Discussion

The specific functions of most of the cells in the body rely on their response to the microenvironment that surrounds the cells. The decision to live or to die, to grow or stay quiescent, to become activated or not, depend at the cellular level on the cell's interaction with extracellular cues. These trigger cell signaling pathways that promote such functions. The extracellular molecules that control the cell response are soluble factors such as growth factors or cytokines, and insoluble molecules such as the extracellular matrix (ECM) (Ingber, 2002). At the cellular level, the particular array of cell receptors that interact with these ligands dictates the nature of the signaling. The aim of this work is to describe some of the mechanisms involved in cell survival triggered by soluble and insoluble factors. In addition, we set out to characterize cell surface components involved in specific cell signaling and describe mechanisms of receptor-dependent cell survival.

One of the features of the immune system is the production of a large amount of many types of cells. Later, most unnecessary cells die through apoptosis. Like other cells of the immune system, macrophages are produced in large amounts and most of them die through apoptosis (Xaus et al., 1999). Macrophages originate in bone marrow and migrate to body tissues through the blood circulation (Klems et al., 1990). The viability of the immune cells may be regulated by the presence of growth factors and cytokines. In this regard, the ability of IFNγ to induce programmed cell death has been suggested as a role for this cytokine in the negative selection of the peripherial T and B lymphocyte repertoire (Liu and Janeway, 1990; Grawunder et al., 1993). Nevertheless, IFNγ is also involved in prosurvival signals in normal and leukemic cells (Mangan et al., 1991; Bach and Brashler, 1995).

As a model for macrophages we have used bone marrow-derived macrophages because they are a homogeneous population of nontransformed cells, that in response to extracellular stimuli are able to proliferate, to become activated, to differentiate, or to undergo apoptosis (Celada et al., 1984; Celada et al., 1994). When macrophages are activated with LPS or IFNγ, the M-CSF-dependent proliferation was inhibited. In fact, the cell cycle was stopped at G1/S interfase. However, whereas these cells undergo apoptosis with LPS, macrophages treated with IFNγ are not apoptotic. The treatment of these cells with IFNγ also protects them from apoptosis induced by LPS, growth factor depravation, or glucocorticoids.

We have shown that IFNγ induces the expression of the cdk inhibitor $p21^{Waf1}$, which is critical for the protective effect of IFNγ on apoptosis. Our results are in agreement with previous works showing an antiapoptotic role of $p21^{Waf1}$. The overexpression of $p21^{Waf1}$ in glioma cells prevents apoptosis (Gomez-Manzano et al., 1997). In differentiating myocytes, the expression of $p21^{Waf1}$ confers resistance against apoptosis (Wang and Walsh, 1996). In differentiating neuroblastoma cells, antisense oligonucleotides to $p21^{Waf1}$ enhance cell death by apoptosis (Poluha et al., 1996). Finally, the expression of p53 in a series of colorectal cancer cell lines resulted in growth arrest in some lines and apoptosis in others. The inactivation of $p21^{Waf1}$ in these cells by homologous recombination changed a cell line with growth arrest to an apoptotic cell, suggesting that, despite inducing growth arrest, $p21^{Waf1}$ could protect against apoptosis (Polyak et al., 1996). Thus, $p21^{Waf1}$ seems to be a critical molecule for the survival of cells, even when apoptosis was induced through incubation with stimuli.

Although $p21^{Waf1}$ was first described as a p53-induced gene (El-Deiry et al., 1993), a p53-independent expression of $p21^{Waf1}$ has also been described (Parker et al., 1995; Zhang et al., 1995). We have found that $p21^{Waf1}$ induced by IGF-I does not protect macrophages against apoptosis, which suggests that the expression of $p21^{Waf1}$ by itself is not sufficient and complementary mechanisms are necessary. c-Myc expression has been involved in both IFNγ-mediated inhibition of macrophage proliferation (Vairo et al., 1995) and in regulation of apoptosis (Evan et al., 1992). However, since c-Myc expression is also inhibited by LPS, which induces apoptosis, c-Myc does not seem to be involved in the protective effect of IFNγ.

The role of $p21^{Waf1}$ in IFNγ-mediated growth arrest is unclear (Chin et al., 1996; Sharma and Iozzo, 1998). Here, using macrophages from $p21^{Waf1}$ KO mice, we showed that its expression does not play a role in the inhibition of macrophage proliferation and therefore other mechanisms may be involved. These results are in agreement with

those of Sharma and Iozzo (1998). In addition, it has also been shown that $p21^{Waf1}$ can interact with procaspase 3 forming a complex, procaspase $3/p21^{Waf1}$ that inhibits the caspase activity by masking the cytoplasmic serine proteinase-cleavage site and therefore preventing the cells from undergoing apoptosis (Suzuki et al., 1998).

In summary, our results show that IFNγ prevents apoptosis in macrophages through the induction of both $p21^{Waf1}$ expression and cell cycle arrest at G1/S boundary. In addition, we have shown that $p21^{Waf1}$ is absolutely required for prevention of apoptosis but not for the cell cycle arrest, suggesting that additional mechanisms mediated by IFNγ contribute to block cell cycle and prevent apoptosis. This is an example of interaction between proliferation, cell cycling, and apoptosis.

Regulated cell proliferation and growth does not require only the interaction of growth factors with their cellular receptors as a prerequisite for the completion of the cell cycle. Cell proliferation is also linked with changes in the interactions of cells with their macromolecular environment, with cell migration, and differentiation. Hence, the extracellular matrix surrounding the cells is of similar importance for growth control as are the interactions between soluble, growth-regulating molecules and their cellular receptors.

The extracellular matrix is a complex alloy of macromolecules capable of self-assembly, predominantly via noncovalent bonds. It is composed predominantly of collagens, non-collagenous glycoproteins, elastin, hyaluronan, and proteoglycans. This matrix, however, is not only a scaffold for the cells of a given tissue. It also serves as a reservoir for growth factors and cytokines, and modulates their activation status and turnover. By interacting with matrix molecules, the growth factors may become sequestered from their signalling receptors; they may become activated, e.g., by proteolytic processing; and they may be presented to the cells in a manner significantly altering their bioactivity. In addition, there is increasing evidence that extracellular matrix molecules may exhibit a direct signalling function, either via interactions with matrix receptors like integrins or via signalling through growth factor receptors themselves (Ornitz, 2000; Schönherr and Hausser, 2000; Couchman et al., 2001; Yamaguchi, 2000).

All classes of extracellular matrix molecules may now be considered as macromolecules involved in growth control. However, the " information network" in which the proteoglycans of the extracellular matrix are involved has only recently been intensively studied. There are two classes of proteoglycans, which at present appear of be capable to directly affecting growth. Members of the lectican family of proteoglycans represent one class; the other one is presently represented only by two members of the small proteoglycan family containing leucine-rich motifs in their core proteins. From other reports, it became evident that in some systems, growth-related effects appear to be mediated via the glycosaminoglycan chain regardless of the biological properties of the core proteins themselves. In these cases, the abundance of a given saccharide structure at the place of action is obviously of greater importance than the fine-tuning of the expression of the proteoglycan itself. It should also be stressed that in order to understand a proteoglycan's role in growth control, it is certainly an oversimplification to only consider the interaction with a cell surface receptor. The temporal and spatial regulation of proteoglycan expression and the possible interactions with other molecules, such as cell adhesion molecules, have to be taken into account as well. Hence, the multifaceted extracellular milieu created by the matrix macromolecules together with the multitude of soluble growth mediators, which are contained within this matrix, yield a level of complexity that cannot be traced back to the interaction of a proteoglycan with a signalling membrane receptor alone. It is therefore not surprising that divergent results may be obtained when correlations between proteoglycans and growth are made in a different biological context (Bandtlow and Zimmermann, 2000).

