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Functional role of extracellular matrix proteins and their receptors in apoptosis and cell survival

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Contents

Abbreviations

ADCC, antobody-dependent cell-mediated cytotoxicity
AIF, apoptosis inducing factor
AMPc, adenosin monophosphate cyclic
APC, antigen-presenting cells
BFGF, basic fibroblast growth factor
BIR, baculovirus IAP repeat
BMBM marrow-derived macrophages
BRASIL, Biopanning and rapid analysis of selective interactive ligands
CAD, caspase-activated DNase
CARD, caspase-recruitment domain
CAM, cell adhesion molecules
CAS, crk-associated substrate
Cdk, cyclin-dependent kinase
CHO, chinese hamster ovary
CSF, colony-stimulating factor
DD, death domains
DED, death effector domain
DFF, DNA fragmentation factor
DOCK-180, dedicator of cytokinesis 180
DR, death receptors
EC, endothelial cell
ECM, extracellular matrix
EGF, epidermal growth factor
EGFR, epidermal growth factor receptor
Erb
ERK, extracellular-signal-regulated kinase
FAK, focal adhesion kinase
FGF, fibroblast growth factor
Flk-1, VEGR2
FN, fibronectin
GAG, glycosaminoglycan
GEMM-CFU, granulocyte-megakaryocyte-macrophage colony-forming unit
GM-CSF, granulocyte- macrophage colony-stimulating factor
GM-CFU, granulocyte-macrophage colony-forming unit
Grb 2, growth-factor-receptor-bound protein 2
GST, glutathione S-tranferase
HUVEC, human umbilical vein endothelial cell
IAP, inhibitor of apoptosis
Ig, immunoglobulin
IGF, insuline-like growth factor
IFNα/β, interferon alpha/beta
IFN γ, interferon gamma
IL, interleukine
ILK, integrin-linked kinase
IMD, integrin mediated cell death
iNOS, inducible NO synthase
kDA, kilo dalton

KO, knock-out
LPS, lipopolysaccharide
LRR, leucine-rich repeats
MAPK, mitogen-activated protein kinase
M-CSF, macrophage colony-stimulating factor
MEK, mitogen-activated protein kinase kinase
MHC, major histocompatibility
MLCK, myosin-light-chain kinase
MMP, matrix metalloproteinases
NO, nitric oxid
NRP, neuropilin
PAK, p21-activated kinase
PECAM, platelet-endothelial cell adhesion mocelule
PG, proteoglycans
PGE, prostaglandin
PI3K, phosphatidylinositol-3 kinase
PKC, protein kinase C
PS, phosphatidylserine
RGD, Arginine-glycine-aspartate
ROI, reactive oxygen intermediates
RT, replicative form
SAGE, serial analysis of gene expression
SPARC, secreted protein acidic and rich in cystein
TGF-β, transforming growth factor-beta
Tie, tyrosine kinases with Ig like loops and epidermal growth factor homology domains
TNF-α, tumor necrosis factor-alpha
TU, transducing units
VEGF, vascular endothelial growth factor
VEGFR, vascular endothelial growth factor receptor
VN, vitronectin
VWF, von Willebrand factor
XIAP, X-inhibitor of apoptosis protein
YS, yolk sac

Introduction

1. CELLS: MACROPHAGES AND ENDOTHELIAL CELLS

1.1 Hemangioblasts: a common progenitor for macrophages and endothelial cells

The simultaneous appearance and close apposition of nascent endothelial and blood cells in mammalian embryos suggest that they may share a common progenitor. Hematopoietic and endothelial cell development has been extensively characterized in the mouse, where hematopoiesis and vasculogenesis can be identified in blood islands of the extraembryonic yolk sac (YS) (Choi et al., 1998). The blood islands consist of nucleated erythroid cells surrounded by ECs. Macrophage progenitors are produced in the YS, and mature macrophages subsequently disperse throughout the embryo by physical migration before circulation begins. Early blood and endothelial precursors share a similar pattern of gene expression, with both lineages expressing CD34, GATA-2, LMO-2, and SCL (Keller et al., 1999) and receptor tyrosine kinases: vascular endothelial growth cell receptor-2 VEGFR-2 (Flk-1), Tie-1, Tie-2, (Yano et al., 1997). Mouse embryonic stem cells cultured under specific conditions express VEGFR-2 and produce clonal populations of blood and ECs (Keller et al., 1999; Nishikawa, 2001). Targeted disruption of the murine VEGFR-2 gene results in catastrophic defects in both YS hematopoiesis and vasculogenesis, suggesting that signaling through VEGFR-2 is important for hemangioblast development (Figure 1).

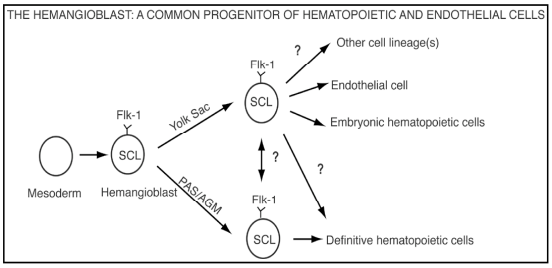

Figure 1. Schematic view of primitive and definitive hematopoietic development in the developing embryo (Choi, 2002).

Although formally not proven from clonal studies, together these data support the development of YS blood and blood vessels from a common hemangioblastic progenitor (Traver et al., 2002). The concept of hemangioblast has gained support from the observation that hematopoietic and endothelial lineages share expression of a number of different genes, known to be expressed in ECs and also expressed in early hematopoietc progenitors.

1.2 Macrophages

Macrophages are members of the mononuclear phagocyte system that also includes monoblast and monocytes, which have phagocytosis as their primary function. Macrophages play an important role in host development and physiology, and pathogenesis of many infectious and degenerative disease processes. Macrophages are motile cells that typically appear at inflammatory sites within 24 to 48 hours. They are relatively long-lived cells that exhibit continuous secretory activity, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, etc, during inflammatory processes, enabling them to destroy a range of cells, antigens, and pathogens (Nathan, 1987, Laskin and Laskin, 1997). Macrophages are also highly phagocytic cells, readily engulfing and digesting a variety of substances including viruses, bacteria, red blood cells, tissue and cellular debris, and some tumor cells (Johnson, 1988). Despite their origin from a common-bone-marrow progenitor population (Van Furth, 1982), macrophages display considerable tissue heterogeneity. Moreover, even within tissues, there appear to be subpopulations of macrophages that exhibit unique characteristics. Studies suggest that the microenvironment of a tissue regulates the phenotype of these cells.

1.2.1 *Macrophage origin*

All blood cells are derived from a small common pool of totipotent cells, called hematopoietic stem cells, located in the bone marrow. The process is strictly regulated by the hematopoietic microenvironment, which includes stromal cells, extracellular matrix molecules and soluble regulatory factors. Several experimental *in vitro* assays have been developed for the study of hematopoietic differentiation and have provided valuable information on the stroma, which includes, among other cell types, macrophages, fibroblasts, adipocytes, and ECs.

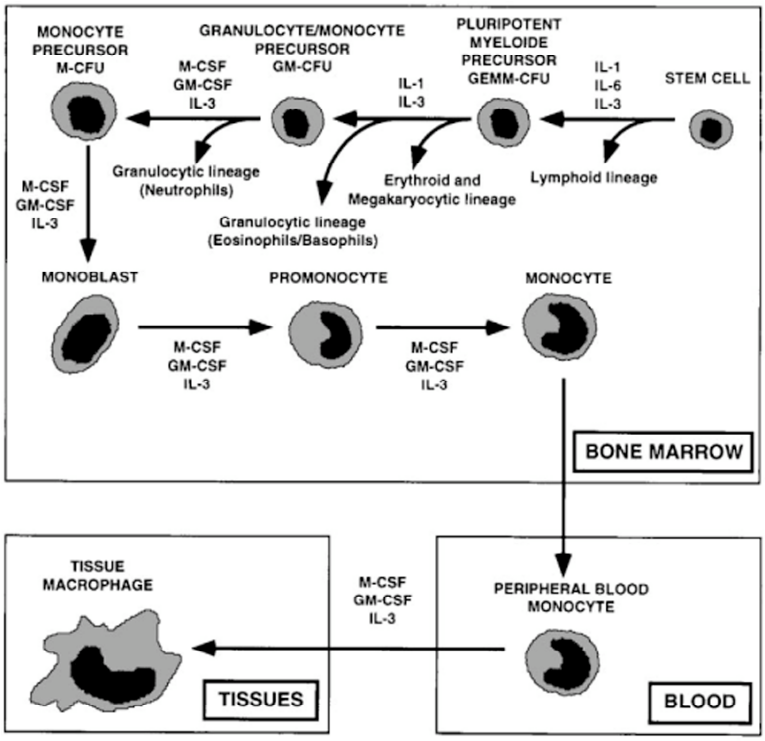

Figure 2. Differentiation of stem cells to monocyte/macrophages. Growth factors involved in each stage are indicated with arrows. Curved arrows indicate the points where derivations to other lineages are generated. The name of each derived lineage is also indicated.

In bone marrow, IL-1 and IL-3 and/or IL-6 induce heteromitosis in stem cells. This division gives rise to a new stem cell and a pluripotent myeloid cell, also referred to as granulocyte-erythrocyte-megakaryocyte-macrophage colony-forming unit (GEMM-CFU). In the presence of IL-1 and/or IL-3, this precursor is committed to becoming a progenitor of both macrophages and granulocytes known as granulocyte-macrophage colony-forming unit (GM-CFU). Thus, both lineages are closely bound together throughout hematopoiesis and commonly referred to as a myelomonocytic lineage. At this point, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce proliferation of these myeloid precursors, whereas macrophage colony-stimulating factor (M-CSF) induces not only their proliferation but also their differentiation to monocytic precursor (Figure 2). The first cell from the terminal monocytic stage that can be detected easily is the promonocyte, which shows a limited phagocytic capability. Maturation of promonocytes and the subsequent generation of monocytes also require the presence of M-CSF. Monocytes are generally smaller (10 to 15 μm) than their immediate precursor, but they have a well-developed lysosomal system and enhanced phagocytic capability. A delicate balance should be maintained between growth factors (M-CSF, GM-CSF and IL-3) produced by their own proliferating cells and the inhibitor agents such as prostaglandin E2, interferon (IFN) α/β and lactoferrin for basal monocyte generation (Celada, 1994; Valledor et al., 1998).

Mononuclear phagocytes are developed in the bone marrow. From there they are released into the blood and then migrate through the blood-vessels walls by means of the interaction of adhesion molecules that allow them to stick to the endothelium, causing the bound monocytes to crawl between the capillary ECs into the tissues. Once settled in the tissue, they differentiate to macrophages by growing and increasing their lysosomal content, the amount of hydrolytic enzymes, the number and size of mitochondria, and the extent of their energy metabolism (Alberts, et al., 1994; Parslow et al.; Valledor et al., 1998). However, although

macrophages have the same origin, their function depends on the tissue in which they reside.

1.2.2 *Macrophage heterogeneity*

Macrophage heterogeneity is a well-documented phenomenon, perhaps first observed by Metchnikoff, who described a progression of infiltrating cell types in inflammatory exudates. Macrophages are a heterogeneous population differing in their site of location, morphology and function. However, most macrophages are large cells (20 to 80 μm in diameter). Macrophages are long-living cells and may survive for months in the tissues. The nomenclature of individual macrophage types (particularly inflammatory) is rather confusing and terms such as *stimulated*, *activated*, *induced*, *elicited* etc., are often used interchangeably. Basically, two main macrophage groups can be distinguished: *resident* and *inflammatory* macrophages. They are present in connective tissue and around the basement membrane of small blood vessels and are particularly concentrated in the liver (Kupffer cells), alveolar spaces of the lung (alveolar macrophages), and linings of splenic and lymph-node medullary sinusoids, where they are strategically localized to filter foreign material. Other examples of macrophages are Langerhan's cells in the skin, mesangial cells in the kidney glomerulus, brain microglia, and osteoclasts in bone. In general, macrophages are characterized morphologically by an enlarged horseshoe-shaped nucleus, significant rough-surfaced endoplasmic reticulum, and large numbers of mitochondria and cytoplasmic vacuoles, although these characteristics vary depending on the tissue origin of the cell.

The major functions of macrophages are the ingestion of particles, endocytosis, digestion of these particles by lysosomes, and secretion of an impressive array of substances that participate in defensive and reparative function. Macrophages ingest a particle by surrounding it with thin extensions of the cell surface called "false hands", or pseudopods, that ultimately fuse, isolating the particle within a phagocytic vacuole (phagosome). Next, lysosomes fuse with the phagocytic vacuole and digest the contents with a large number of different enzymes such as acid phospha-tases, ribonucleases, deoxyribonuclease, proteases, sulfatases, lipases, and β-glucuronidase. Macrophages are antigen-presenting cells and also participate in cell-mediated resistance to bacteria, viruses, protozoa, fungi, and metazoa; in cell-mediated resistance to tumors; and in extrahepatic bile production, iron and fat metabolism, and the destruction of aged erythrocytes. Macrophages can also destroy pathogens without directly targeting them. By moving to an affected area, macrophages can be stimulated to release chemicals toxic to pathogens. A number of oxygen-and nitrogen-related compounds could poison most pathogens.

Because macrophages are responsible for numerous inflammatory processes, it becomes important to distinguish between normal or steady-state haematopoiesis and induced haematopoiesis associated with immunological challenge. Production of the macrophage lineage from bone-marrow progenitors is normally controlled by M-CSF, which is constitutively produced by many cell types. In response to invasive stimuli and inflammation, monocyte numbers increase dramatically, as do serum levels of M-CSF. In addition, GM-CSF appears in the serum. Although there appears to be a large overlap of macrophage progenitors able to respond to M-CSF or GM-CSF, the very different structures and signal-transduction mechanisms of the receptors for M-CSF and GM-CSF suggest that the differentiation pathways they initiate would be dissimilar.

M-CSF-derived macrophages are larger, have a higher phagocytic capacity, and are highly resistant to infection by vesicular stomatitis virus compared to GM-CSF-derived macrophages. Conversely, GM-CSF-derived macrophages are more cytotoxic against TNF-α-resistant tumor targets, express more major histocompatibility complex (MHC) class II antigens, more efficiently kill *Listeria monocytogenes*, and constitutively secrete more prostaglandin-E.

The diversity of the macrophage's morphologic characteristics extends to its metabolism, which also varies according to this cell's functional activity and environment. Functional heterogeneity results from the spectrum of maturational states in a given isolate because of the influx of monocytes and/or local proliferation. Because macrophage function is dependent in part on signals received from the immediate microenvironment, it is suggested that macrophage heterogeneity may arise from unique conditions within specific tissues. Obviously, the sterile, anaerobic environment of the spleen or

peritoneum will impart different constraints on resident macrophages than does the aerobic environment of the alveolar macrophage, which contains numerous external factors.

1.2.3 *Macrophage function*

Macrophages are involved at all stages of the immune response and are also important effector cells in both innate and adaptive immunity. Their effector functions on innate immunity are to phagocytose microbes and produce cytokines that recruit and activate other inflammatory cells. Macrophages serve numerous roles in the effector phase of adaptive immune responses. In cell-mediated immunity, antigen-stimulated T cells activate macrophages to destroy phagocytosed microbes. In humoral immunity, antibodies coat, or opsonize, microbes and promote the phagocytosis of the microbes via macrophage surface receptors for antibodies.

Macrophages act as a rapid protective mechanism that can respond before T cell-mediated amplification has taken place. Activated macrophages play a key role in host defense against intracellular parasitic bacteria, pathogenic protozoa, fungi and helminthes, as well as against tumors, especially metastasizing tumors. After phagocytosis, macrophages prevent intracellularly parasitic organisms from replication by at least three ways: first, intracellular environment is unsuitable for microbial reproduction due to low pH and lack of nutrients in a phagolysosome. Second, a toxic reaction may be activated against dividing organisms. This includes reactive oxygen intermediates, hypochlorite, nitric oxide (NO), myeloperoxidase, neutral proteases and lysosomal hydrolases; and third, macrophages may also produce microbiostatic effector molecules at a steady-state and thus maintain intracellular micro-organisms in the nonreplicating state. This latent infection is generally observed only in individuals whose macrophages cannot be sufficiently activated (Abbas, 1994; Gordon, 1995).

Macrophages are also important killer cells; by means of antibody-dependent cell-mediated cytotoxicity (ADCC) they are able to kill or damage extracellular targets. They also take part in the initiation of T-cell activation by processing and presenting antigen. Activated macrophages become more efficient antigen-presenting cells (APCs) because of increased levels of molecules involved in antigen processing, such as components of the proteasome and cathepsins, increased surface expression of MHC class II molecules and costimulators, and production of cytokines, such as IL-12, which stimulate T lymphocyte proliferation

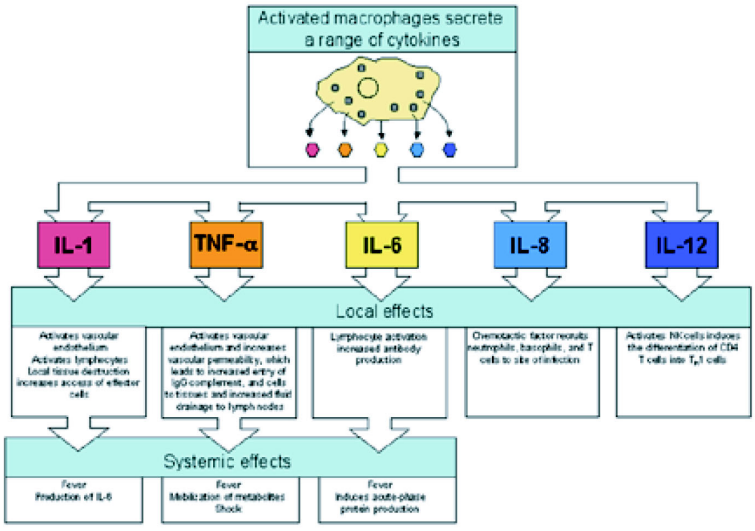

Figure 3. Cytokines secreted by macrophages in response to bacterial products include IL-1, IL-6, IL-8, IL-12 and TNF-α.

and differentiation (Figure 3).

Finally, macrophages are central effector and regulatory cells of the inflammatory response. Activated macrophages stimulate acute inflammation through the secretion of cytokines, mainly TNF, IL-1 and chemokines, and short-lived lipid mediators such as platelet-activating factor, prostaglandins, and leukotriens. To fulfill these functions, macrophages in their activated state are able to produce more than 100 different substances (Table 1).

Macrophages secrete not only cytotoxic and inflammation-controlling mediators but also substances participating in tissue reorganization. They include enzymes, such as hyaluronidase, elastase, and collagenase, inhibitors of some of them (antiproteases), regulatory growth factors and others. Hyaluronidase, by destroying hyaluronic acid, an important component of connective tissue, reduces viscosity and thus permits greater spreading of material in tissue spaces. Hyaluronidase is therefore sometimes designated as the spreading factor.

Table 1. Products secreted by macrophages (Natham, 1987).

Group of substances	Individual products
Microbial and toxic:	
Reactive oxygen intermediates	Superoxide, hydrogen peroxide, hydroxyl radical
Reactive nitrogen intermediates	Nitric oxide, nitrites, nitrates
Oxygen independent	Neutral proteases, acid hydrolases, lysozymes, defensins
Tumoricidal	H_2O_2, NO, TNF-α, C3a, proteases, arginase, thymidine
Tissue damaging	H_2O_2, TNF-α, NO, and neutral proteases
Fever inducing **Pyrogenic cytokines**	Il-1, TNF-α, and IL-6
Inflammation regulators **Bioactive lipids**	Prostaglandins (PGE_2, $PGF_{2\alpha}$), prostacyclin (PGI2), tromboxans, leukotriens (LTB_4, LTC_4, LTD_4, LTE_4)
Bioactive oligopeptides	Glutathione
Complement components	C1, C4,C2<C3<C5, factors B,D,P,I,H
Clothing factor	V, VII, IX, X, prothrombin, plasminogen activator, plasminogen activator inhibitors
Cytokines	IL-1, IL-6, IL-8, TNF-α, INF-γ Macrophage inflammatory proteins (MIP-1, MIP-2, MIP-3) Regulatory growth factors (M-CSF, GM-CSF, G-CSF, PDGF) Elastase, collagenase, angiostatin convertase, stromelysin
Neutral proteinases	α2-Macroglobulin, α-1-proteinase inhibitor, plasmin and collagenase inhibitors, plasminogen activator inhibitors
Proteases inhibitors	Acid proteases (cathepsin D and L), peptidases, lipases, lysozyme and other glycosidases, ribo-nucleases, phosphatases, sulphatases
Acid hydrolases	Heat shock proteins
Stress proteins	
Participating in tissue reorganization	Elastases, collagenase, hyluronidase, regulatory growth factors, fibroblast growth factor (FGF), transforming growth factors (TGF-α, and TGF-ß), angiogenesis factors
Other	Apolipoprotein E, IL-1 inhibitors, purine and pyrimidine derivates (thymidine, uracil, neopterin)

Elastase and collagenase are enzymes capable of splitting collagen and elastin, the basic members of connective proteins.

