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Preface

This book contains the contributions presented at the Workshop on "Methods for Studying Angiogenesis", held in Bari on January 2003 and jointly organized by the Italian Association of Cell Cultures (AICC) and by the University of Bari Medical School.

As it is well known, angiogenesis is a very important phenomenon related to numerous physiological and pathological events in human beings. Its regulation depends on the balance of a number of stimulating and inhibiting factors and the possibility of modulating the action of these factors could help for the control of angiogenesis-dependent diseases. Recently, anti-angiogenesis, i.e. inhibition of blood vessel growth, has obtained renewed interest since it might represent a way to prevent the progression of tumors. Consequently, this experimental strategy may open a new and promising front in the war against cancer.

Therefore, the knowledge of mechanisms underlying angiogenesis has a particular importance and the development and the applications of more and more reliable methods is pivotal to pursue this aim. Angiogenesis is a typical exemple of a scientific field of the biomedical sciences which necessitates both in vivo and in vitro investigation.

The results obtained thus far demonstrate that these two approaches are complementary to gain insight into mechanisms involved in this crucial phenomenon. For this reason, the Italian Association of Cell Cultures decided to organize this Workshop and to assign its coordination to two distinguished and well-known experts in the field: Prof. Domenico Ribatti, from the Department of Human Anatomy and Histology, and Prof. Angelo Vacca, from the Department of Biomedical Sciences and Human Oncology of the University of Bari. The AICC wishes to thank sincerely these two Colleagues for the perfect organization of the Workshop and

for their excellent presentations. The AICC is also very indebted to all prominent scientists who, with their presentations, contributed to the success of the initiative and to the realization of this volume.

Giuseppe Arancia
President of the Italian Association
of Cell Cultures (ONLUS-AICC)

THE CORNEAL ASSAY FOR ANGIOGENESIS

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Introduction

Continuous monitoring of angiogenesis in vivo is required for the development and evaluation of drugs acting as suppressors or stimulators of angiogenesis. In this respect, there are concerted efforts to provide animal model for more quantitative analysis of in vivo angiogenesis (1). In this chapter the avascular cornea assay and its advantages and disadvantages in different species will be discussed. The cornea assay consists in the placement of an angiogenesis inducer (tumor tissue, cell suspension, growth factor) into a micropocket produced in the cornea thikness in order to evoke vascular outgrowth from the peripherally located limbal vasculature. This assay, on the contrary of the other in vivo assays, has the advantage of measuring only new blood vessels, since the cornea is initially avascular.

Experimental procedures

Rabbit cornea assay

The corneal assay performed in white rabbits of New Zealand was firstly described by Gimbrone et al. (2). It was chosen for the absence of a vascular pattern and for the easy manipulation and monitoring of the neovascular growth. This technique, extensively used during the years, has been substantially modified to fulfil different experimental requirements.

Surgical procedure:

- The rabbit size (2-3 kg) lets an easy manipulation of both whole

animal and eye to be easily extruded from its location and to be surgically manipulated.

- Use sodium pentothal (30 mg/kg, i.v.) to anaesthetise animals.
- Under aseptic conditions produce a micropocket (1.5 x 3 mm) using a pliable iris spatula 1.5 mm wide in the lower half of the cornea. A small amount of the aqueous humor can be drained from the anterior chamber when reduced corneal tension is required.
- Locate the implant at 2.5-3 mm from the limbus to avoid false positives due to mechanical procedure and to allow the diffusion of test substances in the tissue, with the formation of a gradient for the endothelial cells of the limbal vessels (Fig. 1). Implants sequestering the test material and the control are coded and implanted in a double masked manner.

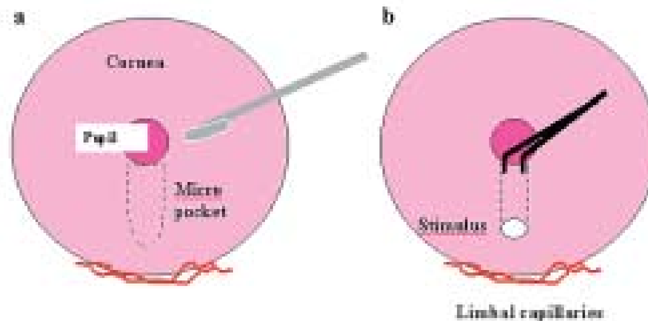

Figure 1. Schematic representation of the corneal micropocket assay. A micropocket is surgically produced in the corneal stroma of anaesthetized animals by a surgical scalpel and a pliable spatula (a) and the test substance is inserted in the micropocket (b).

Sample preparation:

The material under test can be in the form of slow-release pellets incorporating recombinant growth factors, cell suspensions, or tissue samples.

Slow release preparations: Recombinant factors of growth are prepared as slow-release pellets by incorporating the substance

under test into an ethylen-vinyl-acetate copolymer (Elvax-40) (DuPont de Nemours, Wilmington, DE) (3).

Elvax-40 preparation and testing:

- Weight 1 gr of Elvax-40, wash it in absolute alcohol for 100 fold at 37°C, and dissolve in 10 ml of methylen-chloride to prepare 10% casting stock solution.
- Test the solution prepared for its biocompatibility (3). The casting solution is eligible for using if any implants performed with this preparation induces the slightest or histological reaction in the rabbit's cornea. For testing, a pre-determined volume of Elvax-40 casting solution is mixed with a given amount of the compound to be tested on a flat surface and the polymer is allowed to dry under a laminar flow hood. After drying, the film sequestering the compound is cut into 1 x 1 x 0.5 mm pieces. Empty pellets of Elvax-40 are used as controls.

Cell and tissue implants:

- Prepare a cell suspension by trypsinization of confluent cell monolayers or concentrated cell suspensions. Introduce 5 µl containing $2x10^5$ cells in medium supplemented with 10% serum in the corneal micropocket. When the overexpression of growth factors by stable transfection of specific cDNA is studied, one eye is implanted with transfected cells and the other with the wild type cell line (4-6).
- When tissue samples are tested, samples of 2-3 mg are obtained by cutting the fresh tissue fragments under sterile conditions. The angiogenic activity of tumor samples is compared with macroscopically healthy tissue (5).

Drug and gene therapy

When drug solutions, which are incompatible with Elvax polymerisation and genes transduced by viral vectors have to be locally tested, microinjection of concentrated solutions is per-

formed by the use of insulin syringes equipped with 30G needles. After the removal of aqueous humor, a volume of 10 ul is injected within the corneal stroma in the space between the limbus and the pellet implant.

Quantification:

- Subsequent daily observation of the implants is made with a slit lamp stereomicroscope without anaesthesia. Angiogenesis, edema and cellular infiltrate are recorded.
- An angiogenic response is scored positive when budding of vessels from the limbal plexus occurs after 3-4 days and capillaries progress to reach the implanted pellet in 7-10 days (Fig 2). Implants which fail to produce a neovascular growth within 10 days are considered negative, while implants showing an inflammatory reaction are discarded (7).

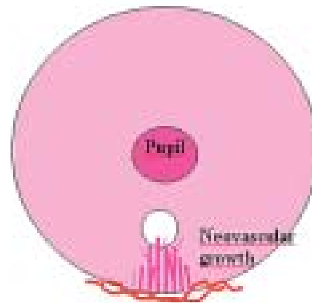

Figure 2. The new formed vessels start form the limbal vasculature and progress toward the implanted stimulus (representative positive implant at 7 days).

- During each observation the number of positive implants over the total implants performed is scored. The potency of angiogenic activity is evaluated on the basis of the number and on the basis of the growth rate of newly formed capillaries, and an angiogenic score is calculated by the formula [vessel densi-

ty x distance from limbus] (4,8). A density value of 1 corresponds to 0 to 25 vessels per cornea, 2 from 25 to 50, 3 from 50 to 75, 4 from 75 to 100 and 5 for more than 100 vessels. The distance from the limbus is graded with the aid of an ocular grid.

- Computerized image analysis: at each observation pictures are taken from each eye. Images are digitalized and analyzed by an ad hoc software after the extraction of the newly formed vessels form the background. The total number of vessels, the area occupied by vessels and the branching of the neovascular net are measured and statistically analysed (Fig. 3).

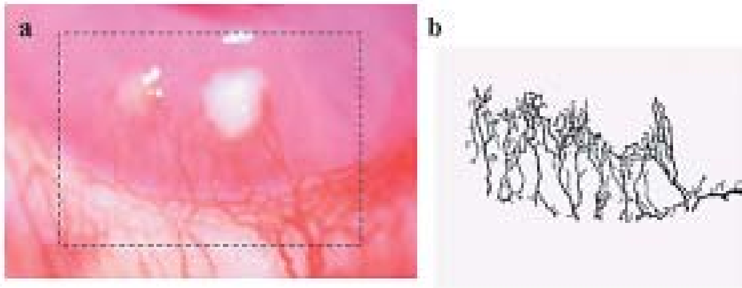

Figure 3. Computerized image analysis of the angiogenic response induced by the implant of two adjacent pellets releasing two angiogenic stimuli (a). Following digitalisation, the new vessels are extracted from the background (b). The image, converted in black and white, is analysed for the number of vessels, the area occupied by vessels and the degree of branching.

Histological examination:

- Remove the cornea and fix it in formalin.
- When required, freeze the cornea in cooled isopentane for 10 seconds and store at -80°C or in liquid nitrogen.
- New formed vessels and the presence of inflammatory cells are detected by hematoxylin/eosin staining or specific immunohistochemical procedure (i.e. anti-rabbit macrophages (RAM11), anti-CD-31 for endothelium) (4). Double staining (i.e. anti-CD-

31 for vascular endothelium and specific markers for tumor cells) could be useful to label new formed vessels of host and proliferating tumor cells implanted in the cornea.

For details and crucial points of the rabbit cornea micropocket assay see table 1.

Table 1: Crucial points of the rabbit's cornea assay

Animals	1) Body weight: in the range 1.8 - 2.5 kg for an easy handling and prompt recovery from anaesthesia. 2) Immobilisation during anaesthetic procedure and observation is important to avoid self-induced injury.
Sterility	1) Sterility of materials and procedures is crucial to avoid non specific responses. 2) Elvax-40 beads should be carefully washed in absolute alcohol as indicated to avoid inflammatory reactions.
Materials	1) Leave Elvax-40 in methylen-chloride at 37° C for 30-60 min. to dissolve it faster. 2) Polyvinylalcohol and Hydron can be used instead of Elvax-40. In our experience, a polymer of hydroxyethyl-metacrylate, gave less satisfactory results than Elvax-40.
Surgical procedure	1) Make the cut in the cornea in correspondence of the pupil and orient the micropocket toward the lower eyelid. 2) When two factors are tested make two independent micropockets. 3) Drain a small amount of the aqueous humor when implanting cells or tissue fragments to reduce corneal tension.

Mouse corneal micropocket

The mouse cornea micropocket assay was firstly described by Muthukkaruppan and Auerbach (9).

- Anaesthetise animals with methosyflurane.
- Make a corneal micropockets in both eyes reaching within 1 mm of the limbus and pellets containing substances to be tested coated with Hydron (Interferon Science, New Brunswick, NJ) are implanted.
- Use Hydron as a casting solution (12% (w/v)) solution, prepared dissolving the polymer in absolute alcohol at 37°C (3)). When peptides are tested, sucralfate (sucrose aluminium sulphate, Bukh Meditec, Copenhagen, Denmark) is added to stabilize the molecule and to slow its release from Hydron (10, 11).
- The vascular response measured as the maximal vessel length and the number of clock hours of neovascularization is scored at a fixed time (usually on postoperative 5 and 7 days) using a slit-lamp biomicroscopy and photographed. To quantify the section of the cornea in which new vessels are sprouting from the pre-existing limbal vessels, the circumference of the cornea is devided into the equivalent of 12 clock hours. The measure of the number of clock hours of neovascularization for each eye is performed during each observation.

Rat corneal assay

- Purified growth factors are combined 1:1 with Hydron as described by Polverini and Leibovich (12).
- Pellets are implanted 1-1.5 mm from the limbus of the cornea of anaesthetised rats (sodium pentobarbital, 30 mg/kg, i. p.).
- Neovascularization is assessed at fixed days (usually 3, 5 and 7 days): animals are perfused with colloidal carbon solution to label vessels, eyes are enucleated and fixed in 10% neutral buffered formalin overnight. The following day, corneas are excised, flattened and photographed. A positive neovascularization response is recorded only if sustained directional.

- ingrowth of capillary sprouts and hairpin loops toward the implant is observed. Negative responses are recorded when either no growth is observed or when only an occasional sprout or hairpin loop showing no evidence of sustained growth is detected.

Advantages and disadvantages in different species

Species: The rabbit's cornea has been found avascular in all strains examined so far. In some strains of rats the presence of preexisting vessels within the cornea and the development of keratitis are serious disadvantages. Furthermore, rabbits are more docile and more amenable to hand and experimentation than mice and rats. In case of inflammatory reactions, these ones are easily detectable in rabbits by stereomicroscopic examination as corneal opacity.

Measurements: In mice and rats it is possible to obtain time-point results. The evolution of the angiogenic response in the same animal is not recommended because each time the cornea is observed the animal has to be anaestesized since it is not easy to keep it quiet. Experiments are made with a large number of animals and vessel growth during time can be visualised by perfusion with colloidal carbon solution in individual animals. Multiple observations are instead possible in rabbits. The use of slit lamp stereomicroscope and of not anaestesized animals allows the observation of new formed vessels during the time with long time monitoring, even for 1-2 months.

Different experimental procedures: In the rabbit's eye, due to its wide area, stimuli in different forms can be placed. In particular the activity of specific growth factors can be studied in the form of slow-release pellets (6, 13-16) and of tumor or non-tumor cell lines stably transfected for the over-expression of angiogenic factors (4,6). The modulation of the angiogenic responses by differ-

ent stimuli can be assessed in the rabbit cornea assay through the implant and/or removal of multiple pellets placed in parallel micropockets produced in the same cornea. The implant of tumor samples from different locations can be performed both in corneal micropockets and in the anterior chamber of the eye to monitor angiogenesis produced by hormone-dependent tissues or tumors (i.e human breast or ovary carcinoma in female rabbits) and it allows the detection of both iris and corneal neovascular growth (4,13).

Treatment with drugs

The effect of local drug treatment on corneal neovascularization con be studied in the form of ocular ointment (17) or microinjection in the corneal thickness. The effect of systemic drug treatment on corneal angiogenesis can be also evaluated (4, 5, 8, 18). However, when considering the size of the animals, systemic drug treatment in rabbits requires an higher amount of drugs than smaller animals.

Interestingly, the use of nude mice allows the study of angiogenesis modulation in response to effectors produced and released by tumors or tumor cell lines of human origin growing subcutaneously. Treatment of mice with antiangiogenic or antitumor drugs allows the simultaneous measurement of tumor growth and metastasis and corneal angiogenesis.

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THE CHICK EMBRYO CHORIOLLANTOIC MEMBRANE

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Introduction

As pointed out by Auerbach et al. (1) 'perhaps the most consistent limitation (to progress in angiogenesis research) has been the availability of simple, reliable, reproducible, quantitative assays of the angiogenesis response'.

Classical assays for studying angiogenesis in vivo include the hamster cheek pouch, the rabbit's ear chamber, dorsal skin and air sac, the chick embryo chorioallantoic membrane (CAM) and iris and avascular corneal of rodent eye (see 1-2 for review). Several new models have been recently introduced including subcutaneous implantation of various three-dimensional substrates including polyester sponge (3), polyvinyl-alcohol foam disc covered on both sides with a Millipore filter (the disc angiogenesis system; 4), and Matrigel, a basement membrane rich extracellular matrix (5). The most reliable in vivo angiogenesis techniques use the CAM and the rabbit cornea. In contrast to the CAM assay, the others in vivo techniques are more complex, consume a large quantity of angiogenic factors, are not feasible for numerous samples and are expansive.

The chick embryo chorioallantoic membrane (CAM) assay

The CAM assay facilitates the testing of multiple samples and the generation of dose-dilution curves. It has been used to identify almost all of known angiogenic factors. The allantois is an extraembryonic membrane, derived from the mesoderm in which primitive blood vessels begin to take shape on day 3 of incubation. On day 4, the allantois fuses with the chorion and forms the chorioallantois.

Until day 8 of incubation, primitive vessels continue to proliferate and to differentiate into an arteriovenous system and thus originate a network of capillaries that migrate to occupy an area beneath the chorion and mediate gas exchanges with the outer environment.

The CAM vessels grow rapidly up to day 11, after which the endothelial cell mitotic index decreases just as rapidly, and the vascular system attains its final setup on day 18 of incubation, just before hatching (6).

Utilization of CAM

Fertilised chick eggs are incubated at 37°C, at a constant humidity. On day 3 of incubation, after aspirating 2-3 ml albumen at the more pointed end of the egg, so that the CAM can be detached from the shell itself, a window is cut into the shell with the aid of scissors and the underlying CAM vessels are demonstrated (Fig. 1).

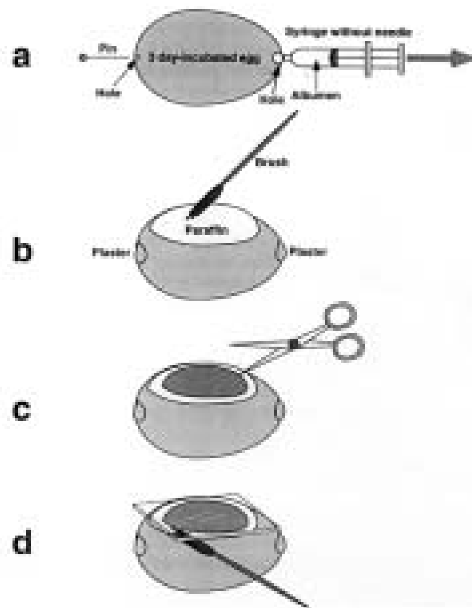

Figure 1. Preparation of the egg for the CAM assay. On day 3 of incubation, 2-3 ml of albumen are aspirated at the acute pole of the egg (a) to detach the developing CAM from the shell; the holes are closed with plaster. The upper surface of the egg is brushed on with paraffin (b) and cut with scissor kept parallel to the surface so as not to damage the embryo (c). The window is covered with a glass slide and sealed with paraffin (d).

CAM was first used to study tumor angiogenesis by grafting tumor samples onto its surface on day 8 of incubation; after 48-72 hours the tumor-induced vasoproliferative response occurring as new-formed vessels which converge towards the graft is evaluated in vivo by means of a stereomicroscope (7-9). On day 12, CAMs are processed for light or electron microscopy. Briefly, the embryos and their membranes are fixed in ovo in 3% phosphate-buffered glutaraldehyde, dehydrated in serial alcohols, postfixed in 1% phosphate-buffered OsO4, and embedded in Epon 812. Semithin and ultrathin sections are cut on a ultramicrotome. The semithin sections are stained with a 0.5% acqueous solution of

toluidine blue and they are observed under a light microscope. The ultrathin sections are stained with uranyl acetate followed lead citrate and examined under a transmission electron microscopy. It should be noted that CAM was found to be ideal for investigating the tumor-induced angiogenesis, because at that point in time the host's immunocompetent system was not yet fully developed, hence the conditions for rejection were not yet established (10).

CAM is also used to study different macromolecules developing angiogenic and anti-angiogenic activity (11). To do it, inert synthetic polymers soaked with the macromolecule someone wants to test, are laid upon the CAM surface. Elvax 40 and hydron are commonly used. They were first described and validated by Langer and Folkman (12): both proved to be biologically inert when implanted onto the CAM and both were found to polymerize in the presence of the test substance, allowing its sustained release during the assay. When polymers are used in combination with an angiogenic substance, a vasoproliferative response will be recognizable 72-96 hours after implantation: the response takes the form of increased vessel density around the implant, with the vessel radially converging towards the centre like spokes in a wheel (13). Conversely, when polymers combined with an anti-angiogenic substance are tested, the vessels become less dense around the implant after 72-96 hours, and eventually disappear (14).

Alternatively, when testing a fluid substance, or cell suspensions, they can be directly inoculated into the cavity of the allantoic vesicle so that their activity covers the whole vascular area in a uniform manner (15-16).

Wilting et al. (17) used culture coverslide glasses 4-5 mm in diameter on which the angiogenic factors were placed. Glasses were turned over and placed onto the CAM on day 9 of incubation and the angiogenic response was evaluated 96 hours later.

Another method has been proposed by Nguyen et al. (18): the testing substance is placed into a collagen gel between two parallel nylon meshes which align the capillaries for counting.

The resulting “sandwich” is then placed upon the CAM on day 8 of incubation. A major advantage of this method is due to the fact that it does not require histological sections, thus facilitating the screening of a large number of compounds.

Still other methods have been proposed whereby the CAM vascular networks can be displayed in greater detail, except that the embryo with the extra-embryonic membranes and yolk sac must be transferred to an in vitro system during the early stages of development (day 3 or 4 of incubation). The system consists of a large diameter (10 mm) Petri dish (19) (Fig. 2), plastic pipe, plastic wrap and tripod (20), plastic cup and plastic wrap (21) and plastic cups alone (22).

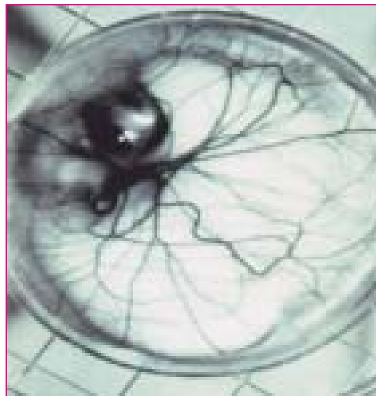

Figure 2. CAM assay in a Petri dish.

Shell-less culture of avian embryos facilitates experimental access and continuous observations of the growing embryos almost to the term of hatching. Hence it has a range of applications in developmental biology, angiogenesis and pharmacology research. Importantly, differentiation of the CAM of cultured embryos appears to follow the same time course as that in ovo. De-

spite these advantages of shell-less culture over eggshell windows, existing shell-less cultures are expensive, occupy large amounts od space and long-term viability is low. For example, ex ovo culture of chicken embryos in Petri dishes amd plastic wrap/cup apparatus is associated with 40% and 20% two-weeks survival rates respectively.

We have developed a new method for the quantitation of angiogenesis and anti-angiogenesis in the CAM, based on the implantation of gelatin sponges treated with a stimulator or inhibitor of blood vessel formation on the top of growing CAM on day 8 of incubation (23). Blood vessels, growing vertically into the sponge and at the boundary between sponge and surrounding CAM mesenchyme, were counted by a morphometric method on day 12 (Fig. 3).

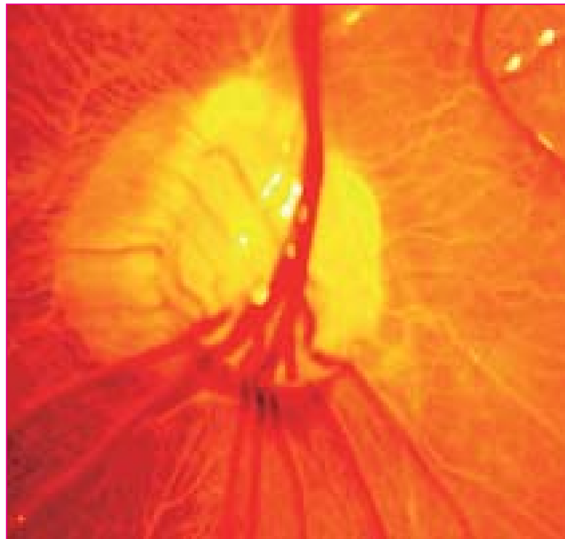

Figure 3. A gelatin sponge loaded with FGF-2 observed in ovo at day 12 of incubation. Many vessels radially arranged converge around the sponge.

The gelatin sponge appears to be also suitable for the delivery of cell suspensions onto the CAM surface and for the evaluation of their angiogenic potential (23-26). Many techniques can be applied within the constraints of paraffin and plastic embedding, including histochemistry and immunohistochemistry (Fig. 4).

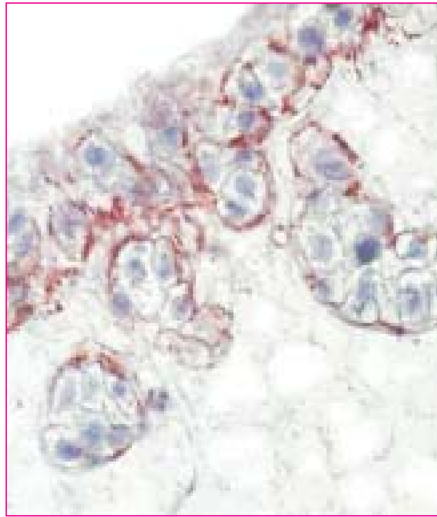

Figure 4. CAM's blood vessels stained with an anti-FVIII antibody.

Electron microscopy can also be used in combination with light microscopy. Moreover, unfixed sponges can be utilized for chemical studies, such as the determination of DNA, protein and collagen content, as well as for RT-PCR analysis of gene expression by infiltrating cells, including endothelial cells.

