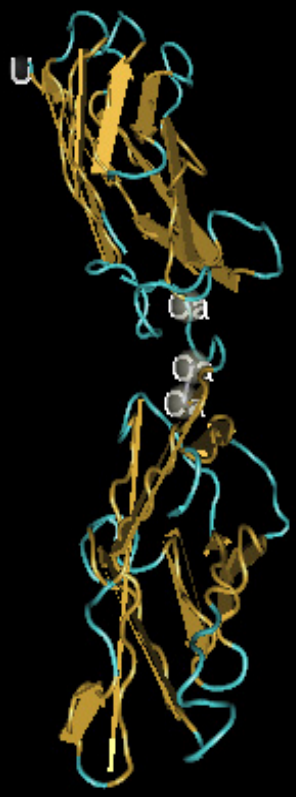
University of Ghent

Faculty of Medicine and Health Sciences

Laboratory of Experimental Cancerology

The N-cadherin ectodomain: fate and function outside the cancer cell



Lara Derycke

Promoter: Prof. M. Bracke

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Aan mijn ouders.

Aan Stefan en Maxime.

Promotor

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Zonder jullie allemaal zou ik hier nooit gestaan hebben, BEDANKT!

Abbreviations

3D: three dimensional
ADAM: a disintegrin and metalloproteinases
APC: adenomatous polyposis coli
CAD: cadherin
CAM: chorioallantoic membrane
Caspase: cysteinyl aspartic acid-protease
CD: cadherin domain
CEA: carcino embryonic antigen
CTN: catenin
EC: endothelial cell
ECM: extracellular matrix
EGF: epidermal growth factor
EMT: epithelium to mesenchymal transition
erbB: erythroblastosis receptor B
ER: estrogen receptor
ERK: extracellular regulated kinase
Ets-1: v-ets erythroblastosis virus E26 oncogene homolog 1
FAK: focal adhesion kinase
Fer: fes-related protein
FGF: fibroblast growth factor
FIGO: federation of gynecology and obstetrics
HAV: histidine alanine valine
IGF: insulin-like growth factor
IL: interleukin
kD: kilodalton
MAPK: mitogen activated protein kinase
MCF: michigan cancer foundation
MMP: matrix metalloproteinases
MT-MMP: membrane type-MMP
NED: no evidence of disease
Neu: erbB2
NF-κB: nuclear factor-kappa B
PASI: Psoriasis Area and Severity Index
PDGF: platelet-derived growth factor
PECAM1: platelet/endothelial cell adhesion molecule (CD31)
PI3K: phosphatidylinositol 3-kinase
PS1: presenilin 1
PSA: prostate specific antigen
Rac: ras related C3 botulinum toxin substrate
Rb: retinoblastoma
Rho: ras homologue
S1P: sphingosine 1 phosphate
S1P1: endothelial differentiation gene 1 (EDGF1)
s-CAD: soluble cadherin fragment
Src: tyrosine kinase homologue to viral Rous sarcoma oncogen
STAT: signal transducer and activators of transcription
TGF: transforming growth factor
Tie2: angiopoetin 1 receptor
TIMP: tissue inhibitor of metalloproteinases
TNM: tumour size, lymph node spread, metastases
Trp2: tryptophan 2
uPA: urokinase-type plasminogen activator
VEGF: vascular endothelial growth factor

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Part I Introduction

1.1. General Introduction

Each year in the European Union nearly 2,9 million people are diagnosed with cancer and there are over 1,7 million deaths from the disease. Cancer remains an important public health problem in Europe (Boyle and Ferlay 2005). The past two decades our knowledge of the genetic and epigenetic events involved in the early event of cancerogenesis increased considerably. By contrast, despite the appreciation of the clinical relevance of tumour metastasis, there is a lack of therapies that can efficiently prevent metastasis (Christofori 2006).

During tumour progression, a multitude of cumulative alterations modulates the transition from a normal to a malignant state. Tumour cells are not isolated but they are present in a micro-environment where stromal cells, like fibroblast, immune cells and endothelial cells; extracellular matrices, like collagen and laminin, proteases and growth factors are present. There exists a constant cross talk that modulates several cellular activities such as growth, differentiation, invasion, ectopic survival and metastasis. Invasion, the hallmark of malignancy, implicates the penetration of cancer cells through the basement membrane and their survival outside their original tissue. Cancer cells also invade into the circulation (lymph or blood) to reach distant organs where they can metastasise (Hanahan and Weinberg 2000). Different factors of the ecosystem are implicated in tumour progression, and some will be highlighted here: cell-cell adhesion (part II), proteinases and their released fragments (part III), angiogenesis (part IV), external or autocrine factors influencing tumour progression (part V).