1.3. Endothelial cells

The endothelium has long been viewed as an inert cellophane-like membrane that lines the circulatory system, with its primary essential function being the maintenance of vessel-wall permeability. As such, endothelial cells (ECs) were thought to participate in tissue reactions, essentially as targets for injurious agents. This point of view has changed radically; it is now evident that the endothelium is a dynamic, heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic and immunologic functions (Fishman, A.P., 1982).

EC coat the entire inner surface of the blood vessels in an adult human. The endothelium is composed of approximately 1 to 6 x 10^{13} cells, weighs approximately 1 kg, and covers a surface area of approximately 1 to 7 m^2 (Augustin HG et al., 1994). Depending on the type of vessel (artery, vein, capillary) and vascular bed, activated ECs that are migrating and proliferating, forming anastomotic connections with each other, become variably surrounded by layers of periendothelial cells - pericytes for small vessels and smooth muscle cells for large vessels. EC are the key cellular mediators of vascular development, and the embryonic vascular system is critical for the transport of metabolic waste, nutrients, and humoral signals to and from developing tissues through the presence of membrane-bound receptors for numerous molecules, as well as through specific junctional proteins and receptors that govern cell-cell and cell-matrix interactions. New vessels in the adult arise mainly through angiogenesis, although vasculogenesis also may occur. The existence of a postnatal vasculogenesis is supported by the evidence that both ECs and endothelial precursor cells co-exist in the circulation. Moreover, EPCs are also recruited to sites of neovascularization in mature mammals from a circulating, marrow-derived population of progenitor cells (Asahara et al., 1997). Although circulating ECs have been suspected of having the capacity to colonize vascular grafts (Shi, 1992; Rafii, 2000), the contribution of these cells to postnatal vasculogenesis is not known. The possibility that endothelial precursor cells, showing properties similar to those of embryonic angioblasts, persist into adult life, whereby they may circulate, differentiate, and contribute to the formation of new blood vessels, remains to be determined.

1.3.1 *Origin of endothelial cells*

The relationship between hematopoietic cells and ECs has been seen as an indication that a common progenitor, the hemangioblast (Choi et al., 1998, gives rise to both cell types in the yolk sac, the initial site of hematopoiesis and blood-vessel formation during mammalian development. Angioblast-circulating endothelial precursor cells exist in adults humans; they are defined as a cell type that has the potency of differentiating into ECs but has not yet acquired all the characteristic markers (Table 2). An important and still incompletely solved question is how ECs take different pathways of differentiation. One of the determinants is the local environment in which ECs differentiate, and especially their interaction with surrounding cells. This interaction may occur through the release of soluble mediators, cell-to-cell adhesion, and the synthesis and organization of matrix proteins on which the endothelium adheres and grows. The activation of flk-1, which is the VEGF receptor tyrosine kinase expressed in EC, triggers the differentiation of angioblast in the endothelial-specific pathway (Millauer et al., 1993). Fibroblast growth factor (FGF) may act at the level of hemangioblasts or at an earlier stage, but it is not responsible for differential commitment into endothelial or hematopoietic lineage.

Embryonic ECs seem particularly "plastic." Most of the specialized characteristics of ECs are induced during development, whereas adult endothelium is not equally susceptible to differentiation factors (Risau W. 1995; Engelhardt and Risau W 1995). Despite its stable constitutive properties, the adult endothelium can reversibly change its functions on activation. For instance, exposure of EC to inflammatory cytokines, such as IL-1 and TNF, or to growth factors, such as VEGF or FGF, induces a complex functional reprogramming, which implies the neosynthesis of some genes and the repression of others. ECs can be activated several times during their life span by the same or different cytokines and thereby display different and reversible phenotypes (Mantovani et al., 1992). Cell senescence may also

influence endothelial responses. Unlike young endothelium, senescent ECs display different properties, such as an

Table 2. Surface markers of endothelial cells.

Surface markers	Endothelial progenitor cell	Vessel wall endothelium
VEGFR-2 (flk-1)	+	+
VEGFR-3 (flt-4)	?	+
VEGFR-1 (flt-1)	?	+
Tie-2	?	+
VE-cadherin	+	+
CD34	++	+
Ac. LDL uptake	+	+
PECAM (CD31)	+	+
AC133	+	-
vWF	-	+
CD105	+	+
CD36	?	+
PIH12 (CD146)	?	+
Trombomodulin	?	+
E-selectin	?	+
Weidel-Palade bodies	+	+
Tie-1	+	+
Thy-1	+	+
UEA-1	+	+
αv integrin	+	+

inability to properly respond to growth factors due to defective signaling pathways. (Garfinkel et al., 1996) The actual mechanism regulating cell senescence is still incompletely understood; current research suggests a correlation between senescence and intracellular IL-1 accumulation; elevated levels of intracellular IL-1 in the senescent human umbilical vein endothelial cells (HUVEC) population are biologically active and may be involved in the induction of the senescent HUVEC phenotype (Garfinkel et al., 1997). In addition, the ability of IL-1 to promote tissue degradation appears to be intimately associated with its ability to stimulate the synthesis of matrix metalloproteinases (MMPs) such as collagenase and stromelysin; and IL-1 not only induces tissue destruction, but also inhibits tissue repair (Ganu et al., 1998).

1.3.2 *Endothelial cell heterogeneity*

ECs are a heterogeneous population (Table 3). There are differences between the endothelium of different species, between large and small vessels and between ECs derived from various microvascular endothelial beds. Typical ECs are elongated (approximately 30 μm long, and 0.3 μm deep) and carry a negatively charged glycosaminoglycan layer (approximately 10 nm deep) on their surface (Ribatti et al, 2002, review). Many human vascular diseases are exquisitely restricted to specific types of vessels. For example, the contribution of platelets to the pathogenesis of arterial and venous thrombosis differs, as does the susceptibility of these two types of vessels to atherosclerosis. Tumor cells may show similar predilection to metastasize through particular vascular beds (Zetter, 1997). Even when systemic risk factors are clearly evident, such as is the case with inherited disorders of lipoprotein metabolism or proteins that control coagulation, there is marked regional variation in disease expression. Furthermore, clinical events such as thromboses are generally episodic and often localized to single vessels. The basis for variation is poorly understood, but may lie, in part, in the heterogeneity of ECs themselves (Augustin et al., 1994; and McCarthy et al., 1991 for reviews).

Variation in the appearance of capillary endothelium from different vascular sites has long been recognized and appears well suited to postulated differences in function. On the basis of morphology, microvascular endothelium in normal adult organisms has been divided into different phenotypes: continuous, fenestrated, and discontinuous (Table 4).

Table 3. Levels of endothelial cell heterogeneity.

Structure	Expression patterns	Function
Size and shape	Protein	Hemostasis
Nuclear orientation	mRNA	Vasomotor tone
Thickness	Transcription networks	Barrier function
Microvilli	Signaling pathways	Leukocyte trafficking
Filaments		Cell survival
Vesicles		Cell migration/proliferation
Junctions		Antigen presentation

ECs line vessels in every organ system and regulate the flow of nutrient substances. Continuous endothelium is characteristic for the brain and retina, where lined ECs are connected by tight junctions that help to maintain the blood-brain barrier: the liver, spleen, and bone marrow sinusoids are lined by discontinuous ECs; the intestinal villi, endocrine glands, and kidneys are lined by fenestrated ECs that facilitate selective permeability required for efficient absorption, secretion, and filtering (see Dejana, 1996 for review). Venules have the most and arterioles the least elaborated junctional system, whereas capillaries have no communicating junctions and can also vary from ECs > 2 μm (thick) to ECs < 1 μm (thin; Simionescu et al., 1975, 1976).

EC from diverse tissues are also heterogeneous with respect to their surface phenotype and protein expression. Among many others, the following examples illustrate the heterogeneity of ECs at the protein expression levels: 1) von Willebrand factor (vWF), used commonly as a marker for ECs, is not expressed uniformly in cells from all types of vessels (Kumar et al., 1987; Turner RR et al., 1985); 2) the expression of tissue type plasminogen activator is limited *in vivo* to approximately 3% of vascular ECs (Levin and Osborn, 1997), and the constitutive expression of u-PA is reportedly confined to renal ECs (Wojta et al., 1989; Louise and Obrig, 1994), which are also uniquely susceptible to injury by verotoxin (Obrig et al., 1994); 3) the induction of tissue factor after infusion of cytokines or endotoxin

Table 4. Heterogeneity of endothelial cells in different adult tissues.

	Tissue or Organ	Properties	Function
Continuous	CNS	low number of vesicles, complex tight junctions	blood-brain barrier
	lymph nodes	high endothelial venules	lymphocyte homing
	muscle	high number of vesicles	exchange/transport
Fenestrated	endocrine glands	fenestrae	secretion
	gastrointestinal tract	fenestrae	absorption
	choroids plexus	fenestrae	secretion
	kidney glomeruli	pores	filtration
Discontinuous	liver	large gaps	exchange of particles
	bone marrow	marrow sinus	hemopoiesis, delivery of blood cells
	spleen	splenic sinus of red pulp	blood-cell processing

is similarly restricted to specific vessels (Drake et al., Am J pathol 1993). Microvascular ECs cultured from the brain, liver, and other organs each express distinct patterns of cell-surface markers, protein transporters, and intracellular enzymes (Owman and Hardbeo, 1988). These tissue-specific phenotypic differences can be maintained for some time under identical tissue-culture conditions (Grau et al., 1997; Smith et al., 1996). Distinct subsets of EC often exist within a single organ. In situ studies of adult human liver show two distinct sinusoidal ECs phenotypes: hepatic periportal vessels express PECAM-1 and CD34, whereas sinusoidal intrahepatic ECs do not (Morin et al., 1984).

ECs can also acquire malignant properties. They can form angiosarcomas, which are highly invasive and usually fatal. There is strong evidence that Kaposi's sarcoma has an endothelial origin; however, it expresses markers from both ECs and macrophages, suggesting that it may originate from a special type of EC similar to macrophages present in lymph nodes (Uccini et al., 1994).

1.3.3 *Endothelial cells' function*

General aspects of endothelial function are regulating hemostasis, vascular tone and growth, as well as the inflammatory response. A particular role in inflammation is played by cell-adhesion molecules (CAMs), expressed both on endothelial and blood cells. It had been shown that cytokine-induced up-regulation of CAM in the endothelium may involve signal-transduction pathways. Finally, the phenomenon of angiogenesis is a characteristic of EC activity, with central importance on both physiology and pathology and the ability of individual ECs to form vessel-like structures. Likewise, the role of ECs in pathological processes as diverse as atherosclerosis, tumor intravasation and multiple organ failure depends of the physiology and cell biology of the endothelium.

Vascular development consists of two different processes, vasculogenesis and angiogenesis. In both, bidirectional signaling between ECs and the surrounding mesenchymal cells is critical (Folkman and DAmore, 1996). The earliest stages of vascular development, termed *vasculogenesis*, include the differentiation, expansion, and coalescence of vascular endothelial cell precursors (angioblasts) that migrate to discrete locations to form the initial vascular network (Risau, 1995; Conway et al., 2001). Angiogenesis is a biological process by which new capillaries are formed from pre-existing vessels. It occurs in physiological and pathological conditions, such as tumors, where a specific critical turning point is the transition from the avascular to the vascular phase. (Figure 4). When angiogenesis is initiated, however, there is a rapid change in the EC-extracellular matrix (ECM) interactions (Conway et al., 2001). The ECs of an angiogenic sprout need to degrade and invade through different types of ECM, detach from various ECM components, and attach to new ones. With alterations in the ECM and ECs invasiveness, the ECs need to receive the appropriate signals from changing microenvironment. These functions are carried out by specific classes of molecules: those that mediate the recognition of ECM components by ECs, i.e. endothelial matrix receptors, and those that participate in the proteolytic degradation of the components, i.e., matrix proteinases.

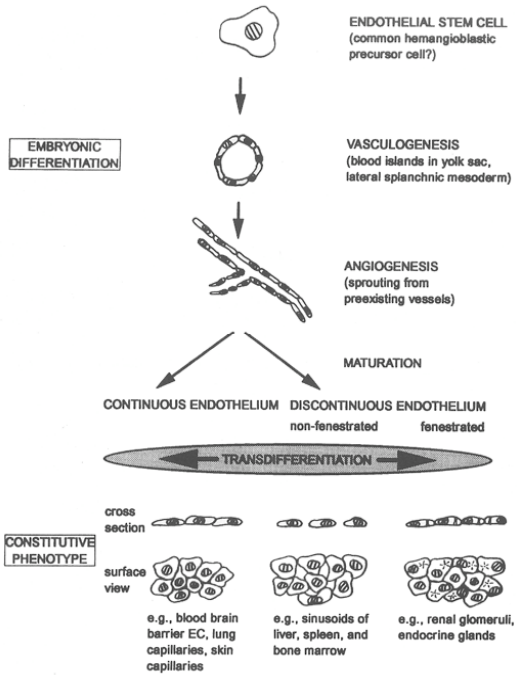

Figure 4. Differentiation of ECs. Putative endothelial stem cells migrate, proliferate and differentiate during vasculogenic and angiogenic processes to form the various EC phenotypes found in different vascular beds. Stimulation of resting ECs leads to the situation-specific expression of ECs molecules, which are characteristic for the activated phenotype. Likewise, transdifferentiation of ECs characterizes the process through which a specific constitutive EC phenotype is altered in response to changes in the microenvironment that control a specific constitutive phenotype.

Differentiation of the EC phenotypes appears to follow similar mechanisms to the differentiation of hematopoietic cells, with the exception that ECs maintain transdifferentiating competence.

ECs, the major cellular components of the vasculature, are metabolically active cells capable of the synthesis and secretion of a variety of biologically active molecules important in regulating vascular tone and blood flow, in the modulation of inflammatory and immune responses, and regulating vascular growth. In particular, ECs synthesize and release a number of growth factors including IL, colony stimulating factors (CSF), and IFNs, to modulate the growth and proliferation of hematopoietic cells. EC also synthesize matrix components to induce the adhesion of blood cells to vascular EC for the migration of these cells to peripheral organs.

1.3.3.1 ***The activated phenotype of endothelial cells***

EC activation in response to the micro-environment is associated with a number of distinct phenotype changes that, much like the transdifferentation process of the constitutive phenotype, serve their need to locally adapt to specific functional requirements (Figure 5).

Surface molecules associated with the activated cytokine-inducible endothelial phenotype play a critical role in pathological conditions, including inflammation, tumor angiogenesis, and wound healing. Early consequences and clinical signs of endothelial activation and damage result from microvascular coagulopathy, increased microvascular permeability, leukocyte adhesion and migration, activation of reactive oxygen species, vasodilatation, and cytokine production. Endothelial activation and damage may occur not only during sepsis but also in all clinical settings that involve systemic inflammation and biochemical alterations such as shear stress and increased hydrostatic pressure (Reinhart K et al., 2002). The cytokine-induced phenotype of ECs during inflammation has been characterized in the last few years. Inducible EC adhesion molecules, such as E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are expressed on the cell surface following cytokine exposure enabling them to direct circulating cells to sites of inflammation. Angiogenesis is another example of EC activation that induces such changes. It involves degradation of ECM combined with sprouting and migrations of ECM from existing capillaries. One of the first events that occurs is the weakening of stable cell-cell contacts between ECs in

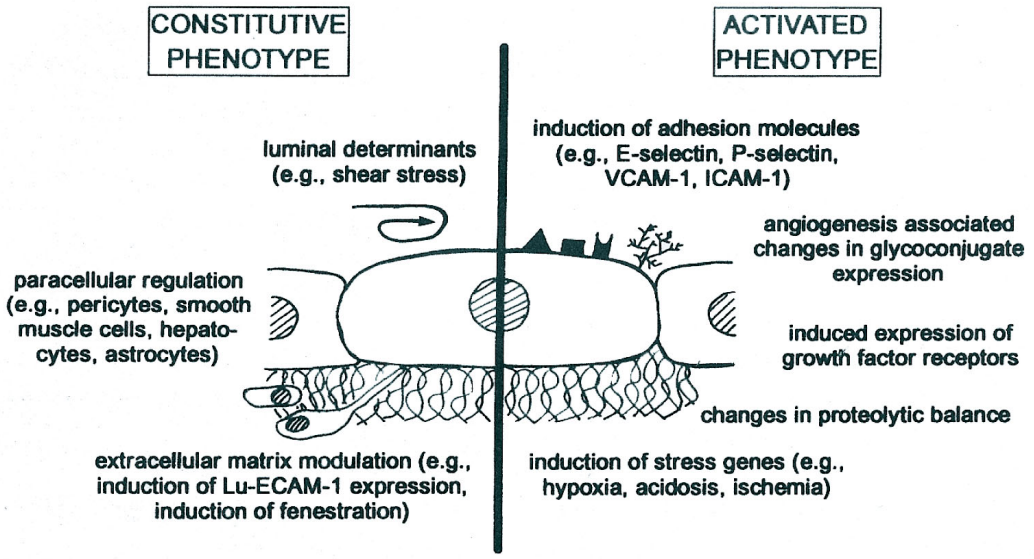

Figure 5. Microenviromental control of the constitutive (left) and activated endothelial cells phenotypes (right) (Augustin et al., 1994).

the parent vessels and the transition of ECs from quiescent stationary to dynamic migratory (Ausprunk and Folkman, 1997). The regulation of motility and adhesion of ECs to the underlying ECM and each other is therefore an important aspect of angiogenesis. Furthermore, areas of replicating ECs exhibit increased vascular permeability (Padera et al., 2002; Caplan and Schwartz, 1977), a critical step in angiogenesis. Adhesion molecules are involved in shaping the angiogenic phenotype of ECs (Brooks et al., 1994).

1.3.3.2 *Endothelial cell-cell interactions and vessel formation*

Two major processes-vasculogenesis and angioge-nesis- are responsible for blood-vessel formation *in vivo* (Hanahan, 1997; Carmeliet and Jain, 2000; Patan, 2000; Conway et al., 2001). These events require EC interactions with ECM through integrins, and signaling events involving cytoskeletal elements that control EC shape and cell-cell interactions that dictate the 3D structure of tubes of the blood vessels. Cellular activities associated with invasion implicate reduced cell-cell adhesion, altered cell-matrix adhesion, migration, ectopic survival, and lysis of extracellular matrix. Endothelial cell contacts control the permeability of the blood-vessel wall. This allows the endothelium to form a barrier for solutes, macromolecules, and leukocytes between the vessel lumen and the interstitial space. The formation and maintenance of ECs contacts require the complex interplay of plasma membrane proteins, cytoskeleton components, and associated signaling molecules. Some of these molecules are specifically expressed in specialized cellular junctions. EC-cell contacts do not show a clear spatial organization and morphological differentiation with respect to tight junctions, desmosomes, and adherens junctions. The endothelial junction of mammalian blood vessels represents a continuous belt system related to but different from the adherens junctions of polar epithelial cells (Franke et al., 1988). In such junctions, the adherens junction protein vascular endothelial (VE)-cadherin and the desmosomal protein desmoplakin can be co-localized (Kowalczyk et al. 1998;). Classical desmosomes are not present in ECs (Schmelz and Franke, 1993). Cell contacts seem to be present along the complete region of the lateral membranes between endothelial cells.

Cell-cell adhesion is mediated by a distinct series of cell-surface receptors that includes PECAM-1 (Albelda et al., 1991), a member of the Ig superfamily, and VE-cadherin (Risau and Flamme, 1995). ECs express two isoforms of PECAM that mediate cell adhesion. They differ in their requirement for divalent cations and sulfated proteoglycans. VE-cadherin, also known as cadherin-5 and found almost exclusively on ECs, promotes cell-cell adhesion by a calcium-dependent homotypic mechanism (Dejana, 1996), i.e., in the presence of calcium, VE-cadherin from one cell binds to the VE-cadherin expressed on an adjacent cell. Beyond cell-cell adhesion, these molecules are important signal-transduction ligands, which influence diverse cellular processes, including proliferation (Conacci-Sorell et al., 2002) and EC survival (Carmeliet et al., 1999).

1.3.3.3 *Endothelial cell-matrix interactions*

The ECM is a key player in activities crucial for normal cell behavior and tissue maintenance (Lukashev and Werb , 1998). In invasion, the ECM and its cellular integrin and nonintegrin receptors are implicated as a barrier, a signal, and a substrate for invasion; as a source of growth and motility factors; and as a regulator of survival (Van Hoorde et al., 2000). For cancer-cell migration, a dynamic formation and dissolution of cell-substrate contacts are needed; and ECM receptors are expected to have a dual function as they provide adhesion to the matrix necessary for migration, as well as arrest of cells inside the matrix. To coordinate all these functions, multiple extracellular and intracellular networks as well as fine-tuning signaling are necessary. Structurally, cell-substrate adhesion is manifested by smaller focal complexes at the leading edge of migratory cells, larger focal adhesions at the end of actin stress fibers all over the surface of static cells, and hemidesmosomes between epithelial cells and the basement membrane. The interactions of ECs with the ECM can regulate the ability of ECs to form capillary-like tubes. For example, HUVEC exposed to transforming growth factor-β (TGF-β) grow as a rapidly dividing monolayer if cultured on a flat surface coated with type I collagen (Sankar et al., 1996). However, under similar TGF-β exposure, but within a type I collagen gel, ECs spontaneously organize into capillary-like tubules and continue to divide. Studies indicate the ECM receptors expressed by EC are critical to maintain a proper ECs function. The best-studied family of receptors that

mediate cell-matrix interactions are the integrins (Schwartz et al., 1995), which serve both a tethering and an information transfer function. Integrin-ligand binding triggers cytoskeletal organization at specific sites on the surface membrane to facilitate cell movement or maintain tissue stability. Binding also activates intercellular pathways that can result in either cell replication or programmed cell death (Varner et al., 1995). Together with these functions, ECs in their activated state are able to produce chemokines that allow a tight regulation of the microenvironment (Table 5).

