides a significant benefit on recovery of left ventricular contractile function in patients with successfully revascularized AMI. Subgroup analysis revealed that the benefit of intracoronary progenitor cell therapy is most pronounced in patients with large myocardial infarctions. Moreover, intracoronary infusion of BMC prevented left ventricular endsystolic volume expansion indicating a beneficial effect on post-infarction remodeling processes.
Finally, BMC administration significantly (p=0.002 versus Placebo) improved blood flow reserve in the infarct artery documenting profoundly enhanced neovascularization within the infarct region. The beneficial effects on cardiac remodeling processes were paralleled by a reduction in the combined clinical endpoint death, myocardial infarction and rehospitalization due to heart failure.
Conclusions: Intracoronary infusion of autologous bone marrow-derived progenitor cells in patients with reperfused acute myocardial infarction is associated with improved global cardiac pump function, prevents left ventricular endsystolic volume expansion, and profoundly augments blood flow in the infarct artery within 4 months. Large-scale clinical endpoint trials are warranted to assess the potential of bone marrow-derived progenitor cell therapy to limit the development of postinfarction heart failure.

17. ANGIOGENIC GENE THERAPY IN PATIENTS WITH SEVERE CHRONIC LOWER LIMB ISCHAEMIA

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ABSTRACT
Aim: The present study focuses on the application of a therapeutic strategy in patients with chronic severe lower limb ischaemia using a plasmid vector encoding the vascular endothelial growth factor (VEGF 165). It has been shown that VEGF promotes neovascularization and blood vessel network formation and thus might have the ability to improve blood-flow at the level of the affected limbs.

However, little information is available regarding the necessary level of expression of VEGF and its possible related adverse effects.
Material and methods: We have sub-cloned VEGF 165 isophorm into pCMV-Scriopt expression vector (Stratagene) under the control of CMV promoter. Three patients with chronic ischaemia of the lower limb, considered as not suitable for surgical revascularization received intramuscular injection with 0.5 ml saline solution containing 10" copies of VEGF165 plasmid
Results: The clinical evolution has been monitored by angiography and estimated by walking time on the rolling carpet (Gardner protocol). Two month after therapy all three patients showed complete relief of rest pain, improvement of ischaemic ulcer lesions and increased walking distance on the rolling carpet most probably due to appearance of newly formed collateral vessels.
Conclusions: Given the failure of previous conventional therapy we are tempted to state that over-expression of VEGF at the level of the thight and calf muscles prevented the amputation which would have been the only alternative left for the three patients

18. CHARACTERISTICS AND POTENTIAL OF MSC FROM CHILDREN FOR SAFE CLINICAL APPLICATION

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ABSTRACT
Mesenchymal Stromal Cells (MSC) became the focus of cellular therapeutics but little is known regarding bone marrow (BM) MSC derived from children. Since MSC are constituents of the bone marrow microenvironment which participates in the regulation of hemopoietic stem/ progenitor cell proliferation and differentiation we examined properties and potential, and if any, differences in BM MSC coming from various hematological diseases.
BM Mononuclear cells (MNC) from children with idiopathic thrombocytopenic purpura, autoimmune neutropenia of childhood and B cell acute lymphoblastic leukemia (at diagnosis and after the end of treatment) were isolated and expanded. MSCs were immunophenotypically characterized and their functional characteristics were assessed by CFU-F assay and cell doubling time calculation. Their ability for tri- lineage differentiation was verified by molecular and histochemical methods. Apoptosis was evaluated and clonal analysis was performed. MSCs were isolated from BM of all groups. They acquired the mesenchymal-related markers from the first passage with simultaneous decrease of haematopoietic markers. A very low percentage of apoptotic cells were detected in all passages. The proliferative and clonogenic capacity did not differ among groups with the exception of ALL at diagnosis in which they were defective. Histochemical and molecular analysis of differentiated MSCs yielded characteristics for adipocytes, osteoblasts and chondrocytes. Clonal analysis in a number of BM samples revealed a highly heterogeneous population of cells within each clone.
For the study of spontaneous MSC apoptosis throughout sequential passaging, BM MNC cultures from children with benign hematological disorders and solid tumours without BM involvement were initiated and grew for 10 passages. The expression of CD105, CD146 and CD95 as well as apoptosis by 7AAD staining, were evaluated by flow cytometry. In every passage CFU-F assay was performed and the cell doubling time was calculated. Cell cycle characteristics were assessed by propidium iodide staining at P2 and P6.