Evaluating the vasoproliferative response by semiquantitative methods

Several semiquantitative methods have been used to evaluate the extent of the vasoproliferative response. One method con-

siders variations in the distribution and density of CAM vessels next to the site of grafting: these are evaluated in vivo by means of a stereomicroscope at regular intervals following the grafting procedure. The response is rated equal to 0 when no change with respect to the time of grafting can be appreciated; the score is +1 when few microvessels converging toward the implant are observed; +2 when a cosiderable change in the number and distribution of converging microvessels is appreciated (8).

By another method, the degree of vasoproliferative response is defined as a vascular index based upon photographic reconstructions. All the vessels converging toward the implant and contained inside a 1 mm in diameter ring superposed to the CAM are enumerated: the ring is drawn around the implant in such a way that it will form an angle of less than 45° with a straight line drawn starting from the implant's centre. Vessels branching dichotomically outside the ring are counted as 2, while those branching inside the ring are counted as 1 (27-29 and Fig. 5).

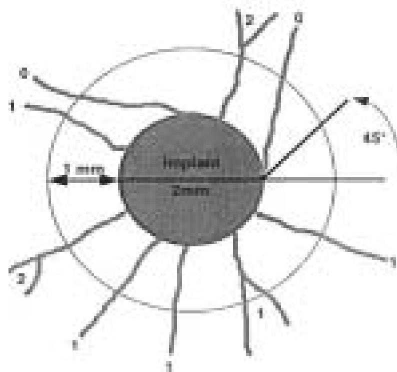

Figure 5.

A third method expresses the degree of vasoproliferative response as evaluated in vivo under the stereomicroscope by means of a 0-to-5 scale of arbitrary values. Zero describes a condition of the vascular network which is unchanged with respect to the time of grafting; 1 marks a slight increment in vessel density associated to occasional changes in the course of vessels converging towards the grafting site; 2, 3, 4 and 5 correspond to a gradual increase in vessel density associated with increased irregularity in their course; a 5 rating also highlights strong hyperemia. A coefficient describing the degree of angiogenesis can also be derived from the ratio of the calculated value to the highest attainable value; thus, the coefficient's lowest value is equal to 0 and the highest value is 1 (29 and Fig. 6).

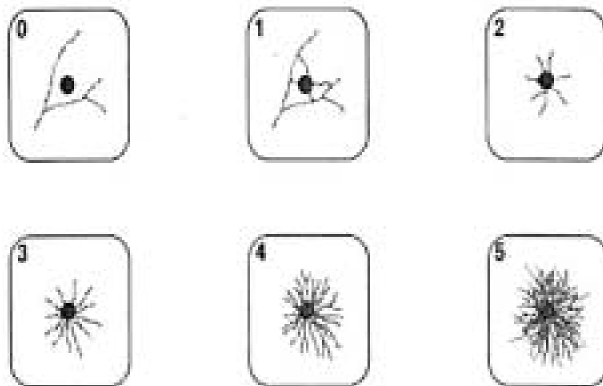

Figure 6.

When an angiostatic compound is tested, a semiquantitative evaluation of the anti-angiogenic response is performed by two independent observers, which determine the radius of the growth inhibition zone as 0-4 grades vessel growth from the center of each disk soaked with the angiostatic compound to the furthest

contiguous area in which tertiary vessels are absent. Zones with a radius greater than 1 mm are interpreted as evidence of significant inhibition of angiogenesis (30).

Evaluating the vasoproliferative response by quantitative methods

Quantitative evaluation of vascular density can be obtained by applying morphometric and planimetric methods to the observation of histologic CAM specimens.

The angiogenic response can be evaluated as a microvessel area by using a morphometric method of ‘point counting’ (23). With a double headed photomicroscope, two investigators simultaneously identify transversally cut microvessels (diameter ranging from 3 to 10 (μm), and each identification is agreed upon in turn. Microvessels are studied at magnification of 250 x, with a square mesh inserted in the eyepiece. The mesh consisted of 12 lines per side, giving 144 intersection points. Six random chosen microscopic fields of each section (every third section within 30 serial slides from an individual specimen are analyzed) are evaluated for the total number of intersection points which are occupied by microvessels. Mean values, are determined for each analysis. The microvessel area is indicated by the final mean number of the occupied intersection points expressed again as percentage of the total number of intersection points. Statistically significant differences between the mean values of the intersection points in the experimental CAM and control ones are determined by Student’s t test for unpaired data. Automatic image analyses have been also suggested for CAM (31).

The limitations of CAM assay

The main limitation of CAM is represented by non specific inflammatory reactions which may develop as a result of grafting, and in turn induce a secondary vasoproliferative response eventually

making it difficult to quantify the primary response which is being investigated (31,32). Inflammatory angiogenesis per se in which infiltrating macrophages or other leukocytes may be the source of angiogenic factors cannot be distinguished from direct angiogenic activity of the test material without detailed histological study and multiple positive and negative controls. In this connection, a study of histological CAM section would help detecting the possible presence of a perivascular inflammatory infiltrate together with a hyperplastic reaction, if any, of the chorionic epithelium. However, the possibilities of causing non specific inflammatory response are much lower when the test material is grafted as soon as CAM begins to develop since then the host's immune system is relatively immature (10). This problem may be overcome by using the yolk sac vessels of the 4-day chick embryo because this system has a markedly reduced inflammatory and immune response (33, 34).

The CAM assay has other drawbacks: i) False-positive angiogenesis may be induced by any test material which causes cell damage by virtue of abnormal osmolarity, pH, or toxicity. This kind of substrates may induce an inflammatory response or cause focal contraction of the CAM. ii) Angiogenesis may be induced by degradation product of fibrin (35) which can leak from CAM vessels in response to injurious test substances. iii) The test material is placed on preexisting vessels and it derives that the actual neovascularization can hardly be distinguished from a falsely increased vascular density due to a rearrangement of preexisting vessels that follows contraction of the membrane (36). iv) Timing of the CAM angiogenic response is essential. Many studies determine angiogenesis after 24 hours, a time at which there is no angiogenesis, but only vasodilation. It would be worthwile to point out that measurements of vessel density are really measurement of new-formed vessels, and that the distinction between vasodilation and neovascularization is not easy to make. To circumvent this drawback it is useful to utilize sequential photography to document new vessel formation.

Concluding remarks

The in vivo assays of angiogenesis have enabled to make up important progress in elucidating the mechanism of action of several angiogenic factors and inhibitors. It is reasonable to reserve the term "angiogenic factor" for a substance which produces new capillary growth in an in vivo assay.

A variety of animal models have ben described to provide more quantitative analysis of in vivo angiogenesis and to characterize pro- and anti-angiogenic molecules.

The principal qualities of the in vivo assays are their low cost, simplicity, reproducibility and reliability which, in turn, among the different in vivo assays are important determinants dictating the choise of method.

However, they are also very sensitive to environmental factors, not readily accessible to biochemical analysis and their interpretation is frequently complicated by the fact that the experimental condition inadvertenly favours inflammation, and that under these conditions the angiogenic response is indirectly elicited, at least in part, through the activation of inflammatory or other non-endothelial cells. On the basis of these limitations, ideally two different assays should be performed in parallel to confirm the angiogenic or anti-angiogenic activities of test substances.

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THE MATRIGEL IMPLANT ASSAY: A QUICK TEST FOR IN VIVO ANGIOGENESIS

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Introduction

Angiogenesis has become one of the most promising targets for the tumor therapy. Starting from the early reports by Folkman and Liotta, biomedical researchers have been involved in the setting of suitable in vitro and in vivo tests, used to identify the angiogenic molecules and their inhibitors.

As in vitro angiogenesis is usually studied utilizing endothelial cells, in absence of important components like pericytes, smooth muscle cells. The infiltrate, the confirmation of these data by in vivo observations is a fundamental issue for reliable results. At present the subcutaneous implant of inert sponges in the mouse, the eye pocket assay in the rabbit's cornea and the chick embryo chorioallantoic membrane assay are widely the more used and accepted.

The most subcutaneous implant models were developed with the aim to trap a putative angiogenic compound into a suitable carrier, mostly an avascular sponge-like structure, which can slowly release the factor at the site of implant. The final result of this process is the recruitment of new blood vessels. These tests can also be used to study the in vivo activity of antiangiogenic agents. Several assays have been established to date: the aorta

ring (1) the dorsal skin flaps (2), the sponge implant assay (3, 4), the sodium alginate beads (5), and the air sac model (6); progress and problems for each method are described in this review (7).

Passaniti et al. described a simple adaptation of the cutaneous implant assays using reconstituted basement membranes (8). Our group has extensively used reconstituted basement membranes to study specific steps of the angiogenic process in vitro and assess angiogenesis in vivo (9, 10, 11). The use of reconstituted basement membranes has several advantages both in vitro and in vivo (12).

The reconstituted basement membrane: one tool for several tests

Three-dimensional matrix substrates have been extensively used in vitro to study differentiation of endothelial cells into vessel-like structures: fibrin and collagen gels have proven good substrates (13, 14) however, the extensive time required for these assays is a major limiting factor.

These problems can be widely bypassed by using a reconstituted basement membrane, the most used material is known as Matrigel(tm), an extract of a murine tumor which produces massive amounts of basement membrane (15).

Matrigel is a mixture of basement membrane components (mostly laminin, perlecan, type IV collagen, nidogen) prepared from the murine EHS tumor of C57/black mice grown in vivo. Once extracted from the tumors with urea-containing buffer, the matrix mixture is dialyzed in DMEM at 4°C and stored frozen (-20°C) at concentrations ranging from 12-18 mg/ml. Commercially prepared Matrigel is generally supplied in aliquots at 10-12 mg/ml (Becton Dickinson, Bedford, MA). A commercial growth factor-reduced Matrigel (Becton Dickinson, Bedford, MA) is also available, but this preparation is obtained reducing the content of heparan sulfate proteoglycan, thus altering Matrigel composition. Matrigel can be frozen and thawed several times without any problem, it

is convenient to prepare small aliquots to shorten the thawing procedure in ice/water bath. As Matrigel quickly polymerizes at a room temperature, all the material used for its manipulation (pipettes, tips, tubes, multiwells) must be pre-chilled.

Matrigel is often used in three different tests related to angiogenesis: the chemoinvasion assay, the morphology assay and the in vivo sponge model. The in vitro models are briefly described from an "angiogenic" point of view and a detailed protocol for the in vivo test given.

The chemoinvasion assay

This is a modification of the classic Boyden chemotaxis assay in Boyden chambers (16). PVP-free polycarbonate filters (12 µm pore size for endothelial cells, Nuclepore, Concorezzo, Milan, Italy) are coated with Matrigel diluted with cold wateron ice (15-40 µg/50 µl/13mm diameter filter for endothelial cells). Filters are placed on the tissue culture plates and the liquid Matrigel is pipetted onto the filter. During this step care must be taken to insure that the Matrigel solution is homogeneously applied on filter surface, leaving a small border of uncoated membrane to prevent leakage off the filter. The Matrigel coated filters are dried under a laminar flow and the plate tightly closed with parafilm (they can be maintained in this form up to 48 hours at 4°C). The filters are re-hydrated just before performing the assay using serum free DMEM at 4°C. Numerous substances can be placed as chemoattractants in the lower chamber. Kaposi's Sarcoma cell conditioned medium is a good chemoattractant for endothelial cells, mimicking a highly angiogenic environment (17, 18); purified angiogenic growth factors are also frequently used, i.e. vascular endothelial growth factor (VEGF) or basic fibroplast growth factor (bFGF) (19, 20). Both chemoattractants and cells are suspended in serum-free medium (SFM) containing 0.1% BSA. Medium with BSA alone in the lower chamber serves as a negative control. Chemotaxis and chemoinvasion assays are often done in parallel to determine if

treatments affect chemotaxis itself or if they are specific for invasion. A control of chemotaxis in a chemoinvasion assay is also useful to verify that Matrigel concentration is sufficient to act as a real barrier to cell migration. An invasion index (invasion/chemotaxis) can be calculated and indicate the specific contribution of matrix degradation using the following formula:

$$\text{Invasion index} = \frac{\text{Invaded Cells}}{\text{Migrated Cells}} \times 100$$

The upper chamber is filled with an endothelial cell suspension (usually 120.000cells/800 µl/chamber). The chambers are then incubated at 37°C in 5% CO^2 for 6 h. While tumor cells usually do not migrate in response to SFM alone, endothelial cells show a variable degree of random unstimulated migration due to the loss of cell-cell contact; once determined, this value is usually subtracted to the final data as "background migration". Invasion inhibitors acting on the chemoattractant are added to the lower compartment of the Boyden chamber, while inhibitors acting on the cells or cell products are generally added along with the cells. At the end of the incubation time, cells remaining on the upper surface of the filter are mechanically removed by wiping them with a cotton swab or stripping on a glass slide. The cells migrated to the under surface are quantitated after staining (with toluidine blue, hematoxylin/eosin or others). Assays are generally performed in triplicate and repeated two or three times. In the original assay quantitation was performed by microscope counting five to ten random fields for each filter. Various alternatives have been proposed such as colorimetric detection of staining, image analysis, and metabolic labeling with MTT or similar compounds (21).

Morphogenesis on Matrigel

Matrigel can also be used for morphological studies on vascular cell organization (22). Matrigel is thawed at 4°C in an

ice/water bath, and 0.3-0.4 ml of a concentrated solution (10 mg/ml) carefully pipetted into 13 mm/diameter tissue culture wells (48-wells chambers), avoiding even small bubbles. The matrigel is then polymerized for 1 h at 37°C. Once polymerization is occurred, $7x10^4$ endothelial cells in 1 ml of medium are carefully pipetted on top of the gel. The plates are then incubated at 37°C in a 5% CO^2, humidified atmosphere. The assays are photographed and monitored with an inverted microscope, a modification using MTT as a dye has been recently described (23). HUVEC cells suspended in 10% FCS-containing medium with heparin and ECGS organize into capillary-like networks within six hours, this structure is maintained for about 48 hours and then lost. The kinetics of network organization and cell viability is modulated by angiogenic factors and anti-angiogenic drugs.

The in vivo sponge model

Although some synthetic polymers have proven suitable systems to trap angiogenic factors in vivo, they are an extraneous material implanted in a living organism. In addition, these polymers frequently need protein enrichment to obtain a slow release of the trapped material (24). The subcutaneous Matrigel implant model has several advantages over many other in vivo implant models. Matrigel is fluid at 4°C but quickly polymerizes at 37°C, thus the material is readily implanted by a simple injection, avoiding slow and complex surgical procedures. For this reason it is an ideal tool to trap cytokines, cells or cell supernatants subcutaneously in mice by simple injection. In addition the EHS tumor which produces Matrigel is syngeneic for the C57/black mice generally used for the experiments. This fact allows the use of a biological matrix in immunologically competent mice without any immunologic reaction. Another important observation regarding the use of Matrigel is its content of Perlecan, the heparan-sulphate proteoglycan of the basement membrane, which is involved in the storage and correct presentation to the receptor of angiogenic growth factors (25, 26).

We have also used the Matrigel sponges model for the study and evaluation of inflammation (10). The presence of tumor associated macrophages (TAMs) and polymorphonuclear (PMN) cells within the tumor was described by Virchow more than one century ago; however, the potential role of these inflammatory cells in promoting/modulating angiogenesis and tumor growth has become appreciated only in recent times (27).

Materials and methods

This assay was originally described by Passaniti (8) who studied the angiogenesis induced by bFGF added with heparin. This method has been extensively used to study the angiogenic potential of Kaposi's sarcoma cell, supernatants and HIV Tat protein (28) and to test numerous anti-angiogenic compounds (TIMP-2, IFNs, Somatostatin...) (28, 29, 30, 31).

The test is performed using at least 5-6 C57/black mice per point (two implants/mouse), Matrigel should have a concentration ranging from 12 to 14 mg/ml to allow a further dilution by the addition of cytokines or cell supernatants (in PBS or DMEM) to reach a final concentration of 11 mg/ml. More dilute Matrigel does not polymerize well in vivo. The ideal final volume to be injected is 500 to 600µl. Cell supernatants need to be concentrated 10x so that once diluted 1:10 with Matrigel the original concentration is restored. Serum-free Kaposi's sarcoma supernatants are concentrated using the Centriprep centrifuge concentrators (Amicon) with a size cut-off of 3kDa, and used as a 10x stock solution stored at -80°C. Most of the angiogenic growth factors are heparin binding, and often use heparin/heparan-sulphates for a correct presentation to the receptor. Matrigel contains perlecan (32, 33) which is able to bind these factors, this interaction constitutes a perfect angiogenic environment when the tested supernatants have high titers of such cytokines, as mouse endothelial cells are easily exposed to this stimulus. The angiogenic growth factors or the cell supernatant should be mixed with heparin (use concen-

trated stock to reach a final concentration of 20-30U/ml) to favor their diffusion and their presentation to the low and high affinity receptors of endothelium. The concentration of heparin is crucial and should be assessed for each new commercial stock and Matrigel batch used, since low heparin concentration does not allow for heporin binding, while high heparin can be angiogenic *per se* (in fig 1 is reported the histological analysis of a VEGF-containing Matrigel sponge and a control heparin gel).

It is convenient to prepare the samples (i.e. Matrigel + angiogenic growth factors + heparin, total volume 600μl) in single 1.5 ml sterile tubes (eppendorf) and use one insulin syringe for each injection (keeping everything in ice): the sample needs to be mixed with the syringe three-four times, just before the injection, avoiding bubbles. The mix is then slowly injected subcutaneously in the dorsal area of C57bl6/black mice (it is better to use only male specimens which appear more prone to the angiogenic response). Matrigel takes about 1 minute to polymerize, after this time the animal can be returned. After 4 days the animals are sacrificed, the implants collected and immediately photographed.

Quantification of the angiogenic response

Matrigel sponges can be processed by two different ways: 1) by an histologic examination and 2) for hemoglobin content. The first approach provides qualitative data regarding the presence and organization of vessels and the immunohistochemical characterization of recruited cells. The second gives a numeric value proportional to the extent of vascularization. Each method excludes the other, because for hemoglobin quantification the Matrigel pellet needs to be minced.

Histologic evaluation: the gel is routinely processed for paraffin embedding by para-formaldehyde fixation (4% in PBS) progressive dehydratation with ethanol/water solutions (30%, 50%, 70%, 80%, 90%, 100% twice, 30' each step), xylol treatment (20', twice), xylol-paraffin (1:1) incubation (1h, 52°C), double paraffin incuba-

tion (40', 52°C) and finally embedded. Note that for the high content of water the Matrigel pellet usually shrinks so that the number of potential sections is limited.

Hemoglobin Content: Hemoglobin content is determined with the Sigma Drabkin solution Kit 525, the principle of the assay is based on the oxidation of hemoglobin to cyan-meta-hemoglobin which is detectable spectrophotometrically at 540 nm. The Matrigel pellets are weighed and minced in 1ml of distilled water; after centrifugation (13,000 g/5') 200 µl of each sample is diluted in 800 µl of Drabkin solution. The kit contains a standard hemoglobin solution to prepare a titration curve. The spectrophotometric measure at 540 nm indicates the relative hemoglobin concentration (RHC) of the sample in g/dl. The absolute hemoglobin concentration (AHC) can be calculated using the following formula:

$$\text{AHC (g/dl)} = \frac{\text{RHC(g/dl)} \times 100}{\text{sample weight (g)}}$$

Additional notes

If it is necessary to inject allogenic cells along with Matrigel the test can be modified using nude mice. Nude mice usually exhibit a lower angiogenic response as compared to C57/black mice; it is necessary to titrate the cytokines and heparin concentration. The appearance of purulent yellow pellets indicates a contamination of the injected material (by bacteria or yeast) as the color is linked to PMN phagocyte activation, these samples must be eliminated.

Figure 1. Histology of VEGF+heparin and heparin alone -containing Matrigel pellets after 4 days in vivo.

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IN VIVO GENE TRANSFER WITH AAV VECTORS AS A TOOL TO UNDERSTAND GENE FUNCTION IN BLOOD VESSEL FORMATION

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Molecular mechanisms of neovascularization

The study of the molecular and cellular mechanisms determining the formation of new blood vessel is both a challenging basic research goal and an essential pre-requisite for the development of new therapeutic strategies.

At least two mechanisms exist to explain the de novo formation of blood vessels (Isner and Asahara, 1999) (Figure 1).

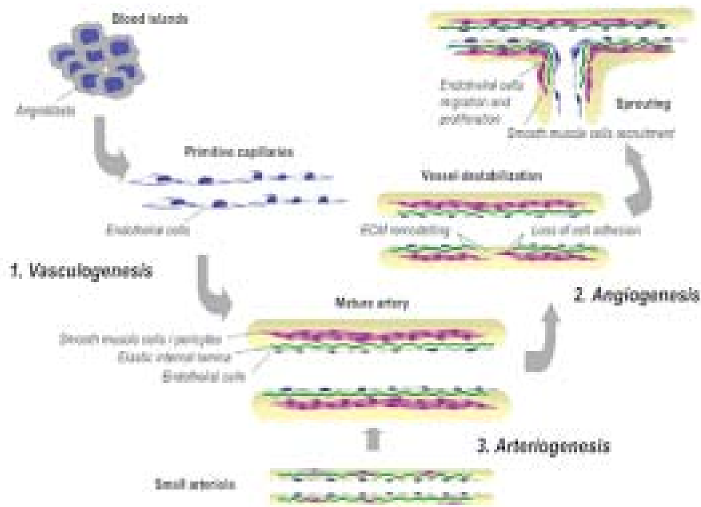

Figure 1. Mechanisms of blood vessel formation. Blood vessels can arise by three distinct mechanisms: vasculogenesis, angiogenesis and arteriogenesis. During embryonic development, a population of endothelial cell precursors (angioblasts) starts to proliferate within the blood islands, and to organize into a primitive capillary network. The consequent establishment of strict cell-to cell contacts, with the recruitment of pericytes, gives rise to a functionally competent vessel.
On the contrary, angiogenesis is the main process by which blood vessels can arise in adult life. This relies on the proliferation and migration of endothelial cells from a pre-existing vessel (angiogenic sprouting) to form a new branching capillary. A transient destabilization of the vessel wall, with profound extracellular matrix degradation and loss of cell-to-cell and cell-to matrix interactions are required for angiogenesis to occur. The latest stages of the process involve the strengthening of endothelial cell adhesion to matrix components and enrollment of smooth muscle cells to the newly formed vessel wall. Arteriogenesis also exclusively occurs in adults as a consequence of a major arterial narrowing and consists in the remodeling of pre-existing arteriolae to form larger arteries. This is probably the main mechanism explaining the development of a compensatory collateral network in the heart and muscles of patients affected by chronic arterial stenosis.

The first one, occurring during embryonic development, is vasculogenesis and consists of the proliferation and subsequent differentiation of pluripotent stem cells (angioblasts). This results in the formation of blood islands, which then fuse to create a primordial vascular network. Increasing evidence suggests that vasculogenesis also occurs in adult organisms, where endothelial

progenitors cells might be mobilized from the bone marrow and contribute to physiological and pathological neovascularization processes. Moreover, several surface markers are shared between angioblasts and hematopoietic stem cells, suggesting the existence of a common precursor - the hemangioblast - able to give rise to both blood and endothelial cells (Asahara et al., 1999).

An alternative mechanism by which blood vessels can arise during adult life is angiogenesis and consists of the sprouting of endothelial cells from pre-existing vessels (Risau, 1997). This requires a profound remodeling of the vascular structure, with cellular migration and proliferation, together with a complex reorganization of the extracellular matrix. Most probably, the angiogenic process in ischemic tissues is promoted by hypoxia, which drives the expression of several pro-angiogenic cytokines. The transcription factor HIF-1 (Hypoxia Inducible Factor-1) is the main regulator of oxygen homeostasis in mammalian cells, being able to induce the expression and secretion of different angiogenic cytokines as a response to hypoxia. These include the vascular endothelial growth factor (VEGF), as well a large series of other factors that control or participate in all phases of the angiogenic process (see below).

Beside vasculogenesis and angiogenesis, there is some evidence that a third mechanism of vessel formation might operate in the adult, which is responsible for the development of angiographically visible collaterals in patients with advanced obstructive atherosclerotic disease. This event is usually referred to as arteriogenesis, since it appears to be consequent to the remodelling of pre-existing arteriolae to form major arteries. Even if some of the stimuli that trigger this process have been defined (for instance, shear stress and endothelial activation with monocyte recruitment), the molecular details of this event still remain largely unknown. In this respect, it cannot be excluded that the three mechanisms leading to new blood vessel formation could share at least some common molecular players, the action of which could

be somehow regulated by local stimuli. It is therefore essential in the next future to develop new molecular tools to identify the function of each specific angiogenic factor in different experimental settings in vivo.

Angiogenesis

Members of the VEGF gene family participate in virtually all phases of the angiogenic process (Ferrara, 1999). The VEGF family is composed by six different members (VEGF-A,B,C,D,E and placental growth factor); in particular VEGF-A exists as a series of different isoforms produced by alternative splicing. Binding of VEGFs to their specific cell surface receptors is a key event in the endothelial cell activation process. Until now, three VEGF receptors have been characterized (VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1 and VEGFR-3/Flt-4), whose relative affinity and tissue distribution determines the biological effect (Carmeliet and Collen, 1999; Neufeld et al., 1999). VEGF-A is almost ubiquitous and mainly binds to VEGFR-1 and -2 on endothelial cells, while VEGF-B is particularly expressed in the myocardium and shows a high affinity for VEGFR-2 (Bellomo et al., 2000). Finally, VEGF-C and -D present selective affinity for both VEGFR-2 and -3, thus exerting a relevant role in the formation of lymphatic vessels (Achen et al., 1998; Mandriota et al., 2001). VEGF-A has been shown to be upregulated under hypoxic and ischemic conditions in vitro and in vivo at both the transcriptional and translational levels (Liu et al., 1995). Interestingly, its main receptor KDR/flk-1 is also upregulated by hypoxia.