The cadherins are important target in several processes implicated in tumour progression, while some are assigned as invasion suppressors others are invasion promoters (Mareel and Leroy 2003). The cadherins are a super family of cell surface molecules that require calcium in order to establish cell-cell adhesion. The cadherins are transmembrane proteins which have in common the presence of several cadherin domains (CD, ± 110 amino acids) (Tepass, 2000). Calcium ions form a complex with the different domains and are necessary for the 3D configuration. The cadherins form two types of dimers: cis and trans. Cis is when cadherins are both present on the same cell and trans is in opposing cells. The cadherin family consists of more than 80 members, which are usually indicated by a prefix letter that refers to the

tissue or organ in which the molecule was found originally. Some examples are epithelial (E-) cadherin is expressed by all epithelial cells, neural (N-) cadherin is expressed in neuronal tissue but also by oocytes, spermatocytes, retina, endothelial cells and fibroblasts. Placental (P-cadherin) is expressed by basal cells of the epithelial tissue for example the myoepithelial of the breast, and Vascular Endothelial (VE)- cadherin is expressed by endothelial cells. During embryogenesis and cancer progression cells can change the type of cadherin they express. During my work in the laboratory of Experimental Cancerology, I focused on N-cadherin. In part II an overview is presented about its function during embryogenesis, cell-cell adhesion and tumour progression (article 2; Derycke and Bracke 2004).

In the micro-environment of cancer, cells secrete elevated levels of several proteases, like matrix metalloproteinases, serine proteinases and cysteine proteinases (Egeblad and Werb 2002; Riddick *et al.* 2005). They are responsible for the cleavage of transmembrane proteins, like tyrosine kinase receptor c-erBb2 and cadherins. The ectodomains of these proteins are detected in different human biological fluids and can be used as tumour markers, to use for diagnosis, prognosis, follow up or therapeutic monitoring of cancer patients (Villanueva *et al.* 2006). From 1994 till now several articles (part III) describe already the detection of sE-cadherin in serum and the correlation with diverse types of cancers, like gastric carcinoma, bladder carcinoma, colorectal carcinoma, melanoma and prostate carcinoma and with the stage of the tumour (Katayama *et al.* 1994; Velikova *et al.* 1997; Griffiths *et al.* 1996). However sE-CAD is not specific for cancer (Pittard *et al.* 1996) and is so far not used in daily practice for the follow up of cancer patients. During my PhD we tried to define soluble N-cadherin as a more useful tumour marker, reasoning N-cadherin is upregulated in many carcinomas (Derycke and Bracke 2004). However until now only one article describes the presence of soluble N-cadherin, namely in the vitreous humour (Paradies *et al.*, 1993). We developed a detection method which detects the N-cadherin ectodomain in different biological fluids, like blood and seminal fluid (article 3). Furthermore, we compared the concentration of the N-cadherin ectodomain present in serum from persons with no evidence of disease, cancer or other diseases.

Another important process during tumour progression is angiogenesis, the formation of new blood vessels. The process of angiogenesis is necessary for the growth of the tumour but also for the dissemination of cancer cells to distant organs (Carmeliet and Jain 2000). N-cadherin plays here also an important role because it is expressed by the endothelial cells and by the

pericytes (Dejana 2005). Recently the group of Luo and Radice concluded that N-cadherin controls vasculogenesis upstream of VE-cadherin. Because specific knock-down of N-cadherin in endothelial cells results in embryonic lethality at mid-gestation due to severe vascular defects. The knock-down of N-cadherin caused indeed a significant decrease in VE-cadherin expression (Luo and Radice 2005). We investigated the role of the N-cadherin ectodomain in the process of angiogenesis (article 4). N-cadherin as well as its ectodomain is able to stimulate neurite outgrowth in a Fibroblast Growth Factor Receptor (FGF-receptor) dependent manner (Williams *et al.*, 1994, Utton *et al.* 2001). However, about the mechanism of the N-cadherin ectodomain during the different processes of tumour progression nothing is known.

As discussed above the cadherins have a cell-cell adhesion function and are deregulated in cancer (part V). The downregulation can happen at different levels: by mutation, by hypermethylation of the promoter, transactivation of other cadherins, phosphorylation of the catenins, sterical hindrance, proteolysis or endocytosis (Van Aken *et al.*, 2001). Some examples of inhibitors of the E-cadherin/catenin complex are epidermal growth factor (EGF), which induce tyrosine phosphorylation of β-catenin (Shiozaki *et al.* 1995) and the matrix metalloprotease matrilysin, which induces shedding of the ectodomain (Noë *et al.* 2001). Insulin-like growth factor (Bracke *et al.* 1993) and 17-β estradiol (MacCalman *et al.* 1994) and natural products like tangeretin (Bracke *et al.* 1994) are activators of the E-cadherin/catenin complex. Another natural product, xanthohumol, originating from hop bells is also upregulating the function of the E-cadherin/catenin complex (article 6). The N-cadherin/catenin complex is regulated by cytokines like EGF (Ackland *et al.* 2003) and interleukin-6 (Gil *et al.* 2002), the pharmacological agent thalidomide (Thiele *et al.* 2000) and proteases like a disintegrin and metalloproteinase 10 (ADAM10) (Reiss *et al.* 2005). Conditioned medium of cells are a source of cytokines and other paracrine and autocrine factors which can modulate the cadherin/catenin complex. Conditioned medium of the human squamous carcinoma cells, COLO 16, induces internalization of E-cadherin and scattering of human mammary carcinoma cells (Boterberg *et al.* 2000), while conditioned medium of Bowes melanoma cells has the ability to stimulate the aggregation of breast carcinoma cells by activation of the E-cadherin/catenin complex (Stove *et al.* 2005). Heregulinβ1 is a growth factor produced by melanocytes and melanoma cells, and is one of the factors responsible for stimulated aggregation of the cells. Heregulinβ1, present in the conditioned medium of Bowes melanoma, works also as an autocrine factor; it stimulates the proliferation of the melanoma

cells (article 5). In this part we discuss also the upregulation of the P-cadherin/catenin complex by ICI182,780 in breast carcinoma cells (article 7, Paredes *et al.* 2004).

