CLU "In and Out": Looking for a Link

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Cancer cells need to interact synergistically with their surrounding microenvironment to form a neoplasm and to progress further to colonize distant organs. The microenvironment can exert profound epigenetic effects on cells through cell-derived interactions between cells, or through cell-derived factors deposited into the microenvironment.

Tumor progression implies immune-escaping and triggers several processes that synergistically induce a cooperation among transformed and stromal cells, that compete for space and resources such as oxygen and nutrients. Therefore, the extra cellular *milieu* and tissue microenviroment heterotypic interactions cooperate to promote tumor growth, angiogenesis, and cancer cell motility, through elevated secretion of pleiotropic cytokines and soluble factors.

Clusterin (CLU), widely viewed as an enigmatic protein represents one of the numerous cellular factors sharing the intracellular information with the microenvironment and it has also a systemic diffusion, tightly joining the "In and the Out" of the cell with a still debated variety of antagonistic functions. The multiplicity of names for CLU is an indication of the complexity of the problem and could reflect, on one hand its multifunctionality, or alternatively could mask a commonality of function. The posited role for CLU, further supported as a cytoprotective prosurvival chaperone-like molecule, seems compelling, in contrast its tumor suppressor function, as a guide of the guardians of the genome (DNA-repair proteins Ku70/80, Bax cell death inducer), could really reflect the balanced expression of its different forms, most certainly depending on the

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intra- and extracellular microenvironment cross talk. The complicated balance of cytokines network and the regulation of CLU forms production in cancer and stromal cells undoubtedly represent a potential link among adaptative responses, genomic stability, and bystander effect after oxidative stresses and damage. This review focuses on the tumor–microenvironment interactions strictly involved in controlling local cancer growth, invasion, and distant metastases that play a decisive role in the regulation of CLU different forms expression and release. In addition, we focus on the pleiotropic action of the extracellular form of this protein, sCLU, that may play a crucial role in redirecting stromal changes, altering intercellular communications binding cell surface receptors and contributing to influence the secretion of chemokines in paracrine and autocrine fashion. Further elucidation of CLU functions inside and ouside ("*in* and *out*") of cancer cell are warranted for a deeper understanding of the interplay between tumor and stroma, suggesting new therapeutic cotargeting strategies. © 2009 Elsevier Inc.

I. INTRODUCTION

Cooperation through the sharing of diffusible factors of tumor microenvironment and the redirection of some specific guardian pathways raises new questions about tumorigenesis and has implication on designing new therapeutic approaches. Tissue microenvironment strongly influences tumorigenesis and neovascularization, redirecting some pathways versus a persisting prosurvival state. The tumor microenvironment consisting of tumoral, immune, stromal, and inflammatory cells, all of which produce cytokines, growth factors, and adhesion molecules, may promote tumor progression and metastases. Among the tumor-associated cells are endothelial cells and pericytes that together form the neovasculature, which supplies tumor cells with nutrients and oxygen. In addition, mesenchymal cells of the tumoral stroma are already apparent in tumors at its earliest stages suggesting a coevolution between stromal and cancer cells that leads to clonal expansions. This cancer stromal coevolution leads to support additional microenvironmental changes to foster tumor growth. Given the striking similarities between the phenotypes of aggressive tumors and embryonic stem cells particularly with respect to specific signaling pathways underlying their intriguing plasticity is not surprising that the gradients of growth factors leads long distant final target. The cytokines produced by cancer cells function to create optimal growth conditions within the tumor microenvironment, while cytokines secreted by stromal cells may influence behavior of malignant cells (Lewis *et al.*, 2006). While the normal development of tissue and organs in the embryo is coordinated by a complex equilibrium between positively and negatively acting cues and signals given by the microenvironment, the dysregulated expression of potent embryonic morphogens in some aggressive cancer cell type in absence of normal negative regulators has demonstrated a strong induction of uncontrolled proliferation, increased survival, dedifferentiation, and plasticity that promotes malignant

transformation and contributes to metastasis. These effects could be transient, as seen in signaling pathways regulating cellular proliferation, or associated to more stable events, such as cell fate determination or differentiation.

In tumor microenvironment as in a microecosystem, all cell population intimately interacts with one another and plays an important role in inflammatory and proangiogenic processes, promoting tumor cell proliferation. Interestingly, there is a tightened association between chronic inflammation and tumor insurgence or progression.

Chronic inflammation is mediated via a persistent and continuous stimulus and the resulting prolonged exposure to inflammatory cytokines has the potential to promote tumor growth through the induction of angiogenesis, DNA damage, events that favor tumor invasion. Inflammatory and stromal cells communicate via cytokines and chemokines. Cytokines regulate growth, trafficking, signaling, and differentiation of both stromal and tumor cells. It has been observed (Soufla *et al.*, 2006) that the different steps of cancer progression are signed by a gradient of differential cytokine cocktails that, in turn, epigenetically activate different pathways, either in cancer and in stromal cells, influencing primary genes transcription. The epigenetic influence of the microenvironment on tumoral cells is curiously observed, not only on the induction of *trans*-signaling cascade pathways affecting gene acetylation and transcription, but also on the fine regulation of tumor-suppressor microRNAs and also on the alternative RNA splicing pattern, leading to the expression of specific protein isoforms. Alternative splicing is a major mechanism for modulating the expression of cellular and viral genes and enables a single gene to increase its coding capacity. An explicative example in cancer is the changes in the microenviroment mediators induced by hypoxia, a hallmark of cancer progression. In fact, hypoxia induces preferential expression of one isoform of vascular endothelial growth factor (VEGF-A165) (Panutsopulos *et al.*, 2003). The different products of VEGF-A, (120, 183, 165a, 165b) are produced by alternative splicing between exon 5 and 8 of the VEGF-A gene, are differently conditioned by microenvironment, as a result of cytokines and growth factors influence. The differential splicing in cancer and stromal cells is also strongly connected with the preferential clusterin (CLU) forms production, and also in this case it could be influenced by external cytokines, growth factors, and environmental condition (pH, ROS, Ca^{2+},etc.), that guide the production of the predominant form needed in a particular district depending on the specific tumoral context. It has been demonstrated that the different CLU forms production is influenced by different soluble factors activating different signaling cascade (Criswell *et al.*, 2005; Patterson *et al.*, 2006; Pucci *et al.*, 2004a,b, 2008, 2009) which in turn may be involved in orchestrating the differential step

of carcinogenesis, inducing a prosurvival response, membrane remodeling, cell–cell adhesion, and cancer cell motility.