VEGF-A is a powerful inducer of endothelial cell activation, proliferation and migration. However, considering the whole complexity of the angiogenic process, it is not surprising that its function is modulated and complemented by the concerted action of several other cytokines. On the surface of endothelial cells, VEGF receptors associate with different co-receptors, such as integrins, caderins and neuropilins, which are involved in the mod-

ulation of VEGF signalling (Bussolino et al., 2001; Soker et al., 1998). In addition, endothelial cells also express at their surface receptors for the ephrin family members, which appear to play a role in the differentiation process of arteries and veins (Gale and Yancopoulos, 1999). Ephrins and semaphorins, which are the ligands for ephrin-receptors and neuropilins respectively, have been initially recognized as the key molecules controlling the spatial distribution of growing axons in the central nervous system. Recent evidence strongly supports the notion that the same molecules, by binding their specific receptors on endothelial cells, also play an essential role in determining the spatial guidance of the developing vessels (Gale et al., 2001).

Other different growth factors appear important to determine the structural organization of new vessels and the achievement of functional competence. Among these, members of the angiopoietin family appear as critical factors in modulating VEGF activity, allowing the proper maturation of new capillaries to form more stable and competent vessels. At the moment, four members of the family have been identified and characterized (Ang-1, Ang-2, Ang-3 and Ang-4), which mainly interact with the Tie-2 receptor on endothelial cells (Suri et al., 1998). The most interesting property of Ang-1 is its capacity to reduce the leakiness of VEGF-induced blood vessels, probably by stimulating the interaction of endothelial cells with extracellular matrix components (Asahara et al., 1998; Davis and Yancopoulos, 1999; Thurston et al., 2000).

Several other molecules, while not having a specific effect on endothelial cells, have been recognized to be able to sustain an angiogenic response in vivo. Among these molecules, particular interest is reserved to some members of the FGF (Fibroblast Growth Factor) family. By interacting with different receptors present on a variety of cell types, FGF-1, FGF-2 and FGF-5 stimulate endothelial cell proliferation and increase the production of proteases responsible for matrix degradation, thus promoting cellular migration (Battler et al., 1993; Giordano et al., 1996). Never-

theless, the precise mechanisms by which these factors induce angiogenesis and their actual role in different physiological and pathological conditions still remains to be fully explored.

Given the complexity of the processes leading to new blood vessel formation, it appears essential to develop experimental conditions allowing the in vivo assessment of the properties of any given angiogenic factor. Two options are currently available for this purpose. On one hand, the use of very simple animal models, such as the rabbit cornea assay and the chicken chorioallantoic membrane assay, permit a relatively easy assessment of the pro-angiogenic activity of recombinant proteins, and have been extensively used for this purposes over the last several years. On the other hand, a more thorough understanding of the function of individual components of the cardiovascular system can be obtained by experimental studies based on transgenic and knock-out animals. Both these experimental approaches appear not fully satisfactory. In the first case, the factor under investigation acts in a very specific condition, which is very favourable to new blood vessel formation. This condition may not actually mimic the physiological situation in which the factor may be found to exert its natural activity. For example, factors that are powerful inducers of angiogenesis in these assays might act differently if expressed in old animals or in conditions of chronic ischemia - an important issue for therapeutic purposes. Conversely, homologous or site-specific recombination in embryonic stem cells actually allows studying the consequences of deficiencies, mutations, and conditional or tissue-specific expression of gene products. However, the procedures involved in this technology, including embryonic stem cells manipulation and selection, as well as gene targeting by homologous recombination, still remain cumbersome and technically demanding.

A new complementary approach for studying angiogenic gene function in vivo stems from the recent development of gene transfer technologies based on the use of recombinant viruses.

Besides their application in the gene therapy field, these viral vectors also appear to represent highly efficient tools for the delivery of genes to several tissues in virtually all mammalian species.

Gene transfer to muscle and heart with viral vectors

Recombinant adenovirus (rAd) and Adeno-Associated virus (rAAV) are among the most extensively used vectors for gene transfer in non proliferating tissues in vivo, including skeletal muscle and heart (Fisher et al., 1997; Guzman et al., 1993). These two vectors share some similar features, such as the ability to transduce a variety of proliferating and quiescent cell types. However they also possess their own unique set of properties that render them particularly attractive for different gene transfer applications. rAd vectors can accommodate larger inserts, mediate transient but high levels of protein expression, and can be easily produced at high titers, although their application in vivo is notably limited by their strong immunogenicity and the stimulation of a potent inflammatory response (Yang et al., 1996). In the gene therapy arena, major safety concerns about a wide use of these vectors have recently been raised by the death of young patient recruited in a gene therapy trial for the treatment of a rare metabolic disorder (OCT deficiency). The lethal event was most probably due to a systemic inflammatory response to the delivered adenoviral vectors (Lehrman, 1999). Starting for these considerations, we can predict that adenoviral vectors will probably find their specific niche only in cancer gene therapy, where a robust inflammatory response against adenoviral-transduced cells is highly desirable.

A constantly increasing number of pre-clinical and clinical gene therapy studies exploit vectors based on AAV, a parvovirus that owes its name to the fact that it has been originally discovered as a contaminant of an adenoviral preparation. The gaining popularity of AAV vectors can be attributed principally to their ability to mediate prolonged transgene expression in a variety of

target tissues and to their lack of pathogenicity (Favre et al., 2001).

The safe profile of AAV vectors stems from two major properties: first AAV have never been associated to any human diseases, and, second, they are completely replication-deficient.

As introduced above, the gene transfer technologies should be considered not only for their therapeutic applications, but also for their utility in basic research, since viral vectors represent a unique tool to investigate the molecular pathways responsible for the different pathologic conditions.

In this context, AAV vectors, allowing long-term transgene expression in the absence of inflammation, stand as an interesting alternative to the use of transgenic, knock-in and knock-out mice. Moreover, the specific tropism of these vectors for muscle cells and cardiomyocytes underscores their huge potential utility in the cardiovascular field, and in particular in the induction of therapeutic angiogenesis (Fisher et al., 1997; Su et al., 2000).

AAV-mediated overexpression of a specific angiogenic factor in a normal or ischemic muscle is a simple and straightforward way to examine the biological effect of that molecule in vivo (Figure 2).

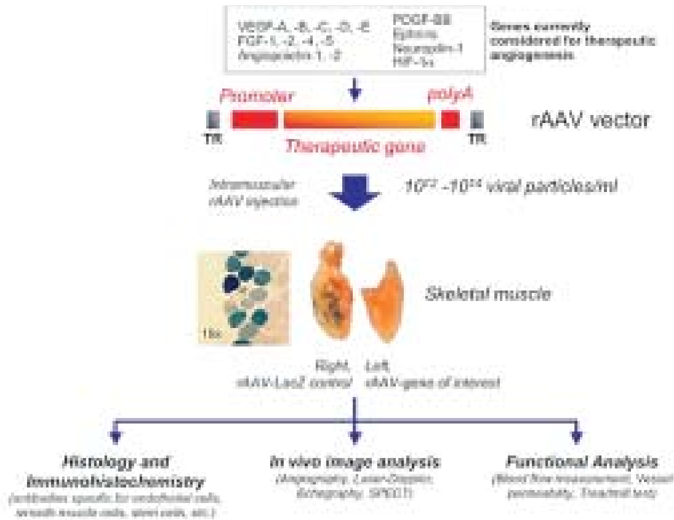

Figure 2. AAV vectors as a tool to study therapeutic angiogenesis. AAV vectors offer the possibility to study the effect of several angiogenic molecules in vivo. The list in the top part shows the main candidate genes currently considered for the induction of therapeutic angiogenesis. The cDNA of almost every interesting gene can be cloned in the AAV plasmid backbone to obtain high titer preparation of rAAV vectors. The recombinant vector is thus suitable for intramuscular injection. The high tropism of AAV for both skeletal and cardiac muscle cells offers the remarkable possibility to obtain high levels of expression and thus to assess the biological effect of the factor under investigation over time. This can be done by different methodologies (microscopic, morphological and functional analysis). While histological examination is usually the procedure of choice when evaluating experiments conducted in rodents, in vivo image analysis and functional tests are generally preferred in studies accomplished on bigger animals and even humans. Experiments can be performed by injection in muscle or heart and by comparing the effects of the AAV vector under investigations with that expressing the reporter gene LacZ, expressing the marker gene (beta-galactosidase), as shows on the left side of the figure.

Moreover, these vectors offer the unique possibility to define possible synergistic, antagonistic or complementary effects exerted by different angiogenic molecules (Arsic et al., 2003). In fact, since they infect cells at high multiplicity, they allow the simultaneous delivery of several combinations of genes in the same tissue. In contrast, this goal would never be possible by using genetically modified animals or other viral vector systems, such as retrovirus-

es, whose genome is integrated in the host genome at a frequency of one copy per cell. The possibility to deliver simultaneously or subsequently different combinations of interesting genes has a great significance from both the biological and the clinical points of view. For instance, it will be possible to define which cocktail of cytokines has the greatest efficacy in the induction of a new functional vascular network, as well as the most appropriate timing for their administration (Athanasopoulos et al., 2000).

Molecular properties of AAV vectors

AAV is a small, helper-dependent human Parvovirus, whose linear single-stranded, non-enveloped 4.7 kb genome requires co-infection with adenovirus or herpes simplex virus to enter a lytic growth cycle and be packaged into particles of plus or minus polarity. Although AAV can replicate also in helper-free conditions, most frequently, and in the absence of superinfection, it preferentially integrates its DNA in a non-random manner into a 4 kb region of human chromosome 19, designated AAVS1, thus allowing a latent infection to occur. In this case, no AAV gene expression is required to maintain latency, and the provirus is stably propagated for several cell passages (Berns and Linden, 1995). This could be a very useful feature for a safe and long-term gene therapy in humans.

The viral genome contains two open reading frames (orf), whose expression is under the control of three promoters. From the first orf, four different transcripts are produced by alternative splicing, coding for the non structural Rep proteins. The two major forms of Rep (Rep78 and Rep68) bind to specific sites within the inverted terminal repeats (ITR), and are required for both viral DNA replication and site-specific integration. In addition, the AAV Rep proteins participate in the regulation of gene expression. In particular, Rep induces the up regulation of the homologous AAV promoters in the presence of adenovirus infection, while it exerts an inhibitory effect when adenovirus is absent. Other heterologous promoters, including viral and proto-oncogene pro-

moters, are also down regulated by Rep, suggesting a pleiotropic effect of this protein on gene expression (Marcello et al., 2000). Another property if Rep is the inhibition of replication of a number of DNA viruses, such as adenoviruses, herpesviruses and papillomaviruses. This effect could partially be ascribed to the above mentioned down-modulation of transcription, but it is probably also due to a more general effect of Rep on DNA replication.

The 3' side of the AAV genome encodes for the capsid Cap protein. Because of the alternative usage of three different translation start sites and of a common polyadenylation signal, three capsid proteins are produced (VP1, VP2, VP3).

The whole coding region is flanked by two 145 bp interted terminal repeats (ITRs), which show complementarity within the first 125 bp and form a T-shaped hairpin at both ends of the genome. This palindromic sequence is the only cis-acting element required for all the major functions of AAV (viral DNA replication, assembly of the viral particles, integration/excision from the host genome).

The life cycle of AAV strictly depends on the presence or absence of a helper virus superinfection in the host cells. Under non-permissive conditions (i.e. without helper virus), the AAV genome mainly integrates into the AAVS1 region, where it establishes a latent infection for indefinite periods of time. A crucial role in latency persistence is played by Rep 68/78, which is synthesized at basal levels and negatively regulates AAV gene expression and DNA synthesis. The latency state, although not altering cell viability, does affect the phenotype as well as the expression of specific cellular genes, conferring more sensitiveness to UV-light, genotoxic agents and heat, enhancing serum requirement and reducing the cellular growth rate. It is assumed that all these effects are somehow related to a low-level production of the Rep protein (Marcello et al., 2000).

Under permissive conditions (i.e. after superinfection with helper virus), the regulation of AAV gene expression becomes

rather complex, depending upon both the helper virus and the presence of Rep68/78 proteins. In these circumstances, the integrated genome can be rescued from the host and packaged into infectious particles.

The mechanism underlying the integration process, depending on the specific recognition between AAV sequences and the AAVS1 region on chromosome 19, is not yet fully understood. Recent evidence indicates that this region is located close to the human troponin T gene, displaying an overall GC content of 65%, a 35-mer minisatellite tandemly repeated for 10 times and a putatively transcribed orf; but the putative role of these features in the integration process still remains unclear (Dutheil et al., 2000). A pivotal role in the integration and rescue mechanisms is exerted by the viral ITRs, and in particular by two short sequences (Rep Binding Site, RBS and Terminal Resolution Site, TRS) in the stem of the T-shaped structure. These sequences drive the binding of Rep 68/78 and the subsequent nicking of the viral DNA, a process essential for viral DNA replication. Since the same RBS and TRS are present also within the AAVS1 sequence, a model has been proposed which suggests the involvement of an oligomeric complex of Rep to juxtapose the RBS and TRS from the cellular and viral DNAs.

The exact elucidation of the molecular mechanisms underlying the site-specific AAV integration process is of extreme importance in the light of the utilization of these vectors for gene therapy purposes. However, it should be emphasized that site-specific integration is strictly dependent on the rep gene, or at least on one of its products, the Rep68 or Rep78 proteins. Since the gene coding for this protein is never present in the recombinant AAV vectors, it can be reasonably concluded that integration of vector DNA either occurs in a random manner or does not occur at all.

Production of AAV vectors

The characteristics of AAV life cycle, including its defective-

ness and ability to persist in infected cells as a latent viral genome, early suggested that this virus could be an excellent tool for in vivo gene transfer. Since the AAV genome cloned into a plasmid is still infectious and able to produce viral particles, any exogenous gene (less than 4.5 kb in length) can theoretically be placed within the two 145 bp ITRs to obtain a circular backbone suitable for vector production. Unlike other delivery systems that have evolved into several generations, the original composition of the AAV vector plasmid (a transgene expression cassette flanked by the two ITRs) is essentially the same as in the current version.

The traditional method for rAAV production is based on co-transfection of the vector plasmid together with a second plasmid, supplementing the rep and cap gene functions, into helper-infected cells (usually HeLa or 293 cells) (Figure 3).

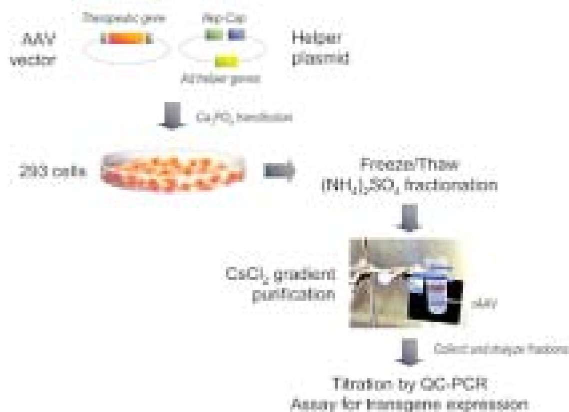

Figure 3. Production of recombinant AAV (rAAV) vectors. The diagram illustrates a revised version of the traditional method for rAAV production, currently used in many laboratories. The procedure starts with the co-transfection of the AAV vector plasmid (carrying the cDNA of the therapeutic gene), together with the helper plasmid (supplementing the rep and cap gene functions, as well as adenoviral genes supporting helper activity) into 293 cells. A cell lysate is obtained by repeated freezing and thawing, and subsequently fractionated by $(NH_4)2SO_4$ precipitation. rAAV particles are then purified by $CsCl_2$ density gradient ultracentrifugation, collected and dialyzed in order to eliminate $CsCl_2$ toxicity. The

viral preparation stock can be finally titered by competitive PCR and assessed for transgene expression in cultured cells.

Packaging efficiency seems to depend principally on the cellular system used and on the size of the packaged genome. A "head-full" mechanism appears to be used by the packaging machinery, with upper and lower limits of 4.9 and 4.1 kb respectively for optimal packaging, even if constructs up to 5.2 kb are tolerated.

One of the most critical problems in the traditional AAV preparations is the presence of the contaminant helper virus, and occasionally also of wild-type AAV. Since recombinant particles are usually assembled in cells infected by adenovirus, they have to be purified, which may negatively interfere with the amount and the activity of the recombinant particles. In fact, even marginal amounts of infectious adenovirus or adenoviral proteins can result in relevant host immune response, while the presence of the wild-type virus poses the risk of mobilization of the recombinant AAV vector (provided that simultaneous infection with a helper virus also occurs in the same organism). Several improvements of the conventional protocol for rAAV vector production have been proposed over the last few years, as a consequence of the growing understanding in the biology of the AAV life cycle (Grimm et al., 1998).

AAV vector particles are traditionally separated from adenovirus virions by repeated cesium chloride ($CsCl_2$) density gradient ultracentrifugation. However, this separation is far from perfect, having a negative impact on the success of rAAV production. To decrease toxicity from residual hyperosmotic $CsCl_2$, the substitution of density centrifugation with the iso-osmotic and inert iodixanol has recently been adopted. Alternatively, the identification of heparan sulfate proteoglycan as a cellular receptor for the attachment of the virions, has resulted in the transition to ligand affinity matrix chromatography purification as the standard of rAAV production in most core facilities.

The risk of adenovirus contamination has been recently solved by the identification of the adenoviral genes essential to provide helper activity to AAV replication in packaging cells. Actually, it is now clear that this effect is not direct. Proteins expressed from the adenovirus early region 4, open reading frame 6 (E4ORF6) stimulate still unrecognized cellular activities that are essential to stimulate AAV replication. This notion led to the development of new protocols that allow for vector manufacturing in a setting totally free of helper virus, based on plasmids containing part of the adenoviral genome. These plasmids are completely non-infectious, carrying only the subset of adenoviral genes that is essential for rAAV production. More recently, both the AAV and adenoviral genes were assembled on one plasmid, thus reducing the number of plasmids required to transfect for recombinant particles production (Grimm et al., 1998). In this way, it is now possible to obtain rAAV preparations free of contaminating helper virus and unwanted adenoviral protein at a yield even higher than that achieved by using infectious helper virus.

A particular goal in trying to overcome the dependency on transient transfection is the establishment of packaging cell lines, similar to those already developed for retroviral and adenoviral vector production, but stably containing multiple copies of the rep and cap genes. Expression of these genes would be moderate in basal conditions, but could be triggered by infection with adenovirus. Nonetheless, these efforts have met very limited success so far, mainly due to the toxicity of the constitutive expression of Rep, which, even at very low levels, is very badly tolerated by proliferating cells. Therefore, transient transfection still remains the method of choice to obtain AAV vector preparation for both investigation and gene therapy purposes.

The increasing popularity of rAAV vectors relies on the results of a number of in vivo transduction experiments that have demonstrated efficient and long-term persistent infection of a series of tissues and organs in vivo (Xiao, 1996). For some still com-

pletely unexplored reasons, however, transduction occurs essentially only in cells of muscular origin (including skeletal and smooth muscle cells and cardiomyocytes), in neuronal cells (both in the central and peripheral nervous system and in the retina), in retinal pigmental cells, and, at a lesser extent, in hepatocytes. The reasons for this selective tropism are most likely unrelated to viral penetration into the cells, since AAV particles are able to bind efficiently and enter a large number of cells, due to the usage of widely expressed molecules as receptors, including heparan sulphate proteoglycans, FGFR-1 and alpha-V beta-5 integrin (Bartlett et al., 2000). After infection, however, different and unknown cellular factors influence the outcome of viral transduction that limit the in vivo efficiency. In adenovirus-infected cells, AAV DNA colocalizes in the adenovirus replication centers within the nucleus. In contrast, in the absence of adenovirus, AAV essentially has a perinuclear localization, whereas it is quickly internalized in the nucleus after helper viral superinfection. A peculiar molecular event limiting the infection efficiency is the synthesis of the complementary strand of the viral genome, which converts the single-stranded AAV DNA into its transcriptionally active, double-stranded form (Ferrari et al., 1996). Intriguingly, this event often takes a long time to occur: even in highly permissive tissues, the maximum levels of transgene expression is detected only after several weeks, being preceded by a lag period during which some still not completely understood molecular events take place (Zentilin et al., 2001).

Potential of vector-mediated gene transfer in cardiovascular medicine

The specific tropism of AAV vectors for tissues of muscular origin opens the way to a number of gene transfer applications to the myocardium, the skeletal muscle and the arteries for both basic research investigation or for human gene therapy. In particular, AAV vectors are likely to be more and more exploited in the

cardiovascular field, with the specific purpose of delivering genes encoding growth factors and cytokines that are secreted in order to exert a biological effect in the whole tissue and beyond the individual transduced cells. We anticipate that three of the most striking applications that will exploit the potential of gene transfer with AAV vectors will be the induction of therapeutic angiogenesis, the treatment of heart failure, and the local recruitment of stem cells to the ischemic heart and muscle.

Ischemic disease and therapeutic angiogenesis

Ischemia refers to a lack of oxygen due to inadequate perfusion, which results from an imbalance between oxygen supply and demand. The most common cause of myocardial ischemia is atherosclerosis of epicardial coronary arteries. Slowly developing, high-grade coronary artery stenosis usually does not precipitate acute infarction, because of the progressive development of a rich collateral network over time. Myocardial infarction generally occurs when there is an abrupt decrease in coronary blood flow, following the thrombotic occlusion of a coronary artery, previously narrowed by atherosclerosis. This event is associated with the almost instantaneous failure of normal muscle contraction and relaxation. The events following myocardial infarction often have a devastating impact on the patient conditions, principally because of the irreversible death of the cardiomyocytes and their substitution by a scar of akinetic fibrous tissue. The extent of the infarction depends on the duration and the severity of the perfusion defect. However, the entity of the myocardial injury is also modulated by a number of factors, including development of a functional collateral circulation, medical therapy and ischemic preconditioning. Beyond scar contraction, progressive ventricular remodelling can further reduce cardiac function in the weeks following the initial event (Pfeffer et al., 1991).

Despite remarkable progress in terms of early diagnosis and prevention, coronary artery disease is the most common, serious,

chronic, life-threatening illness in Western countries, causing more death, disability and economic costs than most other disorders. Most of the currently available therapies - for instance angioplasty and thrombolysis - can significantly relieve the cause of the infarction, notably improving the prognosis of patients. Conversely, after the occurrence of the irreversible injury, no medication or procedure has so far shown efficacy in restoring adequate supply of blood and replacing the fibrous tissue with new contractile fibres. As a consequence, most of the patients experiencing a myocardial infarction inexorably progress toward heart failure.

From all these considerations, we can state that for the next future there is an absolute need of new therapeutic approaches to induce the development of new blood vessels and new contracting fibres at the site of the infarction. Obviously, these goals will become realistic only after a deeper comprehension of the molecular pathways sustaining vessel formation and muscle regeneration. The detection and subsequent modulation of growth factor-related signals might thus represent an appealing novel strategy for the management of cardiac and vascular diseases.

After the identification of VEGF and FGF as powerful inducer of angiogenesis, a limited series of studies has been conducted in order to evaluate a possible therapeutic effect achievable by these factors when delivered as recombinant proteins (Post et al., 2001; Sato et al., 2000). Despite the relatively good results obtained on animal models, this approach produced only modest results in clinical trials, probably as a consequence of the short half-life of these cytokines in vivo (Hendel et al., 2000; Henry et al., 2001).

In contrast, much more enthusiasm has been generated by the preliminary results of clinical trials of several gene therapy conducted over the last few years (Ferrara and Alitalo, 1999). Clinical applications of cardiovascular gene therapy began in 1994, identifying patients with critical limb ischemia, who had exhaust-

ed all conventional options for revascularization (Isner et al., 1996). The treatment consisted of the injection of a plasmid coding for VEGF-A. The success of this treatment has been evaluated by angiography and nuclear magnetic resonance, revealing the formation of new collateral vessels and a significant perfusion improvement in the VEGF-treated group (Baumgartner et al., 1998). The only major side effect was the occurrence of a remarkable edema of the leg, probably due to the increase in vessel permeability caused by VEGF (Baumgartner et al., 2000).

After establishing proof of concept in this population, the same plasmid strategy was extended to patients with myocardial ischemia, similarly proved refractory to available medical therapy; the plasmid was injected directly into the ischemic myocardium via a mini left anterior thoracotomy (Losordo et al., 1998). The first treated patients did not present any complication, showing a clear improvement in symptoms and perfusion, as documented by SPECT and electromechanical mapping techniques (Vale et al., 2000). The major concerns about this study arose from the invasiveness of the surgery and the lack of experimental data accumulated before the transition to the clinics. Moreover, these trials included uncontrolled, open label designs; this implies that the results should be interpreted with caution, considering the significant placebo effect observed in patients with coronary artery disease. Other criticisms came from the lack of transparency, particularly referring to the unnoticed death of some patients and the recruitment of people bearing neoplasia (which is a major contraindication to any kind of pro-angiogenic therapy). For all of these reasons, in 1999 the FDA announced the temporary suspension of the cardiovascular trials, which nonetheless restarted few months later (Simons et al., 2000).