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Molecular Targets of Growth, Differentiation, Tissue Integrity, and Ectopic Cell Death in Cancer Cells

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ABSTRACT

Cancer cells continue to grow, lose their differentiation, and are found beyond their tissue boundaries, where they survive. These phenomena lead to cancer invasion and metastasis and are responsible for the outcome of the disease in cancer patients. Different factors determine where and when the cells will metastasize. The surrounding host cells, such as fibroblasts, macrophages, leukocytes, et cetera, and the extracellular matrix play an important role in the creation of the microenvironment for the cancer cells to invade. Blood and lymph vessels are not only the transporters of nutrients and metabolites for the primary tumor, these vessels also transport cancer cells to distant sites, where they metastasize. Angiogenesis and host cells are targets in cancer treatment. To monitor therapy or to predict cancer relapses, circulating tumor markers are used that reflect the molecular cross-talk between cancer and stromal cells.

Key words: cancer, microenvironment, tumor markers, host cells, angiogenesis

INTRODUCTION

In adult multicellular organisms, the number and the differentiation state of the cells is strictly controlled, and tissue integrity is maintained because cells stay between their tissue boundaries. When normal cells are brought beyond these boundaries into an ectopic tissue context, they are even unable to survive, a phenomenon known as anoikis.[1] In cancer, however, genetic alterations allow cells to escape from these control mechanisms. Cancer cell populations continue to grow progressively in time and space and tend to lose or change their differentiation characteristics. In several epithelial cancers, a transition from normal epithelial to pathological mesenchymal marker expression has been described. This switch is commonly designated as the epithelial to mesenchymal transition (EMT).[2] Most important for the clinical outcome is that cancer cells ignore tissue integrity and have the ability to overcome ectopic cell death.[3] The latter two phenomena lead to cancer invasion and metastasis and thus compromise the prognosis of the cancer patient. In this paper, we will focus on molecular targets that are relevant in the pathogenesis of cancer invasion and metastasis.

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Metastasis Promoter Genes Can be Activated Early During Tumor Progression

Invasion and metastasis are responsible for the fatal outcome of the disease in cancer patients.[4]

While local invasion by the tumor into the surrounding tissue is the cause of death in brain cancer patients, distant metastases are the life-threatening events in the course of melanoma and prostate cancer progression. Colorectal and bladder cancer patients die from both locoregional or distant tumor invasion and metastasis.

Local tumor invasion is characterized by at least two changes of function by the cancer cells. Firstly, these cells express higher levels of membrane-type and secreted proteolytic enzymes in comparison with their normal epithelioid counterparts. Two families, the matrix metalloproteases[5] and the plasminogen activators,[6] have been studied extensively. Their contribution to invasion ranges from breakdown of the extracellular matrix, over release of proinvasive factors, to cleavage of cell-cell adhesion molecules. Secondly, cancer cells are more motile than normal epithelial cells. Control of their actin cytoskeleton dynamics by small GTPases (Rac, Rho and Cdc42) appears to be crucial for motility,[7] while assembly of their cytoplasmic microtubule complex is implicated in direction finding.[8] Local tumor invasion is also made possible by disruption of epithelial cell junctions. E-cadherin, which is part of the adherens junctions, plays an important role in maintaining the epithelioid cell organization and in preventing invasion.[9] Cadherins not only serve cell-cell adhesion by means of homophilic interaction between cadherins from neighboring cells, they also allow outside-in signalling through the cytoplasmic catenin complex toward the actin cytoskeleton. Downregulation of the expression or the function of E-cadherin is a constant finding in invasive carcinomas,[10,11] and the causal relation between this downregulation and the onset of invasion has been demonstrated in models *in vitro*[12] as well as *in vivo*.[13] Although E-cadherin is considered as an invasion suppressor, other classical cadherins, such as N- and P-cadherin, now show up as potential invasion promoters. In particular, the switch from E- to N-cadherin expression can accompany the EMT phenomenon in invasive tumors derived from mammary gland epithelium.[14] The shedding of the extracellular part of these cadherins by proteolytic cleavage is intriguing and offers new diagnostic candidates as circulating tumor markers.[15]