In order to test if CD95 which is highly expressed on MSCs as previously described is functional, induction of apoptosis was triggered by the addition of an agonistic antibody (anti-Fas CH-11) under standard culture conditions. No apoptosis was induced suggesting no leading role of this molecule in the apoptotic event of MSC. Additionally, serum deprivation of MSCs for up to 72 hours revealed no substantial apoptotic effect although the number of cells after trypsinization was slightly decreased in serum deprivation conditions. Furthermore, MSC deprived of serum for 72 hours were sequentially set in culture under standard culture conditions for 2 additional passages and tested as to their functional and cell cycle characteristics and spontaneous apoptotic events in two experiments. The

number of CFU-F and doubling time was not affected and no apoptosis was observed while cell cycle pattern was maintained irrespectively of the serum free or serum complemented condition initially used. This finding could be very useful in the setting of transplantation as long as serum deprivation for up to 72 hours does not seem to affect in vitro expanded MSC while it favors their use in the clinical setting avoiding the possible risk of animal contaminants and immune responses attributable to serum contamination.

According to the cell cycle analysis, cells at P2 are mostly at GoG1 phase while at P6 there is a slight increase in the cells that have entered cell cycle.

We also studied the expression profile of oncogenes and tumour suppressor genes (TSG), known to play an important role in the malignant transformation of cells since recent studies have shown that MSCs can become targets of neoplastic transformation leading to cells with malignant potential. We assessed the expression levels of p53, p16, Rb and H-Ras genes in BM-MNCs and compared them to the respective ones in MSCs as well as their levels in different MSC passages by RT-PCR.

MNCs and MSCs were isolated from BM of children diagnosed with idiopathic thrombocytopenic purpura autoimmune neutropenia, solid tumours without BM involvement and B cell acute lymphoblastic leukaemia (ALL) at diagnosis. MSCs were cultured for 6 passages. RNA was isolated from MNCs as well as from MSCs at P2 and P6. GAPDH housekeeping gene was used as internal control and HUVEC cells as normal control for adherent cells.

In all groups studied, MSCs at P2 expressed higher levels of p53, Rb and H-Ras and lower levels of p16 in comparison to MNCs from the same patients. However, there were no differences in expression levels between passages P2 and P6 and between P2 and HUVEC. In ALL samples though, expression levels were similar between MNCs and MSCs at P2.

These results indicate that despite the differences in oncogene and TSG expression levels between MNCs and MSCs, the profile of the same genes does not change during cultivation of MSCs up to passage 6. Additional experiments of MSC growth on soft agar showed no colony formation, confirming the anchorage dependence of cells.

As cord blood (CB) constitutes an easily accessible source of hematopoietic progenitors suitable for transplantation and recently its feasibility as a source of mesenchymal stromal cells (MSC) is assessed we tried to establish the optimal culture conditions for the expansion of CB MSC, to assess their functional and immunophenotypic characteristics and to compare them with bone marrow (BM) MSC. Culture conditions were initially based on the technique used for the isolation of BM MSC and sequentially different culture conditions were tried in order to optimize MSC expansion. They included different cell concentrations, enrichment of the culture medium with FGF-2, different concentrations of the lot selected FCS, different culture surface area and pretreatment of the culture plate with FCS.

Isolation of CB MSC was achieved in 25% of the samples cultured under optimal conditions which were found to be the higher initial concentration of cells ($3x10^6$ cells/cm^2), the enrichment of the culture medium with FCS (20% compared to 10% of the BM protocol), the addition of 5ng/ml FGF-2 (1ng/ml in the BM protocol), the pretreatment of the culture surface with FCS and the initial volume of the sample (> 40 ml). MSC were morphologically similar to the ones derived from BM, but appeared late in culture requiring 34 days to reach confluency and 55 days from P1 to P2. Immunophenotypic analysis of P1 cells showed no expression of CD34 while CD45 ranged from 0-17.83% and CD105 ranged from 49-83%. CFU-F colonies were developed in one case.

Conclusion

These findings suggest that CB cannot be regarded so far as a sufficient source of MSC for clinical use. MSC from bone marrow of children with haematological disorders -with the exception of ALL at diagnosis –though, can be isolated in sufficient number and quality and moreover retain their functional characteristics throughout serial passages and are very stable under conditions that usually cause apoptosis although progression in passages results in a lower number of cells maintaining quiescence. The constant oncogene and TSG expression levels for a number of serial passages combined with the similar expression levels between MSCs and HUVEC cells, imply that MSCs may not be transformed during long term culture and thus they can serve as a potential source for safe clinical applications.