Both forms of CLU are involved in cancerogenesis. The prevailing data suggest that in the first step the cell could attempt to block the transformation, therefore the prodeath form nCLU functions as a tumor suppressor, while its repression during cancer progression could favor the prosurvival sCLU isoform, that could be extracellularly released in the microenvironment. In normal cells, the balanced production could be affected also by ionizing radiation (IR) in a dose-dependent manner (Klokov *et al.*, 2004). In fact, high doses induce the proapoptotic CLU isoform, whereas low doses ($>$0.02 Gy) induce the prosurvival isoform involved in the adaptative response. This particular function of CLU in tumors, as demonstrated by D.A. Boothman and his group, is strongly involved in the acquisition of radio- and chemoresistance to various chemotherapeutic agents, including docetaxel, cisplatin, doxorubicin, and camptothecin used for treatment of breast, colon, renal, bladder, lung, and prostate cancer. The prosurvival function of sCLU is on the basis of current phase I/II clinical trials in prostate, lung, and breast cancer.

In tumor progression, the induction of sCLU requires its transcriptional activation and *de novo* synthesis, which is strongly influenced by growth factors and cytokines, such as TGF-β1, TGF-β2, IL-6, IGF-1, and VEGF-A165 prevailing in the tumoral context (Criswell *et al.*, 2005; Pucci *et al.*, 2009). In the present review, we shall concentrate on the molecular cross talk between cancer cells and stroma that influences the differential production of CLU forms (*in* the cell) and the potential action that sCLU could exerts *out*, in the microenvironment, to favor tumor growth and tumor cell migration. Taken together, these observations underscore the influence of the *host* microenvironmental changes on the survival and the behavior of malignant tumors, adhering to the older Paget's concept that the soil could influence the success and the behavior of the seed.

II. NORMAL AND CANCER MICROECOSYSTEM

Development and homeostasis of tissue is dynamically and finely modulated by a complex network of cellular communication that influences cell behavior and fate, throughout the embryogenesis and beyond. During gastrulation, epithelial cells in the ectoderm change into mesenchymal cells, which invade the primitive steak and insert themselves between ectoderm and endoderm. Some of these mesenchymal cells will participate to epithelial stroma establishment, maintaining a lifelong relationship with citotype. The organ and tissue formations are coordinated by epithelial–mesenchymal

reciprocal interactions and the epithelial response to systemic hormones and growth factors are often mediated by the mesenchyme. Many data demonstrated *in vitro* the cross talk between these cell types. Among tumor-associated mesenchymal cells, are endothelial and pericytes that together form the neovasculature, which is fundamental to supply nutrients, oxygen, evacuating wastes, and carbon dioxide. Moreover, fibroblasts and myofibroblasts overshadowed. This communication could be physically supported by the heterotypic cell–cell interactions for a short-range signal that do not need a prompt diffusion in the surrounding area, or by secreted molecules such as cytokines, chemokines, growth factors, proteinases and their inhibitors, and lipid products.

There is growing evidence that tumors are promoted and sustained by active inflammatory signals from the surrounding microenvironment as an alteration of the normal tissues homeostasis. In 1850, Rudolf Virchow was the first to describe the tumor-promoting effect of chronic irritation or inflammation. Prominent examples include the association between infection with *Helicobacter pylori* and gastric cancer, discovered by the Nobel prize laureates Barry Marshall and Robin Warren; papilloma virus and cofactors like Chlamydia or herpes simplex 2 infection and cervical cancer; and the predisposition of patients with Chron's disease to colorectal cancer, prostate chronic inflammation, and prostate cancer. The persistence of these pathobiological factors concur to determine an active chronic inflammation acting in a paracrine manner to induce angiogenesis, as well as activation of surrounding stromal cell types, fibroblasts, smooth muscle cells, and adipocytes, leading to the secretion of growth factors and proteases.

Activated fibroblast in the stroma promotes tumor progression by producing stroma-modulating growth factors. In particular, members of VEGF family, platelet-derived growth factors (PDGF), epidermal growth factors receptors (EGFR) ligands, interleukins such as IL-6, IL-1, IL-8, and transforming growth factors-β (TGF-β). These factors disrupt the normal tissues and act in a paracrine manner to induce stromal reaction, angiogenesis, and inflammatory response. The altered expression of growth factors is associated, as a result of their autocrine effect on tumor cells, with the production of proteolytic enzymes and matrix metallo-proteinases (MMPs) which start the remodeling of the promigratory cell matrix components.

TGF-β, VEGF, PDGF, and fibroblast growth factor signaling pathways are involved in the process of neoangiogenesis, whereas insulin-like growth factor-1 (IGF-1), epidermal growth factor, CXC12, and IL-6 play active roles in the cancer progression and in the formation of distal metastasis of many epithelial cancers.

It seems that the different phases of cancer progression are accompanied by gradients of different components of the microenvironment that prelude and induce the subsequent step and cooperate to confer different

aggressiveness to the tumor. CLU is one of the targets of this alteration of the balanced production of cytokines and growth factors, that signs the transition between normal and malignant phenotype.

In 2003, De Wever and Mareel (2003) proposed two closely interactive pathways in their model of the cross talk between cancer cells and stromal tissue, namely the efferent and afferent pathways. In the efferent pathway, cancer cells trigger a reactive response in the stroma by releasing soluble factors such as TGF-β and PDGF. These factors can directly or indirectly *trans*-differentiate fibroblasts into myofibroblasts or induce epithelial–mesenchymal transition (EMT) in the surrounding cancer-associated stroma, resulting in cells that exhibit increased expression of vimentin, unchanged levels of smooth muscle α-actin, and decreased expression of calponin, which together constitute the characteristic myofibroblast phenotype. Cancer cells are also capable of inducing neoplastic transformation in the stromal cells of the host organ. This efferent pathway seems necessary and may serve as an early event in cancer progression.