The role of surrounding host cells in the creation of a microenvironment for the cancer cells is often underestimated. Fibroblasts, macrophages, leukocytes, platelets, and the extracellular matrix are sources of proinvasive and anti-invasive molecules that affect the balances between proteases and their inhibitors, motility factors and their antagonists, and up- and downregulators of the cadherin/catenin complexes (Fig. 1). One example of the cross-talk between cancer cells and host elements are myofibroblasts. They are recruited from fibroblasts by colon cancer cells via transforming growth factor-β (TGF-β), and secrete proinvasive molecules for the cancer cells (scatter factor/hepatocyte growth factor and tenascin C).[16] Enterobacteria such as *Listeria monocytogenes* demonstrate that environmental factors may contribute to the production of proinvasive peptides for the colon cancer cells.[17]

For most tumor types, invasion of cancer cells at the primary site eventually leads to metastasis formation. The latter is a multistep process involving intravasation of cancer cells into the blood and lymph vessels, transportation through the circulation, and extravasation at distant sites. At these sites, cancer cells can start to grow and invade again, giving rise to secondary tumors (metastases). Escalation of these phenomena is the basis of a metastatic cascade, which will finally kill the patient. This does not exclude direct seeding from the primary tumor to different organs as an important mechanism.[18]

During cancer progression, including the stages of hyperplasia, dysplasia (carcinoma *in situ*), invasive carcinoma, and metastasis formation, a disequilibrium in promoter and suppressor gene activity is responsible for growth (oncogenes versus tumor suppressor genes), invasion, and metastasis. All of those gene alterations can occur early during cancer development. So, in lobular carcinoma of the breast, mutations of the E-cadherin gene, an invasion suppressor, can be detected as early as in the carcinoma *in situ* stage.[19] A challenge for the future is the recognition of metastasis genes, as recent data indicate that they can be activated in the early stages as well.[20–21] This is of clinical importance, because detection of distinct sets of activated metastasis genes in the primary tumor will predict its metastatic capacity and will be useful to direct the treatment of the individual patient. A concerted action by the European Community (METABRE) is now aiming at defining such a workable set of clinically relevant metastasis genes and gene products.