19. ISOLATION OF BONE MARROW PROGENITOR CELLS ON PERCOLL GRADIENT

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ABSTRACT
Purpose: Bone marrow (BM) represents the main source of progenitor cells for the entire lifespan of adult individuals. Their plasticity is being highly investigated in tissue engineering for restoring the function of damaged organs. We checked whether the Percoll gradient separation of adult BM cells generates subpopulations enriched in c-kit$^+$/ Sca-1$^+$ progenitor cells.
Matherial and methods: BM aspirate isolated from adult mouse tibia and femurs, was layered on a discontinuous Percoll gradient (1,046 - 1,052 - 1,060 - 1,064 - 1,070 - 1,075 g/ml) and 6 cell sub-populations (I-VI) were separated by centrifugation (1500g, 20 min, 4^{o}C). The progenitor cell markers and the potential to generate differentiated cells were checked in each subpopulation through fluorescence microscopy and flow citometry assays before and after cell cultivation for 2 weeks.
Results: Progenitor cells (c-kit$^+$ and Sca-1$^+$) were identified mainly in fractions I-IV. When cultured for 2 weeks, the adherent cells within these subpopulations (mesenchymal progenitor cells) showed a great capacity of proliferation and generated various cell types (fibroblast-like, neuronal-like and endothelial-like cells). Fractions III and IV proved to be enriched in CD 45$^+$ and CD 68$^+$ cells, demonstrating the presence of white lineage cells in these subpopulations. By culturing, some of these cells adhered and accumulated lipids, becoming foam cells, as demonstrated by Oil Red staining. Fractions V and VI contained mesenchymal enlarged cells which stained positive for smooth muscle actin.
Conclusion: centrifugation of BM aspirate on Percoll gradient is a simple and inexpensive technique for isolation of a progenitor cells enriched subpopulation. These cells can be further used in FACS analysis to obtain the pure population of progenitor cells subpopulations needed in cellular therapy. By culturing, some of these cells adhered and accumulated lipids, becoming foam cells, as demonstrated by Oil Red staining. Fractions V and VI contained mesenchymal enlarged cells which stained positive for smooth muscle actin.

In conclusion, centrifugation of BM aspirate on Percoll gradient is a simple and inexpensive technique for isolation of a subpopulation enriched in progenitor cells. These cells can be further used in FACS analysis to obtain the pure population of progenitor cells needed in cellular therapy

20. 5-AZACYTIDINE SUPPORTS THE MYOGENIC DIFFERENTIATION OF BONE MARROW STROMAL CELLS

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ABSTRACT
Background: Bone marrow stromal cells (BMSC) are multipotent cells able to give rise to various cell types, among which the myocytes, which may be highly valuable in cellular therapy of myocardial infarction.
Purpose: In an attempt to increase the myogenic commitment, we investigated whether the exposure of cultured BMSC to the demethylation agent, 5-azacytidine, could improve or accelerate their differentiation, prior to be utilized in cell-mediated therapy.
Materials and methods: BMSC isolated from the adult rat tibia were exposed in culture to 5 microM 5-azacytidine for 24 hours, one day after seeding; the treatment was repeated at weekly intervals for four passages and the expression of muscle specific morphology, proteins and genes was assessed by Western blot, immunocytochemistry and RT-PCR.
Results: Microscopic examination, as well as immunocytochemistry, Western blot, and RT-PCR experiments revealed that cultured cells lost the native expression of Osteocalcin and Alkaline phosphatase as a function of time and began to express Connexin 43. As compared to control

(untreated cells), the exposure to 5-azacytidine of BMSC induced a myocyte-resembling phenotype except the sarcomeric organization, and the expression of muscle specific proteins (Troponin T, alpha-sarcomeric actin, Desmin and GATA-4) and genes (GATA-4, myoD and desmin). Conclusions: Taken together, the results show that in vitro treatment with 5-azacytidine promotes the commitment of BMSC towards cells that express several features of cardiomyocytes; one can safely assume that his agent could serve to prime bone marrow cells prior to their transplantation to improve myocardial remodeling after acute injury.

21. PRACTICAL APPLICATION OF STEM CELL TRANSPLANT IN THERAPY OF MYOCARDIAL INFARCTION

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ABSTRACT

Objectives: The present study tested the safety and feasibility of bone marrow derived autologous cells transplant, using intra-coronary infusion at the level of infarcted artery, in patients suffering from recent and acute myocardial infarction. Different studies suggested that stem cells transplant can have a positive contribution both in regeneration of damaged myocardium, as well as in neovascularization of ischemic area.

Methods: Our study comprised 11 patients suffering from extended acute or recent myocardial infarction (1-28 days), which were already committed to successful reperfusion (PCI and stent implant at the level of the damaged coronary artery) and had a 40% ejection fraction of the left ventricle. The bone marrow derived $AC133^+$ cells were harvested from the posterior iliac crest, isolated, washed and resuspended in order to be injected through a catheter. Cells were gradually administrated by intra-coronary injection at the level of infarcted coronary artery. After transplant and 6 months evaluation comprised clinical and laboratory examinations, ECG, 48 hours Holter monitoring, trans-thoracic echocardiography, stress echocardiography, SPECT – using Technetium 99 sestamibi, left ventricle angiography.