In the afferent pathway, cancer cells respond to modified stromal cells in the surrounding microenvironment. Reactive stroma exerts multiple effects on the behavior of cancer cells. Reactive stromal cells release soluble factors, secrete solid matrix components, repress cell apoptosis, increase motility and invasion, and guide progression and distal spread.

III. MICROENVIRONMENT EFFECTS ON CLU EXPRESSION (THE "IN" EFFECT)

A. TGF-β, the P*rimum* M*ovens*

In normal unstressed tissues, the basal release of TGF-β by local source may suffice for the maintenance of the homeostasis. However, under conditions of tissues injury, TGF-β is abundantly released by blood platelet cells and various stromal components. TGF superfamily includes: TGF-β, bone morphogenic protein (BMPs) and activins. TGF-β plays multifunctional roles in regulating cell cycle, apoptosis, differentiation, and extracellular matrix (ECM) remodeling. The different isoforms of TGF-β seems to play different antagonistic role during tumor growth and progression, being differentially expressed during tumorigenesis. The controversial role of TGF-β could be attributed to the presence of different isoforms and receptors members and the prevailing expression of one on the others could determine the inhibition or development of tumor formation (Tian and Shiemann, 2009). The inhibition of the signal transduction of TGF-β receptor II has been shown to induce the progression to malignancy in epithelial

cells (Browmick *et al.*, 2001). Cancer cells that lose the tumor suppressive arm of the TGF-β pathway accrue tumorigenic effects that directly enhance tumor growth and invasion (Massaguè, 2008). Conversely, the overexpression of TGF-β in skin papillomas of a transgenic mouse model is associated with progression to metastatic tumors, mediated by both autocrine and paracrine signaling.

Experimental animal models have demonstrated that cancer invasion is stimulated by wound healing stroma. This observation implies that growth factors involved in wound healing such as TGF-β and PDGF play an important role in tumor growth and invasion as well.

TGF-β and PDGF are two factors secreted by a wide range of cancer cells and mediate the first interaction among tumor cells and stromal fibroblast. Reactive stroma not only play an important role during cancer initiation and progression, but also in determining whether TGF-β suppresses or promotes tumor formation. TGF-β exerts its antitumor activities by regulating the behavior of epithelial cells and adjacent fibroblast, which synthesize and secrete a variety of cytokines, growth factors, and ECM proteins that mediate homeostasis and suppress cancer development. Thus, the inactivation of paracrine TGF-β signaling between adjacent epithelial and stromal compartment promotes cellular transformation, as well induces the growth, survival, and motility of developing neoplasm.

In turn, cancer cells trigger a reactive response in the stroma inducing the release of factors, such as TGF-β and PDGF that directly or indirectly could *trans*-differentiate fibroblast into myofibroblast or induce EMT in the surrounding cancer stroma, with an increased production of vimentin, unchanged expression of α-actin decreased expression of calponin (De Wever and Mareel, 2003).

A controversial correlation between high inflammatory infiltrate and aggressiveness of the tumor has been observed by pathologists in the middle of nineteenth century. The dual and ambiguous role of TGF-β during cancer progression is further demonstrated by the TGF-β activation of nuclear Foxp3 in the T cell infiltrate, inducing CD25+FoxP3+ T cells the determinant players of tumor immune escaping during cancer progression. These data effort the involvement of TGF-β in immune dormancy in cancer favoring cancer progression. Moreover, TGF-β affects Foxp3 activation and its nuclear translocation also in cancer cells, where its intriguing role has still to be clarified. TGF-β2 treatment but not TGF-β1 induced the upregulation of the forkhead factor Foxp3 and determined its translocation from the cytoplasm to the nucleus of the neoplastic cell. The Foxp3 expression and nuclear translocation induced by TGF-β2 and IL-10 has been associated with worse overall survival in breast cancer (Merlo *et al.*, 2009).

Interesting data were obtained after TGF-β2 treatment to a hepatocarcinoma cell line, Hep-G2. In TGF-β2-treated Hep-G2 cells, we observed an

increased expression of one specific isoform of Foxp3. Moreover, TGF-β 2 treatment induced a nuclear translocation of this factor (Pucci *et al.*, unpublished data). TGF-β2 treatment induces the expression and the nuclear translocation of Foxp3 also in pancreatic ductal adenocarcinoma cells and tumors. The TGF-β2 effect could be mimicked by ectopic expression of a constitutively active TGF-β type I receptor/AK5 mutant (Hinz *et al.*, 2007). Furthermore, the expression of Foxp3 induced by TGF-β2 in cancer cells could affect, by an unknown paracrine action, also the immune response dormancy of the tumor T cell infiltrate. Coculture of Foxp3-expressing tumor cells with naïve T cells, completely inhibited T cell proliferation, but not their activation. This effect indicates that pancreatic carcinoma cells share growth suppressive effects with T-reg and suggest a new mechanism of immune evasion induced by TGF-β2 action underlying the dual role of this growth factor in cancer initiation and progression.

In this complex cross talk, factors that directly and indirectly affect CLU production will be examined. It seems that TGF-β is closely involved in the regulation of CLU different forms expression, depending on the stage and state of neoplastic disease.

The TGF-β input influences the expression of different CLU forms in cancer cells. Hence, TGF-β and its dichotomous nature during tumorigenesis could also be related to the differential induction of the antagonistic CLU different forms.