Cells express more than one integrin and the combination of integrins expressed during embryonic development is constantly changing, suggesting that specific combinations are required as development proceeds. Experimental results in the developing mouse embryo suggest that functional compensation by integrins can occur during embryogenesis.

Angiogenesis is stimulated by a shift in the balance between angiogenic and antiangiogenic factors and depends on specific molecular interactions between the vessels. The formation of new blood vessels, i.e., angiogenesis, is an important phenomenon during normal development and tissue repair, as well as during various pathological processes, such as tumor growth and ocular neovascularization (Folkman, 1995; Carmaliet and Jain, 2000). ECs and their surrounding microenvironment are composed of neighboring cells and the ECM, which consists of a network of molecules composed primarily of type IV collagen, laminins, and heparan sulfate proteoglycans.

2. EXTRACELLULAR MATRIX

Cells of higher multicellular organisms are surrounded by a highly organized matrix of proteins and glycoconjugates. The ECM was originally defined morphologically as extracellular material visible as fibrils or sheets in the electron microscope. The understanding of ECM functions has been enhanced in recent years by the discoveries of novel extracellular matrix constituents, mapping sites crucial for the interactions of ECM constituents with each other and with cells, characterization of proteases and protease inhibitors that regulate ECM assembly, and turnover and identification of novel receptors and signaling mechanisms that mediate cellular responses in ECM constituents.

Matrix components include ligands that activate intracellular signaling pathways within cells, thereby regulating cell proliferation, survival, and differentiation (Schwartz et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996; Kleiman and Reichardt, 1996; Sanes, 1997; Wary et al., 1996). These functions can be mediated not only through interaction with cell receptors but also through direct interaction with growth factors (Lopez-Casilla et al., 1994; Kaname and Rouslahti 1996). The ECM thereby helps regulate the special and temporal properties of signals conveyed by these molecules.

Major constituents identified initially in the ECMs included collagens, noncollagenous glycoproteins, (i.e., fibronectin), and proteoglycans. Organization of these components into a fibrillar framework provides mechanical strength and

Table 5. Chemokines produced by endothelial cells.

Family	Molecule	Endothelium	Principal inducers
CXC	IL-8	Diverse	LPS, IL-1, TNF, IL-6/IL-6R, IL-4, IL-13, IL-7, thrombin, hypoxia
	ENA-78	HUVEC	Similar to IL-8
	GRO-a	HUVEC	Similar to IL-8
	IP-10	Endothelium in vivo	LPS, IFN-γ
CC	MCP-1	Diverse	Similar to IL-8, IFN-γ
	MCP-3	HUVEC	Similar to IL-8
	RANTES	Diverse, in vivo	TNF and IFN-γ
	Fractalkine	HUVEC	IL-1

elasticity for cell adhesion and migration.

2.1. Glycoproteins or noncollagenous proteins

Many glycoproteins unique to vertebrates are unusually large molecules with extended conformations spanning distances of several hundred nanometers. Two groups of glycoproteins have been described according to function: the first group includes the adhesive glycoproteins, such as fibronectin, vitronectin and laminin, which form connections between other ECM and cells via specific integrin receptors; the second group comprises the matricellular proteins, which are a class of secreted proteins that interact with other ECM constituents, and multiple specific cell surface receptors, as well as growth factors, to modulate cell-matrix interactions. This last group includes osteopondin, tenascin, trombospondin and secreted protein acidic and rich in cysteine (SPARC) (Raines, 2000) (Table 6).

Table 6. Structural glycoproteins of the ECM

Glycoproteins
Adhesive glycoproteins
fibronectin
laminin
vitronectin
Matricellular proteins
osteopontin
SPARC
tenascin
thrombospondin

Fibronectin (FN) is one of the best-characterized ECM proteins (Rouslahti, 1988; Hynes, 1990; Springer, 1994). FNs are high-molecular-weight glycoproteins found in many ECMs and in blood plasma. They promote cell adhesion and modulate cell morphology, migration and differentiation and cytoskeletal organization. They are composed of two similar subunits of 230-290 kDa joined by disulfide bonds at their carboxyl termini. Each subunit is made up of a series of repeating units that in turn form structural and functional domains specialized for binding to cell-surface integrin receptors or other ECM molecules. The polymorphic nature of FN molecules results from alternative splicing of the transcript of a single gene. FNs have been found in all major groups of vertebrates, but there are not convincing reports showing that invertebrates have FNs. Laminins represent a protein family of heterotrimers primarily located in basement membranes (Timpl and Brown, 1996). This family of ECM molecules was first identified in mouse tumor-cell lines. Major activities include the formation of networks and filaments and binding to cells through integrin and nonintegrin receptors. The latter activities determine cell adhesion and migration, differentiation, gene expression and cell fate. Another adhesive glycoprotein, vitronectin (VN) is a multifunctional 75 kDa glycoprotein that constitutes the major adhesive component of serum, and permits the attachment and spreading of cells propagated in serum-containing media in culture (Felding-Habermann and Cheresh, 1993). VN is present in the supporting stroma of a variety of normal tissues but not in the basement membrane per se. *In vivo*, the physiological roles of VN have been shown to include the regulation of thrombosis, fibrinolysis, and complement-mediated cell lysis in the circulation.

There are several forms of matricellular proteins: tenascins represent a family of large multimeric ECM proteins, each consisting of identical subunits built from variable numbers of repeated domains. These include heptad repeats, epidermal growth factor (EGF)-like repeats, FN type-III domains and a C-terminal globular domain. The prototype of tenascins is tenascin C, present in a large number of developing tissues and it is also abundant in adult ligaments and tendons. Tenascin C is overexpressed in many tumors. SPARC binds both to extracellular matrix proteins and to cells and thereby regulates cell-matrix interaction. Addition of SPARC and SPARC-derived peptides to cultured cells modulates cell spreading, cell migration, cell-cell progression, and gene expression.

The thrombospondins are a family of five extracellular calcium- and glycosaminoglycan-binding proteins that function during embryogenesis, angiogenesis and wound healing (Bornstein, 1992; Adams et al., 1995. Thrombospondins are trimeric glycoproteins that appear to facilitate cell-to-ECM contacts that regulate cellular migration, motility, proliferation, and neurite outgrowth (Iruela-Arispe et al., 1993). Osteopontin is a secreted acidic glycoprotein normally found in mineralized matrices.

In tissues undergoing inflammation, fibrosis, oncogenesis, or dystrophic calcification, osteopontin expression is increased due to novo expression by macrophages, epithelial, tumor and/or mesenchymal cell populations responding to injury. *In vitro*, it has been shown that osteopontin regulates cell adhesion, migration, and survival.

2.2. Collagens

The collagen matrix constitutes a major portion of the vascular ECM and imparts blood vessels with tensile. Collagenous proteins usually form supramolecular aggregates (fibrils, filaments, or networks), either alone or in conjunction with other ECM components. Their major function is to contribute to the structural integrity of the ECM, or to help anchor cells to the matrix. The collagen superfamily can be divided into several subfamilies (Table 7) based on exon structures of the genes that encode collagenous proteins, and functional considerations. The fibrillar collagens are a major component of the vascular matrix, and the most abundant are types I and III collagen, which form an interconnected network of cross-banded fibers along with smaller amounts of type VI and XVIII collagen associated with the fibers and the elastic lamellae in the blood-vessel wall (Raines, 2000). Collagens are not only essential for the mechanical resistance and resilience of multicellular organisms, but are also signaling molecules defining cellular shape and behavior. The communication between collagens and cells is achieved by cell-surface receptors. Three types of cell-surface receptors for collagen are known: integrins, discoidin domain receptors, and glycoprotein VI. All three types independently trigger a variety of signaling pathways upon collagen-binding. Novel mechanisms of locally controlled expression of collagen, collagen-binding receptors and collagen-degrading proteases in the cellular

Table7. Collagen types.

Type	Chains	Function
Fibril-forming collagens		
I	α_1(I), α_2(I)	Synthesized as precursos, procollagens, which
II	α_1(II)	are proteolytically processed to collagen in the
III	α_1(III)	extracellular space. Participate in the formation
V	α_1(V), α_2(V), α_3(V)	of fibrils.
XI	α_1(XI), α_2(XI), α_3(XI)	
Basement-membrane collagens		
IV	α_1(IV), α_2(IV)	Interactions with cells indirectly through
	α_3(IV), α_4(IV)	laminin, and binding sites with some integrins.
	α_5(IV), α_6(IV)	
FACIT		
IX	α_1(IX), α_2(IX), α_3(IX)	Serve as a molecular bridge between fibrillar
XII	α_1(XII)	collagens and other ECM molecules.
XIV	α_1(XIV)	
XVI	α_1(XVI)	
XIX	α_1(XIX)	
Multiplexin		
XV	α_1(XV)	Fragment of XVIII: endostatin is a potent
XVIII	α_1(XVIII)	angiogenesis inhibitor.
Short-chain collagens		
VIII	α_1(VIII), α_2(VIII)	Unique networks in basement regions and
X	α_1(X)	hypertrophic cartilage
Collagens with transmembrane domains		
XIII	α_1(XIII)	New class of cellular adhesion molecules that
XVII	α_1(XVII)	are connected to ECM.
Other collagens/orphans		
VI	α_1(VI), α_2(VI), α_3(VI)	Molecules that form specialized structures in a
VII	α_1(VII)	variety of tissues.

Fibril-associated collagens with interrupted triple helices: FACIT

Multiple-triple-helix domains with interruptions: multiplexin

microenvironment have recently been discovered (Vogel, 2001).

2.3. Proteoglycans

Proteoglycans comprise a collection of macromolecules that surround the plasma membrane of a cell and constitute part of the substrate upon which the cells are attached and perform their major functions. These molecules are a separate class of glycoproteins, and they are a set of ubiquitous proteins found on cell surfaces or within intracellular vesicles, and incorporated into intracellular matrices. They are also found in membrane spanning and soluble forms. Proteoglycans are considered to be important in cell-to-cell and cell-to-ECM adherence (Letourneu et al., 1997), and as regulators of cell migration, synaptogenesis, and plasticity (Bandtlow and Zimmermann, 2000).

Proteoglycans are involved in maintaining the transparency of the cornea, the tensile strength of the skin and tendon, the viscoelasticity of blood vessels, and the compressive properties of the cartilage. In addition, proteoglycans play key roles as storage depots for growth factors and cytokines; and by virtue of their multifunctional properties, they alter the biology of these factors. The growth of the proteoglycan gene family has been astounding in the past decade. Before the era of molecular cloning, proteoglycans were named by the type of glycosaminoglycan chain found on them, from where they were isolated, and/or by distinguishing biochemical features. Lately a classification for the proteoglycans was based on the nature, overall structure and biological properties (Iozzo and Murdoch, 1996). According to this classification, the proteoglycans are divided into three broad categories: the small leucine-rich proteoglycans (SLRPs), the hyalectans (proteoglycans interacting with hyaluronan and lectins) and the basement-membrane proteoglycans (Iozzo, 1998) (Table 8).

2.3.1. *Small leucine-rich proteoglycans (SLRPs)*

SLRPs are small, ubiquitous proteoglycans enriched in leucine with 24 amino acid tandem repeats flanked by cysteine clusters. The prototype member of the family is decorin. Based on their protein and genomic organization, three classes of SLRPs can be identified (Table 8). Essentially, they are all characterized by a central domain containing leucine-rich repeats (LRR) and flanked at either side by small cysteine-clusters. Class I comprises decorin

Table 8. Proteoglycan classification.

Proteoglycans	
Small Leucin-rich proteoglycans	
Class I	
decorin	Ubiquous, collagenous matrix, bone, teeth, mesothelia, floor plate
biglycan	Interstitium, and cell surfaces
Class II	
fibromodulin	Collagenous matrices
lumican	Cornea, intestine, liver, muscle, cartilage
keratocan	Cornea
PRELP	Cartilage, kidney, aorta, sclera, liver, skeletal muscle, cornea, skin
osteoadherin	Teeth, bone
Class III	
epiphycan	Ephiphyseal cartilage
osteoglycin	Vascular smooth muscle cells, bone
Modular proteoglycans	
Hyalectans	
aggrecan	Cartilage, brain, blood vessels
versican	Blood vessels, brain, skin, cartilage
neurocan	Brain cartilage
brevican	Brain
Basement membrane	
perlecan	Basement membranes, cell surfaces, sinusoidal spaces, cartilage
agrain	Synaptic sites
bamacan	Renal mesangial, basement of membranes, tumor matrix

and byglican, which show the highest homology (57% identity). Class II includes fibromodulin, lumican, keratocan, and PRELP (~50% identity). Class III comprises epiphycan and osteoglycin with 40% identity.

2.3.1.1. ***Decorin***

Decorin is a small proteoglycan composed of a 38-kDa core protein usually modified with a single chondroitin sulphate (bone) or dermatan sulphate (most soft tissues) glycosaminoglycan chain and two or three N-linked oligosaccharides. The protein has four domains very well differentiated: domain I, which contains the signal peptide and a propeptide; domain II, which contains four evenly spaced cysteine residues and the glycosaminoglycan attachment site; domain III, which comprises the 10 tandem repeats of ~25 amino acids characteristically rich in ordered leucines (leucine rich repeats); and domain IV, which contains a relatively large loop with two cysteins residues (Iozzo, 1998) (Figure 6). The most commonly cited functions of decorin are its roles in collagen fibril assembly and stabilization, as well as its ability to bind to TGF-β.

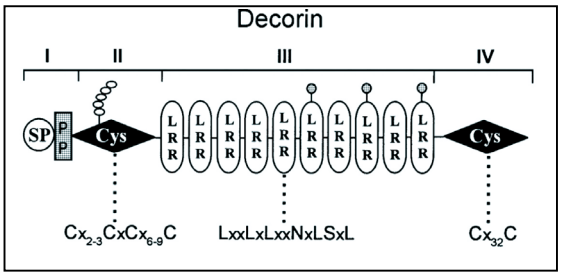

Figure 6. Schematic representation of the structural features of decorin, the prototype member of the small leucine-rich proteoglycans.

The decorin-induced effects are mediated, at least in part, by a specific interaction between the decorin protein core and the EGF receptor (EGFR) (Moscatello et al., 1998; Iozzo et al., 1999). The decorin protein core binds to a discrete region of the EGFR, partially overlapping with but distinct from the EGF-binding epitope (Santra et al., 2003). This interaction triggers a signal cascade leading to activation of mitogen-activated protein kinases (Moscatello et al., 1998), mobilization of intracellular calcium (Patel et al., 1998), up-regulation of $p21^{Waf1/CIP1}$ (Nash et al., 1999, De Luca et al., 1996), and ultimately growth suppression (Santra et al., 1995; Santra et al., 1997, Nash et al., 1999). However, following a transient stimulation of the EGFR kinase, decorin causes a sustained down-regulation of the EGFR (Csordas et al., 2000) and other ErbB members of receptor tyrosine kinase (Santra et al., 2000), an action that would negatively affect tumor growth. Interestingly, EGF suppresses decorin expression in normal fibroblasts, suggesting a negative feedback loop between growth-promoting and growth-suppressing factors (Laine et al., 2000) (Figure 7).

2.3.1.2. ***Effect of decorin on cell proliferation***

One of the more interesting activities ascribed to decorin is as a regulator of cell proliferation. Decorin levels are elevated during quiescence (Asundin and Dreher, 1992), and decorin expression is low in actively proliferating or transformed cells (Coppock et al., 1993), observations consistent with a role for it in growth inhibition. Colon carcinoma cells transfected with decorin become quiescent (arrest in G_1), lose their malignant phenotype (Santra et al., 1995; Nash et al., 1999), and exhibit a marked up-regulation of $p21^{Waf1/Cip1}$ (De Luca et al., 1996) (Figure 7). Decorin also suppresses the growth in other tumor cells derived from genitourinary, skeletal, cutaneous and bone-marrow tissues; and that activity is independent of functional p53 (Santra et al., 1997). Recently, decorin expression has been shown in normal cells such as endothelial (Schonderr et al., 2001), and mesangial cells (Abdel-Wahab et al., 2002). Synthesis of decorin as well as type I collagen is associated with endothelial cord and tube formation *in vitro* and correlates with the cells' functional state (Jarvelainen et al., 1992; Schönherr et al., 2001).

The role of decorin as an antiproliferative agent may be important in different disease states, such as fibrosis and cancer. Indeed, decorin has been used as therapeutic agent for reduction of matrix accumulation in experimental glomerulonephritis in rats (Isaka et al., 1996). The expression of decorin is sufficient to induce differentiation of ECs and to protect them from apoptosis, an important element for the survival of the ECs during angiogenesis (Schönherr, et al., 1999; Schönherr, 2001). Furthermore, decorin expression is followed by an up-regulation of cyclin-dependent kinase inhibitors (DeLuca, et al., 1996), probably, as mentioned above, via interaction with an EGFR family member

(Moscatello et al., 1998). It is therefore also involved in cell-cycle control.

2.3.1.3. *Interaction of decorin with TGF-β*

TGF-β is a multifunctional cytokine that regulates a diverse range of processes important for cell growth. It has been determined that TGF-β participates in the control of cell proliferation, differentiation, adhesion, and deposition of the ECM (Figure 7). These cellular events are critical in tissue growth, remodeling and repair. In the earlier' 90s, it was proposed that decorin and other SLRPs may modulate the activity of this growth factor (Yamaguchi et al., 1990). Overproduction of TGF-β in response to injury and disease is thought to be a major cause of tissue fibroses because it stimulates deposition of ECM by the following mechanism: up-regulating synthesis of matrix components such as collagens, proteoglycans and FN; up-regulation of the inhibitors of matrix-degrading enzymes; and down-regulation of the expression of these degradative enzymes (Border et al., 1992; Isaka et al., 1996).

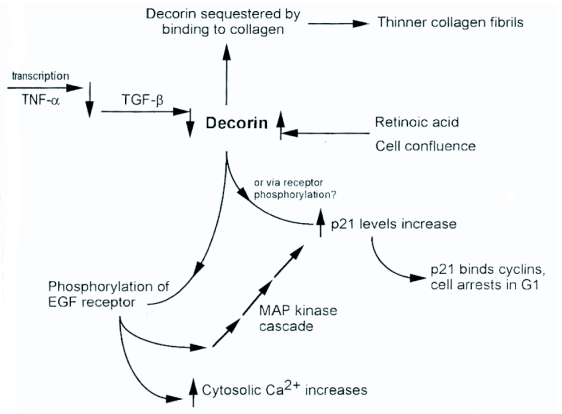

Figure 7. Regulation of decorin and its relationship to cell cycle control. TNF-α and TGF-β both down-regulate decorin, but by different mechanisms. Decorin core protein has a different effect on cells, probably through phosphorylation of a receptor and activation of the mitogen-activated protein (MAP) kinase pathway, resul-ting in upregulation of p21. Outside the cell, increased decorin production inhibits collagen fibrillogenesis, resulting in thinner collagen fibrils.

Decorin, biglycan, and fibromodulin bind to active TGF-β1, β2 and β3 via their core proteins, with both high-affinity and low-affinity binding sites (Hildebrand et al., 1994). The chondroitin/dermatan sulfate chains of decorin and biglycan appear to somewhat hinder this binding, making TGF-β binding to fibromodulin more effective than binding to decorin or biglycan within the tissue. This binding was specific for TGF-ß out of several other growth factors tested and suggests an important role for these small proteoglycans in regulating TGF-β capacity to induce the synthesis of matrix components. Studies by Markmann et al., (2000) suggest that decorin must be bound to a matrix to sequester and inactivate TGF-β.
In turn, TGF-β contributes to the regulation of the synthesis of the small proteogylicans. Several investigators have reported that TGF-βs increase the synthesis of small proteogylicans in cultures of isolated chondrocytes. It has also been reported that there is a decorin decrease in mRNA in chondrocytes cultured with TGF-β in monolayers (Roughley et al., 1994) and that TGF-β inhibited decorin synthesis in chondrocyte monolayers, but not in chondrocytes in alginate beads (Demoor-Fossard, 1998). There is no information about the effect of TGF-β on fibromodulin in chondrocytes, with the exception of preliminary data showing two fold increases in fibromodulin at the RNA and protein level in chondrocytes cultured in alginate beads (Burton-Wurster, 2003).

2.3.2. *Hyalectans proteoglycans*

A common feature of these proteoglycans is their tridomain structure: an N-terminal domain that binds to hyaluronan, a central domain that carries the glycosaminoglycan side chains, and a C-terminal region that binds to lectins. Alternate exon usage occurs extensively, and various degrees of glycanation and glycosylation make these proteoglycans appropriate bridges between surfaces and extracellular matrices. This family currently contains four distinct genes: versican, aggrecan, neurocan and brevican.