An increasing number of recent literature data

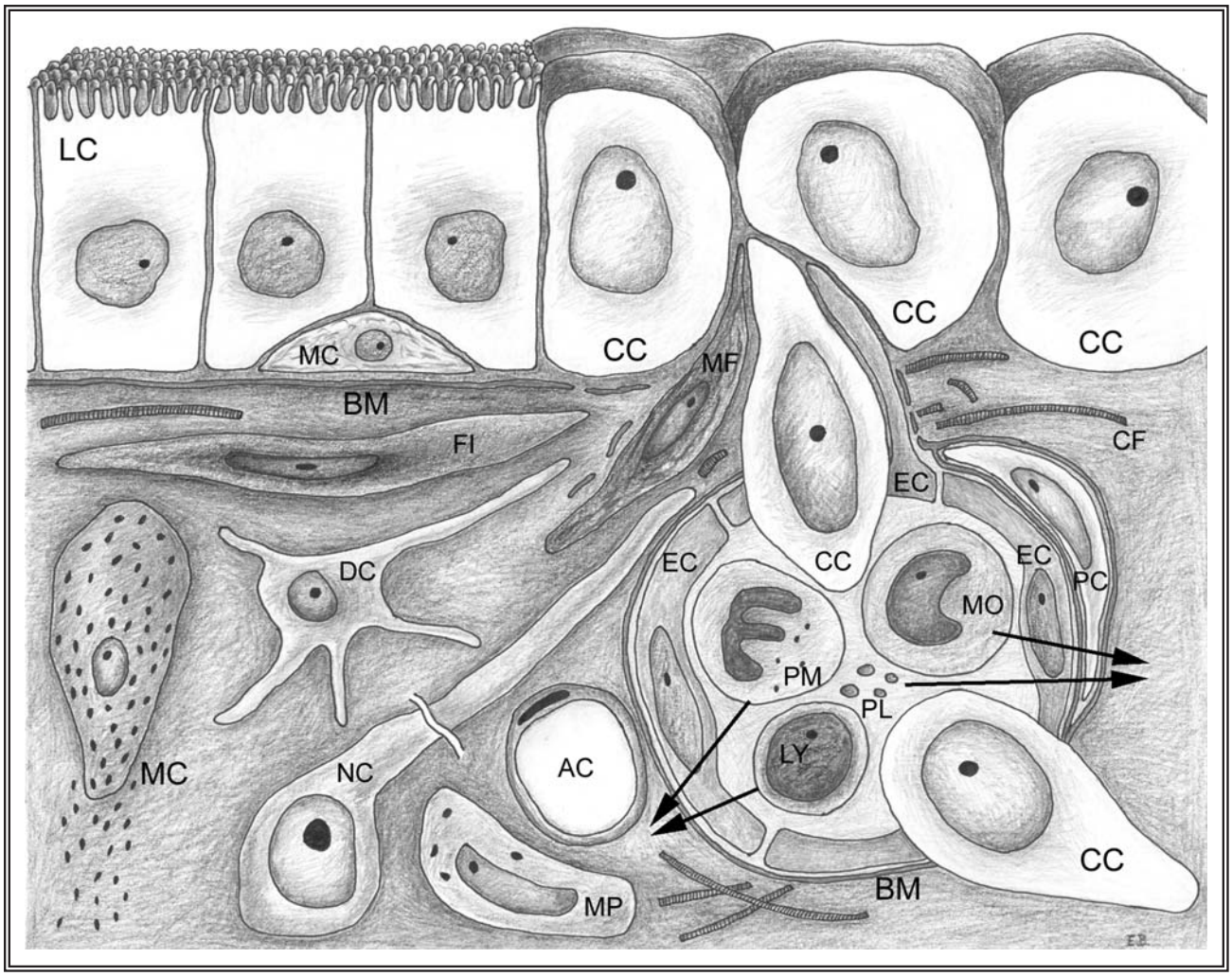

FIG. 1. Cellular components of the breast cancer micro-ecosystem. In normal breast epithelium, luminal cells (LC) and myoepithelial cells (MC) adhere to an intact basement membrane (BM). In breast cancer, however, the cancer cells (CC) break through this membrane and cleave the structural molecules of the interstitial extracellular matrix, such as the collagen fibers (CF). The cancer cells can invade locally, intravasate, are transported by the circulation, and extravasate to produce distant metastases. In the metastatic cascade, the cells can establish molecular cross-talks with host cells: fibroblasts (FI), myofibroblasts (MF), mast cells (MC), dendritic cells (DC), neural cell extensions (NC), macrophages (MP), and adipocytes (AC). Angiogenesis results from interactions with endothelial cells (EC) and pericytes (PC) and brings in new interacting cells from the circulation, such as polymorphonuclear leukocytes (PM), lymphocytes (LY), monocytes (MO), and platelets (PL).

confirm that metastasis is an early phenomenon in cancer development. In one study, Gerber et al.[22] showed the presence of occult tumor cells in lymph nodes and bone marrow from breast cancer patients staged pT1-2 N0 M0 (no lymph node nor systemic metastases, following current diagnostic methods). Using sensitive immunohistochemical techniques for cancer cell detection, the authors found occult cells in 6.4% of the lymph nodes samples, in 26.0% of the bone marrow samples, and in 4.8% of both. This makes a total of 37.2% of all samples containing occult tumor cells.

The Seed and the Soil Hypothesis can be Translated into Molecular Cross-Talks Between Cancer Cells and the Surrounding Host Cells

What is it that decides what organ shall suffer in a case of disseminated cancer? This question was raised in 1889 by Paget and answered with his "seed and soil" hypothesis.[23] His answer was that the microenvironment of each organ (the "soil") influences the survival and growth of tumor cells (the "seed"). This statement can currently be

translated into molecular mechanisms that govern the interaction between circulating cancer cells and the potentially metastatic organs, and it still inspires current research. Again, metastasis is the result of molecular cross-talks between cancer cells and cells from organs, such as the brain, lungs, bone, liver, and bladder. They decide about specific homing and survival of the cancer cells. We will extend on one example of such a cross-talk that recently caught major attention, namely angiogenesis.

Angiogenesis is the Result of a Cross-Talk Between Cancer Cells and Local Endothelial Cells

Angiogenesis in the primary tumor is not only providing to the tumor an afferent influvium of nutrients and an efferent discharge of toxic metabolites, the endothelial network is also a source of afferent growth and proinvasive factors for the cancer cells. Furthermore, it facilitates the inflow of host cells, such as tumor-infiltrating lymphocytes. Also, the newly formed network enables the intravasation of cancer cells and so the initiation of metastasis to distant sites. The establishment of neovascularization in both the primary tumor and the distant metastases opens the gate for a mutual cross-talk. Folkman's group has shown, for instance, that the primary tumor releases an angiostatin that inhibits the development of distant metastasis.[24]

When cancer cells extravasate into an organ of future metastasis, they are able to turn the local balance between pro- and antiangiogenic factors from mainly antiangiogenic to a net proangiogenic outcome. Tumor secretion of proangiogenic factors, such as vascular endothelial growth factors (VEGFs) and many others, stimulates endothelial cells from neighboring capillaries to proliferate and migrate ("sprouting") toward the cancer cells. This is the initiation of the formation of more stable vessels that involves other cell types such as pericytes as well.[25] Here, the similarity of growth, invasion, and differentiation of angiogenic endothelial cells with cancer cell activities is striking.