Results: There were no complications occurring during the procedures of intra-coronary infusion of bone marrow derived cells, and no malign arrhythmias were recorded during 48 hours Holter monitoring. Angina pectoris was not present in any patient and there were no signs of clinical worsening. Side effects did not appear at 6 months evaluation and neither did malign ventricular arrhythmias when the 48 hours Holter monitoring was used.

Conclusions: Bone marrow derived autologous cells transplant proved to be a secure and feasible method. Long term clinical reports will confirm the benefits of this new therapeutic approach.

22. ANTIANGIOGENIC THERAPY BY M TOR BLOCKADE AGAINST SOLID TUMOR GROWTHS AND METASTASIS

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ABSTRACT

Angiogenesis, the formation of new blood vessels from the endothelium of the existing vasculature, is fundamental in tumor growth, progression and metastasis. There is preclinical as well as clinical evidence that inhibiting angiogenesis will suppress tumor growth and tumor metastasis. Antiangiogenic therapy in general is a concept that targets the vasculature of tumors rather than tumor cells themselves, which has traditionally been the case by chemo- or radiotherapy. Endothelial derived growth factor (VEGF) and its receptors have been the focus of antiangiogenic research.

Other molecular targets are matrix metalloproteinases (MMPs), Cyclooxygenase 2 (COX-2) and the tubulin cytoskeleton.
Over the last 30 years numerous pro- and antiangiogenic molecules, their ligands and intracellular signalling pathways have been identified. Enormous efforts have been undertaken in order to develop antiangiogenic strategies for clinical cancer treatment. Despite numerous promising results in preclinical models, several initial clinical trials gave no convincing evidence for efficient antitumoral therapy by classical antiangiogenic agents as monotherapy. This has led to the development of new antiangiogenic compounds and successful combination of angiogenesis inhibitors with classical cytotoxic chemotherapy and radiotherapy. Combined with chemotherapy, anti-angiogenesis has proven its clinical efficiency in patients suffering from advanced colorectal cancer leading to an improved patient survival time. In 2004 the first antiangiogenic compound Bevacizumab (Avastin) was therefore approved by the FDA as first line therapy in combination with standard 5-Fluorouracil-based chemotherapy in patients with advanced colorectal cancer.
Conventional immunosuppressive drugs have been used effectively to prevent immunologic rejection in organ transplantation. However, cancer development and recurrence are ominous risk factors for these immunocompromised patients. Recently, we showed that the new immunosuppressive drug rapamycin may have a unique ability to reduce the risk of cancer development, while simultaneously providing effective immunosuppression. Experimentally, rapamycin inhibited metastatic tumor growth and angiogenesis in *in vivo* mouse models. Normal immunosuppressive doses of rapamycin effectively controlled the growth of established tumors. From a mechanistic perspective, rapamycin demonstrated antiangiogenic activities linked to a decrease in vascular endothelial growth factor production and to a markedly inhibited response of vascular endothelial cells to vascular endothelial growth factor stimulation. In pancreatic cancer, rapamycin therapy alone inhibited tumor growth and metastasis more than gemcitabine, with remarkable long-term tumor control when the drugs were combined. Mechanistically, H&E analysis revealed tumor vessel endothelium damage and thrombosis with rapamycin treatment. Furthermore, terminal deoxynucleotidyl transferase-mediated nick end labeling/CD31 double staining of orthotopic tumors demonstrated apoptotic endothelial cells with rapamycin treatment, which also occurred with human umbilical vein endothelial cells in vitro. Indeed, it has been shown that the mammalian target of rapamycin (mTOR) inhibitor rapamycin, when administered to tumor-bearing mice, selectively induced extensive local microthrombosis of the tumor microvasculature. Importantly, rapamycin administration had no detectable effect on the peritumoral or normal tissue. Intravital microscopy analysis of tumors implanted into skinfold chambers revealed that rapamycin led to a specific shutdown of initially patent tumor vessels. In human umbilical vein endothelial cells vascular endothelial growth factor (VEGF)–induced tissue
factor expression was strongly enhanced by rapamycin. We further showed by Western blot analysis that rapamycin interferes with a negative feedback mechanism controlling this pathologic VEGF-mediated tissue factor expression. This thrombogenic alteration of the endothelial cells was confirmed in a one-step coagulation assay. The circumstance that VEGF is up-regulated in most tumors may explain the remarkable selectivity of tumor vessel thrombosis under rapamycin therapy. Taken together, the data suggest that rapamycin, besides its known antiangiogenic properties, has a strong tumor-specific, antivascular effect in tumors.