Cells reside in a protein network, ECM, which they secrete and mold into the intercellular space. The effects of the matrix are primarily mediated by integrins, which attach cells to the matrix and mediate mechanical and chemical signals from it. Integrins thus act in a crucial biosensory role, coordinating survival or death responses as a function of ECM composition. Among the ECM components, the involvement of decorin, a proteoglycan, in growth control has been proposed

on the basis that decorin becomes up-regulated together with some other matrix and intracellular proteins in quiescent cells compared with logarithmically growing cells (Coppock et al., 1993; Mauviel et al., 1995; Laine et al., 2000).

Secreted decorin can also modulate the immune response mediated at the inflammatory loci by monocytes and macrophages. Stimulated monocytes and macrophages secrete a diverse set of mediators that modulate cellular immune functions and inflammation (Leonard et al., 1993). These mediators include proinflammatory and antiinflammatory cytokines such as GM-CSF and IFNγ, which are secreted as an integral component of the innate immune response. At the inflammatory sites, proteoglycans are both secreted by activated mononuclear leukocytes and monocytes, and released as a result of ECM degradation. Thus, proteoglycans, which are major constituents of the ECM, are another class of molecules produced by monocytes and macrophages (Laskin et al., 1991; Uhlin-Hansen et al., 1992) that are potential modulators of the immune response.

Since most of the experiments regarding the antiproliferative activity of decorin have been performed using transformed cell lines, we decided to study the role of decorin in survival and cell death in primary cultures. We have shown that decorin inhibits macrophages proliferation in a dose- and time-dependent manner. Previous work had shown that decorin interacts with components of the EGFR family and the effects that decorin produces in macrophages mimic those induced by IFNγ. However, we have shown that decorin does not use EGF or IFNγ receptors to inhibit macrophage proliferation.

After decorin treatment of macrophages we observed the induction of two cdk inhibitors, $p21^{Waf1}$ and $p27^{Kip1}$. Using mice with these genes disrupted by homologous recombination we demonstrated that $p27^{Kip1}$ but not $p21^{Waf1}$ is responsible for the antiproliferative effect of decorin in macrophages. This is interesting because fibrillar collagen, a molecule that closely interacts with decorin *in vitro* (Vogel and Trotter, 1997) and *in vivo* (Danielson et al., 1997), inhibits smooth muscle cell proliferation by inducing $p27^{Kip1}$ levels and, to a lesser extent, $p21^{Waf1}$ levels (Koyama et al., 1996). Moreover, we also observed that adhesion of macrophages to fibronectin, another protein of the extracellular matrix, which also binds decorin, also inhibits macrophage proliferation through induction of $p27^{Kip1}$. Interestingly, it has been proposed that the growth-suppressive properties of decorin are independent of a functional p53 or retinoblastoma protein but require a functional $p21^{Waf1}$, since $p21^{Waf1}$-deficient colon carcinoma cells (Waldman et al., 1995) are totally unresponsive to the action of decorin (Santra et al., 1997). This may indicate a cell type dependent response to decorin effect or perhaps a different response of normal and tumor cells.

$p27^{Kip1}$ inhibits cyclinE-cdk2 kinase activity, and its role in adhesion-mediated G1-phase arrest has been extensively documented (Chen et al., 1997; Jiang et al., 2000; Koyama et al., 1996). That cell-cell contact causes normal cells to stop cell proliferating has been recognized for a long time in normal organ development. More recently, it has become clear that in such contact-mediated growth arrest $p27^{Kip1}$ is required and up-regulated. This is illustrated in mutant $p27^{Kip1}$ mice, which display generalized increased body size and a significantly expanded hematopoietic progenitor pool (Johnson et al., 1998; Nakayama et al., 1996). Using $p27^{Kip1}$ KO mice, we observed that the inhibitory effect on proliferation induced by adhesion of macrophages to extracellular matrix components such as decorin or fibronectin is also mediated through $p27^{Kip1}$ expression.

We have shown that decorin, like IFNγ not only inhibits macrophage proliferation but can also protect macrophages from apoptosis induced by M-CSF starvation. In this case, the effect of decorin is not mediated by the increase in adhesion. The induction of $p21^{Waf1}$ and the block of the cell cycle induced by decorin is responsible for the protective effect against apoptosis. The results obtained using $p21^{Waf1}$ KO mice justify this conclusion. Therefore, as we find with IFNγ, it seems that $p21^{Waf1}$ is a critical molecule for macrophage survival.

The reported adhesion experiments suggest that decorin and fibronectin are recognized by different receptors, mainly integrins, due to the synergistic effect observed on macrophage adhesion induced by both components of the extracellular matrix. Decorin may bind to a fibronectin domain different from that recognized by macrophages. Binding to

fibronectin can promote the differentiation of blood monocytes into tissue macrophages (Armstrong and Chapes, 1994; Lundahl et al., 1996; Rein et al., 1994). In a similar way, our results show that decorin can also modulate macrophage activation (Comalada et al., 2003). We have found that decorin not only inhibits macrophage proliferation but can also enhance both IFNγ- and LPS-mediated activation. This effect was originally linked to the capacity of decorin to increase cell adhesion, based on the fact that fibronectin also inhibits macrophage proliferation. However, we observed that decorin increased the activation mediated by both LPS and IFNγ, whereas fibronectin did not.

The involvement of decorin in growth control has been discussed on the basis of the proposal that decorin becomes up-regulated together with some other matrix and intracellular proteins in quiescent cells compared with logarithmically growing cells (Coppock et al., 1993; Mauviel et al., 1995; Laine et al., 2000). However, a complex regulation of the quiescence-induced genes with respect to cell adhesion and cell type was noted subsequently (Coppock et al., 2000). In CHO cells, recombinant expression of decorin was accompanied by an inhibition of cell proliferation (Yamaguchi and Ruoslahti, 1988). The effect was considered to result from the proposed capability of decorin to inhibit the activity of TGF-β (Yamaguchi et al., 1990). While this explanation may indeed serve to explain the effect in part and/or in selected cell lines, decorin can also directly influence cell behavior in an autocrine or paracrine manner. The discovery that a fibril-associated proteoglycan serves not only as an organizator of the extracellular matrix, but as also a signalling molecule led to a novel level of complexity in cell matrix interactions. Simultaneously, it became apparent how cell-type specific the signalling pathways might be, and to what large extent the final effects may depend on the cellular environment.

In A431 squamous carcinoma cells, decorin suppresses the malignant phenotype (Santra et al., 1995) by activating the EGF receptor (Santra et al., 1997) through its dimerization and increased phosphorylation (Moscatello et al., 1998). This leads to an activation of the MAPK pathway (Moscatello et al., 1998), mobilization of calcium stores (Patel et al., 1998), and an up-regulation of $p21^{WAF1/CIP1}$ (De Luca et al., 1996; Santra et al., 1997), an inhibitor of cyclin-dependent kinases being involved in tumor suppression (el-Deiry et al., 1993). Upon prolonged interaction between decorin and the EGF receptor, e.g., after stable expression of decorin in tumor cells, the signalling pathway becomes desensitized by a reduction of the number of receptor molecules and by an abrogation of the degree of EGF receptor tyrosyl phosphorylation (Csordás et al., 2000). The signal-transducing role of decorin may not rely on interactions with the EGF receptor. Other members of the EGF receptor family may play a role. Thus, in breast carcinoma cells decorin seems to activate ErbB4, which in turn blocks the phorphorylation of heterodimers containing either ErbB2 or ErbB3 (Santra et al., 2000).