In other cardiovascular gene therapy trials, different delivery systems are currently employed, such as liposomes and adenoviral vectors (Laitinen et al., 2000). Adenoviral vectors are in fact widely the most used vectors in the clinics, likely because of

the ease of production, high transduction efficiency and expression in non-proliferating cells (Barr et al., 1994; Laitinen et al., 1998). Despite interesting preliminary results, these vectors exert a potent inflammatory effect, which rapidly switches off transgene expression, as already discussed above. The angiogenic gene therapy (AGENT) study was the first randomized, placebo-controlled trial of therapeutic angiogenesis for myocardial ischemia, based on the intracoronary injection of an adenoviral vector expressing FGF-4. Although this intervention was well tolerated, no significant improvement was detected by echocardiography nor by exercise treadmill testing. The poor efficacy of this approach could indeed be the result of the too short term expression driven by the vector (Gilgenkrantz et al., 1995).

In contrast, AAV vectors not only allow the transduction of quiescent cells, but also trigger a persistent transgene expression in the absence of inflammation, which could be essential for the detection of a relevant biological effect (Byun et al., 2001). These characteristics render AAV vectors a particularly attractive delivery method for the induction of therapeutic angiogenesis (Deodato et al., 2002; Su et al., 2000).

Heart failure

Heart failure represents one of the major challenges in cardiovascular medicine, since its progression can be only minimally counteracted by current therapies. As a consequence of the recent medical progresses that have allowed the survival of a great number of patients with different cardiac diseases, together with a constant increase of the average human life span, the mortality rate for heart failure has more than doubled in the last 25 years. Heart failure may be readily described as a clinical syndrome, characterized by well-known symptoms and physical signs, but a precise physiological or biochemical definition is far more difficult. From a clinical point of view, heart failure is the condition in which an abnormality of cardiac function is responsible for the in-

ability of the heart to pump blood as a rate commensurate with the needs of the different tissues. Several cardiac disoders tend to evolve by causing a progressive loss of the ventricular function and as a consequence of the appearance of the heart failure syndrome. Several new introduced drugs have significantly reduced the progression rate of diseases. However, eventually hear failure often becomes refractory to any kind of treatment, once the natural adaptive responses are not more able to compensate for the effect of prolonged ischemia.

The potential approaches based on gene transfer for heart failure find their rationale in the understanding the biochemical and physiological basis of the disease (Hajjar et al., 1998b). The contractive activity of myocardial cells strictly depends on the intracellular calcium level. At rest, the cardiac cell is polarized, with the sarcolemma largely impermeable to Na^{+} and Ca^{++}. During the plateau phase of the action potential, there is a slow inward current that reflects primarily a movement of Ca^{++} into the cell. This small depolarizing current not only diffuses across the surface of the cell, but deeply penetrates into the cell by the ramifying T system, triggering the release of much larger quantities of Ca^{++} from the sarcoplasmic reticulum. This rise in intracellular Ca^{++} is termed “regenerative release” and is a key regulator of the interaction between actin and myosin, and thus of myocyte contraction. During the repolarizing phase, the SERCA2a ATPase on the sarcoplasmic reticulum re-accumulates Ca^{++} against a concentration gradient. This results in the decrease of the ion concentration in the vicinity of the myofibrils, and thus in muscle relaxation. The SERCA2a activity is normally inhibited by the phospholamban, while this inhibition is lost when the phospholamban become phosphorylated. Substantial evidence supports the view that in the failing heart there is a significant increase in the intracellular calcium level during the telediastolic phase, probably due to the reduced activity of the SERCA2a pump.

The relevance of these molecules in the pathogenesis of

heart failure has been mainly discovered using gene transfer approaches in the rat model of hypertensive heart disease. For instance, a significant improvement in both systolic and diastolic function has been obtained by inducing the SERCA2a pump overexpression or by inhibiting the phospholamban activity, either by the delivery of a specific ribozyme (a catalytic RNA molecule able to specifically cut a target mRNA) or by the transfer of a dominant-negative mutated form of the protein (del Monte et al., 1999; Hajjar et al., 1997; Hasenfuss, 1998; He et al., 1999; Miyamoto et al., 2000).

The application of the gene transfer technologies has also allowed the recognition of the role of adrenergic receptors role during heart failure progression. In particular, the down-regulation of βreceptors) (β–AR) and the increased activity of the β-AR-KI kinase have been well documented in patients with advanced heart failure (Hajjar et al., 1998a). Accordingly, the intracoronary injection of a viral vector encoding β-AR, as well as the overexpression of an inhibitor of the β-ARKI, was able to improve significantly cardiac contractile function, thus slowing the progression of disease in different animal models (Akhter et al., 1997; Rockman et al., 1998).

Gene transfer for stem cell recruitment

Over the last few years, a great excitement in the biomedical community has been generated by the observation that the marrow of an adult bone contains a limited pool of stem cells that can be induced to differentiate into several cell types (Asahara et al., 1999; Jackson et al., 2001; Shintani et al., 2001). In particular, the local injection of bone marrow-derived stem cells in the heart has been proven to be capable of inducing the generation of new myocytes and vasculature (composed of both endothelial and smooth muscle cells) thus promoting myocardial regeneration after infarction (Kocher et al., 2001; Orlic et al., 2001). This and several other similar observations in a variety of other tissues, in-

cluding brain, skin, liver, pancreas and eye, has recently stimulated the start of different clinical trials involving the injection of stem cells derived from the bone marrow or from other sources to promote regeneration of damaged tissues. Although this experimentation is still in its infancy, it already appears that a major problem hampering its success is the difficulty in identifying precisely isolating and expanding in vitro cells with true staminal potential.

The event by which stem cells from a given tissue can acquire morphological and functional properties of different cell types normally residing in a different tissue, is usually referred to as “stem cell plasticity”. This concept implies that local signals can strongly influence the fate of marrow stem cells, promoting their homing to tissues in need, and driving their differentiation along multiple pathways. In light of these considerations, it appears that an alternative possibility to cellular therapies with stem cells would be the use of viral vectors to express the actual genes promoting homing and differentiation of endogenous stem cells. Again, gene delivery to the heart and muscle using AAV vectors appears a very powerful strategy to address the issue of which could be the soluble molecules mediating stem cell recruitment and promoting their terminal differentiation.

Since VEGF, besides its role in the promotion of angiogenesis, is also a main regulator of hematopoiesis during embryonic development, it would be not surprising that it might also act as a growth factor of stem cell in the adult. In this respect, the recent observation according to which that hematopoietic stem cells expresses high levels of VEGF receptors may suggest that this growth factor can have a direct role in the stem cell recruitment, proliferation, and/or differentiation (Gerber et al., 2002). Moreover, recent investigations have demonstrated the existence of postnatal circulating bone marrow-derived endothelial progenitor cells (EPCs) that may home to sites of neovascularization to differentiate in endothelial cells, a finding consistent with vasculo-

genesis occurring during adult life. A key regulator of EPCs mobilization and neovascularization has been again found to be VEGF-A (Asahara et al., 1999; Kalka et al., 2000). Finally, consistent with these findings, we have recently observed that the long-term expression of VEGF-A in the skeletal muscle using AAV not only stimulates the formation of new blood vessel, but also promotes local accumulation of bone-marrow derived stem cells, some of which express markers of undifferentiated stem cells (Arsic et al., 2003).

Given this full set of novel findings, it appears that the use of AAV vectors now offers the unique possibility to further explore the issue of stem cell recruitment in order to identify eventually a potential "cytokine cocktail" that could be expressed from an injured region to promote stem cell attraction, their local proliferation and activation, and their terminal differentiation to regenerate the damaged tissues.

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THERAPEUTIC ANGIOGENESIS FOR THE TREATMENT OF ISCHEMIA

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Introduction

Cardiovascular diseases account for 51% of deaths in Europe at the beginning of the new century. Incidence rate fells by half over the past 30 years, as the result of better prevention and of rational treatments based on the growing knowledge of vascular biology. However, an additional dramatic improvement is unlikely to be achieved without the development of entire new therapeutic and preventive strategies. Here, we present examples of therapeutic angiogenesis in preclinical models of limb ischemia.

The nutrition of normal tissue depends on an adequate supply of oxygen and ofnutrients through blood vessels, so that an acute damage occurs when supplying arteries are occluded by atherosclerotic plaque enlargement or thrombosis. After the occurrence of an acute vascular occlusion, clinical outcome mainly depends on the native potential to develop new collaterals, a defense response aimed to maintain the tissue perfusion and function. However, under certain conditions such as diabetes, hypercholesterolemia, and hypertension, post-ischemic healing is compromised because of a defective modulation of angiogenic growth factors (1-3).

While angiogenesis occurring after an acute vascular occlusion has been extensively investigated, information regarding the mechanisms which modulate collateral vascular growth in chronic obstructive vascular diseases remains elusive. In a chronic peripheral vascular disease, we have found that circulates tissue kallikrein (TK), but not vascular endothelial growth factor (VEGF), is up-regulated and correlate with the number of angiographically recognizable collateral vessels. Interestingly enough, the increase in circulating TK is reversed by a surgical revascularization, suggesting that the growth factor may represent a biomarker of a chronic ischemia (4).

Even more insidious is the evolution of a chronic ischemia deriving from microvascular insufficiency. Rarefaction of microcirculation that typically occurs in lower extremities of diabetic patients leads to delay healing of foot ulcers and contributes to a peripheral neuropathy. Unfortunately, no medical or surgical treatment is available for the treatment of these microvascular complications.

Therapeutic Angiogenesis: Successes and Pitfalls

Recently, therapeutic angiogenesis, whether using viral (53%) or nonviral (47%) vectors, has been proposed as an alternative for the treatment of ischemic diseases (5). Since the first application of the cardiovascular gene therapy in 1994, clinical trials have increased in proportion to the rest of the field to reach the figure of 17% of all gene therapy trials (6). This strategy is based on the concept that a supply-side approach with angiogenic growth factors would overcome the endogenous deficit and result in more robust angiogenic response. Potentiation of microcirculation by therapeutic angiogenesis has been applied in models of myocardial and peripheral ischemia and subsequently exploited for the treatment of wound healing and peripheral neuropathy. Following successful application in animal models, these concepts have been transferred from the bench to the bedside.

Encouraging are the results of the recent randomized clinical trial TRAFFIC using fibroplast growth factor (FGF) for the treatment of patients with moderate-to-severe intermittent claudication. Intra-arterial rFGF-2 resulted in a significant increase in peak walking time at 90 days (7). In contrast, first controlled clinical trials using VEGF-165 or FGF-2 in patients with ischemic heart disease did not result in the level of efficacy for which researchers had hoped (8-10). Furthermore, the serious side effects that might accompany any therapeutic benefit are matter of concern.

Several reasons explain the partial failure of therapeutic angiogenesis to maintain the initial promises. Given the complexity of the angiogenesis program, it appears rather naïve to think that the administration of a single angiogenic molecule would be insufficient to generate a well-tempered and durable neovascularization. It is now clear that VEGF leads to the formation glomeruloid vascular tangles without sufficient pericyte encapsulation (11-12). This chaotic, tumor-like vasculature is functionally inefficient and easily subjected to regression. A concern also exists regarding the possibility that VEGF can accelerate the progression of atherosclerotic plaque, by stimulating intralesional angiogenesis (13). To overcome the above limitations, one may choose another molecule or a combination capable of modulating not only angiogenesis but also arteriogenesis and vascular remodeling. In theory, the members of the angiopoietin family seem to be the most suitable for stabilization VEGF-induced neovascularization (14). Given the phenotypic variability of various vascular beds, different therapeutic factors might be used to stimulate collateral growth in different tissues. Finally, vectors used for the transfer of angiogenic factors have been delivered at high doses thus unmasking a potential intrinsic pro-inflammatory activity. Due to the few titration studies performed so far, little is known regarding the appropriate dosage, the duration of treatment, and the schedule of combinations.

Therapeutic angiogenesis with tissue kallikrein in peripheral ischaemia

The angiogenic potential of the kallikrein-kinin system (KKS) has been documented in pre-clinical models of peripheral ischemia using a gene-transfer approach. To this aim, a adenoviral vector of replication-defective containing the gene encoding human tissue kallikrein (hTK) was injected into the adductor skeletal muscle of mice previously submitted to unilateral limb ischemia (Figure 1) (15).

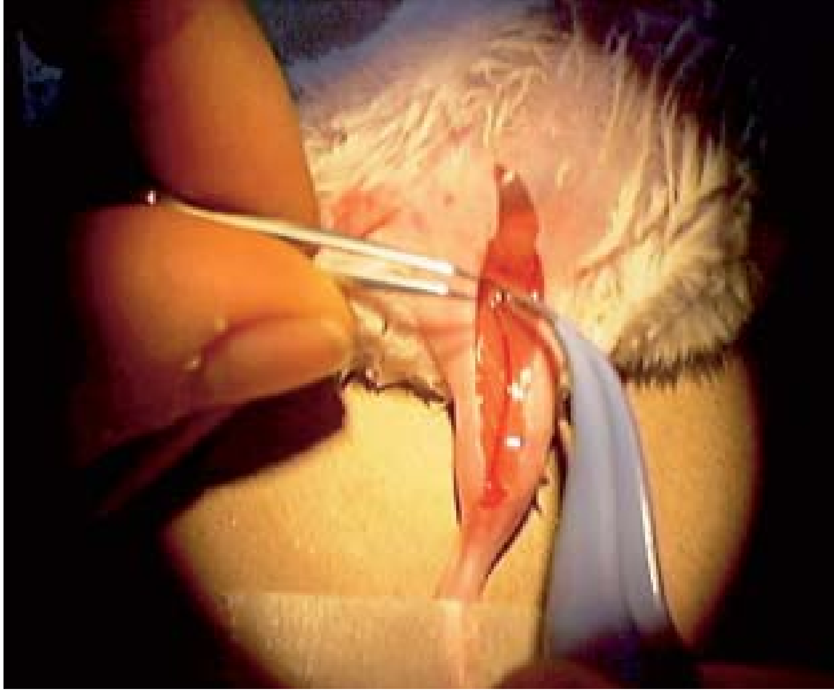

Figure 1. Representative picture of microsurgery approach for isolation and dissection of femoral artery in the mouse. The left femoral artery is separated for femoral vein and for nerve and for electro-coagulated. The wound is closed and animal is allowed to recover. Limb blood flow, as measured by laser Doppler methodology, rapidly decreases after excision of femoral artery. Hemodynamic recovery takes place over time reaching pre-operative levels in 2-3 weeks in otherwise healthy animals. However, the recovery is generally delayed in diabetic, atherosclerotic, or hypertensive mice.

Successful transduction of the transgene was achieved for up 3 weeks, as documented at mRNA level by PCR analysis and a protein level by measurement of hTK by a specific ELISA. Expression of hTK resulted in augmented angiogenic response to ischemia, accelerated haemodynamic recovery and preserved muscular energetic charge. It should be bared in mind that endogenous KKS is already activated in otherwise healthy animal. Thus, the additional protective effect exerted by this gene therapy approach might occur because transduction of the angiogenic protein exceeds, in terms of magnitude and duration, the short-lasting upregulation of endogenous kallikrein by ischemia. Accordingly, conditions characterized by reduced KKS activity might take advantage of therapeutic angiogenesis with hTK (16).

Peripheral ischemia represents a major problem especially among diabetic patients because of the concomitance of accelerated atherosclerosis and microvascular insufficiency. The natural course of disease often requires to proceed to a limb amputation as the ultimate remedy for unbearable symptoms. Recently, we challenged the therapeutic potential of local hTK gene delivery as sole therapy for the treatment of peripheral microangiopathy in the streptozotocin (STZ) diabetic mouse model (17).

First, we characterized if this animal model resembles the human condition in terms of vascular damage induced by the underlying disease. It was found that in limb skeletal muscles of STZ-induced diabetic mice, vascular rarefaction occurs as the result of an abnormally activated death program. During the first 28 days after induction of diabetes, capillary loss occurred at the rate of 1% per day. In the long term, this mismatch significantly contributes to alter the path length for oxygen transport to myocytes, thus ultimately leading to ischemia and additional activation of cell-death mechanisms. Accordingly, from 28 to 120 days, a supplemental 35% decrease in capillarity occurred in association with myofiber rarefaction and atrophy. Rarefaction also occurred at arteriole level with additional detrimental hemodynamic effects and tissue hy-

poxia.

We found that apoptosis is significantly activated in the vasculature of these diabetic animals.

Local angiogenesis gene therapy was intentionally chosen for the diabetic model to avoid endangering distant tissues, especially the retina. The safety of the approach was proven by the absence of obvious side effects or angiogenesis in contralateral muscles and lack of angiogenic effect in the retina (Figure 2).

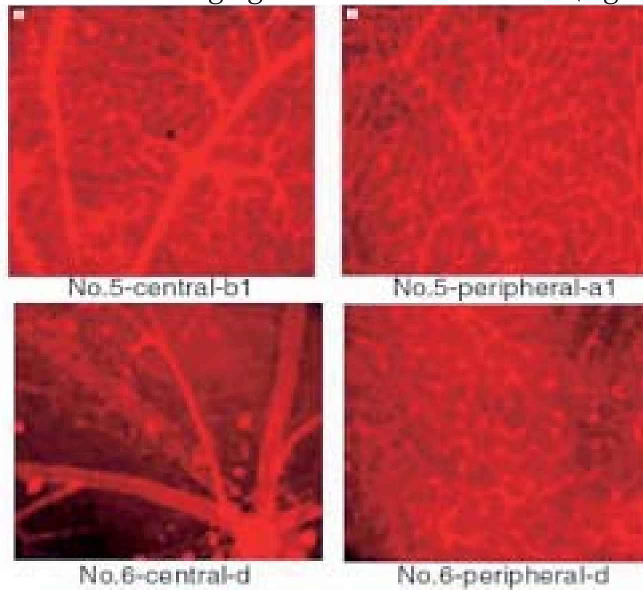

Figure 2. Representative picture of retinal microvasculature. Intramuscular delivery of human tissue kallikrein does not affect retinal microcirculation (A-B) as compared with controls given vehicle (C-D).

Delivery of hTK to the diabetic muscle was able to prevent microangiopathy by promoting capillarization and attenuating EC apoptosis. Another important finding is that hTK supplementation promotes arteriole regeneration in diabetic hindlimb muscles and

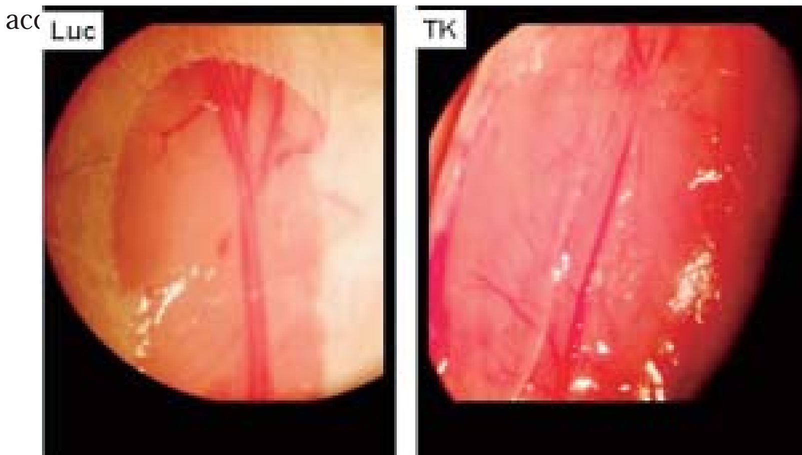

Figure 3. Representative picture of collateralization in diabetic limb muscle injected with kallikrein (TK). Control receiving reporter gene is shown for reference (Luc).

The new generated arterioles may derive from pericyte encapsulation and muscularization of nascent endothelial channels. Furthermore, hTK stimulate the growth and remodeling of preexisting collateral arteries, which is supported by concomitant arteriole luminal enlargement. As late as 106 days from gene delivery, muscular capillarity was in the range found in non-diabetic animals. Thus, a single shut of hKT gene significantly prevents diabetic microangiopathy for long period of time.

The therapeutic benefit of hTK for the treatment of diabetic microangiopathy represents solid progress toward the potential clinical application of this gene therapy approach.

Concluding remarks

Recent studies indicate that the KKS plays a relevant role in reparative and therapeutic angiogenesis. Thus, supplementation with hTK could aid to the cure of myocardial and peripheral ischaemia. Given the complexity of angiogenic program, the strat-

egy could be advantageously complemented in combinatory approaches, but the question of the angiogenic substance most suitable for combination with hTK and the administration schedule remain to be addressed by future studies. The gene encoding TK has been delivered to target tissues using an adenoviral vector. However, since this delivery system might trigger inflammatory responses, we are currently engaged with studies using plasmid vectors as possible alternatives. Administration of recombinant hTK protein could represent another option, but this has obvious disadvantages related to the preparation and the necessity of repeated administration. Long-acting kinin receptor agonists may be envisioned as therapeutic tools rather than hTK. It remains unsolved whether the preferred agent should be a B1 or B2 receptor agonist, or a combination of both. In addition, the same disadvantages of recombinant hTK is applicable to the use of these peptides. By converse, because of their ability to interfere with the angiogenic programme, kinin receptor antagonists in addition to kallistatin, might also be envisaged as therapeutic reagents to combat the pathological angiogenesis in cancer and chronic inflammatory diseases.

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ISOLATION OF MICROVASCULAR ENDOTHELIAL CELLS

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Until the end of Seventy vascular patophysiology were mostly studied using in vivo animal experimental models. However these methods did not permit the investigation of many phenotypic and functional properties of endothelial cells (EC) because of lacking specific markers for endothelium (in particular monoclonal antibodies against endothelial antigens).

In addition EC cultures were poorly developed since the techniques for isolation and purification EC were not enough efficient. The EC cultures were often contaminated by other cell types, in particular fibroblasts (or smooth muscle cells), which usually have an higher growth capacity compare to EC and therefore, they shortly became the predominant cell phenotype in culture.

Some researchers found that the substitution of D-valine with L-valine or the addition of high serum concentration (30-40%) in the culture medium could favor the growth of EC in spite of fibroblasts, but these conditions were still not sufficient to block proliferation of fibroblasts.

Gimbrone and Jaffe bypass this problem and obtained pure culture of EC derived from large vessels like aorta or the human umbilical vein. They prepared EC cultures by detaching the endothelium of vessel wall with an enzymatic solution (containing generally collagenase-dispase) which was used to fill up the lume of the vessel. The EC were then successfully recovered by washing the vessel lume and plated on culture dishes and grown as

primary cultures that were serially passed. Gimbrone and Jaffe have been able to develop a method for EC isolation which is still used in many laboratories.

However, the impossibility to separate EC and stromal cells (particularly fibroblasts) from organs or tissues.did not allow to isolate the EC of small vessels or capillaries. As a consequence of this problem, therefore, did not permit to study microvascular EC (MEC) which are, not only the most abundant EC phenotype present in the body, but overall represent the preferential target for many ormones and growth factors which are involved in angiogenesis, a process that participate to several human pathologies such as inflammation, diabete and cancer.

One of the first publication describing the successfully isolation of MEC was in 1979 in the laboratory of J. Folkman at Children Hospital in Boston (Figure 1). The method lead to isolate and long-term culture of MEC from bovine adrenal gland (cortical part). Substantially the technique developed by J. Folkman and coworkers was based on the identification, under light microscopy, of MEC colonies raising in the gelatine coated plate after gland digestion.

Later all fibroblasts near the MEC colonies were removed from plate by scraping with pasteur pipet tip and using cloning rings, MEC colonies were detached with trypsine and transferred into new culture wells for serial subcultures. In spite of this technique was long and difficult to apply, it was the first procedure described for isolation MEC and leaded to the possibility to investigate many factors involved in tumor angiogenesis.

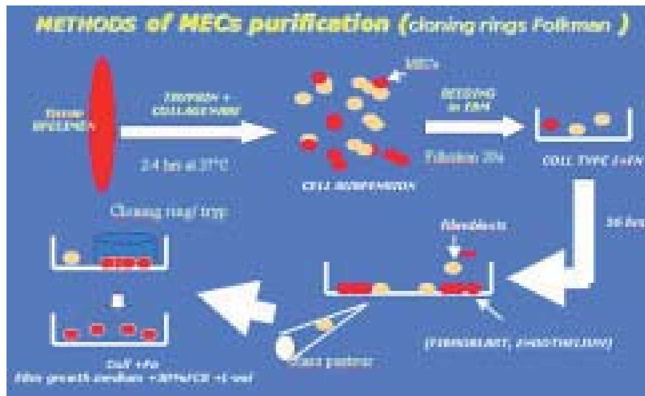


Figure 1.