TGF-β1 signaling pathways are activated by ligand binding to the cell surface transforming growth factor receptor type II (TGFRII), the activation leads to a phosphorylation of the TGF receptor type I. The signaling cascade involved the migration to the nucleus via SMAD (SMAD2 and SMAD3) superfamily proteins inducing the transcription of target genes. However, variant branches of Smad-independent pathways coexist with the canonical paths in response to TGF-β. Smad-independent modes of TGF signaling involve the activation of the transcription factor AP-1 and EGR-1. AP-1 has been proposed to have a role in the CLU upregulation after TGF-β treatment in normal cells (Jin and Howe, 1997; Reddy *et al.*, 1996). The induction of nuclear localization of CLU in normal cells after TGF-β treatment indicates the TGF-β role in maintaining the normal tissue homeostasis and its functional link in the tumor suppressor action of CLU. A striking accordance exists between CLU and TGF-β expression during mouse embryogenesis, cardiac valve morphogenesis, and in various pathophysiological conditions, such as atherosclerosis and Alzheimer's disease. On the other hand, in advanced stage cancers the truncated form of CLU, produced by differential splicing event, is extracellularly released, probably through the stimulus of different cocktail of cytokines indirectly induced in cancer progression.

A reciprocal control exists between CLU and TGF-β signaling. It is known that CLU interacts with TGF-β type II receptor (TGF-βRII). Moreover,

experimental data demonstrated that CLU regulates TGF-β signaling pathway by modulating the stability of Smad2/3 proteins. CLU siRNA repressed TGF-β-induced transcriptional activity and decreased the amount of Smad2/3 proteins in hepatocarcinoma Hep3B cells (Lee *et al.*, 2008). It has been demonstrated that CLU increased Smad2/3 phosphorylation and also stabilized Smad2/3 proteins, probably modulating their turnover via proteosomal degradation (Lee *et al.*, 2008).

In yeast two-hybrid analysis and cell lysates, CLU is able to bind the receptors TGF-βRI and TGF-βRII. However, the sCLU binding occurred in the intracellular portion of the receptor, suggesting that the cytoplasmic, rather than the secreted form may be involved in TGF-β1 signaling. It has been demonstrated that the treatment with TGF-β1 induced an increase of both sCLU and nCLU proteins in TGFRII-proficient VACO-400RII human colon cancer cells, whereas no induction of CLU was found in VACO-400 cells bearing a mutation of TGF-βRI (Boothman, Koklov *et al.*, unpublished data). Moreover, TGF-β1-induced CLU production resulted in growth arrest but not apoptosis. This effect of TGF-β1 is in accordance to its main function during tissue formation and after tissue damage or in the first phases of tumor growth in order to maintain tissue homeostasis inhibiting proliferation and, in case of damaging agents, activating cytoprotective pathways as defense mechanisms. In contrast, the presence of cytokines and growth factors such as IL-6 and VEGF-A165, that secondly becomes abundant in the tumor microenvironment act influencing sCLU production favoring the prosurvival pathways of the neoplastic cell. The increased expression of IL-6 and VEGF-A165 is strongly influenced by oxidative stress and hypoxia.

B. Hypoxia Inducible Factor: Altered of IL-6 and VEGF-A165 Expression in the Microenvironment

The discovery of the hypoxia-inducible (HIF-1) transcription factor (Semenza, 2003) and the finding that it directly controls the transcription of almost every enzyme of glycolysis provided a potential molecular mechanism for the effects of hypoxia on the glucose metabolism of tumors. The accumulating data suggest that the altered metabolism of tumor cells is genetically controlled by the very mutations that give rise to cancer. In recent years, several studies have demonstrated that oncogenic and tumor suppressor mutations found in a wide variety of human cancers can directly activate HIF-1 independently of hypoxia. HIF-1 is a heterodimer transcription factor that consists of two proteins HIF-1α and HIF-1β. The HIF complex binds to HREs (hypoxia responsive elements) in promoters of target genes, stimulating transcription of key angiogenic factors such as VEGF and

angiopoietin-2 which are overexpressed in tumors (and essential for tumor angiogenesis). In addition, nearly all the enzymes of glycolysis are increased by HIF-1. As a consequence, the glycolytic shift would be caused by a specific transcriptional program.

Signaling pathways activated by growth factors and deregulated in cancer lead to an increase in HIF-1α protein levels. Studies on VHL tumor suppressor (which is mutated in familial and sporadic clear cell renal carcinomas) show that VHL encodes for a subunit of ubiquitin ligase protein involved in the degradation of HIF-1α. In particular, HIF-1α is not hydroxylated and can escape polyubiquitylation (Ub–Ub, mediated by the von Hippel–Lindau protein (pVHL) ubiquitin–ligase complex) and degradation even under normoxic conditions. HIF-1α then translocates to the nucleus where, together with HIF-1β, forms the active HIF complex that induces the expression of genes that support tumor growth and spreading and might decrease apoptosis (Semenza, 2006). Presently HIF activation in tumoral tissues is considered a prognostic factor associated with radiation resistance and poor prognosis representing a hallmark of cancer progression.

HIF-1 protein synthesis is regulated by activation of the phosphatidylinositol-3-kinase (PI3K) and ERK mitogen-activated protein kinase (MAPK) pathways. These pathways can be activated by signaling via receptor tyrosine kinases, nonreceptor tyrosine kinases, or G-protein-coupled receptors. In particular, PI3K is directly activated by both Ras and growth factor receptor tyrosine kinase (EGFR-Her2/Neu) mutationally activated in the majority of human cancers. The downstream effector of PI3K is AKT protein kinase. Overall, HIF-1 is overexpressed in human cancers as a result of intratumoral hypoxia as well as genetic alterations, such as gain-of-function mutations in oncogenes (amplification of Her2/Neu) and loss-of-function mutations in tumor-suppressor genes (VHL and PTEN). HIF-1 overexpression in cancer is associated with treatment failure and increased mortality. In preclinical studies, inhibition of HIF-1 activity has marked effects on tumor growth. Efforts are underway to identify inhibitors of HIF-1 and to test their efficacy as anticancer therapeutics. The level of IL-6 in tumor microenvironment and in cancer cells could be strongly influenced by the HIF.