A somewhat different situation was encountered in endothelial cells, which do not express decorin except during angiogenesis (Järveläinen et al., 1992; Schönherr et al., 1999). Using the endothelial cell line EA.hy 926 in an *in vitro* model of angiogenesis, it could be shown that adenovirally mediated expression of decorin is sufficient for angiogenesis-related phenomena to occur and for the prevention of apoptosis in the absence of fetal calf serum. In EA.hy 926 cells, surface receptors other than the EGF receptor must be involved in decorin signaling, because the signal cannot be blocked with tyrphostin AG1478, a specific inhibitor of the EGF receptor (Schönherr et al., 2001). Calcium was not involved in signal transfer either, but the phosphorylation of Akt (protein kinase B) was required to mediate some of the effects, as judged from the effects of expressing a dominant negative form of Akt. Within a few hours upon decorin addition, cyclin A became transiently regulated. As occurs in epithelial cells, decorin expression was followed subsequently by an induction of $p21^{Waf1}$ and additionally of $p27^{KIP1}$. Akt, however, only regulated the expression of $p21^{WAF1/CIP1}$ and cyclin A, and the mechanism(s) of induction are presently not understood. An increase in the two cyclin dependent kinase inhibitors was not only observed in endothelial cells overexpressing decorin, but also in arterial smooth muscle cells which were retrovirally induced to synthesize decorin and in addition stimulated to proliferate. In this experimental setup also a decrease in DNA synthesis by decorin was found (Fischer et al., 2001). Decorin expression in EA.hy 926 cells also influenced the NF-κB signal transduction system. p65, an active component of NF-κB, was translocated to the nucleus, and the

cytoplasmic concentration of I-κBα decreased. Figure 1 shows a compilation of the different modes of signalling of decorin that have been observed to date. However, all these pathways may not be activated in every cell type

From the studies of carcinoma cells one would like to conclude that decorin-deficient animals might frequently develop spontaneous tumors. However, this is not the case. It could be shown instead that decorin deficiency is permissive for lymphoma tumorigenesis in mice predisposed to cancer due to p53 mutations (Iozzo et al., 1999).

The biological implications of the interaction between small proteoglycans and the isoforms of TGF-β have been most intensely investigated. Starting with the observation that decorin expression by Chinese hamster ovary cells inhibits their proliferation via TGF-β inactivation (Yamaguchi et al., 1990), several attempts have been made to clarify the biological importance of the interplay between small proteoglycans, especially of decorin, and TGF-β. However, the cytokine may exhibit both growth promoting and growth inhibitory activities (Sporn, 1999), and in most of the studies the role of TGF-β in matrix production and not in growth control was studied. There is no doubt that all the isoforms of TGF-β interact with the core proteins, at least with decorin, biglycan, and fibromodulin (Hildebrand et al., 1994), exhibiting dissociation constants in the nanomolar range. A centrally located decorin peptide was similarly active, and it could be shown that collagen-bound decorin was also able to interact with TGF-β (Schönherr et al., 1998). Controversy exists about the biological activity of the proteoglycan/TGF-β

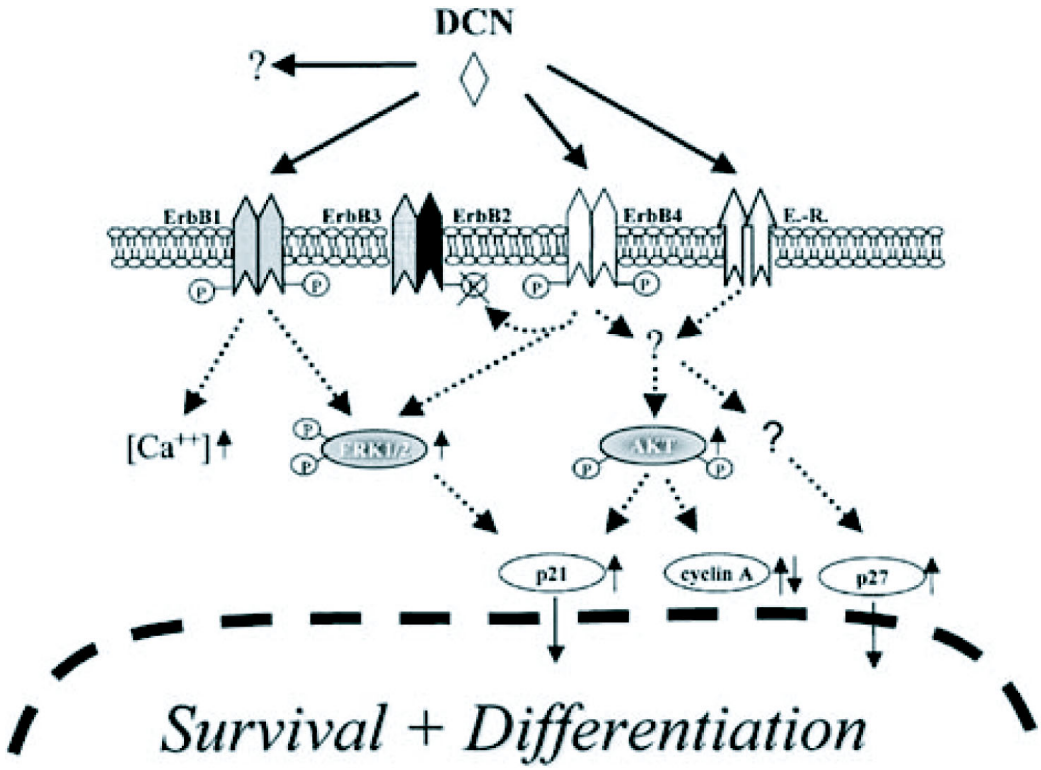

Figure 1. Signaling of decorin via cell surface receptors in epithelial and endothelial cells. Dashed arrows symbolize pathways in which not all links are yet known. DCN, decorin; E.-R., endocytosis receptor for small proteoglycans; p21, p27, inhibitors of cyclin-dependent kinases.

complex. In several models of fibrotic diseases (anti-Thy-1 glomerulonephritis, bleomycin-induced pulmonary fibrosis) decorin administration considerably improved the course of the disease, regardless of whether the proteoglycan was administered parenterally, adenovirally, or whether its synthesis was induced by gene transfer (Border et al., 1992; Isaka et al., 1996; Zhao et al., 1999; Kolb et al., 2001). Similarly, the growth response of arterial smooth muscle cells towards TGF-β was reduced upon retrovirally overexpression of decorin (Fischer et al., 2001) as was the chemotactic response of microglial cells in experimental rat glioma (Engel et al., 1999). Nevertheless, proliferation of decorin-over-expressing smooth muscle cells was not influenced after balloon injury (Fischer et al., 2000). Interestingly, no beneficial effects were seen when, in pulmonary fibrosis, biglycan instead of decorin was adenovirally induced (Kolb et al., 2001). On the other hand, it was shown that in several different experimental systems TGF-β activity remained unchanged in spite of an up to 10,000-fold molar excess of decorin (Hausser et al., 1994). Recent studies indicated that collagen-bound decorin may sequester the cytokine in the extracellular matrix (Markmann et al., 2000). As biglycan is located predominantly pericellularly, this proteoglycan would be much less suited to trap TGF-β in the extracellular matrix, and hence explain the unsuccessful treatments of fibrotic disorders with this proteoglycan. Studies in human diabetic nephropathy led to the conclusion that small proteoglycans might be able to remove the cytokine via the circulation or the urinary tract, again postulating that it is not the inactivation of the cytokine but the different localization of the TGF-β/decorin complex that may explain the beneficial effect of decorin application (Schaefer et al., 2001). Figure 2 shows the different possibilities of interaction of decorin/TGF-β complexes with extracellular matrix molecules and/or cell surface receptors. In other studies, TGF-β/decorin complexes were not inhibitory but they had even greater biological activity than the growth factor alone (Takeuchi et al., 1994; Riquelme et al., 2001). This may indicate that in given cells or in given biological circumstances the proteoglycan/TGF-β complex may be more efficiently presented to the TGF-β signalling receptors than the free cytokine. The potential role of small proteoglycans in TGF-β activation has not yet been investigated.