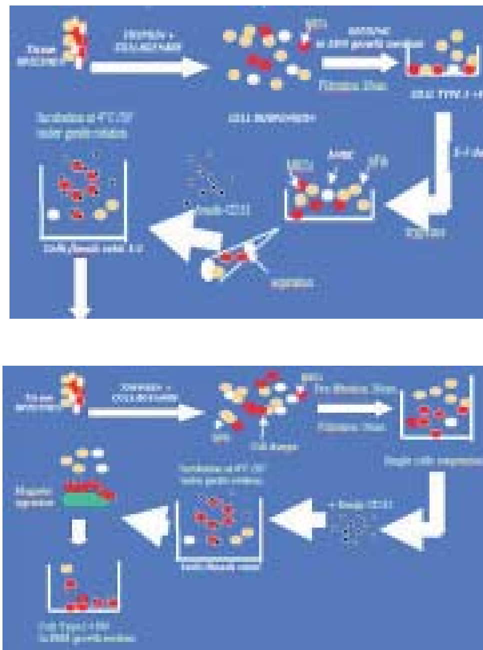
During these last ten years, the methods for isolation of MEC have improved enormously after the discovery of several EC specific markers. At this regard the production of monoclonal antibodies (mAb) against EC antigens have been useful for EC purification. In our laboratory we have developed procedures for purifying MEC from different human normal adult an fetal tissues as well from tumors (Figures 2, 3).

Our methodology can be divided in different steps:

1) human normal, fetal (heart, lung, liver, skin etc...) or neoplastic tissue specimens are finely minced with scissors and then digested by incubation for 2-4 hours at 37°C in medium containing collagenase-dispase (this enzyme composition may change from tissue to tissue) to obtain cells releasing from tissue samples;
2) the cell suspensions are then washed and passed through a 20 μm pore size filter to remove undigested cell aggregates;
3) the cells are then plated onto Petri dishes previously coated with collagen- fibronectin (plasma FN) (substrata that enhance EC adhesion) and cultured in EBM medium containing 10%

FCS, bFGF, EGF, heparin and hycortisone, all these factors are necessary to sustain growth of MEC;

4) after 7-10 the primary culture are harvested by tripsinization, washed and counted, then incubated(30' at 4°C) with magnetic beads (ratio cells / beads = 1:1) bound with mAb against CD31+ the beads may be coated also with other mAb or lectin against EC antigens), an antigen typically expressed on EC membrane and absent on fibroblasts;
5) at the end of incubation the MEC are purified by using a magnetic apparatus, all the cells CD31 negative are washed away; 6) the CD31+ positive cells are then seeded onto collagen-Fn coated plate and growth in EBM medium. This is assumed as the first in vitro passage of MEC.



Figures 2, 3.

This method lead us to prepare very pure MEC culture from different human tissues (Table 1). In addition, the use of onco-foetal FN (this is an FN isoform produced in the culture supernatant of WI38VA cell line) as adhesion substrata, in substitution of plasma FN, is important for purification of tumor derived MEC (Table 2).

Tissues to apply Magnetic beads techniques

Human Tissues/organs	enzymes	magnetic beads techniques After cell culture	direct
Skin	trip+collag+disp.	+	±
Muscle	"	+	±
Fat	collag.+disp.	+	+
Bone marrow	"	-	+
Brain	trip+collagenase	+	-
Liver	collagenase	+	+
Lung	elast.+collag+disp.	+	-
Kidney	collag.+disp	+	-
Heart	trip+collagenase	+	±
Lymphonodes	"	+	-
Mamm. Tiss.	"	+	-
Tonsils	"	+	-

Table 1.

Isolation of TdMECs grown on coll type I+WI38va-FN substrate

Tumor samples	weight	% MECs	primary cultures	Nº of subcult.
Astrocitoma(1)	0.45gr	0.02	No	-
Fibrosarcoma(1)	1.45gr	0.3	yes	1
Glioblastoma(3)	1.45gr	1.0-2.4	yes	3-5
Lung carc. (3)	1.3gr	0.2-0.6	yes	3-5
Liver carc.(10)	1.8gr	0.7-3.6	yes	7-11
Mammary carc.(4)	1.5gr	0.02-0.6	yes	4-7
Meningioma(2)	2.5gr	0.5-2.3	yes	4-6
Neuroblastoma(1)	1.7gr	1.2	yes	5
Ovary carc.(3)	2.2gr	2.0-3.7	yes	6-8
KS sarcoma(5)	0.56gr	2.4-3.2	yes	10-12
Normal liver(12)	1.5gr	15.4-18.3	yes	14-16
Normal skin(14)	0.7gr	10.5-17.6	yes	13-15
Adrenal gland(15)	>5.0gr	29.8-38.7	yes	13-16

Table 2.

Magnetic beads coated with CD34, an EC antigen that can be found also on stem cells, have been used by our laboratory to isolate and purify EC progenitors derived from human fetal tissues (aorta an heart) (Figure 4).

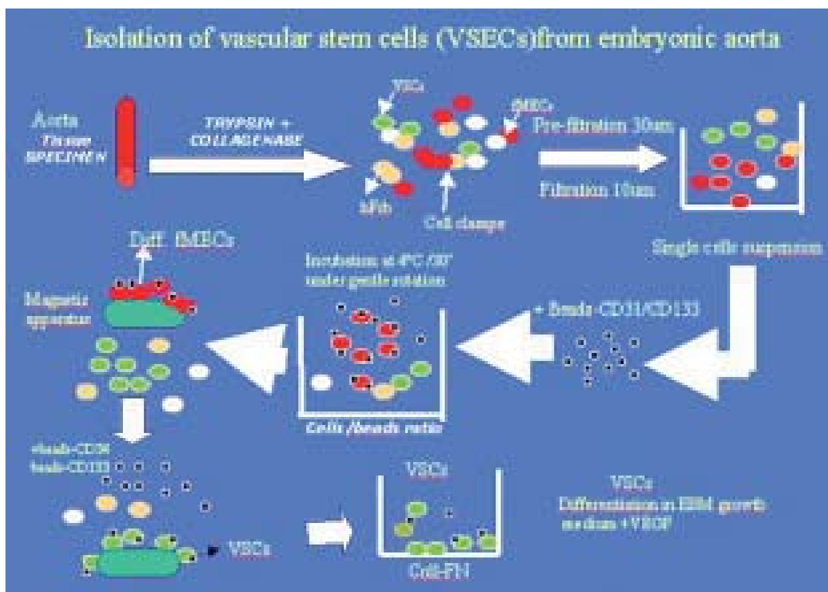


Figure 4.

In conclusion, the methods for EC isolation and cultures form different human organs or tissues represent an important tool to enhance our knowledge in vascular research and in particular in the field of tumor angiogenesis.

THE CHICKEN AORTIC RINGS ASSAY

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Introduction

Both in vivo and in vitro angiogenic assays are made complex by the presence of a poorly defined chemical environment in which the agents to be tested interact with unknown factors derived from tissues, plasma, or serum. In addition, systemic inflammatory reactions can influence blood vessel formation during in vivo angiogenic assays. On the other hand, three-dimensional in vitro models employing isolated endothelial cells do not appropriately recapitulate the angiogenic process because of the lack of mural cells, such as pericytes, smooth muscle cells and fibroblasts. The understanding of the molecular mechanisms of angiogenesis could be facilitated by assays designed to provide a chemically defined environment for the growth of microvessels as well as the presence of both endothelial and mural cells. In this respect, the culture of ring of aorta embedded in a gel of extracellular matrix represents a good reproduction of the in vivo angiogenic process, where endothelial and mural cells interact. Actually, the aorta ring assay initially developed by Nicosia [1], is a good compromise between in vivo and in vitro models. In this ex vivo assay, it is possible to evaluate endothelial sprouting from rat aortas which have cut into rings and embedded in gels of fibrin or interstitial collagen. In a following variant of this method, a serum-free culture of aortic rings was used and this provided a

chemically-defined growth medium, that can be pharmacologically manipulated [2]. In our lab, we further extended this model by developing an assay that employs adult chick aortas and growth factor-reduced Matrigel as extracellular matrix for embedding rings.

Modulation of microvascular growth in different experimental conditions

Different growth media have been tested in order to evaluate their different ability to support both angiogenesis and fibroblast proliferation in the absence of serum supplement. As reported by Nicosia et al. [2], aortic rings embedded in fibrin gels gave rise to microvessels when cultured in the presence of serum-free MCDB 131, an optimized medium for microvascular endothelial cells, or DMEM/HAM F12, in a 1 to 1 mixture. In contrast, MEM failed to support both angiogenesis and fibroblast proliferation. The growth of microvessels in serum-free MCDB 131 in fibrin gel is characterized by: i) an initial lag phase; ii) a growth phase; iii) a plateau phase. The first microvascular sprouts appear after 5 days. The growth phase takes place during the end of the first week and continues into the second week to end 13 to 14 days after the beginning of the experiment (Figure 1A).

Different solid phase substrates have also been tested such as fibrin, collagen, plasma clot and factor reduced Matrigel [3]. As compared with collagen, fibrin promotes angiogenesis inducing a 170% increase in newly formed microvessels. As compared to collagen, fibrin or plasma clot, Matrigel gives rise to a markedly reduced three-dimensional networks of capillary with an inferior mean luminal area (Figure 1B).

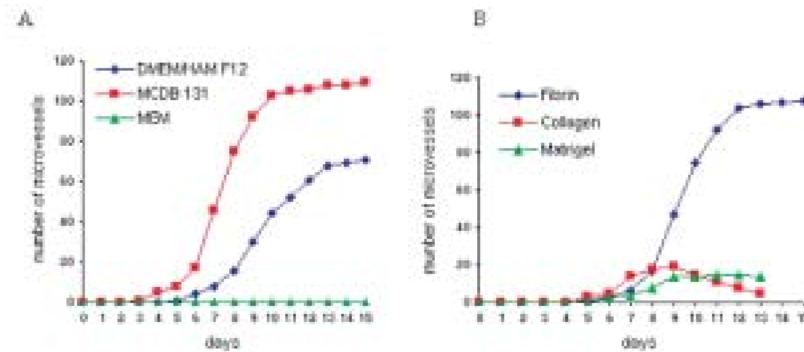

Figure 1. Microvascular growth curves of aortic rings. A. Growth curve of aortic ring-fibrin gel cultures in serum-free MCDB 131 (blue), DMEM/HAM F12 (red), and MEM (green). New vessels forms in MCDB 131 and DMEM/HAM F12, while no growth is observed in MEM. B. Microvascular growth curve of culture of aorta in fibrin (blue), collagen gel (red), and factor reduced Matrigel (green) in serum-free MCDB 131. Fibrin stimulate angiogenesis inducing an increase over collagen and Matrigel in the number of microvessels.

In order to test substances endowed with either an angiogenic or an antiangiogenic effect, it is necessary to choose the right combination between solid phase matrix and growth medium. Serum-free culture of aortic rings are suitable to test the effect of angiogenic substances added to the medium when aortic rings are embedded in collagen or Matrigel. When compared to the control supplemented with serum-free MCDB 131 alone, microvascular growth profile in the presence of an angiogenic growth factor such as bFGF (Figure 2A) had a shorter lag phase and produced a higher plateau due to a relative increment in the final number of microvessels. Inhibition of angiogenesis can be visualized after treating aortic rings cultured in fibrin gel supplemented with MCDB 131 serum-free medium with an antiangiogenic factor. For example, a dramatic inhibition of angiogenesis was seen by adding hydrocortisone to the growth medium. Cultures treated with hydrocortisone produced only a few short,

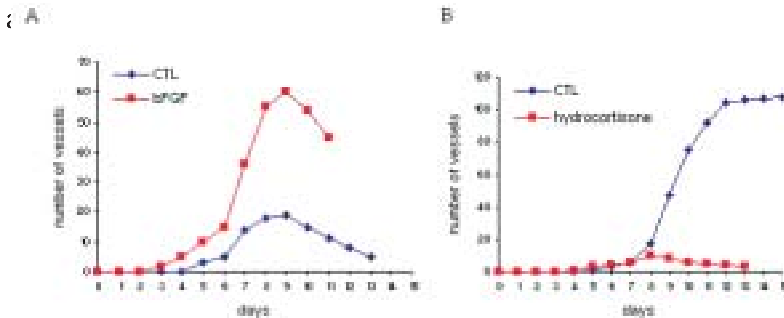

Figure 2. Microvascular growth curves of aortic rings in presence of stimulators or inhibitors of angiogenesis. A. Microvascular growth curve obtained from collagen gel cultures of aorta grown in serum-free MCDB 131 in the presence (red) or absence (blue) of bFGF. bFGF stimulates angiogenesis inducing an increase in the number of microvessels B. Growth curve of aortic ring-fibrin gel cultures in serum-free MCDB 131 containing hydrocortisone (blue), or not containing it (red). Angiogenesis is markedly inhibited by hydrocortisone.

Characterization of sprouting capillaries

The process of vessels formation, in cultured aortic rings in a three-dimensional matrix of clotted chick plasma, has been studied with light microscopy, radioautographic and ultrastructural techniques [1]. On the second day of culture, endothelial cells sprouted from the intima of the aorta and its collateral branches into the surrounding clot, forming solid cellular cords. A complex vascular network was established within the first week by spindling and poorling differentiated endothelial cells. At this stage cells were migrating, branching, and proliferating in a longitudinal fashion. When present, lumens appeared as slitlike spaces enclosed with junctional complexes. By the end of the second week, the migratory activity decreased and proliferation occurred mostly in a cross-sectional plane, with formation of large patent lumens. Vascular channels were lined by prominent endothelial cells rich in rough endoplasmic reticulum, polysomes, mitochondria, Golgi apparatuses, and coated vesicles. A discontinuous

basal lamina was present along the abluminal side. At 28 day of incubation, the still viable endothelium exhibited numerous microfilaments and microtubules, decreased cytoplasmic organelles, and increased pinocytotic activity.

Microvessels during the early stages of angiogenesis were composed primarily of endothelial cells. As vascular proliferation decreased, the microvessels became coated with pericytes. Pericytes migrated from the root to the tip of the microvessels using the endothelium as a surface for attachment, proliferation, and contact guidance [4]. The rat aorta contains a subpopulation of intimal/subintimal smooth muscle cells that differentiate into pericytes during in vitro angiogenesis. These cells have a distinct endothelial tropism and respond to endothelial cues, contributing to the differentiation and maturation of microvessels.

Moreover, the discovery that in fibrin gel culture supplemented with serum-free medium angiogenesis occurs in the absence of exogenous growth factors suggests that the aortic explants, and possibly the endothelial cells, possess a set of chemical signals capable of initiating and regulating the formation of new microvessels. It is thus possible that angiogenesis was promoted by growth factors released by the aorta itself [2].

Experimental procedures

In the chick variant of this assay, rings from three-months old chicken aorta are embedded in growth-factor reduced Matrigel and supplemented with serum-free medium. It can be used as a sensitive assay for the study of soluble or solid-phase angiogenic and antiangiogenic factors and it allows the quantification of the vascular response to different molecules.

Isolating the aorta and preparing the rings

1. 3-month-old sacrificed and plucked White Leghorn chickens were from the local abattoir.

2. In a vertical flow hood, incise the skin over the ventral midline of the ribcage with a scalpel and then cut the sternum with the help of a bone cutter.
3. Grab the aorta with forceps and detach it from the surrounding tissues with a scalpel. Then, dissect and isolate the aortic arch and the descending thoracic aorta, transfer them into a sterile Petri dish, and rinse extensively in PBS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL).
4. Under a stereomicroscope, remove carefully the fibroadipose tissue around the aorta, paying special attention not to damage the vessel wall.
5. When the aorta is completely made free of the fibroadipose tissue, put it in a new sterile Petri dish and lid it with serum-free medium (M199 - Bio Whittaker Europe, Verviers, Belgium) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). Cut then the aorta with the blade of a scalpel in 1 mm-long rings (about 20 rings/aorta).
6. Transfer rings into a 50 ml tube (Falcon) containing ice cold serum-free medium. At this step explants can be stored for until two hours.

Embedding the aortic rings in Matrigel

1. Add 0,2 ml of growth factor-reduced Matrigel (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) to each well of a 48-well plate.
2. Incubate at 37°C for 30 min. to allow gel formation.
3. Transfer one aortic rings to each well onto Matrigel and properly position it in the center of the well.
4. Cover the rings with 0,2 ml of Matrigel and allow it to jellify at 37°C. for 30 min.
5. Add 0,5 ml of serum-free M199 medium to each well. Keep cultures at 37°C in an humidified incubator at 5% CO_2. M199 medium has been chosen for its ability to support vessels' growth without inducing by itself a massive capillary proliferation.

6. Change the medium daily.

Testing angiogenic or antiangiogenic factors

Three-dimensional matrix culture of chick aorta in serum-free medium can be used as a sensitive assay to study soluble or solid phase angiogenic agonists and antagonists. After two days of incubation, the serum-free medium over the aortic ring culture can be replaced with fresh growth medium containing the factor to test. As a positive control it is possible to use M199 medium supplemented with 20% FCS, 50 mg/l heparin and 50 mg/l endothelial cell growth supplement from bovine neural tissue (Sigma), while serum-free medium can be employed for negative control purposes. The medium has to be changed daily. It is also possible to evaluate the angiogenic response to solid matrix proteins incorporated in the gel.

Quantification

In the aortic ring model, the angiogenic response can be quantified by: i) manual processing and measurement [2]; ii) global characterization [5, 6]; iii) determination of the mean length of microvessels emerging from the aortic ring [7]; iv) automatic binary processing of grey level image and determination of the number and the total area of microvessels at a fix distance from the aortic ring [8]; v) the determination of the following geometric and morphologic parameters: number and length of branching microvessels; size and form of aortic rings and the number and spatial distribution of fibroblast-like cells [9].

In our laboratory [7], microvessel outgrowth is visualized by observing specimens in phase contrast with an inverted photomicroscope (model DM IRB HC; Leica Microsystems, Wetzlar Germany) and the mean length of microvessels is measured throughout the course of the experiment. Phase contrast images on different focus planes of 1024 x 1024 pixels are captured using a cooled digital CCD Hamamatsu ORCA camera (Hamamatsu Photonics Italia, Arese, Italy), digitally recorded, and quantify with Image-

ProPlus 4.0 imaging software (Media Cybernetics, Silver Spring, MD). After choosing the appropriate spatial calibration scale, at least 50 capillary sprouts per well were measured as follows. Two point features, one at the base and one at the distal tip of each sprout were generated by means of the "Point" tool of ImagePro-Plus 4.0. The measurement of the distance between these two point features was quantified by means of the "Distance Measurement" tool. Results were expressed as the mean capillary length (in μm).

Advantages of serum-free matrix culture and limitation of the assay

The serum-free Matrigel culture of chick aortic rings bridges the gap between in vivo and in vitro models combining advantages of both systems. The major advantage of this method is that the growth medium and the extracellular matrix are chemically defined and can be manipulated to test different substances. This allows to evaluate the angiogenic or antiangiogenic effects of both soluble factors and solid phase factors or of the combination of different molecules.

Another advantage of this method is the possibility to quantify the angiogenic response to the different factors such as the daily count of microvessels useful to generate curves of microvascular growth. Analysis of the microvascular growth curves provides data on the lag phase, growth, and regression of microvessels. New formed microvessels can be counted daily as in the rabbit corneal assay, however a significant advantage over the rabbit corneal assay is the absence of inflammatory complications.

This assay can be used to study both inhibition and stimulation of angiogenesis. However, when quantification is limited to the evaluation of the number of microvessels and angiogenic stimulation results in the formation of more than 200 to 250 microvessels, the margin of error for the observer who is counting the microvessels become too high, due to the three-dimensional

complexity of the vascular network.

Table 1. Advantages of serum-free matrix culture of chick aorta as a model of angiogenesis

1. Study the effects of angiogenesis agonists or antagonists in the absence of serum factors that my bind, inactivate or simulate the action of the substance being tested.
2. Study the effect on angiogenesis of both soluble factors and matrix factors.
3. Evaluate the synergistic or antagonistic effects of soluble factors or matrix factors on angiogenesis.
4. Quantify angiogenesis generating curves of microvascular growth.
5. Improve reproducibility of results by eliminating serum whose composition varies in different batches.
6. Reduce growth of fibroblasts by eliminating serum.
7. Study angiogenesis in the absence of inflammatory reaction.
8. Prepare several assays from one animal (15 to 20 culture/chick aorta).
9. Study endogenous production of angiogenic molecules at different stages of microvascular development.
10. Matrigel is a matrix easy to manipulate.
11. Matrigel contains a representative mix of basement membrane proteins on which endothelial cells typically reside.
12. Chicken are easily obtainable from an abattoir and do not require euthanasia of many animals.

The lesson of GM-CSF

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that regulates proliferation and differentiation of myeloid progenitor cells and functional activation of mature cells [10]. Several studies have suggested that GM-CSF responsiveness may not be limited to hematopoietic lineages. Endothe-

lial cells respond to GM-CSF and express both GM-CSFR subunits [11]. Moreover, it has been reported that GM-CSFR-mediated endothelial cell activation includes functions related to angiogenesis and inflammation and to mobilization of EC precursor from bone marrow [12]. We had previously shown that in vitro in human endothelial cell GM-CSF elicit JAK-2 tyrosine phosphorylation, as well as activation of its catalytic activity [13]. First we demonstrated that this cytokine has an angiogenic activity in vivo in chick chorioallantoic membrane (CAM). Afterwards, we examined the role JAK-2/STAT pathway during angiogenesis in vivo. Specifically, we investigated the activation of JAK-2 and STATs in the CAM during the angiogenic response to GM-CSF. We demonstrated that GM-CSF promoted an early tyrosine phosphorylation of JAK-2 and STAT-3 which preceded vessel growth.

To get further direct insights about cellular targets of GM-CSF in the avian model, we evaluated the effect of GM-CSF on endothelial sprouting from adult chicken aorta. This assay allowed us to demonstrate that GM-CSF is able to activate its angiogenic effect directly on endothelial cells excluding the contribution of an inflammatory reaction effect. After embedding chicken aorta in growth factor-reduced Matrigel, branching microvessels developed at the periphery of aortic rings treated with M199 medium 20% FCS, used as positive control (Figure 3 C), as well as with serum free medium containing 50 ng/ml GM-CSF (Figure 3 D).

In preliminary experiments, this concentration has been demonstrated to be the lowest one able to give the maximal effect. In contrast no vessel formation was evident in aortic rings cultured with serum free medium alone (Figure 3A). Quantitative analysis of microvessel length at different time points revealed a clear-cut induction of vessel growth after stimulation of aortic rings with GM-CSF (Figure 3 B).

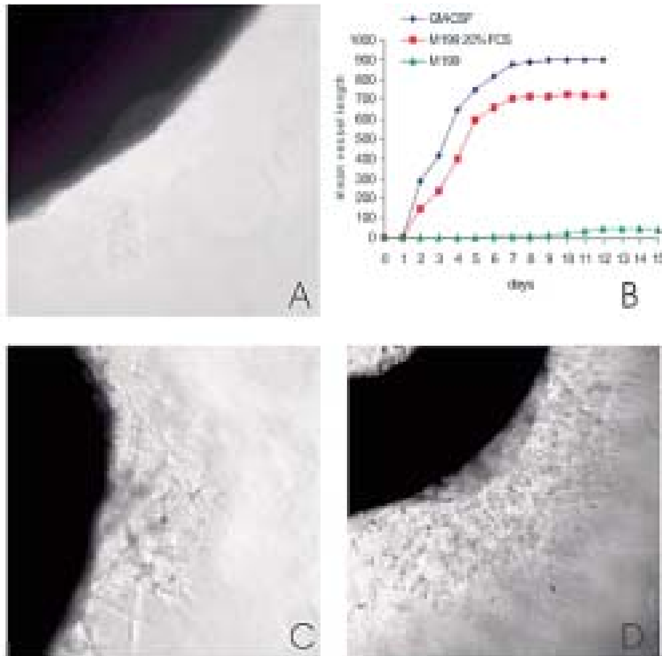

Figure 3. Microvessel formation elicited by GM-CSF in chick aortic rings. Chick aortic rings isolated from 3-month-old White Leghorn chick were embedded in the center of Matrigel gel. Aortic rings were incubated in serum-free M199 (A) or in M199 medium containing GM-CSF at the concentration of 50 ng/ml (C) or in M199 supplemented with 20% FCS (D). Medium was replaced daily. Representative phase contrast pictures taken after three (A, C, D) days of incubation are shown. Note the formation of microvessels branching form the aortic ring in the presence of GM-CSF (D) or FCS (C) as compared to serum-free medium (A). The mean length of outgrowing microvessels after GM-CSF stimulation was measured throughout the course of the experiment (B).

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COMPUTER-ASSISTED ANALYSIS OF ENDOTHELIAL CELL SPROUTING: AN IN VITRO ASSAY FOR THE SCREENING OF ANTI-ANGIOGENIC COMPOUNDS

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Introduction

Angiogenesis is the process of generating new capillary blood vessels. In the adult, the proliferation rate of endothelial cells is very low compared to many other cell types in the body. Physiological exceptions in which angiogenesis occurs under tight regulation are found in the female reproductive system and during wound healing. Uncontrolled endothelial cell proliferation is observed in tumor neovascularization and in angioproliferative diseases (1). Tumors cannot growth as a mass above few mm3 unless a new blood supply is induced. It derives that the control of neovascularization may represent a novel approach to tumor therapy. Also, it is conceivable that the availability of chemical agents able to prevent neovascularization would potentially have broader applicability as a therapy for a wide spectrum of diseases.