Of particular interest is the cooperation of IL-6 and HIF-1 and their action on tumor cells behavior and cell death escape. It has been observed that in critical conditions (hypoxia, oxidative stress), the activation of STAT3 influences the preferential expression of VEGF-A165a, leading to the inhibition of programmed cell death inducing Bcl-2. Moreover, it has been shown that an increased formation of IL-6–sIL-6R complexes that interact with gp130 on the cell membrane (*trans*-signaling) leads to the enhanced expression and nuclear translocation of STAT3, which can cause induction of antiapoptotic genes, such as Bax antagonist, Bcl-x_L (Mitsuyama *et al.*, 2007). In this view,

IL-6, induced by HIF, seems to contribute to a key mechanism of tumor development and progression through the inhibition of cell pathways leading to apoptosis. Moreover, IL-6 could directly or indirectly influence and promote tumor growth and vascularization synergizing with the HIF-1α in the induction of VEGF-A165 expression. These complex network influences also CLU production in neoplastic cells. We will discuss firstly the effect of exogenous IL-6 on CLU different forms expression in colon cancer cells and the IL-6 induced physical interactions among sCLU, Ku70, and Bax. Finally, we report results on the cooperative interaction between IL-6 and VEGF-A165 in sCLU form-mediated cell death escape (Fig. 1).

C. IL-6

IL-6 is involved in several processes, explaining its long list of synonyms (B cell stimulatory factor-2, B cell differentiation factor, T cell-replacing factor, interferon-β_2, 26-kDa protein, hybridoma growth factor, interleukin

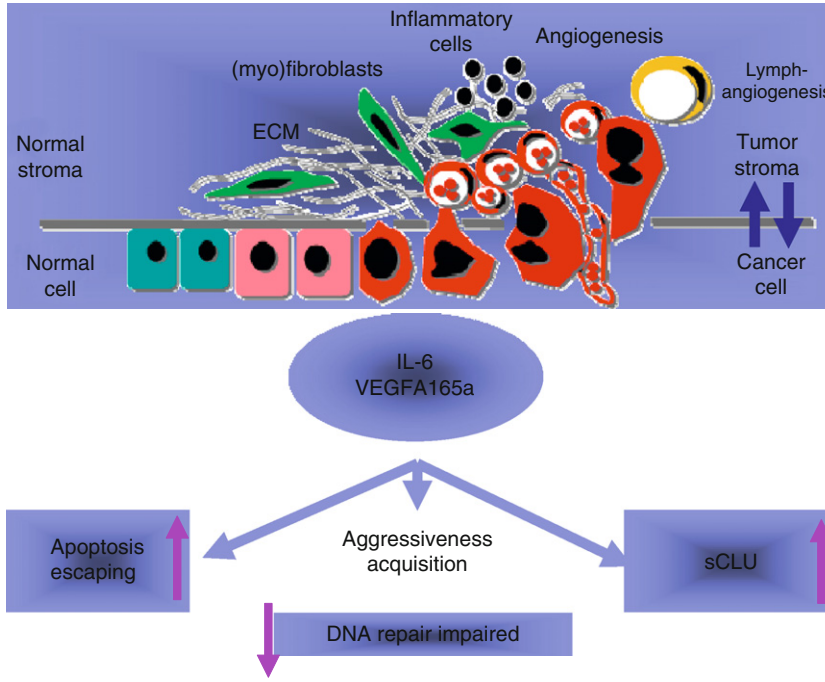

Fig. 1 Tumor microenvironmental factors. IL-6–VEGF-A165a cooperation in cancer progression.

hybridoma plasmacytoma factor 1, plasmacytoma growth factor, hepatocyte-stimulating factor, macrophage granulocyte-inducing factor 2, cytotoxic T cell differentiation factor, and thrombopoietin). IL-6 is now well recognized for its role in the acute-phase inflammatory response which is characterized by production of a variety of hepatic proteins termed acute phase proteins (e.g., C-reactive protein, serum amyloid A, fibrinogen, complement, alpha$_1$-antitrypsin) (Scheller *et al.*, 2006). In addition to its role in the acute-phase response, IL-6 is important for the development of specific immunologic responses. Moreover, it appears to play an important role in bone metabolism through induction of osteoclastogenesis and osteoclast activity. IL-6 induces differentiation of activated, but not resting, B cells culminating in production of immunoglobulin. Along with B cell differentiation, IL-6 stimulates proliferation of thymic and peripheral T cells and in cooperation with IL-1, induces T cell differentiation to cytolytic T cells and activates natural killer cells. These observations emphasize the importance of IL-6 in both nonspecific and specific immune responses. In addition to the activities described above, IL-6 functions in a wide variety of other systems including the reproductive system by participating in spermatogenesis, skin proliferation, megakaryocytopoiesis, macrophage differentiation, and neural cell differentiation and proliferation.

Furthermore, IL-6 levels are directly correlated with aging in a variety of species, thus it may play an important role in the aging process and in the aging-related disorders including, Alzheimer's disease, arteriosclerosis, and thyroiditis. Intriguingly, dietary restriction, the only experimental intervention that reproducibly prolongs maximum lifespan in mammals can restore to the young phenotype a variety of physiologic parameters, including IL-6 secretion and serum levels. IL-6 may be an important mediator of several infectious and autoimmune diseases. These include human immunodeficiency virus, rheumatoid arthritis, Castleman's disease, and the paraneoplastic symptoms associated with cardiac myxoma.