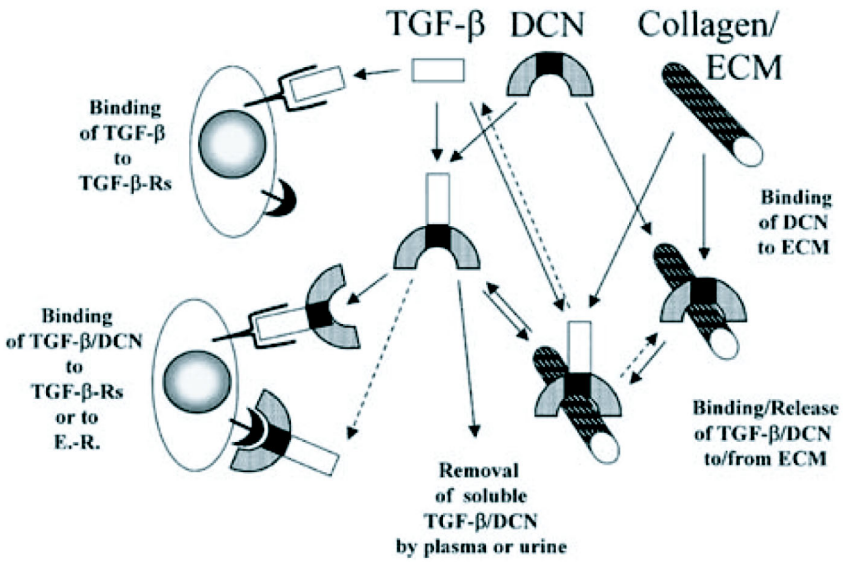

Figure 2. Possible interactions of decorin, TGF-β, and extracellular matrix molecules. Solid arrows show interactions that have already been demonstrated in different experimental systems, and dashed arrows show possible relations that still have to be proven. TGF-β-R, cell surface receptors for TGF-β signal transduction, other abbreviations are explained in previous figures.

Decorin may also exhibit therapeutic effects in inflammatory diseases, which are independent of its interaction with TGF-β. For example, apoptosis of mesangial cells, which is a hallmark of glomerular damage during inflammation, can be prevented by $p27^{KIP1}$ (Ophascharoensuk et al., 1998). As decorin may induce the expression of this protein, decorin administration in glomerulonephritis may also be beneficial because of the up-regulation of this cyclin-dependent protein kinase inhibitor.

Decorin-induced adhesion is not a sufficient mechanism to increase macrophage activation (Schonherr et al., 1998). Therefore, we analyzed the repressive effect of this proteoglycan on the effect of TGF-β on macrophages. This cytokine is produced in an autocrine manner by macrophages and down-regulates activation (Assoian et al., 1987). TGF-β antagonizes IFN-γ-driven processes of macrophage activation such as the production of H_2O_2, NO, the up-regulation of iNOS, the release of TNF-α, or the IFN-γ-induced death of intracellular microorganisms (Ding et al., 1990; Tsunawaki et al., 1988; Vodovotz et al., 1993; Bogdan, 1992). TGF-β also shows an inhibitory effect on IFN-γ-induced MHC class II genes (Reimold et al., 1993). The repressive effect of TGF-β on IFN-γ is based on the crosstalk between the molecules involved in signal transduction pathways. The TGF-β/SMAD signaling cascades are inhibited by the IFN-γ/STAT pathways and vice versa (Pitts et al., 2001; Ulloa, 1999). In addition, TGF-β also inhibits LPS-induced activation of macrophages.

Interestingly, our results also show that the treatment of macrophages with decorin alone is sufficient to induce the mRNA expression of some cytokines, namely TNF-α and IL-1β, but it is not able to induce their secretion. This indicates that although treatment with decorin alone could modulate gene transcription, it does not regulate the post-transcriptional mechanisms involved in cytokine secretion (Wegrowski et al., 1995). Increased expression and secretion of these inflammatory cytokines induced by decorin were only observed in the presence of macrophage activators such as LPS or IFN-γ.

We have found that macrophages bound TGF-β with an affinity and number of binding sites per cell similar to those observed in other cell types (Massague et al., 1990). Decorin blocked the binding of TGF-β to macrophages, which could be due to binding of decorin to TGF-β and inhibition of the interaction with the cell surface receptor, resulting in the inhibition of the TGF-β effects in macrophages. In response to subsaturating amounts of IFN-γ or LPS, decorin may compete with macrophages for the autocrine TGF-β produced. This could explain the beneficial effect of decorin on IFN-γ- or LPS-mediated activation.

Here we show that decorin can block the inhibitory effects of TGF-β in macrophage activation. The presence of decorin in tissues could account for increased macrophage activation. These phagocytic cells play a critical role during inflammation. In the early stages, neutrophils are present at the inflammatory loci but leave after 24 to 48 hours. Later, macrophages reach these loci, where they remain until inflammation disappears (Bellingan et al., 1996), that is, for as long as stimulated Th1 cells produce IFN-γ. In the late phases of inflammation, macrophages eliminate nonself structures, remove all the debris (including apoptotic bodies), and remodel impaired tissues. However, during chronic inflammation, such as rheumatoid arthritis, macrophages play a key role in the pathogenesis (Janossy et al., 1981). In these situations, the persistence of these phagocytic cells may be related to the presence of molecules that block their deactivation. Several soluble mediators, such as TGF-β (Tsunawaki et al., 1988; Vodovotz et al., 1992; Bogdan et al., 1992), IL10 (Bogdan et al., 1992; O'Farrell et al., 1998), adenosine (Xaus et al., 1999b), etc., block macrophage activation. Macrophages are restrained from tissue-damaging activation by CD200R (a myeloid-specific receptor on the phagocytes) when it engages on other cells the glycoprotein CD200 (Wright et al., 2000; Hoek et al., 2000). Depending on the balance between activators and inhibitors, macrophages remain at the inflammatory loci and release enzymes or cytokines that could be deleterious for the articulation (Nathan et al., 2001). In this context, decorin or other molecules of the ECM may contribute to the pathogenesis of chronic inflammation by blocking inhibitors. In this regard, in an animal model of experimental autoimmune encephalomyelitis, systemic administration of antibodies specific for TGF-β identified a role for

endogenous TGF-β in the suppression of the disease (Miller et al., 1992).

We have discussed above some of the mechanisms involved in cell survival mediated by soluble and insoluble factors. These factors modulate the cell response through the interaction with surface receptors. Therefore, characterization of the molecular mechanisms involved in receptor-mediated signaling is key to understanding the ligand-receptor signaling.

ECM proteins mediate their effects upon binding to cell surface receptors (integrins). Previous works have demonstrated a prominent role of integrins in cell survival and apoptosis (Frisch and Francis, 1994; Stupack et al., 2001; Yamada, 1997). However, intracellular signaling responsible for these effects is largely unknown. To date, the integrins αvβ3 and αvβ5 appear to be the most closely associated with tumor angiogenesis, suggesting that modulation of apoptosis by these integrins may be important for neovascularization associated to cancer (Friedlander et al., 1996; Brooks et al., 1994).

Angiogenesis initiated by fibroblast growth factor (bFGF) can be inhibited by an anti-αvβ3 blocking antibody, whereas vascular endothelial growth factor (VEGF)-mediated angiogenesis can be prevented by a blocking antibody against αvβ5. Thus, distinct cytokine-induced pathways that lead to angiogenesis seem to depend on the particular β subunit that associates to αv chains. Moreover, integrin β chains are sufficient to activate downstream signaling molecules (Green et al., 1998). It has been suggested that the cytoplasmic domain of integrins must be involved in integrin signaling; therefore to better understand the intracellular events involved in integrin-mediated apoptosis we decided to look for proteins that bind to the cytoplasmic domain of integrins binding proteins. The β5 cyto-domain is very small, and probably because of this previous attempts to find binding proteins using two-hybrid failed. Because it is more suitable for identification of small molecules interactions, we decided to use phage display. Here, we have identified a new mechanism of apoptosis that is dependent on β5 but not β3 or β1, thus providing additional information regarding the specificity of individual β subunits.