2.3.3. *Basement membrane proteoglycans*

Basement membranes are thin, cell-associated sheets of multimodular molecules, most of which interact with each other to form a barrier of charged molecules, thereby acting as true biological filters. Basement membranes are biochemically complex and heterogeneous structures containing laminin, collagen type IV, nidogen, and at least one type of proteoglycan. Three proteoglycans are characteristically present in vascular and epithelial basement membranes of mammalian organisms:

perlecan, agrin, and bamacan (Table 8). The first two primarily carry heparan sulfate side chains, whereas the latter primarily carries chondroitin sulfate. The chimeric structural design of these proteoglycans suggests that they may be involved in numerous biological processes. It is unclear why only these three seemingly diverse molecules are associated with the basement membranes (Iozzo, 1998).

3. CELL ADHESION MOLECULES

The overall architecture of a tissue is determined by adhesion mechanisms that involve not only cell-cell interactions but also cell-matrix interactions. The interface between cells and ECM molecules is dependent upon cell adhesion molecules (CAM). CAM are cell surface macromolecules that dictate cell-to-cell and cell-to-ECM interactions, thereby mediating the processes of adhesion, migration, differentiation, and intracellular signaling (Gumbiner, 1996). This basic function plays a critical role in normal processes, such as embryonic development and tissue morphogenesis, as well as in pathological processes such as tumor cell invasion and metastasis, thrombosis, and inflammation (Hynes and Lander, 1992). These cellular interactions with the ECM are highly regulated, in part, by a family of

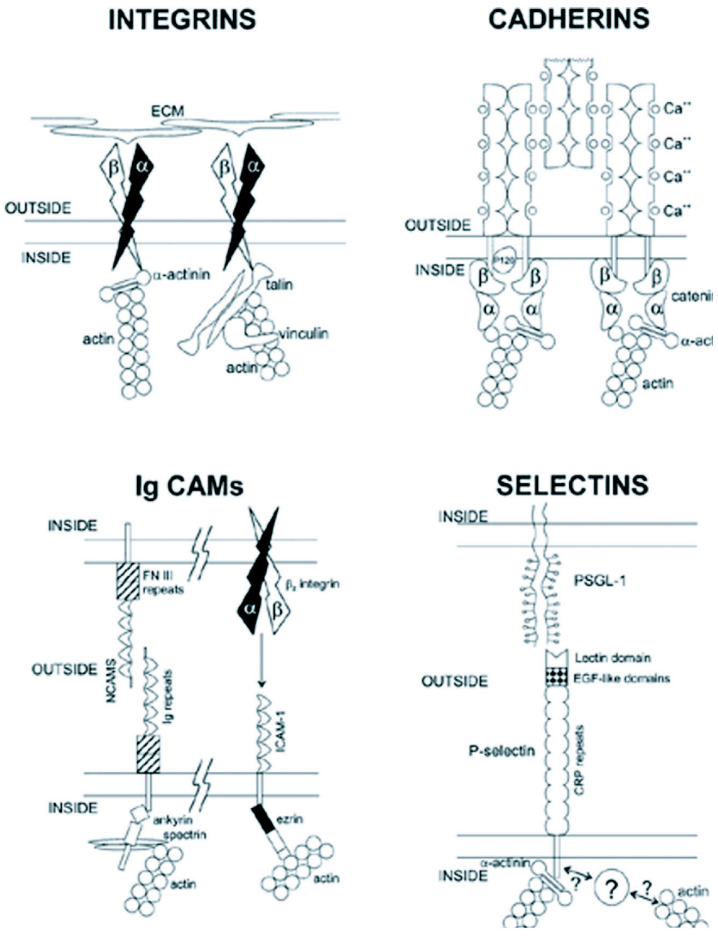

Figure 8. Cell adhesion receptors and associated cytoskeletal components. Integrins, cadherins, selectins and Ig CAM cell adhesion receptors are depicted in association with their typical extracellular ligands and bound to the proteins that link them to the actin cytoskeleton.

catabolic enzymes that selectively degrade ECM proteins, referred to as matrix metalloproteinases (MMPs) (Chen and Wang, 1999). Collectively, these molecules and remodeling enzymes create an environment of chemoattractant and chemorepulsant cues. The proteinase activity targets the extracellular domain of CAM, whereas the intracellular domains interact with cyctoskeletal components. The interactions of cells with neighboring cells and the surrounding ECM are mediated by different classes of cell-adhesion receptors, including integrins, cadherins, and members of the immunoglobulin and selectin families (Figure 8). Integrins primarily mediate interactions of cells with components of the ECM (Hynes, 1999). Following adhesion and clustering, integrins recruit different cytoskeletal and cytoplasmic proteins, which anchor the newly formed complexes to the actin cytoskeleton. This ultimately leads to the local remodeling of the actin cytoskeleton and the formation of specialized adhesive structures, called focal adhesions. In addition to forming a structural link between the ECM and the actin cytoskeleton, focal adhesions are also important sites of signal transduction.

3.1. Integrins

The *integrin* terminology was first applied in a 1987 review article (Hynes, 1987) to describe a family of structurally, immunochemically, and functionally related cell-surface receptors, which *integrated* the ECM with the intracellular cytoskeleton to mediate cell migration and adhesion. Integrins are a large family of heterodimeric cell-surface receptors, composed of an α- and β-subunit, which bind to a wide variety of ECM and cell-surface ligands (Hynes, 1992). The three original β subunits (β1, β2, and β3) identified have now expanded to eight, and the number of α subunits stands at 18. In the recent survey of the human genome, 24 α- and 9 β-subunits have been identified (Venter et al., 2001). However, their existence is not yet firmly established. The 18 α- and 8 β-subunits interact noncovalently in a restricted manner to form 24 different heterodimers (Plow et al., 2000, Hynes, 1992; Table 9). Each of the 24 integrins appears to have a specific, nonredundant function. In part, this is evident from the details of their ligand specificities but also is shown by the phenotypes of knockout mice (Hynes, 2002).

Table 9. The integrin receptor family. The table depicts the mammalian subunit and their αβ associations.

Subunits		Spectrum of ECM protein ligands
β1	α1	Collagen, laminin
	α2	Collagen, laminin, tenascin
	α3a	Collagen, laminin, fibronectin, entactin
	α4	Fibronectin, VCAM-1
	α5	Fibronectin
	α6a	Laminin
	α7	Laminin?
	α8	Basal lamina
	αv	Fibronectin
β2	αL	ICAM-1, ICAM-2, ICAM-3
	αM	C3b component of complement, fibrinogen, factor X, ICAM-1
	αX	Fibrinogen, C3b component of complement
β3	αIIb	Fibrinogen, fibronectin, vitronectin, vWF, thrombospondin
	αv	Fibrinogen, fibronectin, vitronectin, vWF, thrombospondin, osteopondin, collagen
β4	α6	Laminin
β5	αv	Vitronectin, osteopondin
β6	αv	Fibronectin, tenascin
β7	α4	Fibronectin, VCAM-1, Mad-CAM-1
	αE	E-cadherin
β8	αv	Vitronectin

Integrins are Ca^{2+}-dependent adhesive molecules that form a large family of proteins involved in cell-to-cell and cell-to-ECM contacts (Jones, 1996; Hynes. 1992). Integrin receptors are transmembrane proteins that are widely distributed (Figure 8). The extracellular portion of integrins interacts with ECM molecules such as fibronectin, laminin, vitronectin, and tenascin (Plow et al., 2000; Perris, 1997; Varnum-Finney et al., 1995), as well as with various plasma proteins, cell-surface proteins, and microbial pathogens (Sugimori et al., 1997). The intracellular portion makes contact with actin cytoskeleton via intermediate proteins such as α-actinin, talin, tensin, and vinculin. Thus, the binding of a specific ligand to a particular integrin receptor permits a link between the ECM and the actin cytoskeleton through these intermediate proteins, that in turn, trigger intracellular signaling pathways that mediate changes in cellular shape, motility, growth, gene regulation, and apoptosis (Miranti and Brugge, 2002; Frisch and Screaton, 2001; Giancotti and Rouslahti; 1999; Frisch and Ruoslahti, 1997).

3.1.1. *The integrin family*

Different α and β subunits are expressed in limited combinations (Rupp and Little, 2001) and exhibit different ligand specificities (Table 9). The integrins that a given cell expresses therefore control the repertoire of ECM components with which the cell can interact. Integrins bind to ligands in a manner that is dependent upon both affinity and avidity and is influenced by ligand conformation and the capacity to anchor and array (multimerize) within the pre-existing ECM. Thus, different ligands, or different forms of a particular ligand, can transmit distinct signals to a cell through the same integrin (Geiger et al., 2001; Koo et al., 2002; Stupack et al., 1999). Because ECM components may be recognized by more than one integrin, competitive or cooperative binding among different integrin heterodimers adds an additional layer of complexity to cellular responses to the ECM. A further level of complexity is introduced by the existence of alternative splicing.

3.1.2. *The β integrin subunit*

Each β integrin subunit consists of a large extracellular domain that mediates cell-matrix and cell-cell contacts, a transmembrane domain, and a cytoplasmic domain associated with the cytoskeleton. Integrins are capable of transducing information in a bidirectional manner, and the β subunit is now recognized as playing an important role in this process (Green et al., 1998). Most β integrins have relatively small cytoplasmic domains consisting of fewer than 60 amino acids and do not contain catalytic or consensus protein-protein-binding motifs present in various β subunits. An exception is the cytoplasmic domain of the β4, which is approximately 1,000 amino acids long and contains two pairs of fibronectin type III repeats separated by a connecting segment. The cytoplasmic domain of β5 is structurally and functionally unique with regard to other integrin subunits (Table 10) and shares only 38% homology to the cytoplasmic domain of β1 or β3 (Hemler et al., 1994).

It has been proposed that the structural requirements for association with αv prevented further primary sequence divergence between β3 and β5. Although both αvβ3 and αvβ5 integrins bind to vitronectin, they probably mediate different postligand-binding events. For instance, in the absence of exogenous soluble factors, the integrin

Table 10. Alignment of similar β subunit cytoplasmic domains. The main differences between the β3 and β5 cytoplasmic are underlined.

ß1	H D R R E F A K F E K E K M N A K W D T G E N P I Y K S A V T T V V N P K Y E G K
ß2	S D L R E Y R R F E K E K L K S Q W N N - D N P L F K S A T T T V M N P K F A E S
ß3	H D R K E F A K F E E E R A R A K W D T A N N P L Y K E A T S T F T N I T Y R G
ß5	H D R R E F A K F Q S E R S R A R Y E M A S N P L Y R K P I S T H T V D F T F N K S Y N G T V D

αvβ5 fails to promote cell adhesion, spreading and migration. On the other hand, the αvβ3 integrin can induce such events without additional stimulation by cytokines (Klemke et al., 1994; Lewis et al., 1996; Friedlander et al., 1995).

Although many integrins can bind FN (Table 9), the α5β1 integrin is the major FN receptor on most cells. This integrin mediates such cellular responses to FN substrates as adhesion, migration, assembly of ECM, and signal transduction. Integrin ligands, such as FN, are not passive adhesive molecules but are active participants in the cell adhesive process that leads to signal transduction. Fibronectin is the best-characterized integrin ligand is. The biological function of the central cell-adhesive region of FN requires two critical amino acid sequences -an Arg-Gly-Asp (RGD) sequence and a Pro-His-Ser-Arg-Asn (PHSRN) sequence, which function in synergy- for optimal binding to the integrin. The β1, β3, and β5 integrin intracellular domains are sufficient to initiate signal-transduction pathways. Furthermore, alternative splicing can regulate the ability of β integrin intracellular domains to participate in signal transduction.

3.1.3. *Integrin function*

Integrins were initially shown to function only as cell-matrix adhesion molecules (Tamkun et al., 1986), but it has become well accepted that they are important signal transducers (Giancotti and Ruoslahti, 1999; Shattil and Ginsberg, 1997; Clark and Brugge, 1995). Integrins can signal through the cell membrane in either direction: the extracellular binding activity of integrins is regulated from the inside of the cell (inside-out signaling), whereas the binding of the ECM elicits signals that are transmitted into the cell (outside-in signaling) (Clark and Brugge, 1995; Howe et al., 1998). Integrins undergo dynamic changes during the ligand binding process, including relative movements of subunits and conformational changes within domains (Humphries and Newman, 1998; Loftus and Liddington, 1997). Because the integrins themselves do not possess enzymatic activity, to signal they must trigger downstream molecules (Giancotti and Ruoslahti, 1999; Clark and Brugge, 1995; Schoen-waelder and Burridge, 1999). Examples include activation of tyrosine kinases such as pp125 focal adhesion kinase (FAK) or small GTPases such as Rho or Rac and regulation of cytoskeletal components such as talin, paxillin, or p130CAS. Integrin β chains are sufficient to activate downstream signaling molecules, such as FAK, and can regulate cell-cycle progression and actin cytoskeleton assembly. β tails are thus necessary and sufficient to correct subcellular localization of integrins and to activate signaling pathways, and regulate the affinity of integrins for their ligands. The β integrin cytoplasmic domain binds numerous molecules, which are subunit specific. Thus, β1A binds to FAK, paxillin, α-actin, Talin, ICAP, myosin, ILK, Grb2, Shc, melusin, and skelemin. The β2 subunit interacts with ILK and cytohesin-1. Molecules that bind to β3 tail are: β3-endonexin, ICAP, ILK, myosin and Shc (Liu et al., 2000). To date, the theta-associated protein 20 (TAP 20) (Liu et al., 2000) is the only molecule known to interact exclusively with the β5 cytoplasmic domain.

ECM-integrin interactions function in a bidirectional manner across cell membranes. In this regard, they can influence a wide range of activities including alterations in cell morphology, migration, proliferation, differentiation, and survival; gene expression; suppression of tumorigenicity; changes in intracellular pH; or concentration of cytosolic Ca^{2+} (Figure 9).

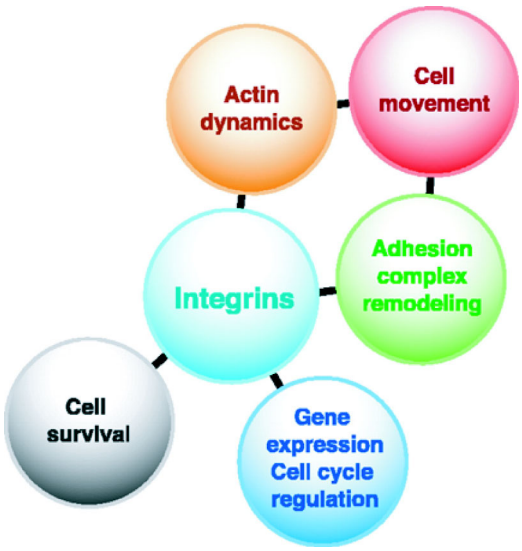

Figure 9. Signal-transduction pathways emanating from integrins (blue) regulate numerous cellular processes, including actin organization (orange) and adhesion complex remodeling (green), which together influence cell movement (red), as well as gene expression and cell-cycle regulation (turquoise) and cell survival (gray).

In addition to their normal cellular function integrins also play a role in the pathogenesis of many major human disease such as: inflammation, thrombosis, metastasis, osteoporosis and infection (Green et al., 1998).

3.1.3.1. ***Integrins in migration***

Cellular migration and invasion are governed at both the extracellular and intracellular levels by several factors, and depend on the cell's carefully balanced dynamic interaction with the ECM. During migration, cells are constantly making and breaking contact; cells project lamellipodia (a broad membrane projection at the leading edge of the cell in the direction of movement) that attach to the ECM, and simultaneously break existing ECM contacts at their trailing edge. This allows the cell to pull itself forward (Sheetz, 1999). Extension of lamellipodia is induced by actin polymerization and facilitated by a localized decrease in membrane tension (Raucher and Sheetz, 2002). Retraction of the cell edge is dependent on the adhesive environment and occurs either by fracturing the cell-ECM linkage in highly adhesive environments (during slow migration) or by simple dissociation of integrins in less adhesive environments (during fast migration) (Palecek et al., 1998; Palecek et al., 1999). During invasion, cells release proteases that degrade and remodel the ECM, promoting cell passage through the stroma and entrance into new tissue. This proteolytic process must be tightly controlled, such that the ECM is sufficiently degraded to facilitate cell passage, but not so degraded that cellular traction is lost.

3.1.3.2. ***Integrins in cell adhesion***

Cell adhesion is one of the defining features of multicellular organisms and underlies the organization of cells into discrete tissues and organs during metazoan development. Adherent cells in culture form specialized structures, termed focal adhesions or focal contacts, at sites of close contact between the plasma membrane and the underlying ECM at which integrins, signaling and cytoskeletal molecules are localized. Adhesion to ECM molecules through integrin heterodimers is a fundamental requirement for cells to acquire the traction necessary for movement. Integrins mediate stable adhesion of cells to their substrate by providing a physical link between the ECM and the cytoskeleton (Hynes, 1992). Interestingly, the maximum rate of cell migration occurs at intermediate levels of adhesiveness (Duband et al., 1991; Palecek et al., 1997). At low levels of adhesiveness, weakly attached cells cannot generate sufficient traction to move efficiently. At high levels of adhesiveness, cells cannot break contact and are therefore immobile. Intermediate levels of adhesion allow traction at the cell front while releasing contacts at the rear, resulting in net forward movement. Integrins not only send signals to the cell in response to the extracellular environment, but they also respond to intracellular cues and alter the way they interact with the extracellular environment (Brown and Hogg, 1996). “Inside-out” signaling regulates integrin adhesiveness by modulating the affinity and avidity of integrins for their ECM ligands, regulating cell invasion and migration.

Integrin-mediated adhesion and signaling regulate a variety of cell functions, including leukocyte homing and activation, bone resorption, clot retraction, cell response to mechanical stress, programmed cell death, tumor cell growth and metastases (Clark and Brugge, 1995). Integrin (α and β) adhesiveness to ECM components is regulated by altering the integrin's affinity for its ECM ligands and/or by altering the integrin's avidity. Small GTP-binding proteins seem to have a prevalent role in these processes. The activation of the small GTP-binding protein R-RAS can lead to increases in integrin affinity for the ECM, whereas H-RAS activation can lead to decreases in integrin affinity (no ECM binding). Similarly, activation of the small GTP-binding proteins RAC and CDC42, and protein kinase C (PKC), can lead to clustering of integrins, thereby increasing integrin avidity. The careful regulation of each of these events is necessary for efficient cell migration.

3.1.3.3. ***Integrins in intracellular signaling***

Integrins are essential for cell migration and invasion, not only because they directly mediate adhesion to the ECM, but also because they regulate intracellular signaling pathways that control cytoskeletal organization, and force generation and survival. During migration, cells are constantly making and breaking integrin contacts. Depending on the composition of the ECM, integrins activate one or more intracellular signaling pathways. These pathways typically involve phosphorylation of FAK,

recruitment of adaptor proteins, activation of small GTPases and subsequent activation of downstream effector molecules (Figure 10).

Integrin ligation induces a complex network of signaling pathways to control cell migration. Integrin (α and β) binding to ligands in the ECM activates FAK, which binds and activates multiple signaling proteins. FAK autophosphorylation at tyrosine 925 causes it to bind growth-factor-receptor-bound protein 2 (Grb2) (Schlaepfer et al., 1994) and activate another small G protein, Ras. FAK activation also promotes Src-dependent phosphorylation of shc (Schlaepfer and Hunter, 1997), leading to Grb2 recruitment and Ras activation.

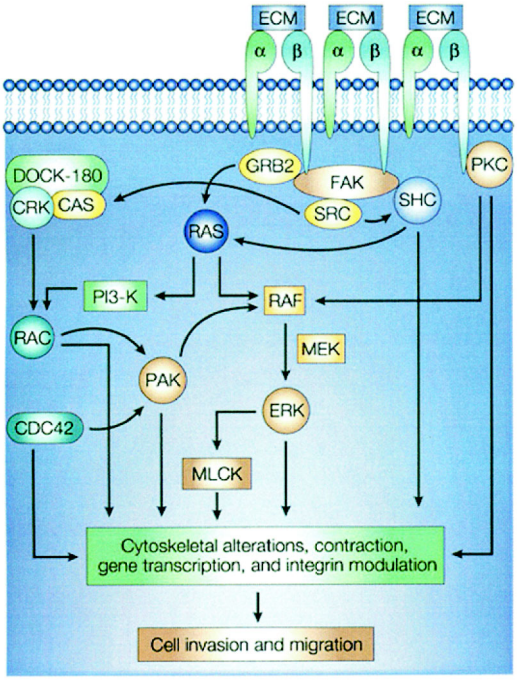

Figure 10. Regulation of intracellular signaling by integrins

Activated Ras recruits Raf to the cytoplasmic membrane, where it can be activated by protein kinases such as Src (Fabian et al., 1993), thereby leading to mitogen-activated protein kinase kinase (MEK) and extracellular-signal-regulated kinase (ERK) activation. Once activated by FAK or shc, Ras can activate phosphatidylinositol 3-kinase (PI3K) and RAF. Activated Src can also phosphorylate CRK-associated substrate (CAS), enabling it to bind CRK, dedicator of cytokinesis 180 (DOCK-180), leading to Rac activation. Activated Rac, in conjunction with activated Cdc42, can regulate numerous biochemical pathways, including activation of p21-activated kinase (PAK). PAK affects numerous pathways and also activates Raf's kinase activity. MEK, once activated by Ras and Raf, can phosphorylate and activate ERK. ERK activation leads to transcriptional activity, alterations in integrin affinity for ligand, and myosin-light-chain kinase (MLCK) activity. Independent of FAK activation, signaling molecules such as SHC and PKC are also activated by integrin adhesion events. Activating these molecules can also eventually lead to Ras and Raf activation, along with the alterations in the cytoskeleton that are necessary for the migratory phenotype.