Because of its multidimensional and complex actions, dysregulation of IL-6 results in a myriad of disorders (Keller *et al.*, 1996) including a variety of neoplastic processes. The involvement of this cytokine in the regulation of neoplastic cell growth has been recently characterized. Levels of both IL-6 and its receptor increase during tumorigenesis in many tissues. Activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription), MAPK and PI3K/AKT signaling pathways has been reported in various cancer cell lines in response to IL-6 (Culig *et al.*, 2005). It may affect cancer progression by its actions on cell adhesion and motility, thrombopoiesis, tumor-specific antigen expression, and cancer cell proliferation. Depending on the cell type and the presence or absence of IL-6R, IL-6 can either inhibit or stimulate cancer cell proliferation. A great variety of tumor types are stimulated by IL-6, including melanoma, renal cell carcinoma,

prostate carcinoma, Kaposi's sarcoma, ovarian carcinoma, lymphoma and leukaemia, and multiple myeloma. In many of these tumors, IL-6R has been detected and a direct proliferative signal has been proposed. Yet, when tumor cells are devoid of IL-6R, a tumor inhibiting effect of IL-6 has been demonstrated, presumably because of its immune enhancing properties. Studies using an anti-IL-6 antibody have reported induction of apoptosis, inhibition of tumor proliferation, and elimination of the progression to androgen-independent status in a prostate cancer xenograft model (Wallner *et al.*, 2006).

D. VEGF-A

The human VEGF-A gene is organized into eight exons, separated by seven introns and is localized in chromosome 6p21.3. Alternative splicing of the human VEGF-A gene give rises to at least six different transcripts, encoding isoforms of 121, 145, 165, 183, 189, and 206 amino acid residues. Multiple protein forms are encoded through alternative exon splicing. All transcripts contain the exon 5, codify for the signal sequence and core VEGF binding or VEGF/PDGF homology domain and exon 8, with diversity generated through the alternative splicing of exons 6 and 7. Exon 6 encodes a heparin-binding domain, while exons 7 and 8 encode a domain that mediates binding to neuropilin-1 (NP1) and heparin exons. Several additional minor splice variants also have been described including VEGF-145, VEGF-162, and VEGF-165b, a variant reported to have an antagonistic effect on VEGF-165a-induced mitogenesis. VEGF-165b recently identified, display different activities in respect of its isoforms 165a, it is not mitogenic and it does not increase proliferation, but its functions are still not well characterized (Pucci *et al.*, 2008).

It is known that IL-6 is involved in regulation of VEGF expression as well as neuroendocrine differentiation in prostate tissue (Culig *et al.*, 2005). It is expressed mainly by stromal cells in the prostate, although both stroma and epithelium express the IL-6 receptor (IL-6R). Key upstream signals for VEGF regulation via HIF-1α may be differentially regulated in cancer and may synergize with other mechanisms of VEGF upregulation.

Overall, the presence of IL-6 induced VEGF by the activation of NF-κb and AP-1. The AP-1 activation resulted in the increased expression of both, VEGF and IL-6 regulated in paracrine and in autocrine fashion. Concomitantly, it was found that increased level of IL-6 and VEGF are closely linked to increased nuclear protein levels of HIF-1α and enhanced nuclear transcription factor DNA binding activity to a hypoxia responsible element located in the VEGF promoter. Especially two isoform are involved in tumor progression: VEGF-A165 and VEGF-120. The characterization of the VEGF-A165 and VEGF-120 variants is a relevant improvement in

discovering the regulatory pathway of this abundant growth factor especially for the design of new targets anticancer chemotherapy.

Recently blocking molecules are successfully used in anticancer therapy. Anti-VEGF therapy with bevacizumab can increase overall survival and/or progression-free survival in patients with colorectal, breast, lung cancer, or glioblastoma multiforme when combined with cytotoxic agents.

The balance and the interaction of IL-6 and VEGF-165 and in particular its isoform 165a has been observed to cooperate in neoangiogenesis and tumor cell survival. The molecular mechanisms involved influences CLU forms balance inducing a strong induction of sCLU form.

E. IL-6, VEGF-A165, and Cell Death Escape in Colon Cancer Cell: Acting on sCLU Induction

There is growing evidence that IL-6 may play a crucial role in the uncontrolled intestinal chronic inflammatory process, leading to colon cancer initiation. IL-6 regulates neoplastic cell growth in autocrine and paracrine fashion, although data on the possible relationship between IL-6 production and tumor progression are conflicting.

In colon cancer progression, we observed that the production of IL-6 released as by the tumor itself as by tumor-associated macrophages, and VEGF-A165, could influence tumor cell proliferation, favor apoptotic escaping and cell migration. The presence of IL-6 influences cell survival acting on the sCLU–Ku–Bax physical interactions.

As we previously reported in the chapter 3 of this volume, Bax is localized in a physiologically inactive form in the cytoplasm of normal undamaged cells, where it heterodimerizes with the C-terminus of Ku70, a protein that participates in the repair of DNA double-strand breaks (DSBs), caused by V(D)J recombination, isotype switching, physiological oxidations, IR, and chemotherapeutic agents that target DNA (Gottlieb and Jackson, 1993).

The ability of Ku70 to sequester Bax is a main determinant in preventing this proapoptotic protein from homodimerizing, thereby abrogating key apoptotic initiation events. The regulation of the ability of Ku70 to sequester Bax in the cytoplasm seems to be regulated by the lysine acetylation state within its C-terminus region (Cohen *et al.*, 2004). Changes in subcellular localization of Ku can, apparently, be controlled by various external growth-regulating stimuli, suggesting biological functions for the nuclear Ku70/86 heterodimer driven by microenvironment-soluble mediators (Pucci *et al.*, 2001; Pucci *et al.*, 2004a,b). Thus, changes in the microenvironment play a central role in redirecting pathways involved in DNA repair and cell death, affecting tumorigenesis. In normal cell Ku86 activation and translocation

into the nucleus could be regulated or stimulated by the induction of nuclear Clusterin (nCLU)–Ku70 interactions. nCLU binds the Ku70 subunit after sublethal damage induction allowing Bax to homodimerize. On the other hand, cytoprotective sCLU seems to play an important role in cell survival pathways stabilizing the Ku70–Bax interaction in the cytoplasm.