Using phage display we identified a peptide that specifically binds to β5 but not to β3 or β1 cyto domains. This peptide, upon internalization, is able to induce apoptosis (a phenomenon that we called "endothanatos" or death from inside). Endothanatos is a β5-dependent process since it cannot be induced in β5-null cells. The mechanisms underlying such process involve caspase activity and protein kinase C (PKC) activation. We raised antibodies against the β5 binding peptide and used it to purify a cytoplasmic protein, reasoning that such a protein would bind to the β5 cyto domain. The purified protein was annexin V, and using immunprecipitation experiments, we showed that β5 interacts with annexin V. Recently, the copurification of annexin V and β5 has been reported by another group (Andersen et al., 2002). Immunofluorescent studies confirmed that β5-annexin V colocalize in endothelial cells.

The mechanism underlying endothanatos is distinct from the other two forms of integrin-dependent apoptosis previously described. First, anoikis is a form of cell death that occurs after cell detachment (Frisch and Francis, 1994). However, cell detachment is not required for endothanatos. In addition, endothanatos is specifically linked to the β5 integrin, whereas anoikis can be mediated by other integrins. Finally, PKC inhibitors can modulate endothanatos in a selective manner. Endothanatos is not related either to another form of integrin mediated cell death (IMD), a process described by Stupack et al. (2001), which is dependent on caspase 8 recruitment to the membrane by the β1 and β3 cyto domains, but not the β5 cyto. However, we have shown that endothanatos is dependent on β5 and does not require caspase-8 activation.

The requirement of PKC activity for endothanatos underscores the importance of phosphorylation for proper integrin function. Protein phosphorylation is one of the earliest events detected in response to integrin stimulation. The ability of tyrosine kinase inhibitors to obstruct the formation of focal adhesions suggests a role for tyrosine phosphorylation in the signaling pathways linked to integrin receptors (Burridge et al., 1992;

Defilippi et al., 1994). Serine/threonine kinase families, such as PKC and mitogen-activated protein (MAP) kinase, are also activated upon integrin stimulation, and inhibitors of PKC block cell attachment and spreading in certain cell systems (Vuori and Ruoslahti, 1993; Nakamura and Nishizuka, 1994; Chen et al., 1994). Our findings suggest a link between cell death and the PKC pathway based on the specific association of annexin V with the β5 cytoplasmic domain.

The importance of PKC activity in αvβ5 function has been highlighted in experiments designed to study the molecular basis for growth factor (VEGF) regulation of αvβ5 function (Varner, 1997; Eliceiri and Cheresh, 2001). Upon binding to immobilized vitronectin, αvβ5 is barely detectable in association with actin, α-actinin, talin, tensin, $p130^{cas}$, and vinculin. In contrast, αvβ3 induces the localized accumulation of such molecules. Upon activation of PKC, αvß5 behaves similarly to αvβ3, but cannot recruit talin (Lewis et al., 1996). Furthermore, calphostin C, an inhibitor of PKC, seems to block angiogenesis mediated by αvβ5 but not by αvβ3 (Friedlander et al., 1995). These observations suggest that PKC activation probably affects the conformation or phosphorylation state of the β5 cytoplasmic domain. The cytokine regulation of αvβ5 integrin is unusual because ligand binding is unchanged, but the events that follow ligand binding differ (Klemke et al., 1994; Filardo et al., 1995; Yebra et al., 1995; Lewis et al., 1996). These results also show that cellular events mediated by αvβ3 or αvβ5 are clearly controlled by different mechanisms (reviewed in Varner and Cheresh, 1996a, 1996b; Filardo and Cheresh, 1994b).

We identified annexin V as a β5 cyto binding protein using an antibody against the β5 binding peptide. The primary sequence corresponding to the peptide is only partially found in that protein, suggesting that it is the structure of the peptide that anables it to bind to ß5. Such structural homology would not become evident in data base searches. Although annexin V is widely distributed, its precise biological function has not yet been established, but it may be an intracellular inhibitor of PKC (Dubois et al., 1996). Annexin V interacts specifically with PKC and can inhibit its activity, but the mechanism of PKC inhibition is poorly understood. It has been speculated that a third interacting partner within the cell would regulate this process within the cytoplasm under physiological conditions. Our results suggest that the third protein may be β5. Also in certain systems, PKC inhibitors have been shown to down-regulate the concentration of annexin V, leading to an antiproliferative phenotype, while PKC stimulators have been shown to have the opposite effect.

Kolanus and Seed (1997) proposed that in the inside-out signal transduction through integrins the signals converge from PKC and phosphatidylinositol 3,4,5-trisphosphate (PIP3). Our findings reveal a structural basis for the regulation of these events. The results presented here also provide evidence that the mechanism of action for β5 antagonists in apoptosis is to disrupt the association with annexin, probably due to a conformational change in the cytoplasmic domain.

Our data provide insights for the mechanism of action of annexin V in the induction of apoptosis. The unexpected result from these studies was that a short synthetic peptide mimic of annexin V induces massive cell death. We propose that the balance between cell survival and cell death after an external stimulus such as VEGF activation is determined by binding of β5 integrin to annexin V. Depending on the intracellular levels, annexin V may promote the survival by inhibiting PKC. We showed that the β5 binding peptide interferes with the union of annexin V to β5 cytoplasmic domain. When this occurs, there are more molecules of annexin V available that can bind to PKC, and the cells undergo apoptosis. We also showed that induction of apoptosis requires stimulation by growth factors, because growth factors are necessary for the apoptotic response. Therefore, PKC activation is required for the binding to annexin V.

Several observations attested to the specificity of the β5-cytoplasmic domain directed peptide and its proapoptotic properties. First, only cells expressing β5 could be killed by the treatment with the peptide-chimera, whereas chimeras carrying another peptide with different integrin specificity did not trigger cell death. Secondly, in the VEGF stimulated endothelial cells there was an impressive increase in cell death. Thirdly, the cell death is caspase dependent and fourthly, PKC inhibitors

could specifically delay cell killing. This last shows that endothanatos is dependent on the PKC pathway, and not on some other coincidental property of the peptide. In addition, the fact that inhibition of the activity of multiple signaling molecules does not affect endothanatos further substantiates the specificity.

Our findings contribute to a better understanding of the biochemical mechanisms that are related to the integrin-mediated signaling events involved in controlling tumor growth and angiogenesis. These processes involve cell adhesion to the extracellular matrix and integrin-mediated signaling. Our work raises the possibility that abnormalities in the expression or function of elements involved in this pathway may play a role in the initiation and progression of certain diseases such as cancer, rheumatoid arthritis, and retinopathies. In addition, these results predict that the intracellular signals mediated by integrin can be efficiently used to induce endothelial cell death, which may be exploited for treatment of these diseases. However, apoptosis is a regulated cellular process and offers several opportunities for therapeutic intervention. In addition to conventional therapeutic agents, which trigger apoptosis by activating cellular stress sensors, new therapeutic agents are being discovered that modulate the key regulators of apoptosis. Among the multiple events involved in apoptosis, caspase activation is the hallmark of programmed cell death. The caspase inhibitor X-liked inhibitor of apoptosis (XIAP) is a widely expressed protein, but abnormally high levels have been observed in some types of leukemias and solid tumors (Tamm et al., 2000).

We have used phage display to isolate peptides that bind to the BIR2 (baculovirus IAP repeat-2) domain of XIAP, a 70-amino acid zinc-binding domain common for all inhibitors of apoptosis (IAP) family members. BIR2 is a domain that selectively targets the active caspase-3 or –7. Therefore we predicted that blocking this domain would result in lack or disruption of caspase activation. The specificity of the motifs for BIR2 was confirmed independently by repeating library screenings using GST-BIR2 as a target, resulting in the selection of phage whose inserts shared a consensus sequence with those obtained originally using GST-full-length XIAP. Interactions of these peptide motifs with BIR2 are biologically relevant, since previous studies indicate that the BIR2 domain is necessary and sufficient for inhibiting caspases-3 and –7 (Takasaki et al., 1998). We also show that an internalizing version of the XIAP-binding peptide identified in our screenings (PFKQ) can induce programmed cell death in leukemia cells in a specific, dose-, and time-dependent manner. These findings, together with the previously described phenomenon of endothanatos, suggest that development of peptidomimetics following affinity maturation strategies to increase binding affinity may lead to tools that can be used as modulators of programmed cell death.