Various angiogenesis inhibitors have been developed so far

and their efficacy has been assessed in different in vitro and in vivo assays (2). The clinical evaluations of some of them in cancer patients are in progress (for further information about angiogenesis inhibitors in clinical trials see the NCI web site: http://cancertrials.nci.nih.gov).

Recently, the hypothesis that anti-angiogenic compounds can be used in combination with cytotoxic drugs for tumor therapy has been advanced (see ref. 3 and references therein). Also, chemotherapeutic agents have shown anti-angiogenic properties in vitro and in vivo (4, 5), leading to the concept of anti-angiogenic scheduling of chemotherapy (5, 6).

The formation of new blood vessel is a multi-step process that begins with the degradation of the basement membrane by activated endothelial cells that will migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space. Then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane. Each step of this process represents a potential target for the inhibitory action of angiosuppressive molecules. To this respect, assays aimed to investigate the effect of natural and synthetic compounds on endothelial cell sprouting may represent useful tools for the screening of potential anti-angiogenic drugs.

Purine analogs were developed in the early 50's as antineoplastic chemotherapeutic agents (7). These antimetabolites inhibit de novo purine synthesis and purine interconversion reactions and their metabolites can be incorporated into nucleic acids (8). 6-Thioguanine (6-TG) and 6-methylmercaptopurine riboside (6-MMPR) also alter membrane glycoprotein synthesis (9). Purine analogs can act as protein kinase inhibitors (10). 2-Aminopurine (2-AP) inhibits proto-oncogene and interferon gene transcription (11). Recent observations had shown that 6-MMPR modulates the angiogenic activity of Fibroblast Growth Factor-2 (FGF2) in vitro and affect blood vessel formation in vivo (12). Also, continuous systemic administration of 6-mercaptopurine ri-

bose phosphate inhibits angiogenesis in the rabbit cornea (5). In contrast, 6-methylmercaptopurine, 2-AP, and adenine are devoid of anti-angiogenic activity (12). More recently, we demonstrated that 6-TG inhibits different steps of the angiogenesis process in vitro and exert a potent anti-angiogenic activity in the chick embryo chorioallantoic membrane assay (13).

Murine aortic endothelial (MAE) cells express undetectable levels of FGF2 (14). We generated FGF2-overexpressing pZipFGF2 MAE cells by stable transfection of parental MAE cells with a retroviral expression vector harboring a human FGF2 cDNA (15). Transfectants are characterized by a transformed morphology, increased saturation density, and an invasive and morphogenic behavior in three-dimensional gels. In vivo, they induce hemangiomas in the chick embryo and opportunistic vascular lesions in nude mice (14, 15).

A FGF2-overexpressing subclone (FGF2-T-MAE cells) was isolated from these lesions: it retained several of the in vitro properties of pZipFGF2-MAE cells but showed an higher tumorigenic capacity when re-injected in nude mice (16). Thus, FGF2-T-MAE cells may represent an unique model in which FGF2 overexpression leads to the activation of a pro-angiogenic autocrine loop of stimulation in endothelial cells.

Aim of the present paper is the description of a methodology that allows the quantification of the inhibitory action of selected compounds on FGF2-*T*-MAE cell sprouting in a three-dimensional fibrin gel. The purine analog antimetabolites 6-TG and 2-AP were used to illustrate the behavior in this assay of positive and negative controls, respectively.

Materials and Methods

Cell cultures

FGF2-*T*-MAE cells represent an highly tumorigenic subclone of FGF2-transfected MAE cells (16). They express high levels of the 18, 22 and 24 kD molecular weight isoforms of FGF2 and form

highly vascularized tumors in nude mice (21). FGF2-*T*-MAE cells were grown in DMEM supplemented with 4 mM glutamine (Gibco) and 10% FCS.

Preparation of three-dimensional fibrin gels

FGF2-*T*-MAE cell aggregates were prepared on agarose-coated plates exactly as described (17). Briefly, cells were trypsinized and seeded at 75,000 cells/cm2 onto 0.5% agarose-coated 35-mm tissue culture dishes in complete medium. After 24 h, aggregates in suspension were recovered and decanted from single cells under gravitational force in conical plastic tubes for 20 min at room temperature. Sedimented aggregates from each dish were then collected and resuspended in 1.8 ml of calcium-free medium containing fibrinogen (2.5 mg/ml).

After addition of thrombin (250 mU/ml), 250 µl/well of cell aggregate suspension were immediately aliquoted in 48 well-plates and allowed to gel for 10 min at 37°C. Then, 500 µl of culture medium with or without the purine analog under test were added on the top of the gel. In all the experiments, the fibrinolytic inhibitor aprotinin was added to the gel and to the culture medium at 10 µg/ml to prevent the dissolution of the substrate (18).

Formation of radially growing cell sprouts was observed during the next 2 days. At the end of incubation, sprouts were photographed using an inverted phase contrast photomicroscope and quantified by computerized analysis of the digitalized images (see below for further details).

Results

In the in vitro sprout formation assay, endothelial cell aggregates are embedded into a three-dimensional fibrin gel in the presence of an angiogenic stimulus and the formation of radially growing endothelial sprouts follows (18). In our model, the angiogenic stimulus is represented by the endogenous production of FGF2 in FGF2-*T*-MAE transfectants. Accordingly, aggregates of

endogenou e gel and form solid 1 and refs 14, 15).

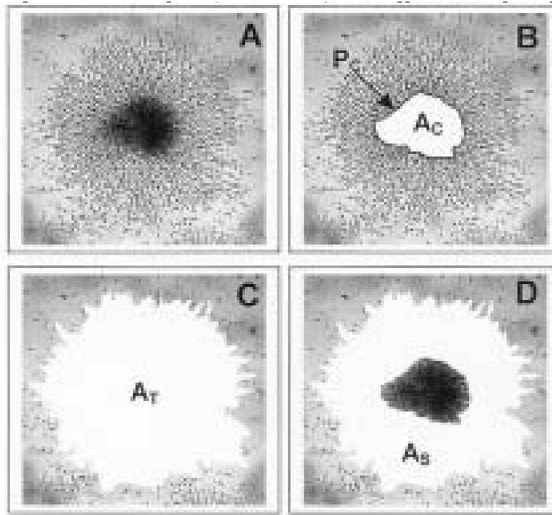

Fig. 1. Endothelial cell sprouting in fibrin gel. Representative control FGF2-*T*-MAE cell aggregate grown within three-dimensional fibrin gel for 2 days (A). The digitized image of the cluster was processed by computer-assisted analysis to highlight the cluster area (A_C) in B, the total area including the cell sprouts (A_T) in C, and the sprouting area (A_S) in D. Cluster perimeter (P_C) is also shown in B.

In an attempt to quantify the sprouting activity of FGF2-T-MAE cells, images of cell clusters were captured at x40 magnifications with a digital TV camera (Sensicam, CCD imaging, Kelheim, Germany) mounted on an inverted phase contrast microscope. Digitized images were then analyzed using the Image-Pro Plus(tm) software (Media Cybernetics, Silver Spring, MD). Next, for each cell aggregate the cluster area (A_C) was subtracted from the total area including the cell sprouts (A_T) using the software tools. The resulting value (in μm^2) corresponds to the sprouting area of the aggregate (A_S) (see Fig. 1). The sprouting activity was then expressed as sprouting units (SU) by calculating the ratio be-

tween the AS area (in µm^2) and the perimeter of the correspon-ding cluster (PC, in µm) according to the formula:

[*1*] Sprouting area (A_S)/cluster perimeter (P_C) = Sprouting units (SU)

Figure 1 shows a typical FGF2-T-MAE cell cluster that invades the surrounding fibrin gel 48 h after seeding. The corresponding digitized images highlight the areas described above. Under standard cell culture conditions, AS area and SU values progressively in-

time	n	A_s area (µm^2)	Pc (µm)	SU
2 h	10	16140 ± 2240	1345 ± 123	12 ± 3
24 h	11	279075 ± 38300^a	1448 ± 162	191 ± 8^a
48 h	8	541319 ± 39320^{ab}	1444 ± 91	374 ± 8^{ab}

Table 1. FGF2-T-MAE cell sprouting in three-dimensional fibrin gel. FGF2-*T*-MAE cell aggregates were grown within three-dimensional fibrin gel for 4, 24, or 48 h. Next, sprouting was quantified by computerized-image analysis as described in the text. Data area expressed as mean ± SE. A_S area, sprouting area; P_C, cluster perimeter; SU, sprouting units. *a*: statistically different from 4 h; *b*: statistically different from 24 h ($p < 0.01$).

Routinely, the effect of inhibitory compounds was evaluated 48 h after seeding. It must be pointed out that the preparation of the cell aggregates results in the formation of clusters heterogeneous in size (P_C values ranging from 733 to 2398 µm, n=11). Nevertheless, the use of the formula [*1*] allows the standardization of the sprouting activity with SU values being independent of the size of the aggregate (Fig. 2).

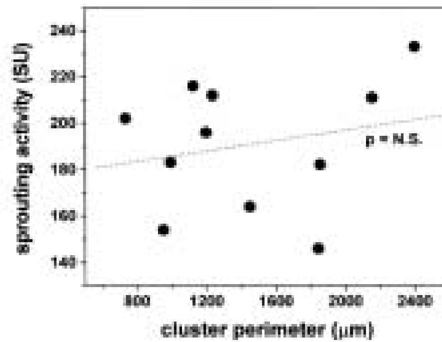

Fig. 2. Sprouting activity as a cluster size-independent parameter. Control FGF2-*T*-MAE cell aggregates were grown within three-dimensional fibrin gel for 24 h. For each aggregate, cluster perimeter P_C and sprouting activity (SU) was quantified by computerized-image analysis. Each point represents one aggregate. The data indicate that SU values are independent of cluster perimeter P_C values, thus allowing the comparison among clusters of different size.

On this basis, the capacity of 6-TG to affect the sprouting activity of FGF2-*T*-MAE cells was investigated. To this purpose, cell aggregates were seeded in fibrin gel in the absence or in the presence of increasing concentrations of 6-TG, or 2-AP, here used as a negative control (12). After 48 h, digitized images of cell aggregates were analyzed as above. As shown in Fig. 3, 6-TG inhibited the capacity of FGF2-*T*-MAE cells to invade the fibrin gel and to generate endothelial cell sprouts whereas 2-AP was ineffective. Quantification of cell sprouting by computerized image analysis confirmed the dose-dependent inhibitory activity of 6-TG (Fig. 4).

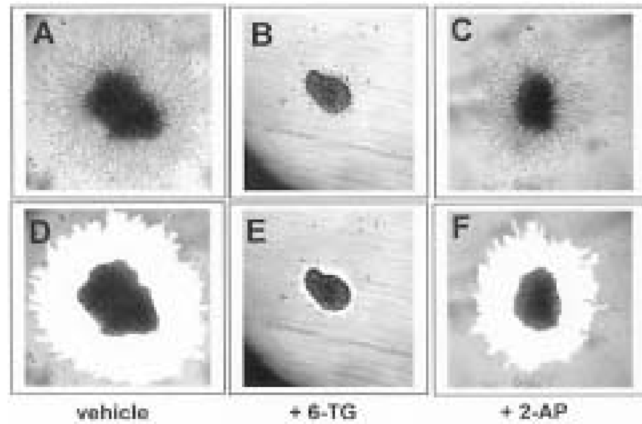

Fig. 3. Inhibition of endothelial cell sprouting by the anti-angiogenic purine analog 6-TG. Representative FGF2-*T*-MAE cell aggregates grown within three-dimensional fibrin gel for 2 days in the absence (A) or in the presence of 10 μM 6-TG (B) or 2-AP (C). The corresponding sprouting areas A_S were highlighted by computerized-image analysis (D, E, and F, respectiv

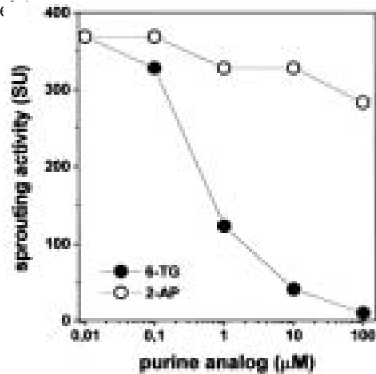

Fig. 4. Dose-dependent inhibition of endothelial cell sprouting by 6-TG. FGF2-*T*-MAE cell aggregates were grown within three-dimensional fibrin gel for 2 days in the absence or in the presence of increasing concentrations of 6-TG (*) or 2-AP (■). Next, sprout-

ing was quantified by computerized-image analysis as described in the text. SU, sprouting units.

Discussion

The invasion of perivascular matrix by sprouting endothelial cells represents a crucial event in angiogenesis. A number of three-dimensional cell culture systems have been developed to study this step of the angiogenic cascade. These systems are aimed to focus delivering aggregates of endothelial cells from which sprouting can occur. The first assay described by Montesano et al. (18, 19) was based on the embedding of bovine endothelial cell clusters in fibrin or collagen gels. Endothelial cell sprouting was induced by treatment with phorbol-ester. This assay was modified to deliver bovine macrovascular endothelial cells grown on gelatin microcarrier beads into a three-dimensional fibrin gel (20, 21). In this assay, both angiogenic FGF2 and VEGF were able to exert a significant response that partially depended upon the structure of the fibrin cloth. More recently, a three-dimensional spheroid model of endothelial cell differentiation has been described (22). So far, all these models have been utilized mainly to investigate the molecular and cellular mechanisms responsible for endothelial cell sprouting.

In the present paper, we describe a new endothelial sprouting assay suitable for the screening of potential anti-angiogenic compounds. The assay is based on the autocrine mechanism of stimulation induced in murine aortic endothelial cells by stable transfection with FGF2 cDNA. Transfectants are characterized by a transformed morphology, increased saturation density, and an invasive and morphogenic behavior in three-dimensional gels (14, 15). Thus, in this assay endothelial cells are continuously stimulated by the endogenous growth factor rather than by the treatment with a single bolus of angiogenic compound, as it occurs in the other assays. This should increase the reproducibility of the results. Indeed, our experience has evidenced a very low intra-

and inter-experimental variability both in terms of kinetics of sprouting in control cultures and in response to various inhibitors.

The data indicate that computer-assisted image analysis represents an easy method for the quantification of the sprouting activity of FGF2-*T*-MAE cell clusters. In particular, the calculation of the sprouting units (SU) expressed as the sprouting area (A_S)/cluster perimeter (P_C) ratio allows the comparison among clusters of different size. This is of importance when considering the high variability in size among the aggregates even within the same experiment.

We have shown that the purine analog 6-TG exerts a significant inhibitory effect on endothelial cell sprouting whereas 2-AP is ineffective. This is in keeping with the different anti-angiogenic activity of the two molecules in vivo ([12]). Experiments performed in our laboratory have shown that various anti-angiogenic compounds, including FGF2-antagonist polysaccharides ([23]), inhibit FGF2-*T*-MAE cell sprouting (data not shown). Given the complexity of the sprouting process that results from the coordinate interplay among various biological events (including cell motility, adhesion, and survival as well as extracellular matrix degradation, activation of intracellular signaling and modulation of gene expression), it is anticipated that potential anti-angiogenic molecules with different mechanisms of action and subcellular targets can be identified by this assay.

In conclusion, the computer-assisted FGF2-T-MAE cell sprouting assay herewith described may represent an useful tool for the screening of potential anti-angiogenic drugs with therapeutical implications in cancer and angiogenesis-dependent diseases.

Acknowledgements

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PROLIFERATION, CHEMOTAXIS AND PROTEOLYSIS ASSAYS FOR ENDOTHELIAL CELLS

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Proliferation assay

It evaluates the effect of angiogenic or antiangiogenic factors. The Kueng et al.' method is a very sensitive procedure for the quantification of cell number in monolayer cultures by crystal violet staining (1).

Endothelial cells (i.e., human umbilical vein endothelial cells [HUVEC]) are plated (2-2.5 x 10^3/well) in a 96-well plate in 200 μl of their complete medium (M199 supplemented with 20% fetal calf serum [FCS]). Peripheral wells are not used to avoid evaporation. Coating with 0.1% gelatine before seeding HUVEC is mandatory. After 18 hours, the medium is removed and replaced with 200 (l of complete medium for positive control, serum-free medium for negative control and complete medium (or serum-free medium) supplemented with the final concentration of the angiogenic (or antiangiogenic) molecule.

The test is performed in quadruplicate. The same stimulations are repeated every 48 hours after removing preexistent

medium. The number of stimulations is variable depending on the features of the molecule; however, at least 2-3 stimulations are carried out. After 48 hours from the last stimulation, the medium is removed, and 2 washings with PBS are carried out to avoid seric resting. Then, cells are fixed in 100 µl/well of 2.5% glutaraldehyde in phosphate buffered saline (PBS) on a vibrating platform for 15 min at room temperature; then, the glutaraldehyde is removed by 3 washings with deionised water in immersion.

Then, plate is air-dried and cells are stained with 100 (µl of 0.1% crystal violet (0.1 g/100 ml of crystal violet in 20% methanol) on a vibrating platform for 20 min at room temperature. Three washings with deionised water in immersion followed by drying are made up. Finally, 100 (l of 10% acetic acid are used for solubilization of adsorbed dye by cell nuclei during staining, and the plate is placed in a spectrophotometer at 595 nm (reference at 655

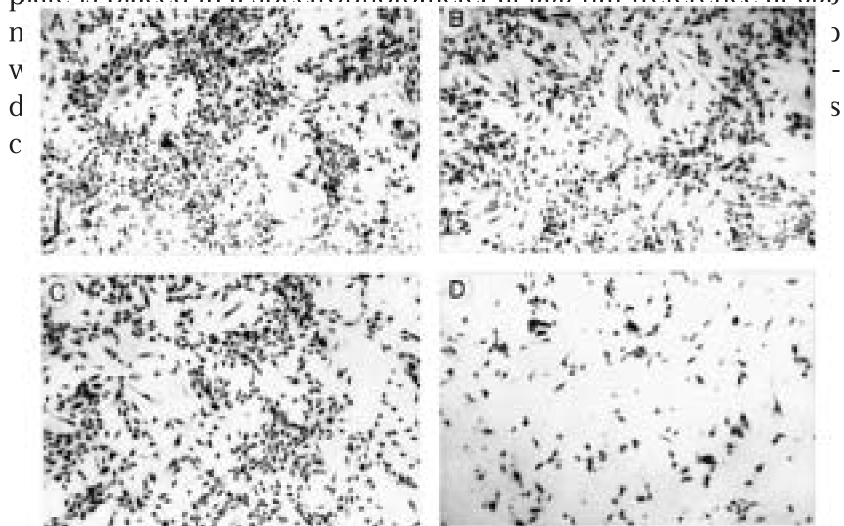

Fig. 1. Proliferation assay effect of TNP-470 on HUVEC proliferation Sub A) positive control; sub B) and C) increasing doses of TNP-470 (0.12 - 0.50 ng/ml) reduce cell proliferation; sub D) TNP-470 1,000 ng/ml is cvtotoxic.

Chemotaxis assay

Chemotaxis is the directional migration of cells in presence of a gradient chemoactractant in suspension. Numerous cells types are able to migrate: leukocytes, smouth muscle cells, neuronal cells, fibroblasts, endothelial and tumor cells. There are various experimental systems to study cell migration, but the most used to evaluate the chemotaxis of endothelial cells (as well as fibroblasts) is the one based on the Boyden chamber assay. The chamber is formed by 2 compartments which are separated by a polycarbonate filter with calibrated pores (8-12 μm for endothelial cells or fibroblasts). The lower compartment contains the chemoactractant, and the other the cells to be tested. The chambers allow a gradient of the agent applied for the chemotactic stimulation.

Coating of filters with collagen type IV: lower surfaces of polycarbonate filters are coated with collagen type IV at a concentration of 5 μg/ml (i.e., 5 ml of PBS with 12.5 μl of a stock solution of collagen type IV at 2 mg/ml in 10 mM acetic acid) for 1 hour at 37°C.

Preparation of endothelial cells: T25 flasks of endothelial cells (i.e., HUVEC or EA.hy926) at 90% confluence are used. Cells are harvested in trypsin/EDTA 0.05/0.02% (vol:vol) in PBS, collected by centrifugation, resuspended in serum-free medium supplemented with 0.1% bovine serum albumin (BSA).

Preparation of the Boyden chamber: a 48-well chemotaxis chamber is used. The filters coated with collagen type IV are numbered on the upper surface. Then, the lower compartement of the chamber is filled with 30 μl of chemoactractant solution in the positive

control, or serum-free medium supplemented with 0.1% BSA in the negative control (to evaluate random migration). The solution should form a small outward-curved meniscus.The filter is placed with the numbered surface side up on the lower compartment, then the rubber gasket and the upper part of the chamber are placed on the top. The chamber is closed and the cells are seeded in triplicate in the upper compartment of the chamber (1.2-1.5 x 10^5cells/50 µl) by avoiding the formation of bubbles. The chamber is incubated for 2 hours in a humidified atmosphere 5% CO_2 at 37°C.

Experiment interruption: the chamber is opened; the upper compartment of the chamber including the rubber and the filter is taken off carefully. The shyne side (the upper numbered surface) is washed in PBS and thus not migrated cells are stripped off. The whole procedure is repeated twice. Afterward, the filter is dried at room temperature for several hours, immersed and thus fixed in absolute ethanol for 30 sec, washed in deionised water, then in toluidine blue for 7-10 min at room temperature. Finally, the filter is washed in tap water, dried, and mounted on slides (a half per glass).

Evaluation of migration: the cells are counted in 5 to 10 400x immersion fields, dependig on the uniformity of the filter, and the number is calculated as a mean ± 1 SD per filter and per medium. It correspondes to the number of cells migrated/field unit.

MMP-2 and MMP-9 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) zymography and activity

The metalloproteases MMP-2 (or gelatinase A) and MMP-9 (or gelatinase B) are proteolytic enzymes able to degrade the collagen type IV, other types of collagens, and fibronectin, that are essential constituents of the extracellular matrix (mainly of the basal membranes). They are secreted by tumor and endothelial

cells, and they are present in their culture medium. To evaluate the gelatinolytic activity of the MMP-2 and MMP-9 the most used method is SDS-PAGE zymography.

Preparation of conditioned media: T25 flasks of endothelial cells (i.e., HUVEC or EA.hy926) at 90% confluence are rinsed twice with serum-free medium and incubated for 24-48 hours in 5 ml of this medium. The conditioned medium is then obtained and collected under sterile conditions, centrifuged sequentially at 1,200 and 12,000 rpm for 10 min to eliminate debris and stored at -20°C.

SDS-PAGE zymography: the total protein content is measured by adsorbance with the Bradford method using a calibration curve set up with known doses of BSA as a standard protein (2.5, 5 and 10 μg/ml). Aliquots of 5-25 (μg proteins of the conditioned medium mixed with non reducing sample buffer are loaded into 7.5% SDS-PAGE gels, co-polymerized with type A gelatine from porcine skin at a final concentration of 0.6 mg/ml.

After electrophoresis, the gel is washed in 100 ml of 2.5% Triton 1x for 1 hour on a vibrating platform at room temperature to remove SDS, incubated for 18 hours at 37°C in collagenase buffer (NaCl 11.68 g, $CaCl_2$ 1.47 g, Tris 4.84 g, Brij 35 solution 30% 0.3 ml in 500 ml of deionised water, pH 7.5) and stained in Coomassie brilliant blue (62.5 ml of 1% Coomassie brillant, 250 ml of methanol, 50 ml of acetic acid in 500 ml of deionised water) for 20 min at room temperature. Then it is treated with a destaining solution (200 ml of methanol, 50 ml of acetic acid in 500 ml of deionised water).

The gelatinolytic regions are observed as white bands against a blue background. MMP activity is measured by scoring the intensity of bands by computerized image analysis. The MMP-2 and MMP-9 induce bands of gelatinolysis at molecular size of 68 kDa and 88 kDa respectively (Fig.2).

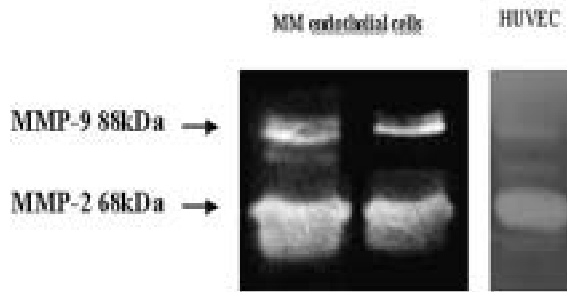

Fig. 2. Zymography of conditioned media of endothelial cells from multiple myeloma and HUVEC MMP-9 and MMP-2 are produced by cell types.