In this scenario, we observed that soluble mediators, in particular IL-6, could actively affect colon cancer progression targeting the prosurvival pathways of neoplastic cell. We found HIF-1α activated in the nucleus of human colon cancer biopsies correlated to an increased level of IL-6. Regarding the expression of VEGF-A165 in advanced colon cancer we found an upregulation of VEGF-A165 a expression with an evident disequilibrium between the production of the two, a and b, isoforms of VEGF-A165 as compared to normal tissues. Notably, the VEGF-A165 isoform *b*, known to display an antagonistic action in respect to the isoform *a*, was completely lost.

Moreover, a tumor-specific modulation of Bax, Ku, and CLU expression and subcellular localization in human colon cancer tissues was reported in chapter 3 of this volume. *In vitro* experiments confirmed that the expression level and the unconventional compartment localization of these proteins could be driven by exogenous factors.

IL-6 and VEGF-A165 treatment of a colon cancer cell line, *Caco-2*, modulated the expression of genes involved in tumor invasion and apoptosis, observed by microarrays. In particular, IL-6 downmodulated Bax expression at mRNA level. Concomitantly, IL-6 exposure influenced Bax also at protein level acting on the Bax–Ku70–sCLU physical interactions in the cytoplasm, by affecting the Ku70 acetylation and phosphorylation state, thus leading to the inhibition of Bax proapoptotic activity. In addition, we found that IL-6 treatment induced a significant downregulation of Ku86 and a strong increase of sCLU. The downregulation or the loss of Ku86 as observed in advanced stage colon carcinomas give raise to an impaired DNA repair process with accumulation of genetic alterations leading to higher aggressiveness acquisition. Concomitantly, the accumulation of sCLU in the cytoplasm cells bound to Ku70 and Bax in IL-6 treated cells, as demonstrated by coimmunoprecipitation experiments (Pucci *et al.*, 2009), inhibits the activation of the proapoptotic process.

In contrast, the *Caco-2* cells treatment with somatostatin, the physiological growth regulatory hormone, was able to restore apoptosis, demonstrating that Ku70–Bax–CLU interactions could be dynamically modulated by microenvironmental factors.

Strikingly, we observed that the cooperation between IL-6 and VEGF-A165 influenced the expression of tumor suppressing miRNAs affecting the epigenetic HDAC-1 activity and the EMT, turning the neoplastic cell from epithelial to mesenchymal, strongly correlated to the aggressiveness

acquisition of many types of cancers (Pucci, unpublished data; Sullivan *et al.*, 2009). The effect of these factors on EMT in cancer cell is strongly supported by sCLU overproduced and released from neoplastic cells versus the stromal component that cooperate to this transformation, as reported in the next paragraph describing the downstream effects of sCLU on the microenvironment.

These still obscure molecular interactions in cancer cell that oppose prosurvival sCLU and proapoptotic factors underlie the relevant role of microenvironmental factors, in the complicated cross talk among molecules that could effectively turn the cell fate (Fig. 2).

IV. sCLU EFFECTS ON MICROENVIRONMENT (THE "OUT" EFFECT): UP- AND DOWNSTREAM SIGNALS

CLU represents one of the numerous soluble factors which share the inner information of cell with the microenvironment. Among the others, one characteristic of CLU is its ability to interact with a wide array of components both in the serum and on the cell surface, such as complement regulatory proteins, lipid molecules, immunoglobulin, β-amyloid peptide (Shinoura *et al.*, 1994). It also binds to the cell surface of *Staphylococcus*

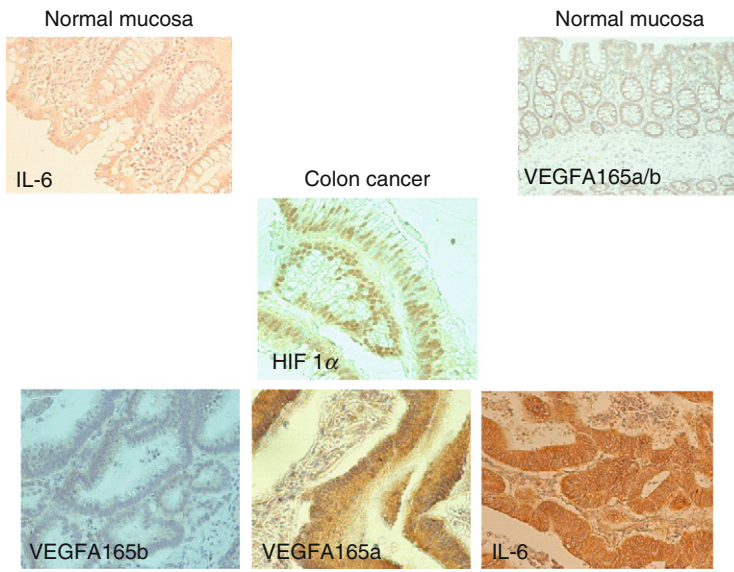

Fig. 2 HIF-1α, IL-6, and VEGF-A165 in colon cancer.

aureus (Partridge *et al.*, 1996) and it seems that such interaction may be an important bacterial virulence determinant for *S. aureus*. On the other hand, CLU binds to membrane-type MMP subgroup MT6-MMP/MMP-25, expressed in neutrophils and in brain tumors. MT6-MMP interacts with CLU forming a complex which regulates the neutrophil function, preventing the destruction of the host normal tissues (Matsuda *et al.*, 2003). Moreover, in CLU-deficient mice affected by autoimmune myocarditis, a more extensive tissue damage caused by immune reaction was observed in the inflammatory site, compared to wild-type mice (McLaughlin *et al.*, 2000).

It was reported that exogenous CLU promotes cell growth. However, how CLU promotes cell growth remains largely unknown. Exogenous CLU stimulates Ras-dependent Raf-1/mitogen-activated protein kinase MEK/ERK activation. Shim *et al.* investigate the signaling pathway and related molecules underlying astrocyte proliferation by CLU. CLU-induced astrocyte proliferation and ERK1/2 phosphorylation were abrogated by an inhibitor of EGFR, or EGFR small interfering RNA. These results suggest that CLU requires EGFR activation to deliver its mitogenic signal through the Ras/Raf-1/MEK/ERK signaling cascade in astrocytes (Shim *et al.*, 2009). However, Reddy *et al.* (1996) showed that Clu/ApoJ did not interact directly with EGFR, whereas it associated with both TGF-β type I and type II receptors.