During programmed cell death, effector caspase zymogens are cleaved at conserved aspartic acid residues, generating large and small subunits, which together constitute the active protease. Activation of effector caspases, such as caspases-3 and -7, is a nearly universal event associated with apoptosis induced by multiple stimuli. Previous observations show that XIAP and other inhibitor of apoptosis (IAP) family proteins directly bind active executioner caspases 3 and 7, resulting in their potent suppression *in vitro* and in cultured cells (Deveraux et al., 1997; Roy et al., 1997), which suggests a general mechanism for IAP-mediated apoptosis inhibition.

Interestingly, we found the sequence EFES, which is embedded in one of the XIAP-binding phage in a cyclic context (CEFESC), occurs in caspase-3, a protease that binds to BIR2 of XIAP (Takahashi et al., 1998). Crystallography studies showed that this sequence is located in the 381-loop of caspase 3, in a region only found in caspases-3 and -7. Since this loop is important in binding of BIR2 to caspases-3 and -7 (Rield et al., 2001; Huang et al., 2001; Chai et al., 2001, reviewed in Stennicke et al., 2002), one might speculate that the EFES-containing peptide corresponds to an important region within the caspase-3-XIAP interacting site. In fact, the recently reported X-ray crystallographic structure of the XIAP-BIR2 domain/caspase-3 and –7 complex shows that the substrate-binding pocket within these caspases is formed by four surface loops, L1, L2, L3 and L4 (Rield et al., 2001; Huang et al., 2001; Chai et al., 2001). The sequence EFES is exposed for binding within the 381-loop, and the second Glu residue in the sequence corresponds to an interaction site between caspase-3 and BIR2 (Rield et al., 2001). This observation underscores the power of phage

display technology for the mapping for biologically relevant protein interacting sites.

As we have mentioned above, ligand-dependent cell signaling relies on the interaction of the extracellular ligands with the corresponding cell surface receptors. Therefore, characterization of cell surface receptors specific for any given cell type under different physiological conditions is important not only to help understand the cell response to the stimuli, but also as an important tool for therapy based on the particular set of receptors expressed in the cell. Angiogenic neovasculature expresses markers that are either expressed at much lower levels or not at all in nonproliferating endothelial cells (Burrows and Thorpe, 1994; Buckle, 1994). The markers of angiogenic endothelium include receptors for vascular growth factors, such as specific subtypes of VEGF and basic FGF receptors (Rettig et al., 1992; Mustonen and Alitalo, 1995; Lappi, 1995; Martiny-Baron and Marme, 1995). In this regard, endothelial cells become activated in response to VEGF stimulation, a process that is essential in angiogenesis. Therefore, characterization of cell receptors expressed in activated endothelial cells is important to understand the molecular mechanisms of cell activation. In addition, this information can also be invaluable for the design of targeted therapy in diseases where angiogenesis plays an important role, including cancer. We have investigated changes in cell surface receptors of endothelial cells in response to VEGF using phage display. The finding of peptides that bind specifically to endothelial cells can be achieved by panning of phage libraries on cells. We have developed a new method for the selection of peptides that bind to cell surface called biopanning and rapid analysis of selective interactive ligands (termed BRASIL). BRASIL result in the separation of phage-cell complexes from the remaining unbound phage; this is accomplished by a differential centrifugation that drives the cells from a hydrophilic environment into a nonmiscible organic phase. Because the organic phase is hydrophobic, it excludes the water and water-soluble materials surrounding the cell surface. Bound phage are recovered from the cell pellet, while the unbound phage stay soluble in the upper aqueous phase, eliminating the need for washes. This method proved to be more sensitive and more specific than techniques that rely on washing or limiting dilution steps to eliminate background during successive rounds of selection. BRASIL relies on organic phase separation and may represent an improvement over conventional cell-panning methods.

Using this method we screened a phage-display random peptide library on $VEGF_{165}$-stimulated endothelial cells (ECs). Stimulation of ECs with VEGF induces cell proliferation and the expression or upregulation of numerous receptors. We selected a novel ligand peptide for the receptor of the VEGF B family ($VEGF_{165}$, VEGF-B_{167}, VEGF-B_{186}). We established that such peptide is a functional VEGF-B mimetope. The sequence of this peptide, CPQPRPLC, carries the motif PRPLC (a neuropilin-1 - NPR-1 - binding site found in VEGF-B_{167}) and the overlapping motif PQPR (found embedded within a 12-residue NRP-1-binding epitope of VEGF-B_{186}; (Makinen et al., 1999). This peptide bound to VEGFR-1 and NRP-1. Thus, the motif PQPRPL appears to be a chimera between overlapping binding sites on different VEGF-B isoforms. Binding assays using individual phage on a panel of purified targets confirmed that the CPQPRPLC phage interacts specifically with VEGF receptors in a pattern consistent with VEGF-B-type ligands (Olofsson, 1999). These results suggest that the C terminal regions of both VEGF-B isoforms bind to VEGFR-1 and NRP-1, in agreement with recent results of deletion and site-directed mutagenesis studies of VEGF-B isoforms (Makinen et al., 1999). Also of importance is the observed difference in the ability of the synthetic peptide CPQPRPLC to block phage binding to VEGF receptors. Our results suggest that the CPQPRPLC peptide is approximately 100-fold more efficient in blocking phage binding to VEGFR-1 than to NRP-1. It is tempting to speculate that our chimeric motif interacts with VEGF receptors differentially. If so, this may be due to differences in the number of peptide-binding sites on each receptor, or in the affinity of the interaction at each binding site. Alternatively, such ligand-receptor interactions may be dependent on the conditions used for the binding assay. Full understanding of binding mechanisms awaits elucidation of the X-ray crystal structures of VEGFR-1– or NRP-1–CPQPRPLC peptide complexes. Although one can not as yet assert that BRASIL will be well suited for every cell-selection application, our data show that vascular targets (Soker et al.,1998) can clearly be found in EC membranes.

We also compared BRASIL and conventional cell-panning methods side by side to test the specific binding of the recently identified β2 integrin-antagonist peptide CPCFLLGCC, which contain the motif Leu-Leu-Gly (Koivunen et al., 2001). We found that BRASIL was again consistently and reproducibly superior to conventional methods used to assess cell binding when selection was performed on β2 integrin-expressing cells (unpublished observations). We are adapting the method for use with phage displaying larger polypeptides or folded proteins such as enzymes or antibodies (unpublished data).

Importantly, we also show that BRASIL can be used as a screening method by panning an unselected phage library on activated endothelial cells, thereby making BRASIL a valuable methodology for targeting and isolating ligand-receptor pairs in cell populations derived from clinical samples. The method may be used in tandem with fine-needle aspirates of solid tumors or fluorescence-activated cell sorting of leukemic cells obtained from patients. Moreover, because multiple samples and several rounds of preclearing and selection can be performed in a few hours with minimal loss, automation for high-throughput screening, and clinical applications are likely to follow. These data establish BRASIL as a bona fide method to probe functional domains of cell surface ligand-receptor pairs. A broad range of practical applications for the method can be envisioned.

Several reports have demonstrated that the array of surface receptors of the vasculature varies according to the host tissue and the physiological conditions of such tissues. Thus, Pasqualini and Ruoslahti reported in 1997 a novel *in vivo phage display* that distinguished between active proliferating microvascular endothelial cells in a tumor and quiescent nonproliferating endothelial cells elsewhere in the vasculature. They identified a peptide sequence that bound to angiogenic tumor endothelium, including a double-cyclic RGD-containing sequence that bound to αv integrin (Pasqualini et al., 1997). The peptide inhibited two metalloproteinases, resulting in inhibition angiogenesis, tumor growth, and invasion (Koivunen et al., 1999; Folkman, 1999). Moreover, when the peptide was conjugated to doxorubicin it delayed the tumor growth in mice significantly (Arap et al., 1998).