23. IMMUNOHISTOCHEMICAL ASPECTS OF SYNCHRONOUS COLORECTAL CARCINOMAS

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Introduction: The synchronous tumours are neoplasias that appear in the same or different organs in a less than 6 months interval. In the literature they are reported as a rare phenomenon and for their identification require detailed investigations.
Material and method: We studied 791 surgical specimens with colorectal carcinomas (2003-2006). We followed the classic clinico-pathological parameters of single colorectal carcinomas and we compared them with those of the synchronous tumours of this organ. In the synchronous tumours we

followed the following immunohistochemical markers: p53 protein, c-erbB2 and bcl-2 oncoproteins, chromogranin A and the markers of angiogenesis (CD31, CD105, VEGF).
Results: We identified 15 cases of synchronous colorectal carcinomas (1.9% of the total number of cases). The peak incidence was in the 71-80 age group. 66% of cases had been associated with polyps. 12 were adenocarcinomas with different grades of differentiation, and 3 were mucinous carcinomas. One of the cases presented an association between a squamous carcinoma arising in the anal mucosa and an adenocarcinoma of the rectum. In 6 cases, one component of the synchronous tumours presented neuroendocrine differentiation. Expression of the p53 protein was notably higher in the tumours of the left colon and in 9 cases the expression of this protein was the same between the tumours. Expression of the c-erbB2 and bcl-2 proteins was demonstrated in 11 and 4 cases, respectively (in 4, respectively 2 cases both tumours were positive for this marker). In 10 cases microvascular density was the same in the tumours, although CD31 expression did not correlate with the expression of CD105, nor VEGF.

24. CORRELATION BETWEEN MICROVASCULAR DENSITY AND DIFFERENTIATION GRADE IN SOFT TISSUE TUMOURS

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ABSTRACT
Introduction: Although there are thousands of publications on the subject of tumour angiogenesis, their great majority refer to carcinomas, and the publications on the subject in soft tissue tumours are very rare and, until present, with discordant results. According to this perspective, we have made the assessment of microvascular density in soft tissue tumours.
Materials and methods: 54 cases of soft tissue tumours (43 malignant and 11 benign tumours) were studied according to the following protocol: surgical samples were fixed in buffered formalin, embedded in paraffin, sectioned at 5 μ, then stained for morphological diagnosis (H&E), and for immunohistochemical detection of microvessels (smooth muscle actin – SMA, CD31 and CD34). Weidnet method of microvessel counting was used.
Results: All the tumours included in the study have presented rich blood vessel network, the majority (>80%) of the blood vessels from within the tumour area being of immature and intermediate type (positive for the endothelial markers and negative for SMA). This aspect was more obvious in the case of liposarcoma and malignant fibrous histiocytoma, but with no correlation with the differentiation degree. The highest values of microvascular density (MVD) were recorded in liposarcoma and malignant fibrous histiocytoma, in straight correlation with the high number of immature and intermediate blood vessels. We have noticed major differences in the blood vessel number between benign and malignant tumours, which were statistically significant ($p<0.02$). We have also noticed significant differences between the group composed of leiomyosarcomas and fibrosarcomas on one side, and liposarcoma and malignant fibrous histiocytoma on the other side ($p<0.05$). The results of microvascular density assessments were concordant for either endothelial marker used (table). Microvascular density was significantly higher in the malignant tumours than in the benign ones.

Tumour	MVD/CD34	MVD/CD31
Benign tumours	10.3	9.4
Fibrosarcoma	34.6	32.2
Liposarcoma	61.2	58.3
Malignant fibrous histiocytoma	58.9	55.7
Leiomyosarcoma	21.5	22.4

Conclusions: Malignant soft tissue tumours have a rich blood vessel network, especially the liposarcoma and malignant fibrous histiocytoma. The majority of the blood vessels from within the tumour area are of immature and intermediate type. MVD does not correlate with the differentiation degree, but have significantly higher values in malignant tumours then in the benign ones.