Extracellular CLU was found to be cytoprotective when cells were challenged with apoptotic stimuli. CLU cDNA transfected into prostate cancer cells increased the resistance to apoptosis induced by tumor necrosis factor-α treatment (Sensibar *et al.*, 1995). Moreover, the increased expression of CLU in prostate cancer correlated with tumor grade. By CLU antisense therapy, Miyake *et al.* (2000) showed that extracellular CLU is an antiapoptotic gene involved in progression of prostate cancer.

sCLU exerts its cell protective function through binding to a cellular receptor. As reported by Koch-Brandt megalin was the first identified CLU receptor (Bartl *et al.*, 2001) and CLU–megalin interaction-induced signaling was the cause of cell protection in prostate cells treated with TNF-α apoptotic stimuli. CLU binding to its receptor activated the PI3 kinase/Akt pathway and produced multiple protein phosphorylation. However, the inhibition of this pathway did not block the protective effect indicating that additional pathways may be involved in the protection by CLU-induced signaling (Ammar and Closset, 2008).

PI3K and its major downstream kinase, Akt, play key roles in many aspects of tumorigenesis, such as cellular proliferation, survival, and migration. Constitutive activation of the PI3K–Akt pathway is closely associated with cancer cell resistance to chemotherapeutic agents. Deactivation of this pathway has been shown to increase the efficacy of many anticancer drugs, targeting a wide range of cellular components. It seems that cancer cell-secreted IGF-1 and extracellular CLU constitute the regulatory system of

PI3K–Akt pathway. The interplay between CLU and IGF-1 produces an effect opposite to that resulting from the CLU/megalin interaction. In fact, Jo *et al.* (2008) showed that sCLU associated with IGF-1 and inhibits its binding to the IGF-1 receptor and hence negatively regulates the PI3K–Akt pathway. This inhibitory function of CLU appears to prefer IGF-1, as it fails to exert any effects on epidermal growth factor signaling. Therefore, CLU represents a positive or negative regulator of PI3/Akt pathway depending on its interacting partner (CLU receptor Megalin and IGF-1, respectively).

Intriguingly, the uptake of glucose is a process mediated by the PI3K–Akt pathway and its availability, often limited in the tumor microenvironment, can be a deciding factor for cell proliferation. Under this condition, those cells with a higher "resistance" would gain growth advantages and thus would be positively selected. We previously reported that CLU level is tightly associated with various cellular stress responses. Therefore, its secretion may reflect the cellular adaptive responses to endure adverse environmental conditions (i.e., by suppressing its own growth and that of surrounding cells). In a mouse model of prostate cancer, it has been shown that epithelial cancer cells initiate and promote the clonal expansion of stromal fibroblasts that lack the p53 tumor suppressor gene (Hill *et al.*, 2005), indicating that the cancer cell-derived factors initially impose selective pressures (i.e., antiproliferation) on neighboring cells. The presence of CLU in the tumor microenvironment can contribute to such selective pressures.

It is of note that PI3 kinase/Akt pathway regulates the HIF-1 protein synthesis and furthermore the activation of MAPK and PI3K/AKT signaling has been reported in various cancer cell lines in response to IL-6 (Culig *et al.*, 2005). It implies that not only IL-6 could promote cancer progression affecting Bax, Ku, and CLU interactions (Pucci *et al.*, 2009), but also CLU could modulate HIF-1α protein levels and enhance IL-6 effects in a positive feedback loop.

Hence, cancer cell-derived CLU may provide a molecular framework to further dissect the complex relationships between cancer cells and their environment.

During EMT, epithelial cells downregulate their intercellular adhesion, lose the apical–basal polarity, and undergo morphological changes from a monolayer of cuboidal-shaped cells to dispersed, spindle-shaped fibroblast-like cells. The expression of differentiation markers switches from cell–cell junction proteins such as E-cadherin to mesenchymal markers including fibronectin and vimentin. Furthermore, the stationary cells convert to migratory cells capable of invasion through ECM. The mechanism underlying EMT involves coordination of multiple signaling pathways, including TGF-β (Heldin *et al.*, 2009), receptor tyrosine kinase/Ras signaling pathways, other autocrine factors (e.g., EGF, HGF, and IGF), Wnt, Notch, Hedgehog, and NF-κB signaling pathways (Bates and Mercurio, 2005). These pathways exert their EMT-inducing effect through modulating

transcriptional regulators, to repress epithelial genes such as those encoding proteins of intercellular junctions, or activate genes pivotal for cells to acquire migratory and invasive properties. It was demonstrated that CLU modulates the EMT in human lung adenocarcinoma cell lines (Chou *et al.*, 2009). CLU-rich cells displayed a spindle-shape morphology while those with low CLU levels were cuboidal in shape. Moreover, CLU silencing by siRNA in highly invasive CLU-rich lung adenocarcinoma induced a mesenchymal-to-epithelial transition (MET) evidenced by the spindle-to-cuboidal morphological change, increased E-cadherin expression, and decreased fibronectin expression.

V. CONCLUSIONS AND FUTURE PERSPECTIVES

In this review, we emphasize the importance of cross talk between stroma and epithelia in carcinogenesis. Reactive stroma is induced after epithelia changes and their coevolution determines the release of the microenvironmental factors, strong determinant of tumor behavior. CLU behaves as an important player of the cell fate within the cancer cell and as relevant soluble factor of the microenvironment still conditioning the fate and the proliferation rate of surrounding cancer cell in a paracrine fashion. Its production and the release are finely regulated by the microenvironment. Further study is required to fully understand the complex interaction between cancer cell and the tumor microenvironment that leads to sCLU production and pro-survival pathways activation. Target therapy to the stromal compartment as well to epithelia is expected to be clinically promising, and further elucidations on the molecular mechanisms underlying tumor–stroma interactions may yield novel therapeutic targets for anticancer therapy.

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