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ANGIOGENIC GROWTH FACTOR-ENDOTHELIAL CELL INTERACTION: RECEPTOR BINDING AND CELL INTERNALIZATION TECHNIQUES

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Abbreviation used: CC, chemokine; CHO cells, chinese hamster ovary cells; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan; FGF2, fibroblast growth factor 2; FGFR, FGF tyrosine kinase receptor; FN, fibronectin; FRET, fluorescence resonance energy transfer; GAG, glycosaminoglycan; GF, growth factor; GFP, green fluorescent protein; GST, glutathione-S-transferase; K_d, dissociation constant; KDR, VEGF receptor II; IL, interleukin; MAPK, mitogen-activated protein kinase; NeuAc, neuraminic acid; PAF, platelet activating factor; PDGF, platelet derived growth factor; PAE cells, porcine aortic endothelial cells; PTX3, pentraxin 3; RGD, Arg-Gly-Asp; RU, resonance units; SPR, surface plasmon resonance; TSP, thrombospondin; VEGF, vascular endothelial receptor; WT, wild type.

Introduction

The study of the interaction of angiogenic growth factors (GFs) and cytokines to their endothelial cellular receptors is an

absolute prerequisite for the comprehension of physiological and pathological processes, including neovascularization, and for the design and production of agonist/antagonist compounds with therapeutic implications.

In this chapter we will attempt to give some guidelines on this field of research, describing the possible approaches and the main advantages and disadvantages that can be faced during cell binding/internalization experiments. This chapter does not want to be a systematical review of known angiogenic GF-receptor interactions. Rather, it represents a collection of selected examples that may suggest novel strategies aimed to study different GF-receptor interactions.

To this purpose experiments performed with two angiogenic growth factors [i.e. fibroblast growth factor-2 (FGF2), and the extracellular form of the HIV-1 Tat transactivating factor] will be described (for reviews about these two proteins see Rusnati and Presta, 1996, and Albini and Noonan, 2000, respectively).

Two approaches can be envisaged for the characterization of G[illegible] the use [illegible] ally excl[illegible] be- caus[illegible] rent intri[illegible]

Cell cultures	Cell free models
Advantages	
more physiological	more accurate (they are elective for the identification of functional domains)
Suitable for studying the biological consequences of the interaction	the labelling of ligand might not be mandatory
	suitable for the fast screening of putative agonist/antagonist
disadvantages	
Possible presence of different classes of receptors that bind simultaneously the GF	GF and receptor must be available in a purified/recombinant form
Presence of soluble molecules that interfere with receptor interaction	Less physiological

Table 1. Cell cultures or cell-free models can be used for studying the different aspects of

GF-receptor in advantages are enlightened.

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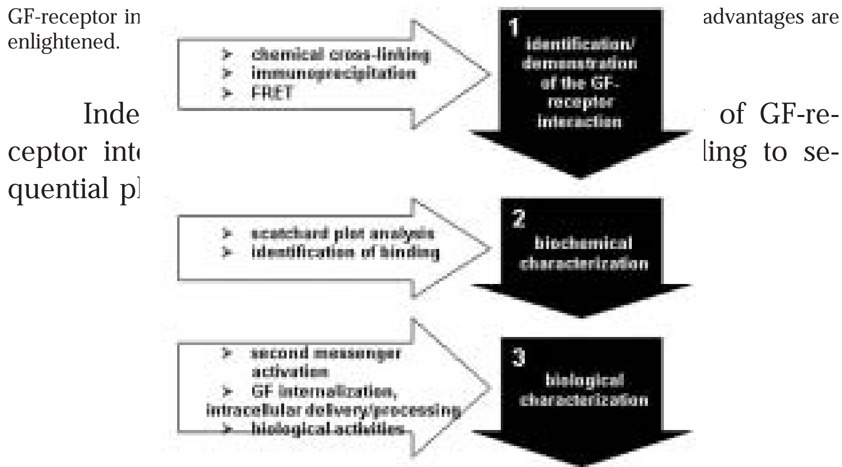

Figure 1. Schematic representation of the different phases for the study of GF-receptor interaction.

The first phase may be represented by the demonstration of the occurrence of GF binding, classically performed by chemical cross linking or immunoprecipitation of the GF-receptor complex. Also, the fluorescence resonance energy transfer (FRET) technique (Truong and Ikamura, 2001) may represent a possible approach despite its awkwardness and expensiveness. FRET relies on the distance-dependent transfer of energy from a donor fluorophore to an acceptor fluorophore allowing the identification of interactions between biological macromolecules both *in vitro* and *in vivo*. In FRET, a donor fluorophore (i.e. linked to a GF molecule) is excited by incident light, and if an acceptor (i.e. linked to

the receptor molecule) is in close proximity (interaction), the excited state energy from the donor can be transferred. This leads to a reduction in the donor's fluorescence intensity, and an increase in the acceptor's emission intensity, that reveals the formation of the GF-receptor complex.

The second phase of the study is based on the biochemical characterization of the interaction that usually consists in the determination of the affinity of the interaction [frequently referred to as dissociation constant (K_d)] and of the number of receptors present on the surface of the target cell. These parameters can be determined by the Scatchard plot procedure (Scatchard, 1949). Also, the biochemical characterization can include the identification of the domains of the ligand/receptor responsible for their interaction. This is functional to the design of receptor and/or ligand agonist and antagonist with possible therapeutic implications.

The third and last phase may consist in the characterization of the biological consequences of the GF-receptor interaction: i) signal transduction and intracellular second messenger activation; ii) GF-receptor complex internalization, processing and/or delivery to different intracellular compartments; iii) biological activities.

Different experimental conditions are required to perform the investigations listed above. Binding experiments usually require relatively short periods of incubation (1-2 hours) at 4°C. This hampers the internalization of the GF-receptor complex and is required to reach the "equilibrium" for receptor binding that allows the determination of Kd values and receptor- number. Shorter periods of incubation (1-30 minutes) at physiological temperatures (37° C) are instead required for studying signal transduction and second messenger activation. On the contrary, longer periods (6-48 hours) of incubation at 37° C are required for studying processes such as cell surface receptor down-regulation, GF internalization, intracellular fate, and biological activities.

Almost all the studies described above requires the labeling of the ligand, that can be obtained by means of radioisotopes or

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Radioisotopes (^{125}I, ^{35}S, ^{3}H)	Fluorochromes
Advantages	
minimal structural modification	high stability
easy quantification	Easy sample handling
high sensitivity	
Disadvantages	
difficult sample handling; special facilities required	large molecules: may modify the GF binding properties
radioactivity decay	Difficult quantification/ Low sensibility
radiolysis of the labeled protein	

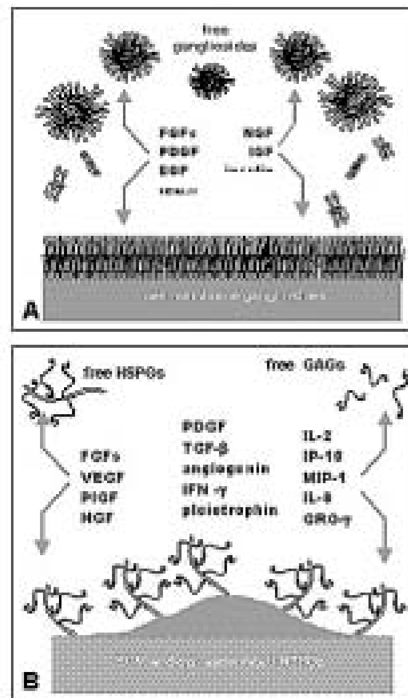
Table 2: GF labeling procedures. A ligand can be labeled with radioisotopes or fluorochromes. These procedures may be performed chemically, enzymatically or biosynthetically. The most common advantages and disadvantages of the two methodologies are listed.

Alternatively, the ligand can be biotinilated and detected by means of streptavidin-coupled fluorochromes or enzymes. Finally, the production of "chimera" proteins in which the GF is genetically fused to a fluorescent tag such as the green fluorescin protein (GFP) (Van Roessel and Brand, 2002) can be envisaged, as in FRET technology.

Studies with cell cultures

A series of considerations may make the use of cell cultures for binding studies more complex than anticipated. Firstly, endothelial cells unlikely express an unique receptor. Frequently, different classes of cell surface binding sites compete for the interaction with the GF under study. Moreover, several components of the extracellular matrix (ECM) of the endothelium can interact with various GFs (Rusnati and Presta, 1996, and references therein).

Gangliosides and heparan sulfate proteoglycans (HSPGs)



are expressed on the surface of endothelial cells (the latter also in the ECM) and have been demonstrated to bind different GFs, cytokines, and chemokines (Fig. 2). Their common feature is the "high binding capacity" that may "mask" other GF-receptor interactions.

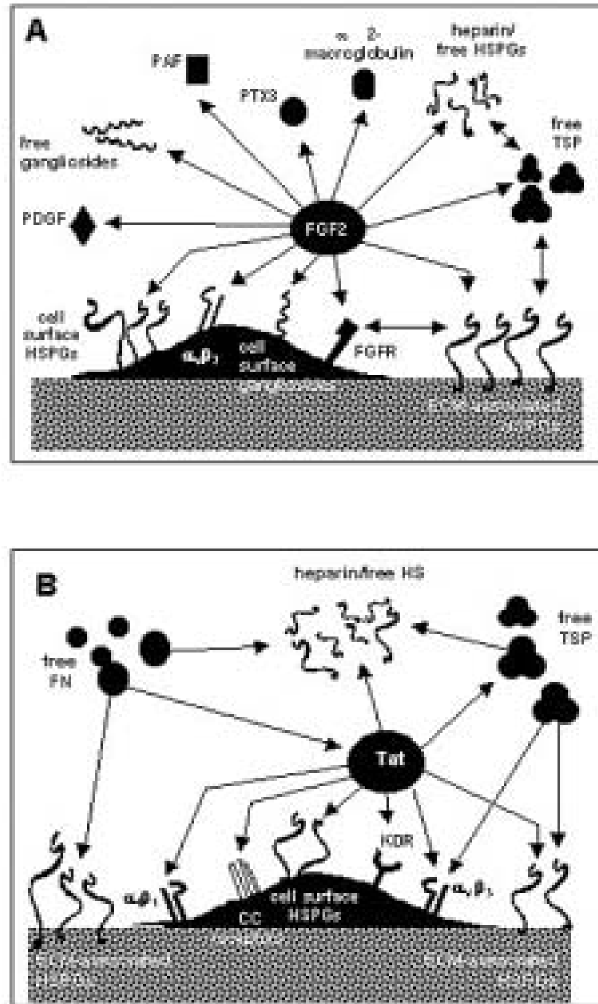
Figure 2. Gangliosides and HSPGs bind GFs, cytokines, and chemokines. Cell-associated gangliosides **(A)** and HSPGs **(B)** act as "high capacity" receptors. ECM-associated HSPGs act as a "reservoir" for GFs. Gangliosides and HSPGs can be shed/mobilized in the extracellular environment where they sequester the GFs, thus preventing their cell interaction.

Gangliosides are neuraminic acid (NeuAc)-containing glycosphingolipids that represent characteristic constituents of the plasma membrane of eukariotic cells including endothelial cells

(Muthing et al., 1999). In some pathological conditions (i.e. tumor growth) gangliosides are shed in the microenvironment and found as free components in plasma (Chang et al., 1997). In turn, free gangliosides are efficiently incorporated into the plasma cell membrane (Sonderfeld, et al., 1985). Depending on their free or cell-associated status, gangliosides modulate the activity of GFs and cytokines inducing opposite effects. The modulating capacity of gangliosides relies, at least in part, on their ability to bind directly the GFs (Rusnati et al., 2002, and references therein) (Fig. 2A).

HSPGs are composed of anionic glycosaminoglycan (GAG) chains covalently bound to a core protein. On the endothelial cell surface they act as low affinity, high capacity receptors for a wide array of GFs, cytokines, and chemokines (Fig. 2B). Binding to HSPGs induces GF oligomerization that, in turn, favours the dimerization of the cognate tyrosine kinase receptors and intracellular signaling. Also, HSPGs mediate internalization of GFs, their protection from lisosomal degradation, and possibly their nuclear delivery. Finally, GFs immobilized to HSPGs of the ECM act as localized, persistent stimula for endothelial adherent cells. Cell- or ECM-associated HSPGs can be mobilized as free proteoglycans or GAGs by the action of proteases and endoglycosidases, respectively. These free molecules retain their capacity to bind to GFs, exerting effects that can be opposite to those of their cell-associated counterparts (Rusnati and Presta, 1996).

The mobilization of cellular gangliosides and HSPGs indicates that cell conditioned media may contain GF-binding components able to interfere with GF-receptor interaction. Exemplificative of this possibility is the interleukin (IL)-1 receptor antagonist that binds IL-1 and sequesters it in the extracellular environment (Dinarello, 1998). Alternatively, fibronectin (FN) and thrombospondin (TSP) may compete with FGF2 (Rusnati et al., 1997), HIV-1 Tat (Noonan and Albini, 1999) and the vascular endothelial growth factor (VEGF) (Soldi et al., 1999) for integrin



binding.

This complex network of interactions has been shown to occur at the surface of endothelial cells for FGF2 and HIV-1 Tat (Fig. 3) and may exist also for other GFs, cytokines, or chemokines.

Figure 3. Circuits of regulation of FGF2 and HIV-1 Tat interactions. FGF2 (A) and HIV-1 Tat (B) bind different free, cell- or ECM-associated molecules. Note that some FGF2 or HIV-1 Tat-binders can interact also with GF receptors. Moreover, some free binders can cross-react thus increasing the complexity of the network.

From the bulk of data it emerges that a simple "single component" interaction is unlike to exist and to be studied in cell cultures. At this regard, some general questions can be raised whose answer may solve at least some of the difficulties generated by the complex scenario described above:

i) Is it possible to avoid the interference caused by free binders?
ii) How to distinguish among the various receptors that bind the GF under study?
iii) How to avoid the "masking" effect caused by "high capacity" binding sites?

The first question can be answered, at least in theory, by the use of media with defined composition devoid of any known (or suspected) binder of the GF under study. Moreover, as already mentioned, cells may affect medium composition by releasing GF binder(s). This effect is negligible for binding or second messenger activation experiments where short periods of incubation and/or low temperatures are utilized. At variance, the same effect becomes relevant during the evaluation of GF internalization or biological activities that require longer period of incubation at physiological temperature.

Also, some free binders are indeed required for GF-receptor interaction. This is the case of heparin that favours the interaction of FGF2 with its tyrosine kinase receptor (FGFR) (Ornitz et al., 1992). On the other hand, heparin may exert opposite effects depending on its concentration, the cell type under investigation and the receptor involved (Rusnati and Presta, 1996). Indeed, heparin favors the interaction of HIV-1 Tat with a_Vb_3 (Urbinati et al., manuscript in preparation), whereas it inhibits its interaction with

HSPC

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Fig. 6

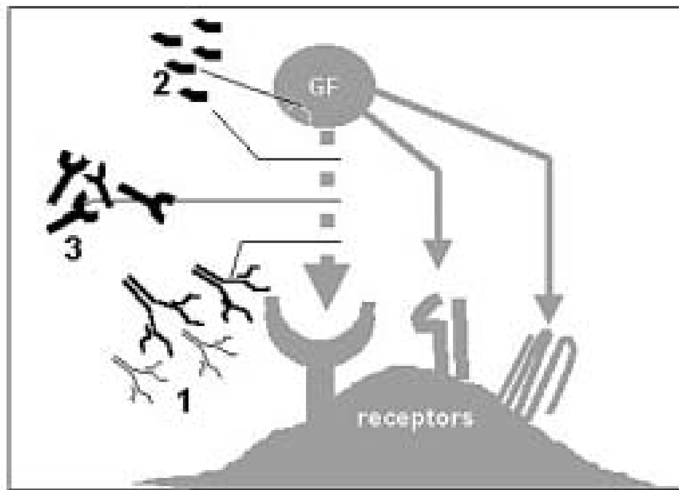

Figure 4. Schematic representation of different strategies aimed to single out a specific GF-receptor interaction: Specific antibodies (1) or synthetic peptides representing functional GF domains (2) can neutralize receptor interaction. Soluble forms of the receptor (3) can act as GF decoys.

Another widely used approach is the over-expression of the receptor of interest following its cDNA transfection (see the example shown in Fig. 7).

When these tools/reagents are not available, different structural requirements of the various interactions must be searched for and exploited. The simplest situation occurs when the GF-receptor interaction under study occurs with high affinity. GFs bind their signaling receptors with very high affinity (with K_d values in the pM range of concentration) when compared to their binding to low affinity HSPGs or gangliosides (K_d values in the nM range

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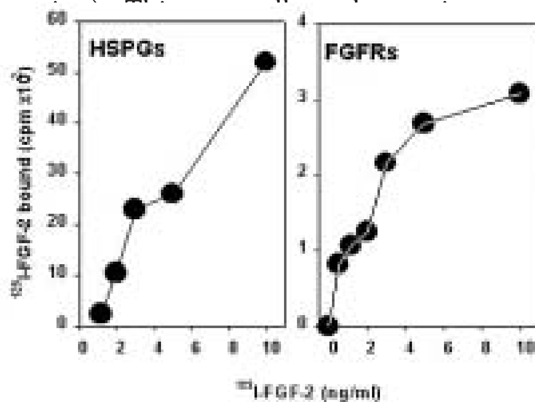

	affinity (K_d)	number of receptors/cell
HSPGs	300 pM	4.4×10^5
FGFRs	20 pM	1.6×10^4

Figure 5. Binding of 125I-FGF2 to endothelial cells in vitro. Fetal bovine aortic endothelial GM7373 cells were incubated for 2 h at 4°C with increasing concentrations of 125I-FGF2. At the end of incubation, unbound 125I-FGF2 was removed by two washes with PBS. Then, cells were washed twice with 25 mM HEPES buffer pH 7.5 containing 2 M NaCl to recover 125I-FGF2 bound to low affinity binding sites (HSPGs) and twice with 25 mM acetate buffer pH 4,5 containing 2 M NaCl to recover 125I-FGF2 bound to high affinity tyrosine receptors (FGFRs) (Moscatelli, 1987). Radioactivity in two fractions was then measured and binding data processed by the method originally described by Scatchard (1949).

In other cases, distinct domains of the GF mediate the binding to different receptors. Alternatively, cofactors (e.g. ions) can be

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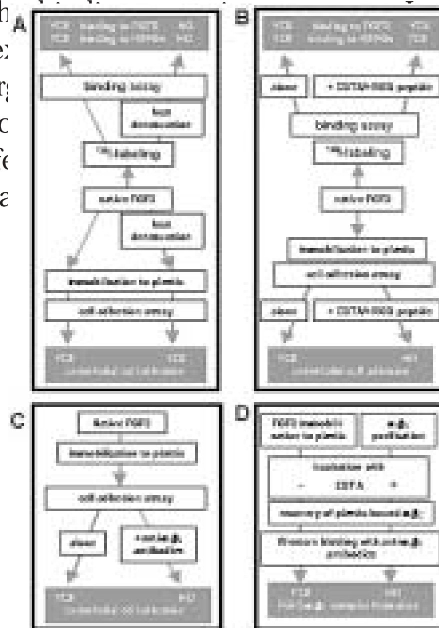


Fig. 6: Schematic representation of the strategies used for the characterization of the $\alpha_v\beta_3$-FGF2 interaction. A) FGF2 was incubated for 10 min. at 80 °C. Then, non tissue culture plastic plates were coated with native or heat denatured FGF2. GM 7373 cells were seeded onto coated plates and allowed to adhere for 2 h at 37°C. Then, the number of adherent cells was evaluated. In parallel experiments, heat denatured 125I-FGF2 was evaluated for its capacity to bind to FGFRs and HSPGs present on the surface of GM 7373 cells as described in Fig. 5. The results demonstrated that, after heat denaturation, FGF2 retains its cell-adhesive capacity despite it loses its capacity to bind to FGFRs and HSPGs, thus indicating that FGFRs and HSPGs are not involved the cell adhesion to FGF2. **B**) Cells were seeded onto non tissue culture plates coated with FGF2 in the absence or in the presence of EGTA or of the peptide GRGDSPK. The number of adherent cells was evaluated after 2 h of incubation at 37°C. In parallel experiments, 125I-FGF2 was evaluated for its capacity to bind to FGFRs and HSPGs in the presence of EGTA or GRGDSPK. The results indicate that cell adhesion to FGF2, but not FGF2 binding to FGFRs or HSPGs, is Ca++ and RGD-dependent. Since these features are typical of integrin interaction (see text),

these data further sustain an involvement of integrins in the cell-adhesive activity of FGF2. **C**) GM 7373 cells were incubated for 30 min at 37°C with a monoclonal anti-$\alpha_v\beta_3$ antibody. Then, cells were seeded onto non tissue culture plates coated with FGF2. The number of adherent cells was evaluated after 2 h of incubation at 37°C. **D**) Purified human $\alpha_v\beta_3$ was incubated onto plastic dishes coated with FGF-2 in the absence or in the presence of EDTA. At the end of incubation proteins bound to plastic were extracted and analyzed by Western blotting with anti-$\alpha_v\beta_3$ antibodies. The results shown in panel C and D demonstrate that FGF2 interacts with $\alpha_v\beta_3$ integrin and that this interaction mediates cell-adhesion to FGF2. For further details about these experiments see Rusnati et al., 1997.

Another way to single out a given GF-receptor interaction is by evaluating its specific biological consequences. This is shown in Fig. 6, where the αvβ3-FGF2 interaction was assessed by cell adhesior … say. Also, HIV-1 Ta … an be distinguishe … evaluating the activ … (MAPK) $ERK_{1/2}$, … e the example sl …

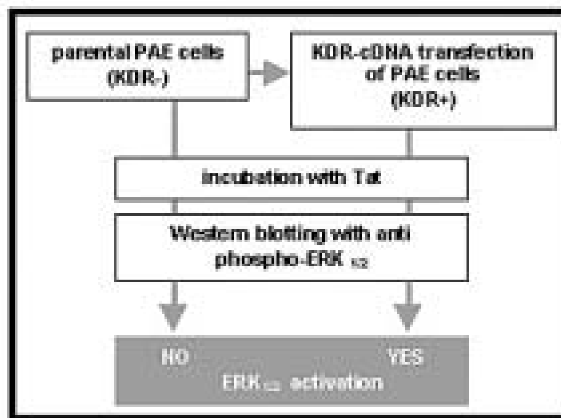


Figure 7. Schematic representation of the strategies used for the biological characterization of Tat-KDR interaction. Parental porcine aortic endothelial (PAE) cells were stably transfected to over-express the VEGF receptor II KDR. Parental and KDR-transfected PAE cells were incubated for 20 min. at 37°C in the presence of Tat. At the end of incubation Western blot analysis of the cell extracts was performed using anti-phospho-$ERK_{1/2}$ antibodies. The results indicate that, to induce $ERK_{1/2}$ activation, HIV-1 Tat requires to interact with KDR (for further details about these experiments see Rusnati et al., 2001a).

A major advantage of this latter approach is that ligand labeling is not required. However, it provides only an indirect evidence for the interaction, so that additional experiments (i.e. by cell free models, see below) are usually required to confirm the interaction.

The use of “naked” cells genetically deficient for the expression of high capacity binding sites may represent a very effective approach. B-lymphoid Namalwa cells do not express HSPGs (Zhang et al., 2001), and different clones of chinese hamster ovary (CHO) cells exist that ... plex gangliosides. (Rosales-Fritz, ... Their transfection to express a gi... nt cell lines (Table 3) suitable fo... nteraction at biochemical and ...

Cell line	FGFRs	HSPGs	GM_1
WT CHO	-	+	+
CHO-K1	-	+	-
CHO-K1-pgsA745	-	-	-
CHOflg7G	+	+	+
CHO-K1flgXB	+	+	-
CHO-K1 pgsA745flg1A	+	-	-

Table 3. Differential expression of cell surface molecules in different mutant CHO cell lines. Wild type (WT) CHO cells express low levels of FGFRs. CHO-K1 cells do not synthesize complex gangliosides, including GM_1 (Rosales Fritz et al., 1997), whereas CHO-K1-pgsA745 cell mutants lack also the ability to synthesize heparan sulfate (Rostand and Esko, 1997). WT CHO, CHO-K1, and CHO-K1-pgsA745 cells were stably transfected with FGFR1 cDNA (Rusnati et al., 1996; Liekens et al., 1999), thus generating the CHOflg7G, CHO-K1flgXB, and CHO-K1-pgsA745flg1A clones, respectively.

For instance, these cell lines were used to demonstrate that

cell-surface HSPGs act as HIV-1 Tat receptor (see the example shown in Fig. 8A, B).

Complementary to this approach is the use of enzymes that specifically remove/inactivate a distinct class of receptors [e.g. heparinase for HSPGs (see the example shown in Fig. 8C) or neuraminidase for gangliosides or sialized receptors].

In the experiments shown in Fig. 8, GF-receptor interaction was demonstrated indirectly by cell internalization of the labeled GF rather than by direc ments. Since GF-internalization allows ıe GF (with consequent amplification can be usefully exploited when cell sı k to be detected directly.