These signals, in concert with signals derived from growth factors, regulate cell behavior in a complex tissue microenvironment.

3.1.3.4. *Integrins in apoptosis*

Finally, we must consider the potential role of integrins as regulators of apoptosis; there are several very distinct versions of this class of models. In the classical view (Fig. 11a), for which there is a great deal of evidence, integrin ligation provides a survival signal (through the PI3 kinase–Akt pathway), and cells are dependent for survival on anchorage (Meredithet et al., 1993). In this view, when cells lose their integrin-mediated adhesion they undergo apoptosis, called anoikis (Frisch and Screaton, 2001) Indeed, in the early experiments that showed LM609 or RGD-based peptides as inhibiting angiogenesis, they induced apoptosis of ECs and this was interpreted as anoikis (Brooks et al., 1994).

Later work showed that RGD-based peptides could induce apoptosis by directly activating caspases, without any involvement (positive or negative) of integrins (Figure 11b) (Buckley et al., 1999; Adderley and Fitzgerald, 2000). Lately, a third model has been described suggesting that unligated integrins promote apoptosis by directly recruiting caspase-8 (Stupack et al., 2001) (Figure 11c) named "integrin-mediated death". These investigators suggest that when inhibitors block αvβ3, unoccupied αvβ3 triggers apoptosis of endothelial cells (Stupack et al., 2001; Cheresh and Stupack, 2002).

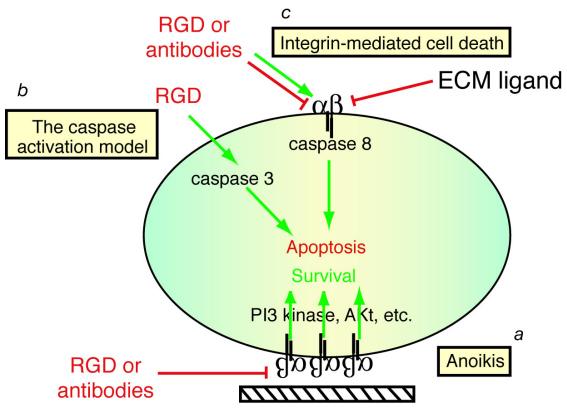

Figure 11. Three models for endothelial apoptosis. ***a***, The classical model, in which integrin engagement by ligand is necessary to provide survival signals. Inhibitors block ligand binding and thus the survival signal. ***b***, The caspase activation model, in which RGD peptides directly activate caspases and trigger apoptosis without any involvement of integrins. ***c***, The unligation model, or "integrin-mediated cell death", in which unligated integrins directly bind and activate caspase-8.

3.2. Cadherins

Cadherins are a family of transmembrane glyco-proteins that mediate calcium-dependent cell-cell adhesion. The adhesion properties (the hemophilic nature activity, the high specificity of the interaction and the tissue specificity of the expression) reveal that various cadherins are involved in the mediation of specific cell-cell adhesion and play a pivotal role in the formation and maintenance of tissues (Suzuki, 1996; Chothia and Jones, 1997). Many cadherins and cadherins-related proteins have been identified in various tissues of different organisms; they are also involved in embryonic morphogenesis and the formation of stable connections among cells in solid tissues and epithelial cell layer (Suzuki, 1996; Chothia and Jones, 1997). Members of the cadherin family can be divided into four subgroups: classic cadherins, desmosomal cadherins, protocadherins, and atypical cadherins. Many tissue-specific cadherins have been identified: these include epithelial (E)-cadherin, neuronal (N)-cadherin, placental (P)-cadherin, vascular endothelial (VE)-cadherin and others (Vleminckx and Kemler, 1999). Proteins are designated as members of the broadly defined cadherins family if they have one or more cadherin repeats, each one consisting of approximately 110 amino acids and containing amino-acid motifs with the conserved sequences LDRE, DXNDN, and DXD (Oda et al., 1994). Regulation of cell–cell adhesion by VE-cadherins is critical during angiogenesis. For example, VE-cadherins mediate endothelial barrier function, and angiogenesis and can also support cross-talk with VEGF receptors (Corada et al., 2001; Liao et al., 2000).

Cadherins serve as homophilic-adhesive molecules revealing a cell-adhesion preference for other cells that express the identical cadherin family member. This feature serves to segregate cells into distinct tissues during development (Takeichi, 1991; Takeichi, 1995). Each form of cadherin shares the characteristic of five homologous extracellular domains (Figure 8), a single membrane-spanning segment, and a cytoplasmic unit attached via catenins to cytoskeletal actin microfilaments (Gumbiner and McCrea, 1993; Yap et al., 1997). The specificity of these homophilic interactions appears to be mediated by the first extracellular domain, at least in the case of E- and P-cadherin. N- and R- (retinal) cadherin can also execute heterophilic interactions, although homophilic is preferred. N- and R-cadherin possess a nearly identical amino-acid sequence in the region implicated in homophilic-binding specificity. This observation suggests that the first extracellular domain contains the binding site necessary for "specific homophilic adhesion" (Wright et al., 2002).

The intracellular portion of the cadherin molecules interacts with cytoskeletal proteins and signal-transduction pathways primarily via α- and β-catenin (Mao et al., 2001; Kintner, 1982; Nagafuchi and Takeichi, 1988). Many studies have confirmed the importance of the cytoplasmic tail, and in particular, the catenin-binding site, for adhesion. Removal of this binding site prevents cadherin-mediated adhesion in cultured cells (Ozawa et al., 1990). Thus, this pathway appears to have a profound influence on the regulation of cell adhesion.

3.3. Immunoglobulin superfamily adhesive molecules

The immunoglobulin (Ig) super family of CAMs is Ca^{2+}-independent cell-adhesion molecules and comprise a large variety of glycoproteins mostly expressed at the cell surface. Typically, Ig CAMs have a large amino-terminal extracellular domain containing the Ig folds, a single transmembrane helical segment, and a cytoplasmic tail (Aplin et al.,

1998). The extracellular domain of these molecules is made up of a number of Ig motifs and FN-III repetitions (Gennarini et al., 1989). Thus, the members of this category differ with respect to the number of Ig and FN repeats in their structures (Figure 8). Many of these molecules evidence several isoforms with specific functions and patterns of expression. The characteristics of attachment to the cell membrane, and the length of the intracellular domain, largely determine the functional consequences of each member of this subfamily. Molecules carrying Ig-like domains are endowed with different functions, including cytoskeletal organization, endocytosis, adhesion, migration, growth control, immune recognition, viral receptors, inflammatory reactions, and tumor progression. Several members of the Ig superfamily, namely, ICAMs, VCAM-1, MadCAM-1, PECAM-1 and CD2, are found to mediate the adhesion events of the immune system.

One of the most important contexts for Ig CAMs is the developing nervous system, where many different members of this superfamily are involved in axon guidance and in the establishment and maintenance of neural connections (Murase and Schuman, 1999). The classic example of a neural Ig superfamily adhesion receptor is NCAM, which contains five Ig folds in its extracellular portion (Crossin and Krushel, 2000). NCAM functions as a homotypic, calcium-independent cell-cell adhesion receptor; however, the precise mechanism of NCAM-mediated cell-cell interaction remains controversial. Another group of Ig CAMs important in neural development are the netrin receptors, such as deleted in colon carcinoma (DCC), that interact with laminin-like netrins in the ECM and provide specific guidance cues to migrating axons (Culotti and Mer, 1998). Yet another group of key Ig CAM receptors involved in neural development is the dozen or so members of the Eph subfamily of transmembrane tyrosine kinases that bind their cognate ligands (ephrins) on neighboring cells (Bruckner and Klein, 1998). Thus, Ig CAMs can be involved in either homotypic (NCAM) or heterotypic (DCC, Eph kinases) adhesive interactions.

3.4. Selectins

The selectins were cloned in 1989 and represent a new family of CAMs primarily involved in the regulation of leukocytes (Kansas, 1996). They are a group of type I transmembrane glycoproteins with an extracellular region consisting of an amino-terminal lectin domain configured to bind carbohydrate ligands. They also possess a single growth-factor-like domain, two to nine short consensuses repeat domains, and a single membrane-spanning region with a cytoplasmic tail. Selectin molecules appear to regulate early leukocyte-EC interactions, especially the tethering and rolling of leukocytes along the venular wall (Lee et al., 1995). At present, this family consists of three molecules: 1) L-selectin which is expressed on nearly all blood neutrophils and monocytes, on some natural killer cells, and on many blood-borne T and B cells; 2) E-selectin expression is limited to endothelium, especially endothelium responding to inflammatory stimuli such as IL-1, TNF-α, and bacterial lipopolysaccharide (Bevilacqua et al., 1989; Springer, 1995; Hartwell and Wagner, 1999); 3) P-selectin is expressed on both endothelium and platelets but in a stored preformed state in secretory storage granules located within α-granules and Weibel-Palade bodies (McEver et al., 1989). P-selectin is quickly expressed at the cell surface upon fusion, initiated by agonists including thrombin, histamine, and activators of PKC. In addition to this fusion-initiated expression of P-selectin at the cell surface, P-selectin expression on endothelium is also transcriptionally regulated (Yang et al., 1999; Gotsch et al., 1994; Sanders et al., 1992). P-selectin gene transcription can be induced by IL-1, TNF-, or bacterial L-polysaccharide with a latency of 2-4 hours. Thus, P-selectin can be expressed during both the early and late stages of an inflammatory response (Kansas, 1996). Although all three selectins are involved in the mediation of rolling of leukocytes along the vessel wall near the point of injury, different selectins appear to be active at different times during an inflammatory response.

4. LIFE AND DEATH OF CELLS

Proper balance of cell survival and cell death in the organism is essential for physiological processes that play a critical role in controlling the number of cells in development, and functioning of the immune system, and throughout an organism's life by removing cells at the appropriate time. One feature of the immune system is the production of a large amount of cells. As approximately 10^{11} to 10^{12} cells are produced every day in healthy adult humans, this rapid proliferation needs to be balanced by apoptosis

to maintain a constant cell number. Changing this balance in either direction has pathological consequences. An abnormally high rate of cell death is found in neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, AIDS and cardiovascular diseases; whereas delayed cell death contributes to a wide variety of human cancers (Thompson, 1995). Numerous cell-death mechanisms are tissue specific and cell type specific. These are regulated by a large number of extracellular and intracellular signals governed by the environment of the cell. The crucial role of cell death in many diseases has spurred intense research to understand the regulation of apoptotic pathways.

The life-and-death decision at the cellular level is controlled by environmental cues, including death-receptor (DR) ligands (Ashkenazi and Dixit, 1999) and growth factors (Dvorak et al., 1995; Eliceiri, 2001), as well as physical stimuli such as mechanical stress (Ingber, 1992), or radiation (Wahl and Carr, 2001). This decision is profoundly influenced by the components of the ECM, which can change dynamically during differentiation, development and other tissue-remodeling events (Figure 12). Cell-adhesion receptors and new ECM proteins, deposited from intracellular stores or synthesized de novo (Brown et al., 1993; Petersen et al., 1998), interact with both pre-existing and proteolytically exposed sites in the assembled ECM (Davis, 1992; Xu et al., 2001).

The ongoing remodeling presents a constantly changing environment, contrasting with the static ECM in "resting" tissues, and presents new information to cells that governs their behavior. The principal adhesion receptors that convey this information are the integrins. In addition, complex regulatory systems are required to form and maintain the array of tissue architectures present in multicellular organisms. Among these, programmed cell death is crucial for developmental and physiological processes, including morphogenesis, wound repair and tissue differentiation. Inappropriately triggered cell death can alter tissue structure or function, compromising embryonic viability (Ranger et al., 2001). Conversely, the failure of cells to undergo apoptosis can result in a range of effects from embryonic death to neoplasia (Lee and Bernstein, 1995; Ranger et al., 2001; Reed, 1999) or autoimmune disease (Eguchi, 2001; Moulian and Berrih-Aknin, 1998).

4.1. Apoptosis

Apoptosis was first described by Kerr et al. (1972), and is defined by the morphological appearance of the dying cell, which includes membrane blebbing, chromatin condensation, nuclear fragmentation, loss of adhesion and rounding (in adherent cells), and cell shrinkage. Biochemical features associated with apoptosis include internucleosomal cleavage of DNA, leading to an

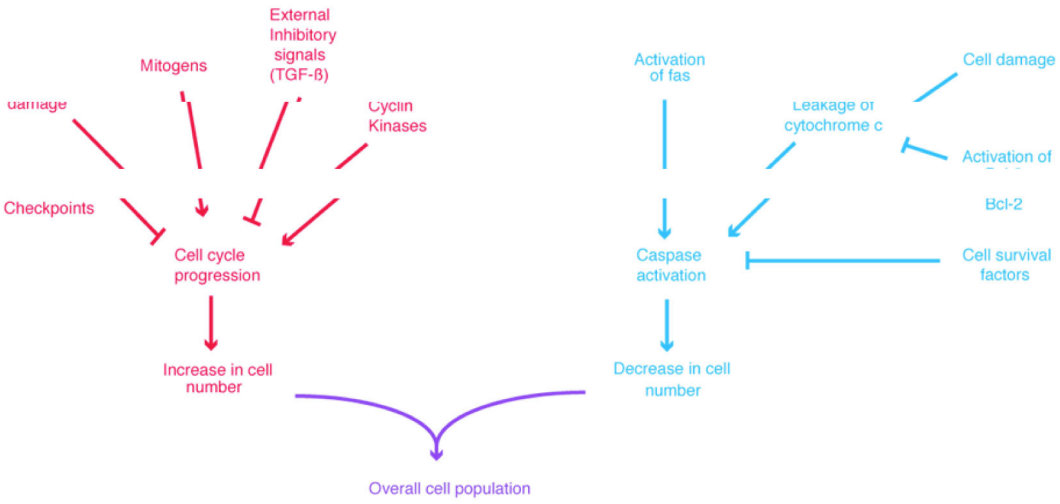

Figure 12. Balance of cell proliferation and cell death. The inputs into control of cell number.

oligonucleosomal "ladder" (Cohen et al., 1994); phosphatidylserine externalization (Martin et al., 1995b); and proteolytic cleavage of a number of intracellular substrates (Martin & Green, 1995). The appearance of phosphatidylserine on the outside of the plasma membrane was assumed to indicate commitment to cell death. It may facilitate recognition by phagocytes, subsequent phagocytosis and degradation of the apoptotic cell. This can be visualized by the binding of fluorescein-labeled annexin V. As a result of the plasma membrane changes, apoptotic cells are rapidly phagocytosed prior to the release of intracellular contents and without induction of an inflammatory response. This is not to be confused with necrosis, where acute environmental changes induce cell swelling and lysis, which are followed by an acute inflammatory response.

Mechanisms that transfer the death signal to the cell's apoptotic machinery fall into two broad categories: 1) the ligation of a death-receptor leads to the recruitment of the adapter protein FADD and procaspase-8, which becomes cleaved and activated at the receptor complex, initiating caspase cascade (Figure 13 Left), and 2) the mitochondria/apoptosome pathway (Figure 13 right) is triggered by a number of apoptotic stimuli. The intracellular protein interactions triggered by the death-inducing receptors can be attributed to two structural motifs within the proteins concerned. Both motifs are able to associate with homologous regions in other proteins, and thus prompt binding of such proteins to one another (Wallach, 1997). One motif, the "death domain" (DD), is found in several death-inducing receptors of the TNF family, including CD95 (also called Fas or Apo-1), CD120a (the p55 TNF receptor) and others. It also occurs in several cytoplasmic adaptor proteins that bind through this domain both to receptors and to each other (as, for example, in the MORT-1/FADD and TRADD adaptors). The other motif, the "death effector domain" (DED), is found in MORT-1/FADD upstream of the DD; it also occurs in duplicate in two caspases, caspase 8 (MACH/ FLICE/Mch-5) and caspase 10 (Mch-4/ FLICE-2). Binding of these two caspases to MORT-1/FADD through association of their DED motifs, and consequent activation of the caspases by their proteolytic cleavage (apparently by self-processing), are thought to be critical steps in the initiation of the killing process. The caspases are believed to cause apoptotic phenotype by cleaving or degrading several important substrates, which ultimately results in cell death. An early, not well understood, step is the mitochondrial release of apoptosis-inducing molecules -including cytochrome c, apoptosis-inducing factor (AIF), HtrA2 and Smac/DIABLO- into the cytosol. Initially, cytochrome c, together with dATP, associates with Apaf1. This event unmasks the caspase-recruitment domain (CARD) motif in Apaf1 and allows binding and activation of procaspase-9. Once activated, caspase-9 propagates the apoptotic signal. Positive feedback loops involving Bid, mitochondria, apoptosome, caspase-9, effector caspases and caspase 8 are able to amplify the death signal. The apoptosome pathway is further potentiated by AIF, through augmentation of mitochondrial release of cytochrome c and procaspase-9. Negative modulators of apoptosis such as FLIPs and inhibitors of apoptosis (IAPs) may negatively influence the transmission of the apoptotic signal. Smac/DIABLO and HtrA2 may abolish apoptosis inhibitory action of IAPs.

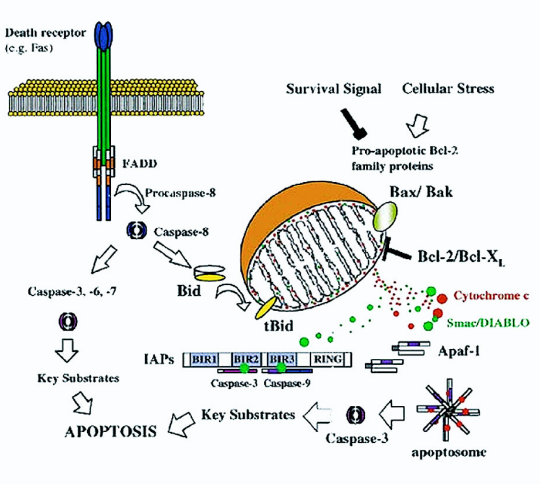

Figure 13. Apoptotic signaling pathways. Cellular stress induces proapoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome *c*. Cytochrome *c* catalyzes the oligomerization of Apaf-1, which recruits and promotes the activation of procaspase-9. This, in turn, activates procaspase-3, leading to apoptosis. Alternatively, the activation of caspase-8 by ligation of the death receptor Fas is illustrated. Ligated Fas recruits FADD to the intracellular region, which in turn recruits procaspase-8. The procaspase-8 transactivates, and the mature caspase now can cleave and activate procaspase-3, leading to apoptosis. Signaling from the Fas receptor to mitochondria involves cleavage of the BH3-only protein, Bid, by caspase-8. Bid subsequently induces cytochrome *c* release and downstream apoptotic events.

4.1.1. *The apoptosis executioners: caspases*

The initiation of programmed cell death can occur in a variety of ways, and the appearance of common morphological effects in cells indicates the presence of common machinery (Green and Evan, 2002; Leblanc, 2003). Caspase activation is central to many, if not all, of the apoptotic pathways. Their ability to proteolytically cleave selected cellular proteins, assures the progress and irreversibility of apoptosis (Slee et al., 1999). Thus far, the caspase gene family contains 14 mammalian members, of which, 11 human enzymes are known. Caspases share similarities in amino-acid sequence, structure, and substrate specificity (Nicho-lson and Thornberry, 1997). They are expressed as proenzymes (zymogens) that contain three domains: an N-terminal prodomain, a large subunit containing the active-site cysteine within a conserved QACXG motif, and a C-terminal small subunit. Caspases are among the most specific proteases, with an unusual and absolute requirement for cleavage after aspartic acid (Asp) residues (Stennicke and Salvesen, 1998). An aspartate cleavage site separates the prodomain from the large subunit, and an interdomain linker containing one or two aspartate cleavage sites separates the large and small subunits. The presence of Asp at the maturation cleavage sites is consistent with the ability of caspases to auto-activate or to be activated by other caspases as part of an amplification cascade.

4.1.2. *Caspase substrates during apoptosis*

The most prevalent caspase in the cell is caspase-3. It is the one ultimately responsible for the majority of the apoptotic effects, although it is supported by two others, caspase-6 and -7. Together, these three executioner caspases presumably cause the apoptotic phenotype by cleavage or degradation of several important substrates. For example, high- and low-molecular weight DNA fragmentation is caused by the action of caspase-3 on a complex of caspase-activated DNase (CAD)/DNA fragmentation factor (DFF) 40, a nuclease, and iCAD/DFF45, its inhibitor (Enari et al., 1998; Liu et al., 1997). In non-apoptotic cells, CAD is present as an inactive complex with iCAD. During apoptosis, caspase-3 cleaves the inhibitor, allowing the nuclease to cut the chromatin. Blebbing is orchestrated via the cleavage and activation of gelsolin (Kothakota et al., 1997), p21-activated kinase-2 (Lee et al., 1997; Rudel and Bokoch, 1997), and most likely through cleavage of fodrin (Martin et al., 1995) to dissociate the plasma membrane from the cytoskeleton. Cleavage of intermediate filaments contributes to disorganize the cytoskeleton (Caulin et al., 1997). The externalization of phosphatydilserine during apoptosis is generally caspase-dependent (Martin et al., 1996), although in some cell types, PS externalization appears to be caspase independent (Vanags et al., 1996).