Aside from *in vivo* phage display, the use of methods such as serial analysis of gene expression (SAGE) clearly shows that the genetic progression of malignant cells is paralleled by epigenetic changes in nonmalignant endothelial cells induced by angiogenesis of the tumor vasculature (St Croix el al., 2000). The complexity of the human endothelium is also apparent from recent studies showing that the profile of certain endothelial cell receptors can vary depending on ethnic background (Wu et al., 2001). In addition, *in vivo phage display* experiments, underscored the heterogeneity of the mouse vascular endothelium. Unfortunately, human vascular addresses cannot always be inferred from rodent information because of species-specific differences in their sequences and location.

In order to address the mapping of the human vasculature we have performed an in vivo screening of a peptide library in a patient for the first time. The circulating peptides containing 47,160 motifs localized to the vasculature of different recovered organs showed that the phage distribution was nonrandom. Furthermore, certain circulating peptides bound specifically to known receptors on the vascular endothelium of the organ from which the peptide was recovered, but not to endothelium from other organs. For example, a prostate homing phage displaying a peptide mimic for interleukin-11 specifically bound to the endothelium and epithelium of normal prostate, but not to other organs such as skin.

In fact, *in vivo* phage-display in humans might reveal diversity of receptors expressed in the blood vessels even at the level of individual patients. However, our validation studies show that at least some ligand–receptor pairs are detectable in multiple unrelated subjects. Another advantage of the method described here is that selected targeted peptides bind to native receptors as they are expressed *in vivo.* Even if a ligand–receptor interaction is mediated through a conformational rather than a linear epitope, it is possible to select binders in the screening. Furthermore, it is often difficult to ensure that proteins expressed in array systems maintain the correct structure and folding. Thus, peptides selected *in vivo* may be more suitable for clinical applications.

Precedent exists to suggest that phage can be safely administered to patients, as bacteriophage

were used in humans during the preantibiotic era (Barrow and Soothill, 1997). Ultimately, it may become possible to determine molecular profiles of blood vessels in specific conditions; infusing phage libraries systemically before resections of lung, prostate, breast, and colorectal carcinomas, or even regionally before resection of limb sarcomas may yield useful vascular targets. Exploiting this experimental paradigm systematically with the analytical tools developed here may permit the construction of a molecular map outlining vascular diversity in each human organ, tissue, or disease. Translation of high-throughput *in vivo* phage-display technology may provide a contextual and functional link between genomics and proteomics. Based on the therapeutic promise of peptide- or peptidomimetic-targeting probes (Latham, 1999), clinical applications are likely to follow.

Resumen discusión

Las funciones específicas de la mayoría de las células dependen de su respuesta al entorno que les rodea. La decisión de vivir o morir, proliferar o permanecer quiescentes, activarse o no, depende, en el ámbito celular, de las interacciones con factores externos que desencadenan señales intracelulares encaminadas a llevar a termino las actividades mencionadas. Las moléculas extracelulares que controlan la respuesta celular pueden ser solubles como los factores de crecimiento o las citocinas, o bien moléculas insolubles como las que forman la matriz extracelular. Estas proporcionan soporte a la célula, polaridad, señales de orientación y potencial de adhesión a los vasos en crecimiento. Los receptores celulares tienen la capacidad de interaccionar con los ligandos y estos son los que determinan la naturaleza de las señales intracelulares que se transmiten. El objetivo de este trabajo fue describir algunos de los mecanismos de supervivencia celular desencadenados tanto por factores solubles como insolubles. Además, nos propusimos caracterizar los componentes de la superficie celular implicados en las señalizaciones específicas y describir los mecanismos de supervivencia dependientes de los receptores celulares.

Los macrófagos son células que juegan un papel clave en el desarrollo de la respuesta inmunitaria. Al igual que otras células del sistema inmunitario, los macrófagos son producidos en grandes cantidades y la mayoría de ellos mueren a través de procesos de apoptosis cuando su presencia deja de ser necesaria. Los procesos de proliferación y supervivencia de los macrófagos están regulados por el factor de crecimiento específico de los macrófagos M-CSF (*Macrophage-Colony Stimulating Factor*). Los macrófagos también pueden ser activados para llevar a cabo funciones tales como la presentación de antígenos, la lisis de células tumorales y la actividad antibacteriana. Los agentes más potentes en la activación de los macrófagos son la citocina IFN-γ (Interferon-gamma) y el LPS (lipopolisacarido).

Con el propósito de analizar los mecanismos que gobiernan los procesos de activación y de apoptosis de los macrófagos hemos utilizado macrófagos derivados de la médula ósea porque se trata de una población homogénea que en respuesta a estímulos externos puede proliferar, activarse, diferenciar o sufrir apoptosis. Sabíamos que los factores solubles como el IFN-γ y el LPS eran capaces de inhibir la proliferación de estas células. Sin embargo, nuestros resultados muestran que mientras el LPS inhibe la proliferación por inducción de la apoptosis, nunca pudimos observar apoptosis en macrófagos tratados con IFN-γ . Este comportamiento diferencial en la inducción de apoptosis tras la activación por IFN-γ o LPS nos llevó a estudiar el papel que podían tener estos agentes en la regulación de la apoptosis en los macrófagos. Así, observamos que el IFN-γ no solo no induce apoptosis en los macrófagos, sino que además protege de la apoptosis inducida por agentes como el LPS, glucocorticoides o la eliminación del M-CSF. Este efecto protector del IFN-γ es dependiente de la expresión del $p21^{Waf1}$ y del bloqueo del ciclo celular en la barrera G1/S. Estos resultados resaltan la relación existente entre proliferación, ciclo celular y la inducción de apoptosis.

La función de los macrófagos no esta regulada únicamente por los agentes solubles. Así, hemos observado que la decorina, que es un proteoglicano pequeño presente en la matriz extracelular, es capaz de inhibir la proliferación dependiente de factores de crecimiento debido a su capacidad de bloquear la fase G_1 del ciclo celular. Además, observamos que la decorina previene la inducción de apoptosis que se produce normalmente tras la eliminación de factores de crecimiento. Nuestros estudios determinaron que la decorina induce la expresión de los inhibidores de la cdk, $p21^{Waf1}$ y $p21^{Kip}$. Los experimentos que realizamos con animales *knock-out* para estos genes nos revelaron que la expresión de $p21^{Waf1}$ era necesaria para la protección de la apoptosis, mientras que la inhibición de la proliferación era mediada por la expresión de $p27^{Kip}$. Los macrófagos para crecer *in vitro* necesitan adherirse a la superficie de las placas de cultivo, y esta adhesión es la que permite la proliferación y la viabilidad celular. Hemos observado que la adhesión de los macrófagos a las placas de cultivo es dependiente de las proteínas de la matriz extracelular que recubren dichas placas. Nuestros estudios demuestran que la decorina aumenta la adhesión a la matriz extracelular y esto es en parte responsable de la inducción de la expresión de $p27^{Kip}$, pero no del aumento en la supervivencia de estas células.

La decorina es secretada por los macrófagos en los puntos de inflamación y allí modula la respuesta

inmunitaria a través de su interacción con las células implicadas. Además, la decorina también puede regular la síntesis e interacción de otras proteínas de la matriz extracelular. Hemos observado que la decorina aumenta la activación de los macrófagos inducida por el IFN-γ y el LPS debido a su capacidad para secuestrar TGF-β (*Transforming Growth Factor*) endógeno, impidiendo así la unión a sus receptores. Nuestros resultados han demostrado que la decorina aumenta tanto la activación de los macrófagos inducida por el LPS como por el IFN-γ, pero sin inducir la expresión del MHC II, de la iNOS o la expresión de citocinas, sino que el efecto de este proteglicano en la activación de los macrófagos es debido a su capacidad de bloquear la unión del TGF-β autocrino a los receptores de la superficie de los macrófagos.