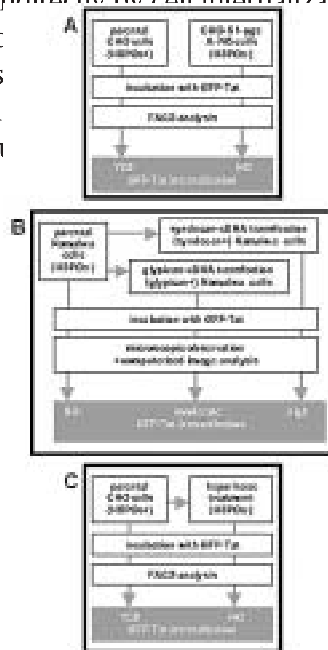
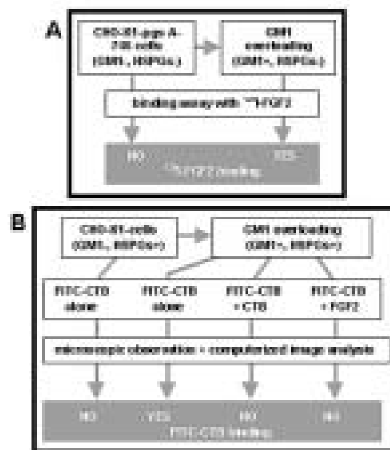


Figure 8. Schematic representation of the strategies used to identify HSPGs as Tat receptors. A) Wild type (WT) CHO-K1 cells and HSPGs-deficient CHO-K1-pgs A-745 were incubated for different period of time at 37° C with GST-Tat-GFP and analyzed by flow cytometry. **B**) WT Namalwa cells were stably transfected to selectively express the HSPGs

syndecan or glypican on their surface. The different clones then were incubated for 24 h at 37° C with GST-Tat-GFP, fixed, and observed under a Nikon photomicroscope equipped for epifluorescence. **C**) CHO-K1 cells were incubated with heparinase III for 40 min and then incubated with GST-Tat-GFP for 5 h and analyzed by flow cytometry; the short incubation time was chosen to minimize synthesis of novel proteoglycans. The results obtained in the different cell models demonstrate that HSPGs are required for cell internalization of HIV-1 Tat, thus acting as HIV-1 Tat receptors. For further details about the experiments described in panel A and C see Tyagi et al., 2001. Panel C: L. Tassone, unpublished data.

It is anticipated that many of the approaches used to single out a given GF-receptor interaction can be used also to avoid the "masking" effect caused by high capacity binding sites. For instance, in cells other than neurons, the complex ganglioside GM1 is expressed at low levels so that its interaction with FGF2 is masked by HSPGs. We successfully exploited the HSPG-deficient CHO cell model to solve this problem (see the example shown in Fig. 9A, B).



Another strategy that we have useful employed to "unmask" a GF-receptor interaction consists in the use of a second ligand that bind the receptor under study independently of the surrounding milieu. The GF can then be evaluated for its capacity to compete with the ligand for the binding to the "masked" receptor (see the example shown in Fig. 9B).

Figure 9. Schematic representation of the strategies used to "unmask" the GM1-FGF2 interaction at the cell surface. A) Naïve CHO-K1-pgsA745 cells were overloaded with GM1 and incubated for 2 h at 4°C with 125I-FGF2. Then, the amount of radioactivity bound to the cells was evaluated. B) Naïve CHO-K1 cells were overloaded with GM1 and incubated for 2 h at 4°C with FITC-cholera toxin B subunit (CTB) alone or in the presence of a molar excess of unlabelled CTB or FGF2. Then cells were fixed and observed under a Nikon photomicroscope equipped for epifluorescence. Both the approaches demonstrate that, independently of the presence of other binding sites, FGF2 can interact with cell associated ganglioside GM1. For further details about these experiments see Rusnati et al., 2002.

Studies with cell free models

At variance with cell cultures studies, cell free models can be accurately planned to avoid any kind of interference with the interaction under study. This kind of study can be performed by using the GF and its binder in a free form or by immobilizing one

Plastic plates	Chromatographic resins
Advantages	
easy procedure of immobilization	recovery of the analyzed protein
easy handling of the samples	high yield of immobilization/ high sensitivity of the method
quick analysis	
limited amount of material required	orientation of the immobilized protein
Disadvantages	
low yield of immobilization/ low sensibility of the method	difficult sample handling
	high amount of material required

Table 4: Substrate-immobilization of proteins. Plastic plates and chromatographic resins represent the most widely used substrata for protein immobilization. The most common advantages and disadvantages of the two substrata are listed. For further detail on protein-immobilization procedures see Rusnati et al., 1997, 1998, 1999.

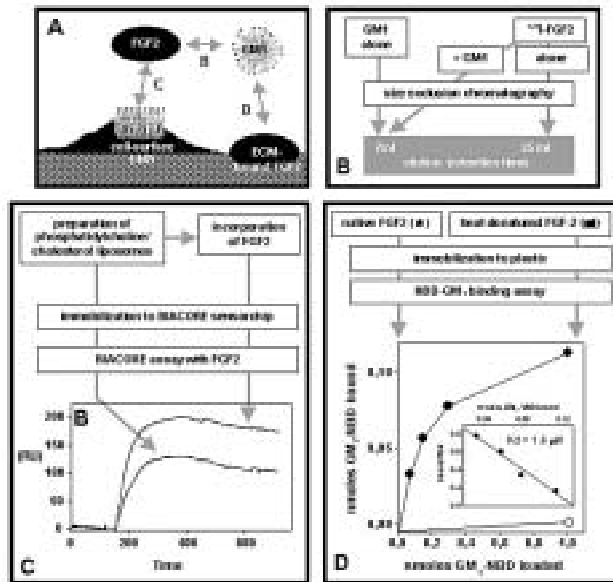


Figure 10. Characterization of FGF2-GM1 interaction by cell free models: A) Schematic representation of the different configurations in which FGF2-GM_1 interaction can occur. Representative experiments for each of these configurations are reported as follows: **B) Free FGF2 versus free GM_1**: ^{125}I-FGF2 alone, GM_1 alone or a mixture of ^{125}I-FGF2 and GM1 were analyzed by size-exclusion fast protein liquid chromatography. Radioactivity or NeuAc concentration were measured in the eluted fraction for the evaluation of ^{125}I-FGF2 or ganglioside content, respectively. The result demonstrated that high molecular weight GM_1 micelles elutes with the void volume (7 ml) whereas FGF2 is retained by the column (elution volume equal to 25 ml). When preincubated with GM_1, FGF2 coelute with the ganglioside in the void volume, attesting the association between the two molecules. **C) Free FGF2 versus immobilized GM_1**: Using BIACORE L1 sensorchip, two surfaces were prepared, with phosphatidylcholine/cholesterol liposomes carring or not GM_1. GM_1 liposomes bound cholera toxin, while no binding could be observed on liposomes without GM1, demonstrating the functionality of the assay (data not shown). Injec-

tion of FGF2 for 5 min over the surface made with PC liposomes gave a signal of 126 RU (lower sensorgram). The incorporation of GM1 into these liposomes lead to an enhanced binding capacity, signal being of 200 RU (upper sensorgram). **D) Immobilized FGF2 versus free GM_1**: GM_1 was coupled to the fluorochrome 4-nitro-7-chloro-benzo-2-oxa-1,3-diazole (NBD). Then, increasing concentrations of NBD-GM_1 were incubated for 5 min at room temperature into wells coated with native (*) or heat-denatured () FGF2. At the end of incu [...] measured with a [...] the binding da [...] ual to 1,6 nM. Fo [...] al., 2002. Panel [...]

[...] lasmon resor [...] mqvist, 1999) [...]

	Chromatography	BIACORE
Radioactive material required	Yes	no
low temperature required during the analysis	Yes	no
Time required for a single analysis	45 min.	10 min.
Amount of compound required	3 µmoles	30 nmoles
Number of steps required for a single analysis	4 (column loading, equilibration, elution, wash)	2 (sample injection, wash)
Complexity of the procedure	High	low
Number of analysis performed with a single column/sensorchip	10-50	50-100
Reproducibility of the results	Moderate	high
Automatic, computerized elaboration of the binding data.	No	yes
Cost	Low	high

Tab. 5: Comparison between classical chromatographic methods and BIACORE technology. These two approaches were used to study HIV-1 Tat interaction with a series of polysulfonated putative antagonists. For further details about these procedures see Rusnati et al., 2001b.

SPR arises when light is reflected under certain conditions at the interface between two media of different refractive index. In the BIACORE system, one of the two molecules participating to the complex is immobilized to a thin layer of gold on the "sensorchip" whereas the other flows over the immobilized one. When the interaction occurs, the concentration and therefore the refractive index at the gold surface changes and an SPR response is detected and quantified as resonance units (RU). Plotting the re-

sponse against time during the course of the interaction provides a quantitative measurement of the interaction. This plot is named sensorgram (Fig 10C).

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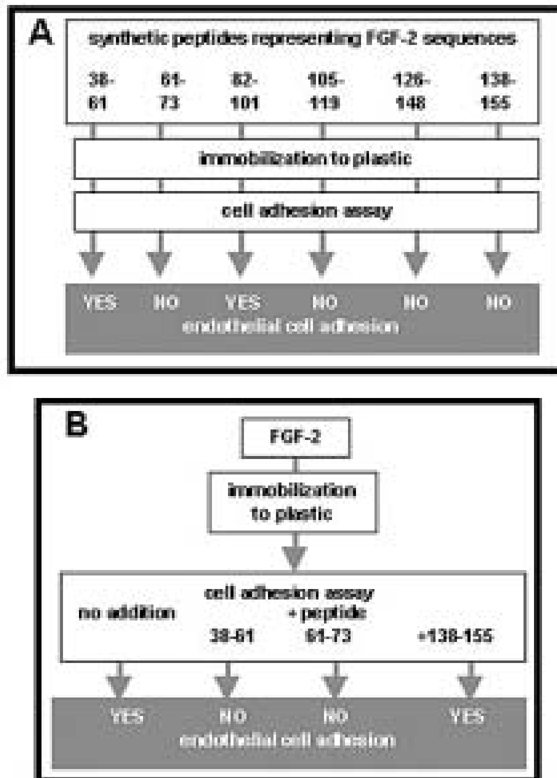

Figure 11. Mapping of FGF-2 cell-adhesive domains. A) GM 7373 cells were allowed to adhere onto non-tissue culture plastic coated with the indicated synthetic fragments of human FGF2. The number of adherent cells was evaluated after 2 h of incubation at 37°C. **B**) GM 7373 cells were incubated in suspension for 90 min at 37°C with peptides FGF2(38-61), FGF2(82-101), or FGF2(138-155). Then, cells were centrifuged to remove unbound pe… ad-he… that FG… GR, sp… ents se…

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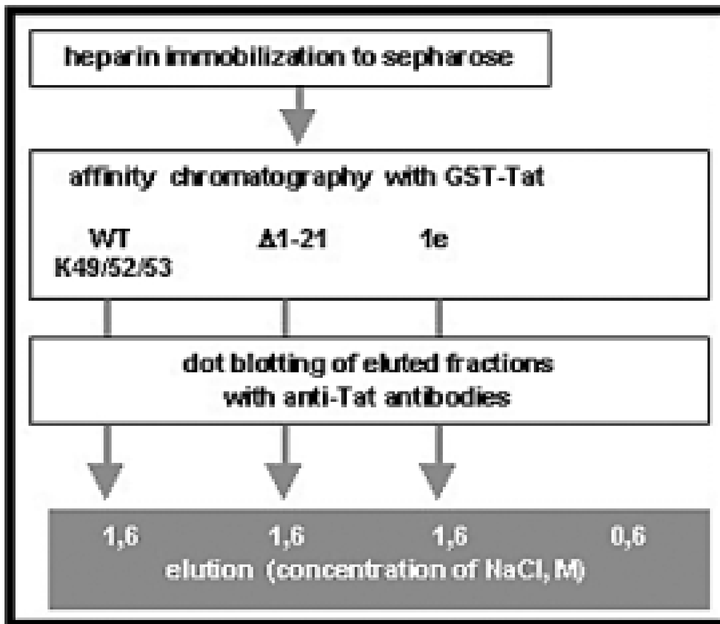

Figure 12. Mapping of the heparin binding domain of HIV-1 Tat. Different mutagenized forms of GST-Tat were used for this study: GST-Tat 1e (containing one-exon Tat and

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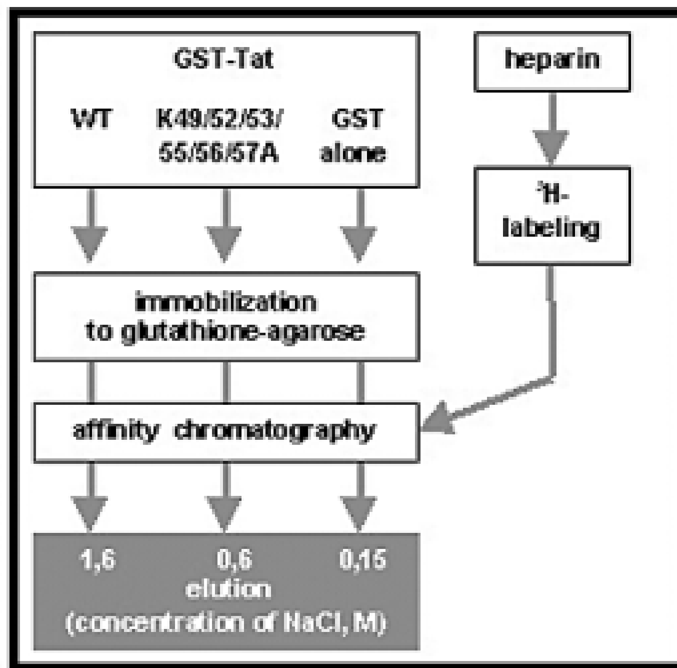

Figure 13. Characterization of ^{3}H-heparin interaction with substrate immobilized WT GST-Tat or GST-Tat R49/52/53/55/56/57A. WT GST-Tat, GST-Tat R49/52/53/55/56/57A or the recombinant GST protein devoid of the Tat moiety were immobilized onto glutathione-agarose columns. Columns were then loaded with ^{3}H-labeled heparin and eluted with increasing concentrations of NaCl in PBS. Radioactivity in the eluted fractions was measured in a liquid scintillation counter. The results confirmed that neutralization of the positive charges in the basic domain of Tat reduces its interaction with heparin. This model allowed also the performing of Scatchard plot analyses that showed that WT and R49/52/53/55/56/57A GST-Tat bind heparin with a dissociation constant equal to 0.3 µM and 1.0 µM, respectively. For further details on these experiments see Rusnati et al., 1998.

Concluding remarks

The mechanisms by which a GF stimulates endothelial cells is much more complex than a simple single "hit" with its receptor. Rather, GF interaction with endothelial cell surface is subjected to multiple modulations and/or interferences. On the other hand, therapeutically-oriented research requires a fine characterization at molecular level of such interactions and the development of methodologies that allow the rapid screening of a large number of putative GF and/or receptor agonists/antagonists.
From the bulk of the data here discussed it emerges that two main approaches to GF-receptor interaction studies can be envisaged: the use of cell free systems and the use of cell cultures. Both approaches possess defined limitations that make them inadequate to an exhaustive study if used by their own. Rather, their combination complements their limitations and allows a deeper understanding of GF-receptor interaction, from the biochemical characterization to the study of its biological consequences.

Acknowledgments

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SHEDDING OF MEMBRANE VESICLES BY ENDOTHELIAL CELLS

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Introduction

Since the first description of the bilayer model of plasma membrane, the concept of the lipid bilayer role has evolved from the static, passive, homogeneous barrier to a highly dynamic, asymmetric, heterogeneous structure, actively participating in metabolic and signal transduction processes. Our study has been focused on morphodynamic and functional studies of endothelial cell membrane undergoing angiogenesis. In particular the interest has been addressed towards a specific phenomenon that occurs on the plasma membrane: "*the vesicle shedding*".

Shedding of vesicles from the plasma membrane into the extracellular environment is a recognized mechanism of communication between the cells and their environment (1, 2). The presence of active molecules associated with the vesicles, including proteolytic enzymes and cytokines, confirms the functional involvement of shed vesicles in the autocrine/paracrine regulation of cell function (3-6). Indeed, vesicles have been described to affect cell functions including the degradation/remodeling of the extracellular matrix, cell differentiation, proliferation, adhesion and invasion(7-10).

This process, observed in a variety of cell types, presents substantial differences among normal and transformed cells (2, 10-13). In tumor cells, shedding of vesicles appears to be constitutive, while in normal cells it is a highly controlled and confined process. In all cases, however, the phenomenon of vesicle shedding has been associated with a migratory/invasive phenotype.

We recently reported that cultured human umbilical vein endothelial cells (HUVEC) rapidly shed vesicles from the cell plasma membrane (14). Shed vesicle contained matrix-degrading enzymes, involved in different phases of angiogenesis. We detected the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP, the latter located on the external side of the vesicle membrane, and the two inhibitors TIMP-1 and TIMP-2. Shedding of MMPs as vesicle components by endothelial cells was stimulated by the angiogenic factors FGF-2 and VEGF. Shed vesicles stimulated in autocrine manner endothelial cell invasion and cord formation. These findings indicate that shedding of membrane vesicles by endothelial cells is a mechanism for regulating invasive and morphogenic events during angiogenesis.

Here we describe the procedures we use to investigate the phenomenon of vesicles shedding by endothelial cells, spanning from the ultrastructural analysis of endothelial cells, to the isolation and molecular/functional characterization of shed vesicles. Similar methodological approaches can also be used to study the shedding of vesicles from tumor cells, and analyze their role as vehicles of angiogenic stimuli for endothelial cells.

Electron Microscopy

To visualize the shedding phenomenon we used scanning electron microscopic (SEM) and transmission electron microscopic (TEM) analyses.

For scanning electron microscopy (Fig. 1) cells are grown on coverslips and fixed with 2% glutaraldehyde in PBS for 30 minutes. Samples are, after critical point dried, glued onto stubs, coat-

ed with gold in a SCD040 Balzer Sputterer and observed using a Philips 505 scanning electron microscope at 10 to 30 kV.

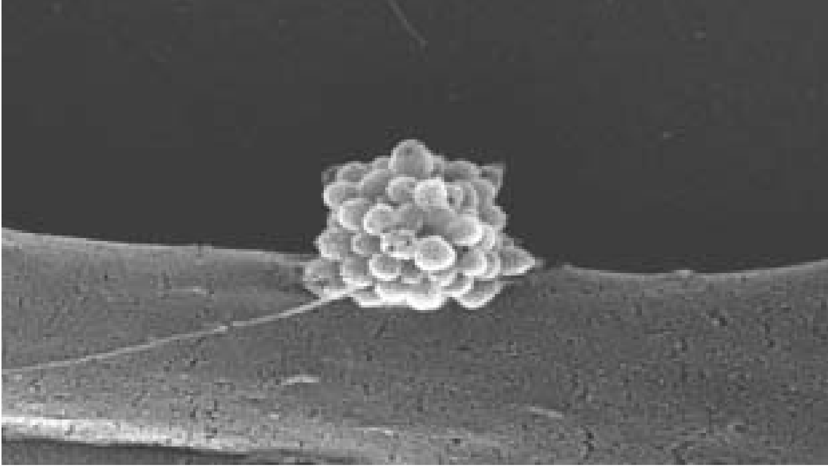

Figure 1. Scanning electron micrograph of HUVEC releasing vesicles. The phenomenon occurs in selected areas of the cell surface.

Transmission electron microscopic analysis (Fig. 2) is performed using a standard technique. Briefly, cells are fixed with 2% glutaraldehyde in flasks, scraped, postfixed1% OsO4, dehydrated with ethanol, and embedded in Epon 812. Samples are then sectioned, poststained with uranyl acetate and lead citrate, and examined under an electron microscope (Philips CM10, Eindhoven, The Netherlands).

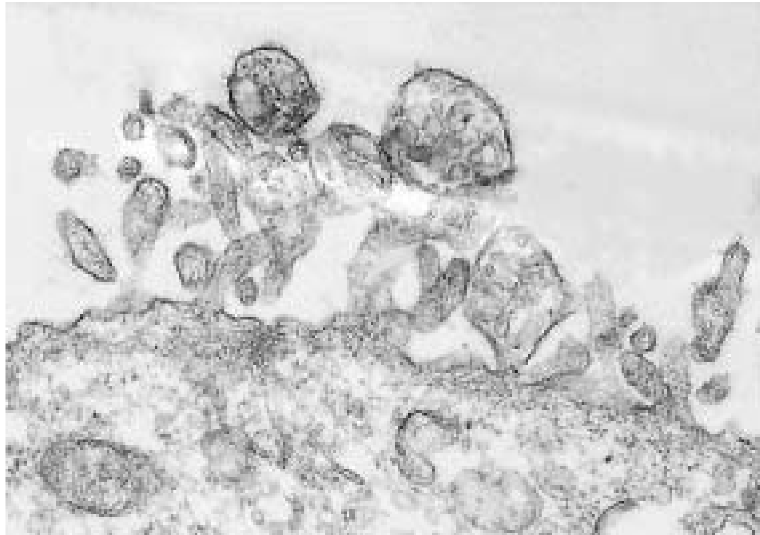

Figure 2. Transmission electron microscopy analysis of the surface of HUVEC, in cross-section, showing vesicle release from the plasma membrane. Insert, morphological picture of an isolated membrane vesicle (250 nm) with negative staining.

For analysis of the ultrastructural morphology of the shed vesicles, the ultracentrifugation pellet containing the membrane vesicles (prepared as described below) is resuspended in PBS and then applied to collodion-coated grids. After washing, the vesicles are negatively stained with 1% phosphotungstic acid, brought to pH 7.0 with NaOH, and examined by transmission electron microscopy (Fig.2).

Analysis of the presence and location of specific molecules can be done by immunogold techniques (14). The immunogold labeling is performed after vesicles are applied to collodion-coated grids: samples are incubated with primary antibodies for 1 hour at room temperature, washed and further incubated with gold-conjugated secondary antibody. After the washings, samples are negatively stained with 1% phosphotungstic acid, brought to

pH 7.0 with NaOH and examined by transmission electron microscopy with a Philips CM100 instrument.

Preparation of supernatants for the collection of shed vesicles

Membrane vesicles are shed in the conditioned medium by endothelial cells, and can be isolated from the supernatant by centrifugation procedures. Vesicles from endothelial cells are recovered in low amount. Therefore, a relevant number of starting cells needs to be prepared, according to the kind of analysis isolated vesicles will be subjected to.

HUVECs are seeded on 1% gelatin-coated plates, in complete medium (M199 supplemented with 10% fetal calf serum, 10% newborn calf serum, 20 mmol/L HEPES, 6 U/ml heparin, 2 mmol/L glutamine, 50 μg/ml endothelial cell growth factor, penicillin, and streptomycin). Cells are used between the fourth and fifth passage. HUVECs are rinsed once with serum-free medium, then exposed to fresh medium for vesicle collection. The process of vesicle shedding by endothelial cells is modulated by serum and by angiogenic factors (14). Therefore the composition of the medium for vesicle collection (percentage of serum, presence of angiogenic factors or inhibitors) can vary depending on the readout and aim of the experiment. Incubation can last for 4 or 18 hours. In optimal culture conditions, shedding is a rapid process, being completed in 4-6 hours.

The supernatant is collected, spun at 600 x g for 10 minutes and then at 1500 x g for 15 minutes to remove cells and large debris. All these procedures are performed in sterile conditions. The supernatants is then kept refrigerated at 4°C until the isolation of vesicles (up to 2-3 days).

Isolation of shed vesicles from the supernatant

The supernatant, prepared as described above, is centrifuged at 100,000 x g for 1 hour at 4°C. Pelletted vesicles are

gently resuspended in phosphate-buffered saline (PBS), pH 7.5. Vesicles are quantified by measuring vesicle-associated proteins, using the method of Bradford, with bovine serum albumin as the standard. Isolated vesicles are now ready for analysis.

Electron microscopy analysis of the ultrastructural morphology of isolated vesicles is described above.

Molecular characterization of vesicles (14) includes analysis of associated matrix-degrading proteases (by zymography, Western blot analysis), TIMPs (reverse zymography, ELISA), growth factors (ELISA, Western blot), following standard procedure (some of them described elsewhere in this book). Immunogold procedure to analyze the presence and location of vesicles-associated molecules is described above. Figure 3 is an example of zymographic analysis of MMP-2 and MMP-9 in vesicles shed by HUVEC.

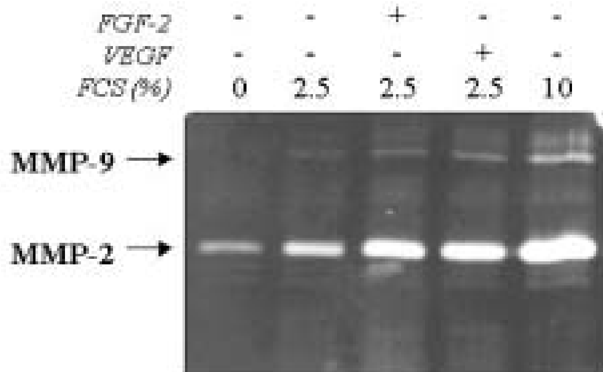

Figure 3. Zymographic analysis of MMP-9 and MMP-2 in vesicles shed by HUVEC stimulated by different concentrations of serum (FCS), FGF-2 and VEGF.

Vesicles can also be used as stimuli for endothelial cells (14) using the functional assays described elsewhere in this volume (including cell adhesion, proliferation, migration, invasion, cord formation on Matrigel). In this case, all the procedures described above are performed in sterile conditions.

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