4.1.3. *Inhibitors of caspases*

The deadly effects of caspase activation can be counteracted in the cell by the presence of caspase inhibitors. A number of proteins that can prevent cell death through caspase activation have been identified, of these, the most well known comprise a family of inhibitors of apoptosis proteins (IAPs) (Salvesen and Duckett, 2002). These were first identified in baculovirus, but subsequently were found in human cells. At least five different mammalian IAPs -X-linked inhibitor of apoptosis (XIAP, hILP), c-IAP1 (HIAP2), c-IAP2 (HIAP1), neuronal IAP, and survivin- exhibit anti-apoptotic activity in cell culture (Roy et al., 1997; Deveraux and Reed, 1999; Deveraux et al., 1998; Liston et al., 1996; Uren et al., 1996; Tamm et al., 1998; and Ambrosini et al., 1997). The spectrum of apoptotic stimuli that is blocked by mammalian IAPs is broad and includes ligands and transducers of the TNF family of receptors, pro-apoptotic members of the ced-9/Bcl-2 family, cytochrome c, and chemotherapeutic agents (LaCasse et al., 1998; Deveraux and Reed, 1999). XIAP appears to have the broadest and strongest antiapoptotic activity. XIAP, c-IAP1, and c-IAP2 are direct caspase inhibitors (Deveraux et al., 1997; Deveraux et al., 1999; Roy; et al., 1997). They all bind to and inhibit active caspase-3 and -7, and also procaspase-9, but not caspase-1, -6, -8, or -10. The binding and inhibition of caspases by IAPs is mediated by the so-called baculovirus IAP repeats (BIR) domain(s) present within the IAP. BIR is a conserved sequence motif of ~70 amino acids that is repeated tandemly in a class of baculovirus proteins (e.g., Op-IAP). All mammalian IAPs have three BIR domains, except survivin, which has only a single BIR. Interestingly, in the case of XIAP, a 136 amino-acid region spanning the middle BIR core was found to be necessary and sufficient for inhibition of

caspase activity (Takahashi et al., 1998). Another region within the IAP, the RING domain, acts as an ubiquitin ligase, promoting the degradation of the IAP itself (Yang et al., 2000) and, presumably, any caspase bound to it. Near the RING finger of both c-IAP1 and c-IAP2 is a CARD domain, suggesting that these IAPs might directly or indirectly regulate the processing of caspases via CARD domain interactions. In this fashion, IAPs put the brakes on the apoptotic process by binding, inhibiting, and perhaps degrading caspases.

4.2. Macrophages in cell survival and apoptosis

Macrophages play an important role in the immune system. They act by means of a number of mechanisms: (a) directly, by destroying bacteria, parasites, viruses and tumor cells; (b) indirectly, by releasing mediators IL-1, TNF-α, etc. which can activate other cells; (c) as accessory cells, by processing antigen and presenting digested peptides to T lymphocytes; and (d) by repairing tissue damage.

Macrophages originate in the bone marrow and migrate to body tissues through blood circulation, and share a common precursor with neutrophils: the GM-CFU. Through the expression of a series of transcription factors, in which PU.1 plays a crucial role, a differentiation process takes place where the M-CSF receptors are expressed, a feature that characterizes macrophages (Klemsz et al., 1990; Valledor et al., 1998; Lloberas et al., 1999).

The bone marrow produces approximately $5x10^9$ macrophages per day, which are released to the circulating blood and exhibit a half-life of 18 hours (Van Furth, 1992). Most of the macrophages that arrive to the tissues disappear, and the few that prevail can exhibit a very long half-life, up to years. In the absence of cytokines or growth factors, most macrophages die by apoptosis (Figure 14). In tissues, a small number of macrophages differentiate under the influence of cytokines and, depending on the tissue type, they may become osteoclasts (bone), Kupffer cells (liver), microglia (brain), etc. Macrophages proliferate in the presence of growth factors, such as GM-CSF, M-CSF or IL-3. Macrophages are critically important in inflammatory reactions and, more specifically, in hypersensitivity reactions. When macrophages are needed at the inflammatory loci, in order to become activated and fully functional, these cells need to interact with IFN-γ, a cytokine released by activated T lymphocytes (Celada and Nathan, 1994) that acts through interaction with the specific receptor (Celada and Schreiber, 1987; Celada et al., 1989; Celada and Maki, 1991). Murine models with an IFN-γ gene that has been inactivated by homologous recombination have shown that this cytokine is necessary for the development of hypersensitivity reactions and the formation of granuloma in response to intracellular microorganisms. Lipopolysaccharide (LPS), a component of the cell membrane of gram-negative bacteria, could induce some macrophage activities.

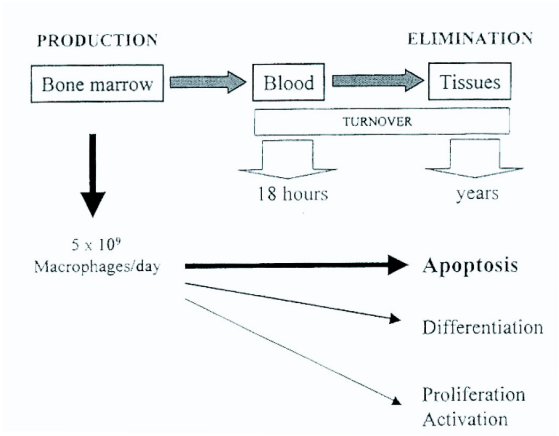

Figure 14. Production versus elimination of macrophages

4.3. Endothelial cells in cell survival and apoptosis

A number of studies have shown that ECs can undergo apoptosis in response to a variety of stimuli, including serum growth-factor withdrawal, Fas activation, and radiation injury (Gratton et al., 2001; Nofer et al., 2001; Suhara et al., 2001). During both normal development and pathology, the formation of new vessels and the regression of pre-existing ones are dependent on the balance between EC proliferation and apoptosis. In mature vessels, EC turnover is also under the control of these tightly regulated phenomena. Since the vascular endothelium is involved in various physiological processes, EC apoptosis (and dysfunction) may constitute an initial step in a variety of pathological situations such as atherosclerosis and hypertension (Nofer et al., 2001). Shed apoptotic membrane particles that are highly procoagulant are found in the blood of patients with acute coronary events (Nofer

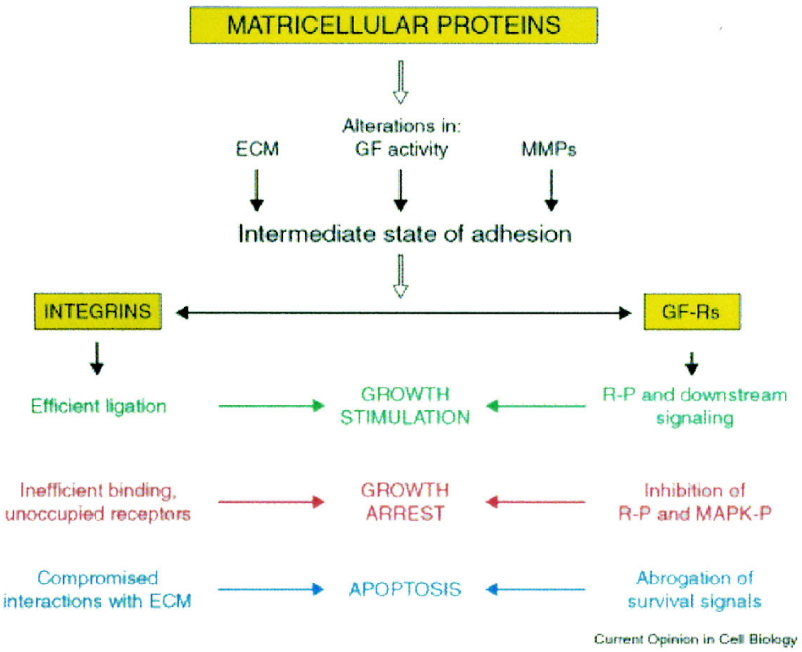

Figure 15. Matricellular proteins affect receptors that mediate both proliferation and adhesion.

et al., 2001; Suhara et al., 2001). As for other cell types, it has been hypothesized that interactions of ECs with their microenvironment may be critical for their survival.

Angiogenesis is strongly dependent on the suppression of EC apoptosis. Many of the proangiogenic growth factors promote the survival of ECs. Both angiogenesis and EC survival also are dependent on the attachment of ECs to the ECM and to cell-cell contacts. Inhibition of growth-factor signaling or adhesion-dependent signaling can induce apoptosis directly and concomitant angiogenesis inhibition. Moreover, a common property of many angiogenic inhibitors is the induction of EC apoptosis. Therefore, the events that induce survival or apoptosis of ECs affect angiogenesis. It is conceivable that ECs integrate exogenous angiogenic and antiangiogenic stimuli and transform them intracellularly into conflicting survival and apoptotic signals. The prevailing signals may determine the fate of the ECs and, subsequently, the fate of the growing vessel. Elucidation of the molecular mechanisms that are involved in EC apoptosis and survival may lead to the development of new therapeutic approaches to enhance angiogenesis in the case of tissue ischemia (e.g., revascularization of ischemic tissue) or to inhibit angiogenesis in the case of neovascularization-dependent disease (e.g., tumor, diabetic retinopathy).

4.4. Cell matrix regulation of endothelial cell survival

Cell-to-cell and cell-to-matrix contacts are necessary for the maintenance of survival of anchorage-dependent cells such as ECs (Brooks et al., 1994; Levkau et al., 1998; Meredith et al., 1993; Re et al., 1994; Scatena et al., 1998). Loss of cell-to-cell contact leads to the activation of a default death programmed in a variety of cell types.

For instance, deficiency or truncation of VE-cadherin mediates adhesion between ECs (Hermiston and Gordon, 1995), which induces endothelial cell apoptosis and abolishes transmission of the endothelial survival signal by VEGF-A to Akt kinase and Bcl2 (Carmeliet et al., 1999). ECs can also enter into apoptosis after growth-factor deprivation and this correlates with cleavage and disassembly of intracellular and extracellular components of

adherens junctions. Caspase-3 appears to initiate proteolytic processing, leading to the cleavage of FAK (Levkau et al., 1998), ß-catenin and plakoglobin and to the shedding of VE-cadherin (Herren et al., 1998), actively interrupting extracellular signals required for EC survival. On the other hand, platelet-endothelial cell-adhesion molecule-1 (PECAM-1) homophilic adhesion rescues ECs from serum deprivation-induced apoptosis, whereas it has no effect on EC migration and proliferation (Bird et al., 1999).

The importance of the interaction of EC with ECM is highlighted by the fact that in absence of any ECM interactions, ECs rapidly undergo apoptosis (Meredith et al., 1993; Frisch and Francis, 1994; Frisch, 2001). Integrins mediate the adhesion of ECs to the ECM proteins (Stromblad and Cheresh, 1996), and this interaction also provides a potent survival signal that is important for angiogenesis (Figure 15). There is also crosstalk between integrin- and growth factor-mediated signaling, which may act synergistically to promote survival.

Various signaling cascades have been considered to mediate the antiapoptotic effect of integrins (Fig L&D-6). The ECM may generate survival signals aiming at the suppression of a p53-regulated cell death pathway. Survival signals from fibronectin are transduced by the FAK, phosphorylation of which leads to p53 inactivation and maintenance of cell survival (Ilic et al., 1998). Interaction with ECM proteins via the integrin αvβ3 inhibits p53 activity, decreases the expression of $p21^{Waf1}$ and Bax (Stromblad et al., 1996), and activates NF-kB (Scatena et al., 1998), therefore promoting cell survival. This is particularly important during migration of ECs in angiogenesis as well as in the maintenance of tumor vasculature (Rüegg et al., 1998).

However, integrins also transmit signals directly through ligation-dependent recruitment of nonreceptor tyrosine kinases from the FAK and Src families, leading to the activation of several major cell-signaling pathways. The consequent downstream signals, especially via the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, are critical for regulation of the cyclin-dependent kinases (cdk) and cell-cycle progression (Schwartz and Assoian, 2001). Since disruption of CDK signaling can result in cell-cycle arrest, leading to apoptosis, and integrin-sustained cell-cycle signaling presents a basic mechanism by which integrins promote cell survival. At the same time as inducing antiapoptotic proteins, integrin-mediated signals block the induction of death by proapoptotic Bcl-2 proteins. In addition to triggering the PI 3-kinase/Akt pathway, integrin-mediated Ras activation also results in the activation of the Raf/Mek/ERK pathway (Giancotti and Ruoslahti, 1999; Schlaepfer et al., 1999).

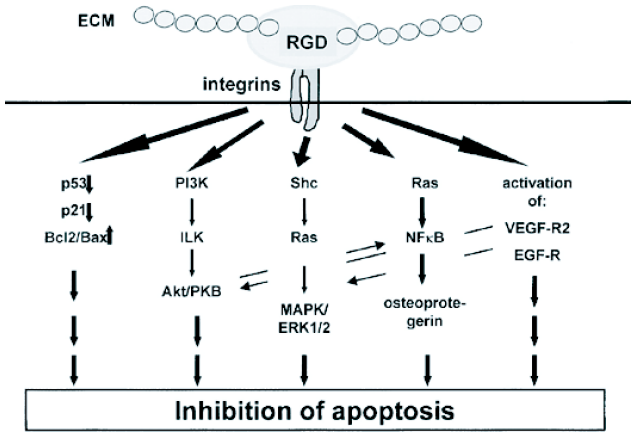

Figure 16. Antiapoptotic signaling pathways activated by integrins. Binding of integrins to ECM promotes EC survival by regulation of the expression of apoptosis-related proteins and activation of protein kinase cascades and of growth-factor receptors (Chavakis E and Dimmeler S, 2002).

The ECM may also generate survival signals aiming at the upregulation of an anti-apoptotic pathway mainly by promoting the anti-apoptotic activity of the Bcl-2 family of proteins. Exposure of human ECs to TNF-α and IFN-γ results in suppression of EC αvβ3 activity, leading to a decreased αvβ3-dependent EC adhesion and survival. *In vivo*, TNF-α and IFN-γ interact with αvβ3 to cause selective disruption of the tumor vasculature in patients with melanoma (Rüegg et al., 1998). Stimulation of human ECs with TNF-α leads to a specific degradation of Bcl-2, a process that is inhibited by specific proteasome inhibitors.

Evidence demonstrates that simple expression of unligated integrin is sufficient to initiate cell death among adherent cells. This "integrin-mediated death" (IMD) was similarly induced by the cytoplasmic domain of β1 or β3 integrins, but not β5, resulting in the recruitment of caspase-8 to the cell membrane

and its subsequent activation (Stupack et al., 2001). These results reveal an unexpected role for integrins as proactive mediators of cell death, and document a novel mechanism for the induction of apoptosis during tissue remodeling and homeostasis. These findings may explain previous observations *in vivo*, where angiogenesis was suppressed in tissues treated with αvβ3 integrin antagonists due to an induction of apoptosis of angiogenic ECs (Brooks et al., 1994b; Stromblad et al., 1996; Storgard et al., 1999).

Finally, angiostatin-induced EC apoptosis (Clae-sson-Welsh et al., 1998; Lucas et al., 1998) parado-xically involves FAK activation (Claesson-Welsh et al., 1998). FAK activation by angiostatin is not associated with Src coactivation and does not involve integrin signaling. In fact, angiostatin probably causes a flawed, integrin-independent activation of FAK that perturbs the ordered turnover of focal adhesion contacts induced by VEGF and FGF-2. Angiostatin-induced endothelial apoptosis may be an important mechanism by which angiostatin modulates the angiogenic process.

5. IDENTIFICATION OF PROTEIN INTERACTING SITES

Understanding protein function is key to know how complex biological systems operate in normal physiological situations and to understanding how these systems are dysregulated in pathological conditions. Identifying the function of a protein can be very difficult to achieve, particularly for novel proteins identified, for instance, by genome-sequencing technologies or by immunological or proteomic approaches. Many clues for functionality can be provided by a careful analysis of the expression patterns of the protein in a variety of circumstances. In a general sense, proteins that directly interact with each other can be expected to participate in the same cellular processes. On this basis, the finding that a protein of unknown function binds to a protein of known function provides a significant clue to the cellular pathway in which the unknown protein participates (Oliver, 2000). A widespread configuration for targeting proteins is that based on direct binding, which consists of immobilizing the target protein on a solid support, mixing the source of possible ligand with or passing it over the immobilized protein and, after a washing step(s), eluting and characterizing it by some other analytical method. The most common form of this analysis is immunoprecipitation (Coligan et al., 1999). Drawbacks of this type of analysis include the possibility of nonspecific binding, a general lack of sensitivity and difficulties of immunoprecipitating noncytosolic proteins complexes or proteins in low abundance.

Phage display is a powerful method for obtaining small-peptide ligands for essentially any protein of interest. In many cases, these binding peptides act as antagonists or even agonists of natural protein functions. This method is accelerating the pace of research by enabling the study of complex protein-protein interactions with simple methods of molecular biology. While most extracellular protein-protein interactions involve large epitopes formed by discontinuous regions of primary sequence, there are numerous intracellular interactions that depend on the specific recognition of small, continuous epitopes within large proteins. Indeed, many distinct families of intracellular protein domains have evolved to recognize linear epitopes with particular characteristics (Pawson, 1995). Proline-rich sequences are recognized by at least two different families: Src homology 3 (SH3) domains (Ren et al., 1993), and WW domains (named for two highly conserved tryptophan residues within the family consensus) (Chen and Sudol, 1995). Another common binding motif consists of phosphotyrosine-containing sequences, which are recognized by Src homology 2 (SH2) (Songyang et al., 1993) and phosphotyrosine-binding (PTB) domains (Kavanaugh and Williams, 1994). PDZ domains (so-called because they were first recognized in the proteins postsynaptic density-95, discs large, and zonula occludens-1) predominantly bind to specific C-terminal sequences (Cowburn, 1997). These and other peptide-binding domains are small compact modules that are usually found imbedded in larger proteins, often with other modules of their own or different types. Acting in concert, multiple modules can bind multiple partners and thus assemble and localize intricate signaling complexes and intracellular architecture. The two-hybrid assay has been proved to be an efficient technique for finding new protein interactions (Fields and Song, 1989; Drees, 1999). However, the system also has a reputation for producing a significant number of false positives that require cumbersome analysis to separate the true interactions from the false positive.

More recently, methods such as phage display have come into wide use to rapidly create extensive genetic diversity and impose highly controlled and focused selective pressures to obtain interesting peptide and protein functions. With all the approaches mentioned, confirmation of candidates by secondary assays is critical.

5.1 Phage Display

Phage display technology has had a major impact on immunology, cell biology, drug discovery and pharmacology. It is an extremely powerful tool for selecting peptides or proteins with specific binding properties from a vast number of variants. The principle that exogenous peptides can be displayed on the surface of filamentous bacteriophage was first described by Smith in the mid 1980s (Smith, 1985). Phage display involves

the expression of proteins, including antibodies, or peptides, on the surface of filamentous phage. This is accomplished by the incorporation of the nucleoid sequence into a phage or phagemid genome as a fusion to a gene encoding a phage coat protein. This fusion ensures that as phage particles are assembled, the protein to be displayed is presented at the surface of the mature phage, while the sequences encoding it are contained within the same phage particle.