25. CD34/AAS TECHNICAL DETAILS OF MALIGNANT TUMORS AT THE LIP AND PHARYNX LEVELS

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ABSTRACT
The immunohistochemical methods identify a certain tisular antigen through an antigen-antibody reaction, revealed with a substance with cromogene role. Histochemical methods provide information about cellular functions and extracellular tisular components. The aim of this study was to perform a double immunohistochemical / histochemical technique, i.e. the differentiating clone (CD34) and alcian blue safranine. Using the immunohistochemical technique we have revealed the endothelial cells from the blood vessels and mastocites in the conjunctive tissue were revealed using the alcian blue safranine method. The immunohistochemical method used the QEBnd 10 differentiating clone.
The principle of the technique consists of the following: sections are deparaphinated, then they are hydrated with alcohols of descending concentrations (100% - 70%), down to distilled water. The principle of antigen unmasking is not well known. It is done using a solution of buffer citrate (phosphate – citric acid) with a pH of 6 for 20 minutes at a temperature of 95-99 degrees Celsius. The blocking of endogene peroxidase, used for eliminating the non-specific coloration of the background, is performed with oxygenated water 3% for 5 minutes, followed by successive washes with distilled water and buffer phosphate, pH 7. The application of the primary antibody (30 minutes) is followed by the En Vision system, based on dextran polymers. The secondary reactive includes a dextran skeleton which binds several molecule enzymes, causing the increase of the method sensibility, the decrease of the non-specific background coloration and work time.
The reaction was revealed using the DAB cromogen. The Lillie hematoxylin countercoloration was not applied; instead the histochemical coloration blue alcyan saphranine coloration was used to reveal the mastocytes. The histochemical technique consisted of the application of the coloring AAS with pH 1,42, followed by a bath of distilled water and one of absolute ethylic alchohol, every few seconds. Sections were clarified in benzene and mounted with Canada balm.
The endothelial cells were identified in dark brown in the normal blood vessels and the cells involved in tumoral angiogenesis. The ancyanophile mast cells were identified in light blue. The vascular microdensity is correlated to the mastocitary vascular with respect to the tumoral type and localization.

26. CORRELATION OF THE CD34 EXPRESSION AND MAST CELL IN VARIOUS TYPES OF MALIGNANT TUMORS

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Angiogenesys is an important prognostic factor mediated by the angiogenic factors released by the malignant cells and the immunitary system cells. Among these we can find mast cells involved in the tumoral progression through angiogenetic promotion.
The aim of the study was to develop a correlation between the identification of endothelial cells in normal and tumoral blood vessels and their possible association to mast cells according to the tumor type and localization.
The study was performed on a series of 17 cases of pharinx carcinoma and 21 cases of lip carcinoma, through the CD34/AAS double coloration using the En Vision/DAB work system and the QBEnd10 clone. Mast cell coloration was performed with blue alcyan safranine. Sections had a width of 5 microns. The normal morphological coloration used to establish the histopathologic diagnostic was the hematoxylin-eosin. 16 cases of scuamo-cellular carcinoma and 1 adenocarcinoma were found in pharinx, and 17 cases of scuamo-cellular carcinoma and 4 basocellular carcinoma were found at the outer lip margin.

Conclusions: A correlation between the number of vessels and mastocitary density was observed in the scuamo-cellular carcinoma of lip and pharinx. Vessels are sinuous, small, with no lumen and with intensely positive endothelial cells for CD34. These are associated to a large number of alcialphile mast cells, fully and partially degranulated. The distribution of vessels and mast cells is at the edge of tumor area. Intratumoral mast cells were not observed. The number of mast cells lowers with the dimension increase of the blood vessels. In case of adenocarcinoma, mast cells are numerous, associated to sinuous mast cells, compared to scuamocellular carcinoma laid around malignant-transformed glands. A lower number of mast cells were seen in the basocellular carcinoma, laid in the vicinity of the tumor. Our findings are consistent with the literature data and our study from the previous year.

27. MAST CELL REACTION IN MALIGNANT LARYNGEAL NEOPLASM

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ABSTRACT
Introduction: Mast cells are normal connective tissue residents; they are present predominantly around vessels. Mast cell densities vary from an organ to other, but are constantly well represented in respiratory tract segments. Mast cell hyperplasia was found in many malignant tumors, but the significance of this phenomenon is stilt unknown. In the literature there are few data about mast cell reaction in malignant laryngeal neoplasm.
Material and methods: We studied archive blocks from 127 cases of laryngeal carcinoma. For histological diagnosis and grading 2 sections were prepared for Hematoxylin-Eozin staining and for identifying mast cells we used Alcian Blue Safranin histochemistry at 0.2 pH. Examination has been performed with Nikon Eclipse 600 microscope, the images were recorded in .jpeg format and the microscopic images were analyzed with Lucia G program. Microvessel density was calculated using the hot spot method.
Results: Most of the cases were squamous cell carcinoma with a great variety of grading (G1 (24.4%, 31 cases), G2 (56.69%, 72 cases), G3 (18.11%, 23 cases) and in 1 case we encountered adenoid cystic carcinoma (0.78%, 1 case)). Invasive squamous cell carcinoma mast cell microdensity was 2.19, in microinvasive squamous cell carcinoma mast cell microdensity was 4.66, in cases of malignant laryngeal papillomatosis the mast cell microdensity was 9.33, and in the case of adenoid cystic carcinoma the mast cell microdensity was 46.66. In carcinoma-associated mast cell hyperplasia, the large majority of mast cells were alcian blue positive.
Conclusions: In early stages the mast cells identified with Alcian Blue Safranin are numerous (microinvasive squamous cell carcinoma mast cell microdensity 4.66) and rare or even absent in late stages (invasive squamous cell carcinoma mast cell microdensity 2.19). In the case of malignant laryngeal papillomatosis mast cell microdensity was 9.33, and in case of the cystic carcinoma the mast cell microdensity was 46.66. Alcianophile mast cells are present in tumor area, and safraninophile mast cells are residents of connective and muscular tissue, at a distance from the tumor.
Key words: carcinoma**,** larynx**,** mast cell, histochemistry, Alcian-Blue Safranin