Both the trigger for the secretion of angiogenic factors by the cancer cells and the way of interaction of these factors with the endothelial cells have been described in molecular detail. Important triggers are hypoxia and low pH in the growing cancer: These conditions increase the intracellular concentration of hypoxia-inducible factors (HIF-1α and HIF-1β, respectively) by inhibiting their ubiquitination at the proteasome complex, a process which is responsible for protein degradation by linking the tag ubiquitin to proteins. While gene transcription is generally shut down in cells suffering from hypoxia, a number of genes possess a hypoxia responsive element (HRE) in their promoter, which is able to interact with the HIF-1s. So, the HIF-1s act as transcription factors for genes implicated in glycolysis, erythropoiesis, CXCR4 expression, and angiogenesis.[26] The latter group of (angiogenic) gene products comprises VEGF, angiopoietin 2, nitric oxide synthase, and platelet-derived growth factor receptor. By this mechanism, the threat of unfavorable conditions for the cancer cells is rapidly corrected by a supply of oxygen and nutrients by newly formed vessels. External conditions, however, are not the only driving force for the generation of proangiogenic molecules, as activation of several oncogenes in the cancer cells is sometimes overruling the cross-talk with the surrounding extracellular milieu. These oncogenes can govern the "oncogenic switch" by overexpression not only of VEGF, but also of basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), placenta-like growth factor (PLGF), transforming growth factor-β (TGF-β), platelet-derived endothelial growth factor (PD-EGF), pleiotrophin, and others.

Although VEGF is probably the most potent angiogenic factor, it owes its specificity to a unique tyrosine kinase receptor (VEGFR), which is present only in endothelial cells. Additional specificity within this system is obtained from the existence of at least 4 types of VEGF (A to D) and 3 types of its receptor (1 to 3). Although VEGFR-1 (or Flt-1) is sensitive to VEGF-A and VEGF-B (and to placental growth factor) and induces new blood vessels, VEGFR-3 (or Flt-4) is sensitive to VEGF-C and VEGF-D and gives rise to new lymph vessels. VEGFR-2 (or KDR/Flk-1) keeps an intermediate position: It is sensitive to all types of VEGF (except VEGF-B) and can induce both blood and lymph vessels.[27–29] This raises the possibility that the choice of an individual tumor to metastasize either through the blood vessels or the lymphatics is at least partly determined by the type of VEGF it secretes: VEGF-A/B secretors would then metastasize mainly through the blood, while VEGF-C/D secretors would occupy the lymph nodes preferentially. It is too early, however, to conclude whether this implicates diagnostic, prognostic, and therapeutic consequences for the individual patient.

Angiogenesis is a Target for Cancer Therapy

Screening and evaluation of potential inhibitors of angiogenesis is done in models *in vitro* and *in vivo*. The effect of compounds on the formation of capillary-like structures by endothelial cells can be tested in cultures of human umbilical vein endothelial cells (HUVEC) on Matrigel® *in vitro*.[30] The effects are easy to evaluate and to quantify, but questions can be raised about the relevance of this rather artificial assay. Somewhat more relevant to the natural situation is the chick chorioallantois membrane (CAM) assay. Here the CAM of the chick egg is impregnated locally with potential modifiers of angiogenesis. Sprouting of new vessels within the zone of impregnation can be quantified and compared with a control zone.[31] This CAM assay is meant as a compromise between the purely *in vitro* HUVEC assay and the angiogenesis assays *in vivo*, which include laboratory animals. An example of the latter group is the rabbit cornea micropocket assay: Here the formation of blood vessels is studied in a normally avascular tissue.[32] In the assays, inhibition of spontaneous and VEGF-induced angiogenesis by test compounds can be quantified, and the results can be compared with the effect of PD 173074, a tyrosine kinase inhibitor of the VEGF receptor.

Two classes of angiogenesis inhibitors can be considered as candidates for cancer therapy: direct and indirect ones. Direct inhibitors, such as vitaxin, angiostatin, and others, prevent vascular endothelial cells from proliferating, migrating or avoiding cell death in response to a spectrum of proangiogenic factors, including VEGF, bFGF, IL-8, platelet-derived growth factor, and PD-EGF. Direct angiogenesis inhibitors are the least likely to induce acquired drug resistance, because they target genetically stable endothelial cells rather than unstable mutating tumor cells. Tumors that are treated with direct-acting antiangiogenic therapy did not develop drug resistance in mice so far.

Indirect angiogenesis inhibitors generally prevent the expression or block the activity of a cancer protein that activates angiogenesis or block the expression of its receptor on endothelial cells. Many of these tumor-cell proteins are the products of oncogenes that drive the oncogenic switch.

The biological effects of angiogenesis inhibitors are expected to be diverse *in vivo*: reduction of tumor blood flow and induction of apoptosis in both cancer and endothelial cells. Of special interest is thalidomide, a synthetic sedative; this drug was found to inhibit angiogenesis induced by bFGF or VEGF in the rabbit cornea micropocket assay.[33] Thalidomide is now being tested in more than 160 clinical trials at more than 70 medical centres in the United States and in Europe. It has become one of the most effective drugs for treating patients with multiple myeloma, either as first-line therapy or for the treatment of patients who are resistant to conventional chemotherapy. In fact, thalidomide is one example of the wide variety of antiangiogenic agents applied clinically.[34] These molecules belong to different classes, possess different targets, and were found to inhibit angiogenesis empirically. Apart from thalidomide, well-known examples are suramin (binder of growth factor), endostatin (naturally occurring inhibitor of angiogenesis), vitaxin (integrin antagonist), marimastat (protease inhibitor), combretastatin-4 (binder of tubulin), and interleukin-6 (cytokine). A better understanding of the molecular mechanisms of angiogenesis now offers rationales for the development of new angiogenesis inhibitors with more specific targets, such as antagonists of angiogenesis factors at the receptor level, inhibitors of the receptor tyrosine kinase, and of their downstream signalling pathways.