5.1.1 *Filamentous phage structure and life cycle*

The filamentous phage particles known as ft include strains M13, f1 and fd. Fd consists of a single-stranded (ss)DNA that is enclosed in a protein coat forming a viral particle and be able to infect E.Coli via F pili. The Fd phage particle is approximately 6.5 nm in diameter and 930 nm in length. The mass of the particle is approximately 16.3 MD, of which 87% is contributed by protein. Bacteriophages infect a variety of gram negatives using pili as receptors. The bacteria are not lysed by the phage but secrete multiple copies of phage displaying a particular insert. The entire genome of the phage consists of 11 genes. Four minor proteins are found at the tips of the virion, of which pIII and pVIII are the most commonly used to display peptide and antibody libraries (Hoess, 2001; Rodi and Makowski, 1999; Vaughan et al., 1998; Griffiths and Duncan, 1998; Zwick et al., 1998; Dall'Acqua and Carter, 1998; Raag and Whitlow, 1995; Winter et al., 1994). A viable phage expresses about 2700 copies of gene 8 protein (g8p or pVIII, a 50 aa residue protein

that is also known as the major capsid protein) and three to five copies of the gene III (g3)-encoded adsorption protein (g3p or pIII, a 406 aa protein that is one of the three minor coat proteins of the filamentous phage) on its tip (Figure 17). The advantage is that quite large peptide and protein inserts can be incorporated into pIII without loss of phage infectivity, and the exogenous peptide is exposed, facilitating high-affinity binding. We have applied phage display technology using an fd-based vector called fUSE5, which consists of a single phage chromosome bearing a single gene III, which accepts

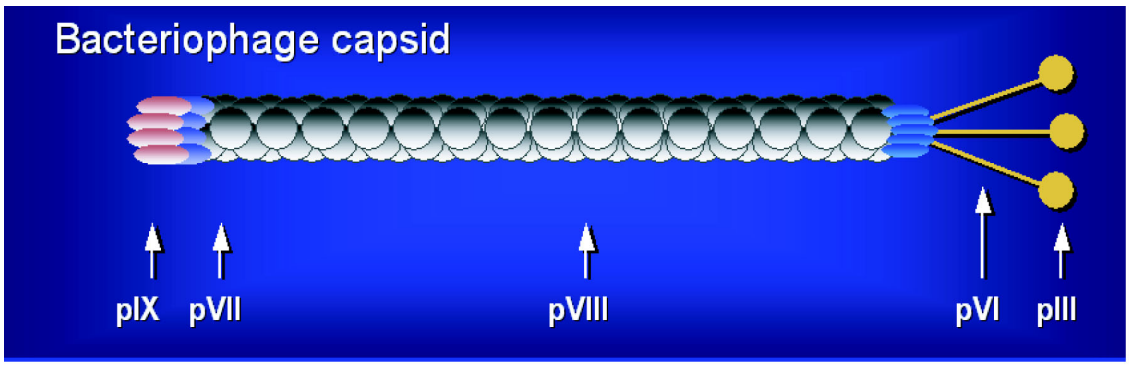

Figure 17. The Fd bacteriophage particle (not a scale). Schematic representation of the phage showing the location of the capside proteins. The pIII coat protein can be u sed as a fusion partner for a limited number of proteins (maximum of five), while thousands of proteins can be ex pressed at the surface of pVIII used as a fusion partner.

foreign DNA inserts and encodes a single type of pIII molecule. The foreign peptide encoded by the insert is therefore displayed on all three to five pIII molecules on a virion (Scott and Smith, 1990).

Filamentous phage do not produce a lytic infection in E. Coli, but rather induce a state in which the infected bacteria produce and secrete phage particles without undergoing lysis. Infection is initiated by attachment of the phage g3p to the f pilus of a male E. Coli. The f pilus is a protein tube assembled from pilin subunits in the cytoplasmic membrane and extends from the membrane out into the medium. Only the circular phage ssDNA enters the bacterium, where it is converted by the host DNA replication into the double-stranded plasmid-like replicative form (RT). The RT undergoes rolling-circle replication to make ssDNA and also serves as a template for expression of the phage proteins g3p and g8p. Phage progeny are assembled by packing of ssDNA into protein coats and extruding through the bacterial membrane to the medium.

5.1.2 *Screening phage display libraries*

Considerable progress has been made in the construction of phage display random peptide libraries and in the development of screening methods (Barbas, 1993). Most screening procedures that use phage are based on affinity selection and involve the following fundamental steps: 1) A library is amplified and phage particles produced, 2) phage particles are exposed to a target protein for which a binding is sought; 3) non-binding phage are removed by washing, and 4) binding phage to the target molecule are eluted and infected into a host bacteria to amplify the selected phage clone (Figure 18). These bio-panning rounds are repeated, typically three to six times until an enriched population of binders is selected. The sequence of a binding peptide is determined by sequencing the part of the phage genome that encodes the peptide. Finally, the insert can be reproduced as a recombinant or synthetic peptide. Using this methodology, specific and selective ligands to target receptors can be found.

5.1.3 *Use of phage display for in vitro and in vivo screenings*

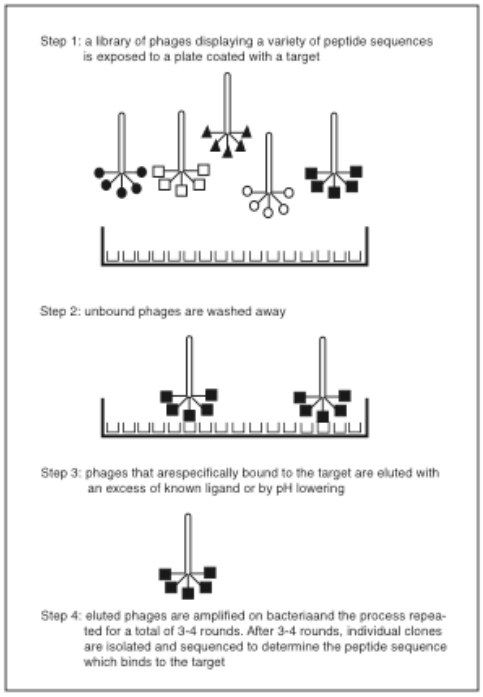

Figure 18.. Biopanning consists of four steps: (1) screening of phage display library on specific target, (2) elution of bound phages from target, (3) amplification of eluted phages, and (4) sequencing of DNA insert (Koivunen et al., 1999).

In recent years, phage display has been widely used for a selection of peptides and proteins that bind target molecules. Extensive data have been generated based on the use of phage libraries to identify extracellular integrin ligands (Koivunen et al., 1999). For example, RGD-containing peptides with high affinity for αv integrins have been isolated by phage display and shown to be useful tools for targeting tumor vasculature *in vivo* (Koivunen et al., 1995; Pasqualini et al., 1997; Zetter, 1997). Phage-displayed peptides libraries have also been used to determine the epitope of an antibody. Antibodies recognize small peptide motifs based on only three or four conserved residues. Based on the epitope motif revealed by phage display, it is possible to delineate the region on a protein recognized by the antibody (Scott and Smith, 1990; Cwirla et al., 1990). Characterization of epitopes of antibodies involved in autoimmune disorders may field important information about the immunogenic mechanisms of

disease (Cortese et al., 1996). There are also other molecules in the immune system that recognize short peptide sequences. The function of the major histocompatibility (MHC) molecules is to display short peptides in the cell surface. Based on peptidic sequences bound to MHC molecules, the immune system can recognize when the cell becomes abnormal or has been invaded by microorganisms harmful to the host. Phage display has been used to delineate the peptide structures recognized by individual MHC molecules (Hammer et al., 1992; Davenport et al., 1996; Kurokawa et al., 2002). In addition, phage antibodies are a special type of phage-display construct in which the displayed peptide is an antibody molecule, or more exactly, a domain of the antibody molecule that includes the site that binds antigen. A phage-antibody library includes billions of clones, displaying billions of antibodies with different antigen specificities (McCafferty et al., 1990; Barbas et al., 1991; reviewed in Rader and Barbas, 1997). Surface display of the antibodies allows for affinity selection by antigen *in vitro*, an artificial analogue of selection by antigen in natural immunity.

Phage display has also allowed the identification of specific ligands to cell surface receptors using as bait cells expressing integrins, the urokinase receptor (Goodson et al., 1994), and the melanocortin receptor-1 (Szardenings et al., 1997). Specific ligands have also been found for fibroblasts and myoblasts (Barry et al., 1996), endothelial cells (Nicklin et al., 2000), neutrophils (Mazzucchelli et al., 1999), T cells (Engelstadter et al., 2000), and head and neck carcinoma cells (Hong and Clayman, 2000), among others (Brown, 2000). Despite this relative success, cell-surface selection of phage-displayed peptides has been plagued by technical difficulties. First, a high number of non binder and non-specific binder clones are recovered when phage libraries are incubated with cell suspensions or monolayers. Second, removal of the background binding by repeated washes is both labor intensive and inefficient. Third, cells and potential ligands are frequently lost during the many washing steps required. For all these reason we pursue a new approach for cell panning that leads to fast identification of selective and specific peptide binders from phage libraries.

Phage display can also be used for *in vivo* selection of phage that home specifically to a target organ by means of a surface-displayed peptide binding. Different peptide motifs binding specific markers have been recovered from brain, kidney, lung, skin, pancreas, intestine, uterus, adrenal gland, retina and tumor xenografts in mice (Aquiline and Roulette, 1996; Remote et al., 1998; Pauline et al., 1997; Burg et al., 1999; Arap et al., 1998; Koivunen et al., 1999; Liu, 2003). These findings have expanded the knowledge of functional phenotypes of cells forming the endothelium based on protein–protein interactions, a field that can be defined as "vascular proteomics" (Trepel et al., 2002). Peptides isolated by phage display can also be peptidomimetic drug leads (Martens et al., 1995; Sparks et al., 1995; Wrighton et al., 1996; koivunen et al., 1999). The isolated peptide ligands have been used as carriers for targeted delivery of cytotoxic drugs (Arap et al., 1998), proapoptotic peptides (Ellerby, 1999), cytokines (Curnis et al., 2000) gene therapy (Wickham, 1997; Trepel et al., 2000), and imaging agents (Hong and Clayman 2000), in tumor mouse models.

5.2 Benefits of phage display in molecular-recognition studies

Phage-display technology has matured to the point where it is now a powerful tool in the postgenome era of biology. Phage-display constructs of genetically tagged peptides, proteins or protein fragments allow researches to convert pools of combinatorial nucleotides, mRNAs or fragmented genomes into populations of viruses that contain the nucleotide coding for the elements displayed on their viral surfaces. In a general sense, proteins that directly interact with each other can be expected to participate in the same molecular processes. The physical linkage of phenotype and genotype intrinsic to a phage-display library makes it possible for the binding properties of the displayed elements to be identified, modified and/or optimized in a matter of weeks with standard laboratory resources.

Yeast and other two-hybrid technologies (Toby and Golemis, 2001) offer an alternative strategy for identifying protein-protein interaction. Two-hybrid techniques share a common shortcoming with phage-display techniques. They are both limited by the biology of the system underlying their implementation. Although yeast two-hybrid has a

Table 11. Phage display versus yeast two-hybrid technology.

	Phage Display	**Yeast two-hybrid**
Screening population	Combinatorial peptides cDNA fragments	Combinatorial peptides cDNA fragments
Sequence diversity of library	Peptides: high cDNA fragments: dependent upon expression levels, unless normalized	Peptides: low cDNA fragments: dependent upon expression levels, unless normalized
Targets	Proteins: available through purification or overexpression Cell surfaces or tissues Small organic molecules (such as drugs or natural products)	Protein: bait is synthesized by the yeast cell Possibility of being folded properly by chaperones
Screening format	In vitro (can vary screening conditions, such as salt and detergent concentrations or on/off rates	In vivo (needs to be transported to yeast nucleus)
High-throughput	Billions of clones can be screened in 1 week	Millions of clones can be screened in 2 to 4 weeks
Range of interactions detected	Below 100 μM	Below 50μM
Positive outcomes	From peptide libraries: isolate peptides ligands, map region of interaction to short peptide sequences, predict interacting protein From cDNA fragment libraries: identify putative interacting proteins	From peptide libraries: isolate peptide ligands From cDNA fragment libraries: identify putative interacting proteins
False positives	Plastic-, GST-,six histine-, and streptavidin-binding sequences	Transcriptional-activating sequences
False negatives	Mimetopes with no homologs in nature UTRs, staggered reading frame products, promiscuous binding by exposed regions of misfolded proteins	Bait or prey proteins fail to fold properly or enter the nucleus UTRs, staggered reading frame products, promiscuous binding by exposed regions of misfolded proteins
Set-up time	Weeks	Months

proven track record in identifying candidate interacting partners in any given proteome (Uetz and Hudghes, 2000; Walhout and Vidal 2001), phage display has some advantages in mapping protein-protein interactions due, in part, to the enormous diversity of sequences capable of being displayed by bacteriophage. Even if it has been possible to generate and screen combinatorial peptide libraries by yeast two-hybrid screening, because of the intrinsically inefficient nature of transforming yeast, the libraries are of lower complexity and have not been of much utility in mapping protein-protein interactions (Table 11).

Another advantage to phage display technology is that it is possible to screen combinatorial peptide libraries for peptide ligands to target not traditionally used in yeast two-hybrid screening. For example, one can affinity-select peptides that bind to small-molecule drugs, such as taxol, 2-methoxyestradiol or doxorubicin (Rodi et al; 1999; Arap et al., 1998). These studies have demonstrated that the selected peptides have primary structures similar to peptide loops involved in drug binding to known targets; such information is invaluable in designing drug candidates that have fewer side effects. Random phage-display peptide libraries, in combination with other combinatorial methods, have offered an excellent approach to search for potent mimetic proteins and minimal peptide sequences retaining activity.

Resumen introducción

CÉLULAS: MACRÓFAGOS Y CÉLULAS ENDOTELIALES.

Los macrófagos que forman parte de las células hematopoiéticas, y las células endoteliales probablemente compartan un progenitor común. A este precursor común se le ha denominado *hemangioblasto*. Las células sanguíneas aun indiferenciadas y los precursores de las células endoteliales comparten los marcadores celulares CD34, GATA-2. LMO-2 y SCL, además de los receptores tirosina kinasa: VEGFR-2, Tie-1 y Tie-2.

Los macrófagos forman parte del sistema fagocítico mononuclear, que a su vez engloba una serie de células (monoblastos, promonocitos, monocitos y macrófagos) y una de sus funciones es la fagocitosis. A diferencia de otras células del sistema inmunitario, tales como los linfocitos B y T, los macrófagos o bien se encuentran proliferando, o bien se activan y dejan de proliferar, pasando así a ejercer sus funciones características.

Los macrófagos son originados en la médula ósea y emigran a todos los tejidos del organismo, donde constituyen junto con los neutrófilos el mayor sistema antimicrobiano del cuerpo. Este proceso de emigración puede estar regulado por las necesidades del organismo (inflamación, isquemia, necrosis, apoptosis, etc.). A nivel local se produce una disminución del flujo sanguíneo y, por tanto, se asegura que los monocitos y las células del endotelio interaccionen más fácilmente, favoreciendo así la extraversación de los monocitos a los tejidos.

Los procesos de proliferación celular son el conjunto de fenómenos que conducen a una célula a dividirse en dos, manteniendo cada una de las células hijas toda la información contenida en su predecesora. Los procesos de división celular están finamente regulados tanto por factores intrínsicos (ciclo celular) como extrínsecos (factores de crecimiento o señales mitogénicas). En el caso de los monocitos, los macrófagos y sus progenitores inmediatos, los procesos de proliferación y supervivencia están regulados por el factor de crecimiento específico que es el M-CSF (*Macrophage-Colony Stimulating Factor*).

La activación del macrófago es un proceso complejo y estrictamente controlado que consiste en una serie de alteraciones morfológicas, bioquímicas y funcionales que culminan en un aumento del potencial de la célula para ejecutar funciones complejas, como la presentación de los antígenos, la lisis de las células tumorales o la actividad bactericida. Los agentes más potentes en la activación de los macrófagos son el interferón gamma (IFN-γ) y el lipopolisacarido (LPS), aunque también otros agentes como el M-CSF, el GM-CSF (*Granulocyte Macrophage-Colony Stimulating Factor*), la IL-1 (Interleucina-1), IL-2, IL-4 y TNF-α (*Tumor Necrosis Factor-a*) pueden inducir algunos aspectos de la activación.

En los macrófagos, el LPS induce la síntesis de citocinas tales como TNF-α, IL-1, IL-6, IL-8, IL-10, IL-12, IFN-α/β, TGF-β (*Transforming Growth Factor-b*), metabolitos del ácido araquidónico y otros lípidos bioactivos, péptidos quimiotácticos como MIP-1α (*Macrophage Inflammatory Protein-1a*), y derivados reactivos del oxígeno y del nitrógeno. La mayor parte de los productos inducidos por el LPS actúan de forma autocrina sobre los propios macrófagos. También se ha descrito un efecto negativo del LPS sobre algunas actividades inducidas por el IFN-γ, tales como la expresión de las moléculas del MHC II (Complejo Principal de Histocompatibilidad de clase II) o la expresión de los receptores *scavenger*. Mientras que una respuesta inflamatoria local generada por el LPS es beneficiosa para la lucha contra las infecciones bacterianas, la repuesta inmunológica generalizada y exagerada debido a la presencia de LPS en la circulación puede conducir a un choque endotóxico, capaz de causar la muerte del individuo.

El IFN-γ es una citocina que juega un papel crítico como modulador de la respuesta inmunitaria. Todas las células del organismo poseen receptores específicos para el IFN-γ, lo que condiciona que esta citocina tenga un cierto efecto pleitrópico sobre distintos tipos celulares. Pese a este efecto pleitrópico, la diana por excelencia del IFN-γ son los monocitos y los macrófagos, sobre los que actúa como principal inductor del proceso de activación. En las células, IFN-γ induce la expresión de los genes del MHC II, promoviendo la función presentadora de antígeno de los macrófagos. Además, induce la síntesis y secreción de citocinas, tales como TNF-α, IL-1β, IL-6 e IL-12, y de los componentes del sistema del complemento. En particular, la inducción de la síntesis de IL-12

representa un mecanismo de retroalimentación positivo en el contexto de la inmunidad específica mediada por células, ya que esta interleucina actúa como un potente inductor de la generación y proliferación de linfocitos T $CD4^+$ del subtipo Th1.

A pesar de que los macrófagos juegan un papel clave en la fagocitosis de los cuerpos apoptóticos o en la inducción de apoptosis sobre otros tipos celulares, poco se conoce acerca de los estímulos que modulan la apoptosis de los propios macrófagos. Estas células requieren factores de crecimiento específicos para mantener su viabilidad. La ausencia del factor de crecimiento M-CSF induce la muerte por apoptosis.

Las otras células derivadas del progenitor común hemangioblasto, son las células endoteliales. Estas células cubren la superficie interior de los vasos sanguíneos formando el endotelio. Se ha hecho evidente en los últimos años que el endotelio es dinámico y enormemente heterogéneo. El endotelio es un órgano diseminado que posee capacidad secretora vital, además de funciones sintetizadoras, metabólicas e inmunológicas.

Las células endoteliales juegan un papel importante como mediadoras celulares controladoras del desarrollo vascular y del sistema vascular embrionario, donde desempeñan un papel crítico en el transporte metabólico de desechos y nutrientes. Además transmiten señales hormonales hacia y desde los tejidos en desarrollo a través de los receptores unidos a la membrana o bien usando los receptores específicos directos que regulan las interacciones célula-célula y de la célula con la matriz extracelular. Los vasos de nueva formación en el adulto se producen principalmente en la angiogénesis. Esta consiste en la formación de nuevos vasos que se ramifican a partir de los ya existentes (generalmente una vénula) y se debe a la actividad propia de las células endoteliales. También puede ocurrir una vasculogénesis.

Las células endoteliales son una población muy heterogénea. Se encuentran diferencias tanto entre los endotelios de distintas especies, como dentro de los diferentes tipos de vasos. También hay diferencias entre las células endoteliales que derivan de las distintas microvasculaturas.

Las principales funciones de los endotelios son la regulación de la homeostasis, el tono vascular y el crecimiento. Además existe una regulación endotelial de la respuesta inflamatoria, donde las moléculas de adhesión celular desempeñan un papel importante, y se expresa tanto en células endoteliales como en células sanguíneas. También, la sobre expresión de las citocinas inducidas por las moléculas de adhesión en el endotelio puede modular la transducción de las señales. Finalmente, las células endoteliales tienen un papel primordial en el fenómeno de la angiogénesis, y se sabe que este fenomeno puede ser el principal en la mayoría de las condiciones patológicas, tal y como ocurre en la retinopatía diabética proliferativa, la artritis reumatoide y en la vascularización de los tumores. La angiogénesis es un factor fundamental para la reproducción, el desarrollo y la reparación tisular. Todo esto depende del crecimiento regulado de los vasos sanguíneos ya que si no se controla conduce a la situación patológica que mantiene la evolución de las enfermedades neoplásicas y no neoplásicas.

Las células endoteliales son uno de los mayores componentes de la vasculatura. Estas células altamente metabólicas son capaces de sintetizar y secretar una gran variedad de moléculas biológicamente activas, que son importantes para la regulación del tono vascular y del flujo sanguíneo. Esto es importante en la modulación de las repuestas inflamatorias e inmunológicas, y en la regulación del desarrollo vascular. En particular, las células endoteliales sintetizan y liberan a la circulación un gran número de factores de crecimiento incluyendo interleucinas, factores de crecimiento e interferones que modulan el crecimiento y la proliferación de las células hematopoiéticas.

La activación de las células endoteliales como respuesta al microambiente en el que se encuentran esta asociada a cambios en el fenotipo de dichas células. Estos son los procesos de transdiferenciación del fenotipo constitutivo, que se producen con el fin de cubrir las necesidades locales y adaptarse a los nuevos requerimientos funcionales. En estos procesos de adaptación están involucradas las moléculas de superficie así como las citocinas, que juegan un papel crítico en las condiciones patológicas como puede ser la inflamación, la angiogénesis tumoral o la cicatrización.

Los procesos de vasculogénesis y angiogénesis requieren la interacción de las células endoteliales con la matriz extracelular a través de las integrinas. La señalización afecta a los elementos del citoesqueleto que controlan la morfología de las células endoteliales y las interacciones célula-célula que condicionan la estructura tridimensional de los tubos de los vasos sanguíneos. Las actividades celulares asociadas con la invasión tumoral implican una reducción de la adhesión célula-célula, una alteración de la adhesión matriz-célula, de la migración, de la supervivencia ectópica, y de la lisis de la matriz extracelular. Esto permite al endotelio formar barreras para la acumulación de solutos, macromoléculas y leucocitos entre el espacio intersticial y el lumen del vaso, facilitando así la posible formación de los nuevos vasos sanguíneos.