28. EXERCISE TRAINING IMPACT ON ARTERIAL RIGIDITY PARAMETERS IN HYPERTENSIVE PATIENTS

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ABSTRACT
Objectives: Assessment of an individualized exercise training program efficacy on hemodynamic parameters, arterial rigidity parameters and cardiovascular risk factors in hypertensive subjects with high normal and stage 1 and mild and moderate additional risk blood pressure (BP).

Patients and methods: We studied 229 hypertensive patients (69% men, 31% women, average age 58.6±1016, recently diagnosed with high normal and stage 1 and mild and moderate additional risk BP, who did not require antihypertensive drugs (ESC/ESH 2003 Guidelines). The patients were included in an individualized comprehensive rehabilitation program, training level representing 80% from maximum achieved in exercise testing. Before and at the end of the 3 month period there were assessed the hemodynamic parameters (automatic ambulatory monitoring of BP) and arterial rigidity parameters through PVWc-r (carotid-radial pulse wave velocity) measured using the Complior method. It was also studied the trend of cardiovascular risk factors.

Results: Pulse pressure (PP) dynamic/analyze measured through BP automatic ambulatory monitoring/24 hours revealed a significant decrease of PP after the 3 month program (from 62.27±8.01 mmHg to 56.18±8.24 mmHg) ($p<0.0001$). We also noticed a reduction of PWVc-r (from 9.95±2.08 m/s to 9.11±1.52 m/s). A significant correlation was found between PP and SBP (systolic blood pressure) values and also between PWVc-r and total serum cholesterol levels (TC) at baseline and after the 3 month training program.

Conclusions:

1. Exercise training has a favorable impact on pulsated component of BP and consequently on arterial compliance of elastic arteries. Due to exercise training impact on arterial compliance, it can slow down/postpone the arterial aging.
2. The favorable results following exercise training were also observed in muscular arteries compartment. Pulse wave velocity measured at distal arteries level can represent a key point in assessment of exercise training outcomes in hypertensive patients.
3. Exercise training has a positive impact on dynamic dysfunction of vascular compartment (endothelial dysfunction) and also on structural changes of arterial wall (arterial rigidity).
4. BP control according to guideline recommendations can have a favorable impact on arterial compliance.

29. BENEFIT OF EXERCISE TRAINING ON CARDIOVASCULAR RISK IN STAGE 2 HYPERTENSIVE PATIENTS TREATED WITH ANTIHYPERTENSIVE DRUGS

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Introduction/Background: Cardiovascular risk represents a major clinical priority for hypertensive patients in choosing the right therapy and life style recommendations. It is well-known the impact of exercise training on cardiovascular risk factors.

Methods: We studied 120 hypertensive patients classified as stage 2, moderate additional risk, following antihypertensive therapy for at least 3 months and no more than 1 year, with blood pressure values bellow the target recommended by the guidelines. Patients were evaluated at baseline and at the end of the study. Hemodynamic parameters, measured using blood pressure Holter monitoring/24h, were: pulse pressure (PP) and pulse wave velocity (PWVc-r). Patients followed a 6 months rehabilitation program. Cardiovascular risk was assessed using Heart SCORE Chart.

Results:

Parameter	At baseline	After 6 months	p
SCORE Risk (%)	7,26 ± 4,70	5,84 ± 4,06	0,01314
PP/24h (mmHg)	56,99 ± 8,82	53,67 ± 9,75	0,00613
PWVc-r (m/s)	12,14 ± 2,35	11,00 ± 2,00	< 0,001
Smoking (number)	39 (32,5)	26 (21,6)	<0,05
Total cholesterol (mg/dl):	194,66 ± 38,41	176,18 ± 29,28	<0,0001
BMI (kg/sm)	27,50 ± 4,47	26,38 ± 3,90	0,01

Mean value ± Standard Deviation (%)

Conclusions:

1. Beneficial interventions on cardiovascular risk factors modify absolute risk of fatal cardiovascular disease to a different extent.