New Targets for Antimetastatic Agents and New Problems Arising

Apart from angiogenesis, many other examples of phenomena contributing to tumor growth, invasion, and metastasis exist that offer possible molecular targets for imaging, follow-up, and therapy in cancer patients. Recently, the role of chemokines and their receptors in metastasis was further elucidated and opened new therapeutic perspectives for antimetastatic treatments. Breast cancer cells were shown to express high levels of a chemokine receptor coined CXCR4, while target organs for breast cancer metastasis, such as the liver, secrete the ligand for this receptor: CXCL12. Signalling through the CXCR4 receptor initiates actin polymerization and pseudopod formation in the tumor cells, preparing them for subsequent chemotactic and invasive responses.[35] So, this chemokine-mediated mechanism determines the metastatic destination of circulating cancer cells. Interestingly, antibodies neutralizing the interaction between CXCR4 and

CXCL12 are able to reduce metastasis formation in laboratory animals, indicating that this interaction may be another target for antimetastatic treatments.

Invasion, however, can be driven by more than one type of cell motility, and cancer cells can switch from one type to another. Cells can, for instance, invade into a three-dimensional matrix by a mechanism that is dependent on the activation of the small GTPase Rac. These cells assume a phosphoinositide-3-phosphate-mediated elongated morphology. Upon inhibition of the extracellular protease activity, the cells in the matrix switch to a mechanism that is dependent on activated Rho, another member of the small GTPase family. They become characterized by a more spherical morphology and migrate by amoeboid movements.[36–38] These findings warn for the dynamic nature of cancer cell mechanisms to maintain the invasive and metastatic phenotype. So, anti-invasive treatments may fail, because cancer cells escape through salvage mechanisms that are not affected by the treatment. Finding new targets for therapy clearly risks to reveal unexpected scenarios in cancer invasion and metastasis.

Proof of Principle for the Target Role of Host Cells in Cancer Treatment

The role of host cells as targets for therapy can be illustrated by the successful application of bisphosphonates in clinical practice. These drugs are used for the treatment of bone metastases, and they do not affect the metastatic cancer cells as such, but inhibit bone resorption by the osteoclasts. These host cells receive stimulatory signals from the cancer cells and from bone marrow macrophages, but bisphosphonates force them to go into apoptosis.[39] Bisphosphonate treatment thus alleviates cancer pain, and, in some cases, reduces the size of osteolytic bone metastases, as measured on radiologic images. Research on the mechanisms of cancer pain has recently revealed new targets for therapy. The sensitivity threshold of the nociceptor from the primary sensory neurons is lowered by inflammatory mediators during invasion and metastasis.[40] Inhibitors have now been developed for proteins involved in nociceptor sensitization: cyclooxygenase-2, endothelin receptor, vanilloid receptor-1, purinergic receptor, and acid-sensing ion channels. Other classes of molecules that increase the sensitivity threshold of the nociceptor are osteoprotegerin and anticonvulsants. The successful introduction of some of these inhibitors in oncology for treating cancer pain confirms that not only cancer cells, but also host cells, express useful therapeutic targets that can be considered for the benefit of the cancer patient.

Circulating Tumor Markers Reflect the Molecular Cross-Talk Between Cancer and Stromal Cells

Molecules such as VEGF and CXCL12 are a few examples of the myriad of tumor markers that have been launched as tools to monitor therapeutic follow-up or to predict cancer relapses. Circulating tumor markers are attractive because they can be determined accurately on a small blood sample, and because they are highly informative, provided the interpretation of the result is considering the different aspects of the cancer environment.

Many circulating tumor markers find their origin in the cancer cells, and they can be divided into three main classes: intracellular, membrane-bound, and secreted molecules (Fig. 2).

Intracellular markers

Some intracellular proteins, such as TdT, NuMA, estrogen, and progesterone receptor, are mainly intranuclear molecules. Others are part of the cytoplasmic cytoskeleton (cytokeratins) and are detected in the blood as CYFRA 21-1, TPA, TPS, or cytokeratin 18. The blood concentration of these nuclear and cytoplasmic markers reflects tumor growth and tumor mass.