Hasta ahora, hemos discutido la importancia de los factores solubles e insolubles en los mecanismos implicados en la activación y la supervivencia celular. Estos factores modulan la respuesta celular a través de la interacción con los receptores de la superficie celular. Por ello, la caracterización de los mecanismos moleculares de la señalización mediada por dichos receptores es importante para entender la señalización que se deriva tras la unión del ligando a su receptor. En este sentido, las proteínas de la matriz extracelular desempeñan sus funciones de adhesión, migración y supervivencia a través de su unión a las integrinas, que son sus receptores celulares específicos. Las integrinas αvβ3 y la αvβ5 parecen ser las que tienen un papel más relevante en la angiogénesis tumoral y se ha sugerido que la modulación de la apoptosis por dichas integrinas podría ser importante en la regulación de la neovascularización. Las cadenas β de las integrinas son suficientes para el inicio de la señalización transmitida a trabes de las integrinas, y se ha sugerido que la parte citoplasmática de esta cadena esta involucrada activamente en la señalización intracelular que modula la supervivencia de las células endoteliales. Con el propósito de estudiar en detalle los mecanismos que gobiernan la señalización mediada por la integrina β5, nos planteamos la búsqueda de las proteínas que se unen al dominio citoplásmico de dicha cadena. Para ello utilizamos la técnica del *phage-display*, método que permite obtener péptidos de pequeño tamaño que interaccionan de manera específica con las proteínas de interés *in vitro* o bien con proteínas de la superficie celular *in vivo,* tanto en células aisladas como en el contexto del órgano intacto. Utilizando esta técnica del *phage display* identificamos un péptido que se unía de manera específica al dominio citoplasmático de la integrina β5. La versión internalizable de dicho péptido era capaz de inducir la muerte en las células endoteliales, fenómeno al que llamamos endothanatos (muerte desde el interior). El análisis bioquímico de este fenómeno demostró que este presentaba las características típicas de la apoptosis, tales como la activación de las caspasas y la formación de la típica "escalera" de DNA. Los experimentos con ratones *knock-out* para la β5 confirmaron que la muerte celular era dependiente la expresión de esta integrina. Con el objeto de aislar la posible proteína que el péptido podía mimetizar, seguimos un proceso de purificación por afinidad que finalizó con la identificación de una proteína cuya identidad se revelo por espectrometría de masas. La proteína purificada resultó ser anexina V. Los posteriores experimentos confirmaron que la anexina V se unía específicamente al dominio citoplasmático de la β5, unión que era dependiente de la presencia de calcio en el sistema. Esta proteína es conocida por inhibir la señalización mediada por la PKC (Proteína Kinasa C). Los experimentos realizados con extractos celulares demostraron que la anexina V se une específicamente a la PKC activada. Además observamos que el uso de inhibidores de la PKC podía revertir la apoptosis inducida por nuestro péptido. Nuestros resultados proporcionan nueva información del mecanismo de acción de la anexina en la inducción de apoptosis, que resulta ser dependiente de su unión al domino citoplasmático de la integrina β5. Los experimentos con modelos angiogénicos mostraron que el péptido inducía una marcada inhibición de la angiogénesis tanto en modelos *in vitro* en células en cultivo como *in vivo* en ratones.

Entre todos los acontecimientos implicados en el proceso de apoptosis la activación de las caspasas es quizás el hecho más importante. El inhibidor de las caspasas ligado al cromosoma X (XIAP) se expresa de forma ubicua en el organismo. Sin embargo, se han encontrado niveles elevados en algunos tipos de leucemias y tumores sólidos, sugiriendo que el bloqueo de la activación de las caspasas pudiera contribuir al desarrollo de estos tumores. En este contexto, pensamos que si consiguiéramos aislar los péptidos que se unen e

inhiben la actividad de las IAP, se podrían utilizar para facilitar la inducción de apoptosis en aquellas situaciones en que pudiera ser beneficioso, como en el tratamiento de ciertos tumores. Así, utilizamos el método del *phage display* para identificar los motivos que se unen de forma específica a la XIAP. Dos de las secuencias peptídicas obtenidas reconocían únicamente uno de los tres dominios de la proteína, el dominio BIR2. La unión del bacteriófago que expresaba esta secuencia a la XIAP era inhibida por el péptido sintético, confirmando así la especificidad de la unión. Los estudios cristalográficos de la proteína XIAP muestran que las secuencias obtenidas guardan homología con el sitio de unión de las caspasas 3 y 7 con XIAP. Los estudios con la forma internalizable del péptido mostraron que una de estas secuencias podía inhibir la viabilidad de las células de leucemia. Estos resultados abren la posibilidad de utilización de esta metodología como nueva estrategia para el tratamiento de este tipo de enfermedades.

La expresión diferencial de las moléculas de superficie de los distintos tipos celulares, así como el hecho de que en estados patológicos la expresión de estas moléculas se altere, ha impulsado la búsqueda y caracterización de las moléculas de superficie asociadas a estados fisiológicos concretos. En este sentido, un buen ejemplo lo constituye la angiogénesis, ya que se han descrito marcadores de superficie en células endoteliales expresadas en los vasos de nueva formación que no se expresan o se encuentran a muy a bajos niveles en las células endoteliales que no proliferan tan activamente. Uno de los factores solubles que regulan la angiogénesis es el VEGF (*vascular endothelial growth factor*), el cual actúa induciendo la activación de las células endoteliales. Así pues, la caracterización de marcadores de superficie expresados en las células endoteliales estimuladas con VEGF puede ayudar a entender mejor el proceso de neovascularizacion y a su vez posibilitar el diseño de terapias encaminadas a bloquear la angiogénesis.

Dadas las limitaciones de las técnicas existentes para la búsqueda de los receptores de superficie celular usando genotecas bacteriofágicas hemos desarrollado una nueva tecnología que permite separar eficientemente los complejos específicos unidos a la célula del bacteriófago no unido. Así, este nuevo método al que hemos denominado BRASIL (*biopanning and rapid analysis of selective interacting ligands*) nos permite incrementar la sensibilidad y la especificidad en la recuperación de los ligandos. Hemos utilizado este método para el aislamiento de ligandos específicos de las células endoteliales estimuladas con VEGF. Un 75% de los péptidos obtenidos presentaban homología con dominios del VEGF que participan en la interacción con sus receptores. Uno de los péptidos seleccionados reconocía dos de los cuatro receptores de la familia del VEGF, el VEGFR-1 (*VEGF receptor-1*) y el NRP-1 (*neuroplin-1*) y además era capaz de impedir la unión del VEGF a su receptor.

La diversidad de los receptores de la superficie celular en la vasculatura ha sido ampliamente ilustrada en los modelos animales. Sin embargo, este hecho ha sido poco explorado en humanos. Aunque se suponía que el aislamiento de las moléculas especificas de la vasculatura de los órganos concretos en los modelos animales podría ayudar a la identificación de homólogos humanos, en realidad esto solo ha sido así en muy pocos casos. Al parecer, la diversidad de los patrones de expresión de las proteínas en los tejidos y la accesibilidad del receptor resulta muy diferente entre humanos y ratones. Para solventar este problema, nos planteamos realizar un estudio de la diversidad molecular de la vasculatura humana analizando ligandos específicos de los vasos sanguíneos de los distintos órganos en un paciente terminal. Para ello, se inyectó a dicho paciente con una genoteca bacteriofágica de péptidos y a continuación se obtuvieron biopsias de la médula ósea, la piel, el tejido graso, el músculo y el riñón. El análisis de los péptidos recuperados en cada tejido reveló que la distribución de los péptidos circulantes no fue al azar y que es posible establecer un perfil específico de las moléculas expresadas en los vasos sanguíneos de los diferentes órganos.

Uno de los bacteriófagos aislado de la próstata expresaba una secuencia peptídica con alta homología con la IL-11. Los experimentos posteriores en ensayos de unión *in vitro* confirmaron que dicha secuencia mimetizaba las propiedades de unión de la IL-11 y además, se vio que el péptido reconocía específicamente el endotelio y el epitelio de la próstata humana, pero no así ningún otro órgano control.