Muchos estudios indican que los receptores de la matriz extracelular expresados en las células endoteliales son críticos para mantener correctamente la función de estas células. La familia mejor estudiada de estos receptores son las integrinas, las cuales tienen dos funciones principales, mantener la unión célula-célula o célula-matriz y transferir información al interior celular que puede resultar tanto en la proliferación como en la muerte celular.

MATRIZ EXTRACELULAR

La mayoría de las células de los organismos pluricelulares están en contacto con una intrincada trama de macromoléculas extracelulares que constituyen la sustancia o la matriz extracelular. Esta es un componente de los tejidos básicos, y comprende toda la sustancia que se encuentra rodeando a las células en los tejidos. En el ámbito molecular la matriz extracelular esta integrada por proteínas y polisacáridos que se segregan localmente y se ensamblan formando una trama organizada en el espacio extracelular de la mayoría de los tejidos. Además de actuar como cemento biológico universal también forma estructuras altamente especializadas como el cartílago, los tendones, las láminas básales, los huesos y los dientes.

Las proteínas que componen la matriz extracelular incluye los colágenos, las glicoproteínas (no-colágenas) como por ejemplo la fibronectina, y los proteoglicanos. Todos estos componentes se organizan formando una red fibrosa que proporcionan la fuerza mecánica y la elasticidad necesaria para la adhesión y la migración celular. Las glicoproteínas se dividen en dos grupos según su función, por un lado están las proteínas adhesivas como la fibronectina, la vitronectina y la laminina, las cuales forman conexiones con otras moléculas de la matriz extracelular y las células mediante las integrinas que son los receptores específicos. El segundo grupo de glicoproteínas esta formado por las denominadas proteínas matricelulares como la tenascina, la SPARC (*secreted protein acidic and rich in cysteine*), la trombospondina y la osteonectina. Estas proteínas son secretadas e interactúan con otros constituyentes de la matriz extracelular, con múltiples receptores de superficie celular y además con los factores de crecimiento, modulando así las interacciones de la matriz con las células.

Los colágenos son una familia de proteínas estrechamente relacionadas entre ellas que pueden agregarse y generar filamentos. Junto con otros componentes de la matriz extracelular inducen la adhesión celular, activan las vías de señalización intracelular y regulan las actividades de varios factores de crecimiento y de otras proteínas.

Los proteoglicanos son macromoléculas formadas por una proteína central, a lo largo de la cual se asocian, por su extremo terminal, numerosa moléculas de glucosaminoglicanos sulfatados. Estas macromoléculas están representadas en todos los tejidos. Existe una enorme variedad de proteoglicanos dependiendo del tipo y tamaño de la proteína central y del tipo, número y longitud de los glucosaminoglicanos asociados a ella. El más pequeño es la decorina, que solo contiene una molécula de glucosaminoglicano y el más grande el agrecan que contiene alrededor de 100. Funcionalmente, los proteoglicanos participan en la adhesión célula-célula y célula-matriz extracelular, en procesos de biomineralización, y son reguladores de la migración y de la plasticidad celular. Recientemente los proteoglicanos se han clasificado en tres grandes grupos, SLRPs (*small leucine-rich proteoglycans*), los hailectanos y los proteoglicanos de las membranas. A su vez, estos dos últimos se agrupan formando proteoglicanos modulares, llamados así porque están estructuralmente compuestos por diferentes módulos.

La decorina es un proteoglicano pequeño de unos 38 kDa. Los efectos inducidos por esta proteína

son mediados en parte por la interacción específica entre la núcleo proteico y el receptor del factor de crecimiento epitelial (EGF), desencadenando una cascada de señalización que da como resultado la activación de las MAPKs (*mitogen activated protein kinases*), la movilización del calcio intracelular y el aumento de los niveles de $p21^{Waf1}$ motivando así una supresión del crecimiento celular.

Una de las funciones más importantes de la decorina es su capacidad para regular la proliferación celular. Se han encontrado niveles altos de decorina en las células quiescentes. Por el contrario, la expresión de la proteína es baja cuando las células están proliferando activamente o en las células transformadas. Estas observaciones son consistentes con el papel inhibidor del crecimiento que ejerce la decorina. Este papel antiproliferativo de la decorina puede tener un significado muy importante en los distintos estadios de las enfermedades tales como la fibrosis y el cáncer. Se ha descrito también que la decorina podría modular la actividad del TGF-β uniéndose activamente a esta citocina, que a su vez participa en el control de la proliferación, adhesión, diferenciación y deposición de la matriz celular.

RECEPTORES DE LA ADHESIÓN CELULAR

Los CAMs (*cell adhesion molecules*) son glicoproteínas ubicadas en la superficie celular que constituyen los receptores celulares, mediante los cuales se efectúan las interacciones específicas célula-célula y célula-matriz extracelular. Todas las funciones biológicas mediadas por estos receptores requieren, o son influenciadas por estas interacciones, especialmente durante la embriogénesis, en la morfología celular, en el desarrollo tisular, en la migración de las células, en los procesos inflamatorios e inmunológicos, la invasión celular en el tumor y en otros muchos. Las interacciones de las células con las células adyacentes y con la matriz celular que las rodea esta mediada por diferentes clases de receptores de adhesión celular, entre ellos las integrinas, las caderinas, y los miembros de la familia de las selectinas y la superfamilia de las inmunoglobulinas.

Las caderinas son una familia de glicoproteínas transmembranales que median la adhesión célula-célula de forma dependiente de calcio. Esta familia esta dividida en cuatro grupos, las caderinas clásicas, las desmosales, las protocaderinas y las atípicas. Muchas de estas se caracterizan por ser especificas de tejido, como la caderina-E que fue identificada en el epitelio, la endotelial caderina-VE en la vasculatura, o bien la neuronal, caderina-N. Las propiedades de adhesión, con actividad homofílica y de gran especificidad de interacción con un determinado tejido revelan un posible papel en la formación y mantenimiento de los tejidos en que se hallan.

La superfamilia de las inmunoglobulinas esta compuesta por una gran variedad de glicoproteínas mayoritariamente expresadas en la superficie celular. Típicamente tienen un dominio extracelular muy largo cuya parte amino terminal contiene los dominios de las inmunoglobulinas. Además, contienen un segmento simple de hélice como dominio transmembranal y una cola citoplasmática. Estas moléculas portadoras de dominios de inmunoglobulinas median la adhesión que se caracteriza por ser independiente de calcio y participan en distintas funciones celulares como la organización del citoesqueleto, la endocitosis, la migración, el control del crecimiento celular, la respuesta inmunitaria, además son receptores víricos, participan en las reacciones inmunológicas y en la progresión tumoral.

Las selectinas es una familia de tres proteínas (selectina L, E y P) que son receptores transmembranales de adhesión y están involucradas principalmente en la regulación de los leucocitos y su unión a las células endoteliales. El dominio amino terminal, que esta expuesto extracelularmente, caracteriza a esta familia de proteínas que unen carbohidratos como lectinas. La selectina L se expresa en los leucocitos y los monocitos, y media el alojamiento de los linfocitos en los nódulos linfáticos. La selectina E se expresa en las células endoteliales activadas por citocinas. La selectina P se expresa en los gránulos secretorios de las plaquetas y en el endotelio estimulado.

Las integrinas son receptores heterodiméricos de membrana, compuestos por una subunidad α y una subunidad β que median las interacciones célula-célula y célula-matriz extracelular. Las 18 subunidades α y las 8 β descritas interaccionan de manera no covalente y restringida para formar 24

heterodímeros distintos. Cada una de estas 24 integrinas cumple una función absolutamente específica. Estas integrinas se unen a su ligando de manera que es dependiente tanto de la afinidad como de la avidez y esta influenciada por la conformación del propio ligando y la capacidad de interaccionar y de hacer multímeros con las moléculas preexistentes de la matriz celular. Por esta razón, los distintos ligandos, o las distintas formas de un determinado ligando pueden transmitir señales diferentes a través de la misma integrina. Las integrinas son receptores transmembranales que están ampliamente distribuidos. La porción extracelular interactúa directamente con las moléculas de la matriz extracelular como la fibronectina, la laminina, la vitronectina y la tenascina, además de diferentes proteínas plasmáticas, y de otras proteínas de la superficie celular y patógenos microbianos. A su vez, la porción intracelular interactúa con la actina de citoesqueleto a través de las proteínas intermediarias que principalmente son la α-actina, la talina, la tensina y la vinculina.

Las integrinas son capaces de transmitir señales de manera bidireccional. Esta señalización esta mediada por las integrinas hacia el interior celular y la señalización que proviene del interior de la célula después de una previa activación de las integrinas por la unión al ligando. La subunidad β es la principal encargada de procesar esta señalización bidireccional. Muchas de las subunidades β tienen un dominio citoplasmático muy pequeño que consta de unos 60 amino ácidos, excepto la subunidad β4 que tiene alrededor de 1000, que además contiene dos pares de repeticiones FN (*fibronectin*) tipo III que están separadas por un segmento conector. Se ha descrito que los dominios citoplasmáticos de las subunidades β1, β3 y β5 son suficientes para iniciar la transmisión de señales. La subunidad β5 es estructuralmente y funcionalmente única respecto a las otras subunidades, además de haber sido descrita como reguladora de la emigración y proliferación celular.

Debido a que las integrinas no tienen por ellas mismas capacidad enzimática, para la señalización se basan en la unión a otras moléculas que sí poseen estas propiedades. La parte citoplasmática de las subunidades β tienen la capacidad de unirse a numerosas moléculas, las cuales en muchos casos son uniones específicas para cada subunidad. Entre ellas cabe destacar la unión de la subunidad β1 a FAK (*focal adhesion kinase*), a paxilina, a α-actina, a talina, a miosina, a Grb2 (*growth factor receptor-bound protein 2*). La subunidad β2 es conocida por interaccionar con ILK (*integrin-linked kinase*) y con citoadhesina-1. La cola citoplásmica de la β3 se une a la β3-endoxina, a ILK, a la miosina y al Shc (*Src homology type 2*). Hasta ahora sólo la TAP20 (*theta-associated prteoin 20*) se ha asociado exclusivamente a la subunidad β5. Todas estas uniones permiten la transmisión de señales de manera bidireccional a través de las membranas celulares.

La función primaria de las integrinas es mantener la adhesion entre las células y de éstas con los componentes estructurales de los tejidos de sostén que las rodean. Dichas uniones se forman y se refuerzan entre sí, en una secuencia que se inicia con el contacto del receptor con una molécula en el exterior de la célula. Al añadirse más uniones de este tipo, se consolida una estructura compleja y firme, llamada placa de adhesión focal. La unión de las integrinas a determinadas moléculas presentes en la matriz celular y las láminas basales, como la laminina y el colágeno modifica la actividad de las células epiteliales, al modular la expresión génica, regulando así los procesos de emigración, la mitosis y la apoptosis. Este mecanismo de muerte celular programada o apoptosis se desencadena cuando la célula pierde sus conexiones con las demás y con el intersticio circundante. Cada vez hay más evidencias experimentales del papel de regulación de la apoptosis en el cual participan las integrinas. En los últimos años se han descrito dos modelos de apoptosis en los que las integrinas actúan de forma directa. El primero se desencadena cuando las células pierden el contacto, fenómeno denominado anoikis. En el segundo, las integrinas no unidas al ligando pueden interaccionar directamente con las caspasas y promover apoptosis, un fenómeno conocido como muerte mediada por integrinas.

VIDA Y MUERTE CELULAR

El balance entre la supervivencia y la muerte celular en el organismo es esencial para procesos fisiológicos como el desarrollo y el funcionamiento del sistema inmunitario en los que es necesario un estricto control del número de células. Las alteraciones de este balance pueden desencadenar una serie de consecuencias patológicas. Las enfermedades como el Alzheimer y el Parkinson

están relacionadas con anomalias en la mortalidad de las células neuronales. Debido al papel que la muerte celular tiene en muchas enfermedades, incluyendo el cáncer, se ha dedicado una extensa investigación a la regulación de estos procesos apoptóticos.

El término apoptosis es usado para definir cómo mueren las células en determinadas condiciones fisiológicas y patológicas. Morfológicamente, la apoptosis se caracteriza por la condensación de la cromatina nuclear, la segmentación del núcleo, la pérdida de la estructura de los orgánulos del citoplasma, y la formación de vesículas en la membrana. Todo ello da lugar a la desintegración celular y a la formación de los cuerpos apoptóticos, los cuales a su vez serán fagocitados por los macrófagos, sin pérdida alguna del contenido celular, aún activo, y evitando así inflamación y daño tisular. Se le da la definición de muerte celular programada por el requerimiento de expresión génica tras la estimulación celular. Las células del sistema inmunitario expresan en su superficie celular los "*receptores para la muerte*" que tras ser activados por sus ligandos específicos transmitirán señales de apoptosis. Esta cascada de señalización incluye la activación de los complejos de cisteino-aspártico proteasas denominadas "caspasas". Se han descrito 10 caspasas en las células humanas que provocan una degradación proteica bien definida hasta llegar a la formación de cuerpos apoptóticos. Algunas caspasas son llamadas "iniciadoras" porque intervienen en la activación de otras caspasas y otras "efectoras" del proceso catalítico, que actúan degradando específicamente múltiples proteínas celulares incluyendo endonucleasas, que son las responsables directas de la fragmentación del ADN. La activación de las caspasas, que existen en calidad de procaspasas inactivas, se produce por diversas vías en las que participan varios complejos moleculares.

Los efectos de la muerte celular derivados de la activación de las caspasas pueden ser contrarrestados por la presencia de los inhibidores de las caspasas. Muchas proteínas que previenen la muerte celular a través de la inhibición de las caspasas han sido agrupadas en la familia IAP (*inhibitors of apoptosis proteins*). Dentro de la familia IAP, la XIAP (proteína inhibidoras de apoptosis ligada al cromosoma X) parece ser el que tiene más importancia y ejerce un estímulo anti-apoptótico más fuerte. Las IAP en mamíferos tienen un amplio rango de bloqueo de la apoptosis que incluye, los ligandos y los transductores de señales de la familia de los receptores del TNF, los miembros proapoptóticos de la familia del ced-9/Bcl-2 y el citocromo c, además de múltiples agentes quimioterapéuticos.

Los macrófagos juegan un papel importante es la supervivencia y la muerte celular. Estas, al igual que el resto de las células del organismo, requieren factores de crecimiento que mantengan su viabilidad. Entre ellos el más importante es el M-CSF que es requerido tanto para la supervivencia como para la proliferación. Por otro lado, existe un gran número de substancias capaces de inducir la muerte de los macrófagos, como son las substancias minerales y ciertas drogas (bleomicina, gadolinium, morfina, etc.,). Al igual que ocurre con otras células del sistema inmunitario, existe una relación entre los procesos de activación de los macrófagos y la modulación de los procesos apoptóticos. También se han descrito un efecto protector de la apoptosis en monocitos humanos y en otras células del linaje macrofágico por el tratamiento con citocinas como el TNF-α, GM-CSF, IL-3, IL-1β.

Por su parte, las células endoteliales también entran en apoptosis si se les retiran los factores de crecimiento o bien en respuesta a otros estímulos (por ejemplo la activación de Fas o el daño por radiación). Como para otros tipos celulares se ha hipotetizado que las interacciones de las células endoteliales con su entorno puede ser crítico para su supervivencia. La angiogénesis es totalmente dependiente de la supresión de apoptosis en las células endoteliales. La inhibición de la adhesión puede inducir directamente apoptosis celular. Una característica común de los inhibidores de angiogénesis es la inducción de apoptosis en las células endoteliales. Por lo tanto, es de gran importancia comprender los mecanismos por los cuales las células endoteliales están implicadas tanto en la apoptosis como en la supervivencia. De esta forma se podran desarrollar nuevas terapias, tanto de inhibición de la angiogénesis para aquellos casos en que la enfermedad sea dependiente de la neovascularización (tumores, diabetes retinopatía, etc) o estimular la angiogénesis en el caso de isquemia en los tejidos.

IDENTIFICACION DE DOMINIOS DE INTERACCION DE PROTEINAS: PHAGE DISPLAY

Para poder comprender completamente la función de las proteínas, es importante entender los complejos biológicos que se forman en condiciones fisiológicas normales y después observar cómo se pierde esta regulación en condiciones patológicas. La identificación de asociaciones de proteínas puede ser una tarea muy complicada de llevar a cabo debido a la complejidad que tales interacciones pueden tener. Una de las técnicas utilizada más comúnmente para este tipo de análisis ha sido la inmunoprecipitación. Sin embargo, la escasa sensibilidad y la dificultad en inmunoprecipitar proteínas no citosólicas y aquellas que son especialmente poco abundantes ha motivado el desarrollo de nuevos métodos para poder llevarse a cabo la identificación.

El *phage display* es un método muy potente para seleccionar pequeños péptidos que actúan como ligandos para cualquier proteína de interés. En muchos casos estos péptidos que se unen específicamente a la proteína pueden actuar de antagonistas o de agonistas de la función de la proteína natural. El principio del *phage display* radica en que péptidos exógenos pueden ser expresados en la superficie de un bacteriófago filamentoso, técnica descrita a principios de los años 80 por Smith. En las genotecas peptídicas normalmente el péptido exógeno esta incorporado a la proteína III del bacteriófago, que es la proteína de infección en la bacteria que le proporciona una gran estabilidad de expresión.

La utilización de esta técnica usando genotecas peptídicas al azar permite la selección de péptidos por afinidad. El proceso de selección conlleva diferentes pasos, que se inicia con la amplificación de las partículas fágicas, las cuales se exponen posteriormente a la proteína de interés para la que estamos buscando péptidos de unión. El fago no unido se descarta y se infectan las bacterias con los fagos que se han unido específicamente a la proteína de interés. Todos estos pasos constituyen el denominado "*panning*". Para obtener mayor especificidad el bio-panning es repetido entre 3 y 6 veces, la secuencia de ADN que codifica por el péptido exógeno insertado en el bacteriófago se determina por métodos de secuenciación y finalmente el inserto puede ser reproducido como proteína recombinante o péptido sintético.

El *phage display* se ha utilizado en los últimos años para identificar un amplio rango de proteínas que se unen específicamente a moléculas diana tanto *in vitro* como en *in vivo*. Existe una amplia variedad de aplicaciones para las que se pueden utilizar las posibilidades de este método. Así el *phage display* permite la identificación de ligandos que reconocen receptores ya conocidos y se puede utilizar para encontrar el epítopo de un anticuerpo que puede proporcionar información importante acerca de los mecanismos inmunológicos de una enfermedad. También se ha utilizado para identificar ligandos de receptores celulares. Sin embargo, esta última aplicación ha tenido muchísimas dificultades para ser aplicada por razones de afinidad del ligando al receptor, además de la gran inespecificidad obtenida de bacteriófago no unido específicamente, o bien la pérdida de posibles uniones al incrementar la astringencia de los lavados para evitar la gran cantidad de bacteriófago unido no específicamente. Por todo ello, nos hemos planteado desarrollar una nueva estrategiaque nos facilite la separación de complejos célula-bacteriófago del resto del bacteriófago no unido a la superficie celular.

Últimamente, debido a la problemática de que no todos los marcadores o moléculas encontradas en modelos de ratones se han podido aplicar a las terapias clínicas en humanos, se ha diseñado un nuevo experimento usando *phage display* en pacientes con el objetivo de poder seleccionar marcadores específicos de los vasos de los distintos órganos e identificar los ligandos *in vivo*.

Objectives

The overall objective of this study focuses on the description of certain mechanisms involved in cell survival. We seek to elucidate molecular mechanisms involving soluble and insoluble extracellular factors and their receptors. To achieve this goal we will use two cellular models, macrophages derived from bone marrow and endothelial cells. Both cell types proliferate in the presence of growth factors and can get activated in response to specific signals such as growth factor withdrawal. We will analyze the response of these cells to soluble and insoluble ligands that modulate cell death and survival. In addition, we intend to characterize cell surface components specifically involved in cell signaling and describe mechanisms of receptor-dependent cell survival. For this purpose we will use phage display, a powerful method used to obtain small peptide ligands to essentially any protein of interest.

The following specific aims are proposed:

Specific Aim 1: To analyze the molecular mechanisms involved in modulation of macrophage death and survival by IFNγ and the extracellular matrix protein decorin:

1.1. To study the ability of IFNγ to protect macrophages against apoptosis induced by different stimuli.

1.2. To analyze mechanisms of cell cycle regulation that mediate decorin-dependent cell survival.

1.3. To analyze mechanisms of decorin-dependent cell survival through interactions with the TGFβ signaling pathway.

Specific Aim 2: Characterization and functional analysis of endothelial cell surface receptors that can modulate proliferation and cell death

2.1. To dissect the integrin β5 signaling pathway by selection and analysis of peptides that bind to the β5 cytoplasmic domain.

2.2. To characterize cell surface receptors involved in endothelial cell activation and proliferation.

2.3. To isolate cell surface markers to the human vasculature by phage display.