2. After a 6 months period we noticed a significant reduction of absolute risk of fatal cardiovascular diseases in 10 years.
3. Absolute risk reduction was due to exercise training impact on associated risk factors as smoking and total cholesterol, even though the final value was still in high additional risk area.
4. The benefits of exercise training were also noticed on arterial rigidity parameters which, even though they are not components of Heart Score chart, have an important significance in assessing hypertensive patient status.

30. SOURCES OF ENDOTHELIAL CELLS FOR STENTS ENDOTHELISATION

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ABSTRACT
Objectives: 1. Culture of endothelial-like cells obtained from $CD34^+$ cells isolated from peripheral blood and umbilical cord blood. 2. Cultivation of endothelial-like cells obtained from bone marrow $AC133^+$ cells.
Material and method: Isolation of human haematopoietic stem cells (hHSC) was performed using a positive selection with immunomagnetic beads directly conjugate with CD34 monoclonal antibodies and AC133 monoclonal antibodies. We used DYNAL Biotech and Miltenyi Biotech separation complete kits. hHSC differentiation to endothelial lineage was performed by cytokines addition: VEGF, SCGF and ECGS. The cells were seeded in multi-well plates coated with fibronectine or gelatin and were maintained at 37°C in a 5% CO_2 humidified atmosphere.
Endothelial character of cells obtained from $CD34^+$ was assessed by identification of the following markers: AC133, CD34, CD31/PECAM, von Willebrandt factor, VE-cadherin, VEGFR2/KDR/flk1, eNOS and several adhesion molecules (CD54, CD62E, CD106) by flow cytometry, immunohistochemistry and RT-PCR.
Results: Flow cytometry analysis of cells obtained from peripheral blood showed that all $CD34^+$ cells exhibit AC133 marker. Approximately 10% of $CD34^+$ cells obtain from umbilical cord blood cultivated in medium supplemented with ECGS expressed morphological characters similar to endothelial cells. Immunohistochemical analysis of endothelial-like cells showed that only 3.25 ± 1.25% of this cells were PECAM/CD31 positive and von Willebrand factor positive.
The flowcytometric analysis of the immunomagnetic separated bone marrow-isolated cells with AC133 MicroBeads showed that 90±5% of the cells are pure. The $AC133^+$ fraction included 95±4% of $CD34^+$ cells. After 3 weeks, all cells were $AC133^-$; in 2/3 of the experiments 5-37% of cells were $CD34^+$. Gene expression analysis by RT-PCR of mature endothelial cells specific markers - VE-cadherin, VEGFR-2, eNOS - showed that most of the cells expressed these markers.
Discussions: Bone marrow and peripheral blood could represent good sources of endothelial cells for stents endothelisation. VEGF is essential for endothelial cells development.

31. PULMONARY VASCULAR REMODELLING IN COPD

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ABSTRACT
Pulmonary vascular remodelling in COPD patients represents more than a simple hypertrophy of media triggered by long-term hypoxic vasoconstriction. The aim of the present study was to characterise structural changes of pulmonary vasculature in both smokers and patients with mild COPD in order to assess the involvement of cigarette smoking in vascular remodelling initiation. Histological examination of tissue sections dissected from exeresis fragments was followed by the morphometric analysis of muscular pulmonary arteries and a semi-quantitative evaluation of elastin and collagen intimal deposits based on a visual scale. Our results suggest that pulmonary vascular abnormalities present in both smokers and COPD patients with early disease are intimal thickening of small muscular arteries (intimal index was 23.11 ± 3.04 in smokers, respectively 25.62 ± 3.06 in COPD group, v. 17.86 ± 2.96 in control group, $p = 0.005$) and arteriolar muscularisation. In patients with mild COPD, the substrate of intimal thickening is determined initially by the local predominant deposition of elastin and further, by colagen deposition. Moreover, intima thickness was found to be significantly associated with cigarette consumption expressed as packs-years ($r = 0.65$, $p = 0.04$) and with vascular diameter ($r = – 0.59$, $p = 0.02$). Immune labelling with anti-CD34 antibodies showed the swelling of endothelial cells as a sign of vascular injury. In conclusion, the direct effect of smoke components on the endothelium may be considered as an important factor responsible for both pulmonary endothelial dysfunction and vascular remodelling in patients with COPD.