Membrane-bound markers

At the apical cell membrane of polarized epithelia, a number of large proteoglycans (mucins) are extending into the extracellular space and are continuously shed from the plasma membrane. Because of their apical localization and their large molecular size, these proteoglycans are unable to cross epithelial cell junctions and the underlying basement membrane, so their concentration in normal blood samples is extremely low. In cancer, cell polarization is lost, cell junctions disappear, and the basement membrane is fragmented. In this way, the large glycosaminoglycans gain access to the circulation, and their blood concentration reflects the invasion of the tumor. They are commonly indicated by "CA" (cancer antigen) followed by a number. In breast carcinoma, these proteoglycans (CA 15-3, CA 549, TAG-12)

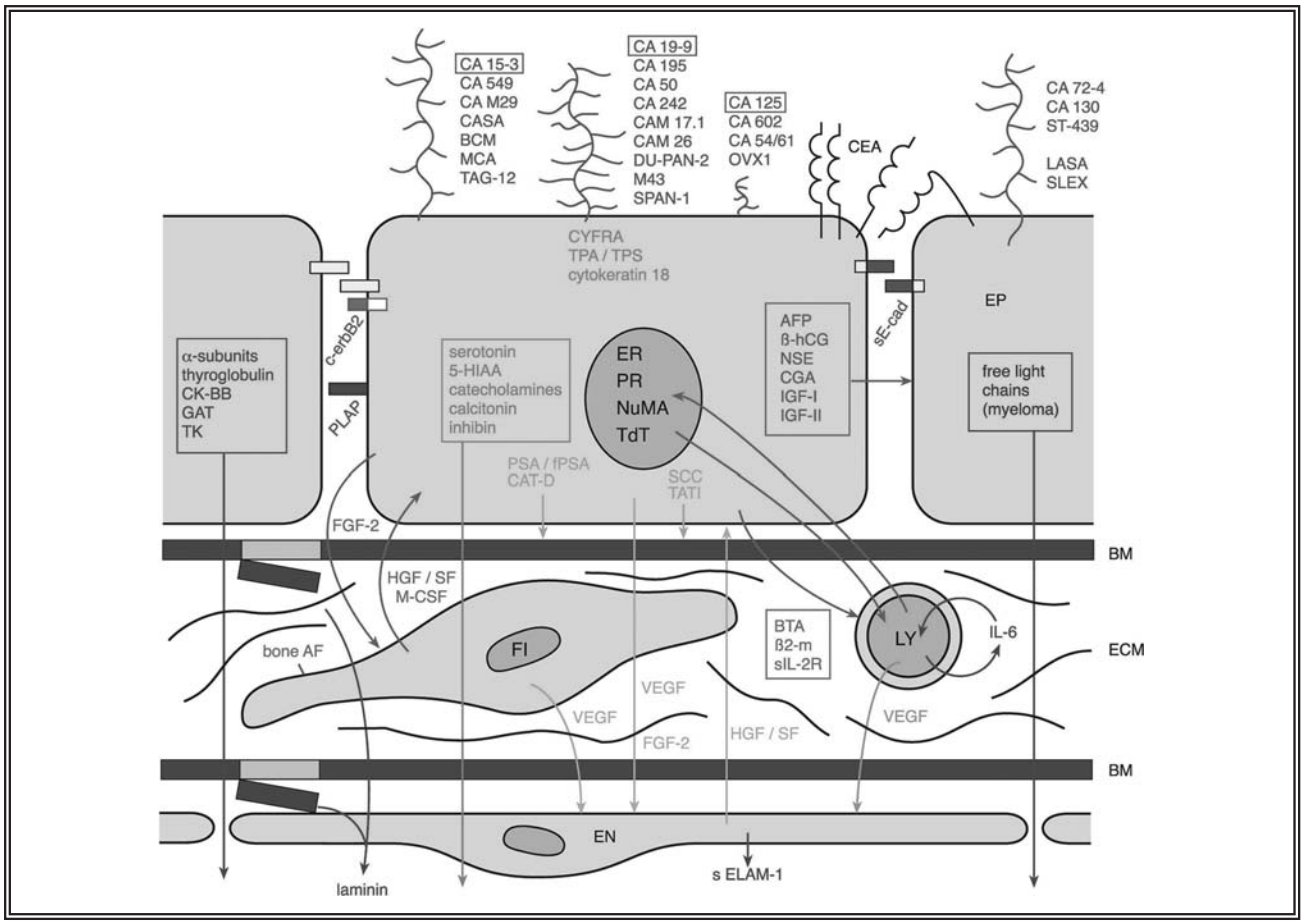

FIG. 2. Schematic overview of cellular origin of circulating tumor markers. Different markers of epithelial carcinoma cells (EP), basement membrane (BM), fibroblasts (FI), lymphocytes (LY), extracellular matrix (ECM), and endothelial cells (EN) are represented. *Intracellular markers* are TdT (terminal deoxynucleotidyl transferase), NuMA (nuclear mitotic apparatus protein), ER (estrogen receptor), PR (progesterone receptor), CYFRA21.1 (cytokeratin fragment antigen 21.1), TPA (tissue polypeptide antigen, TPS (tissue polypeptide antigen), and cytokeratin 18. *Membrane-bound* markers. In breast carcinoma, CA 15-3 (cancer antigen), CA 549, CA M29 (carcinoma-associated mucin), CASA (cancer-associated serum antigen), BCM (breast cancer mucin), MCA (mucin-like cancer-associated antigen), and TAG-12 (tumor associated antigen 12) are found. For gastrointestinal carcinoma, CA 19-9, CA 195, CA 50, CA 242, CAM 17.1, CAM 26, DU-PAN-2 (a sialytated carbohydrate antigen), M43 (mucin 43), and SPAN-1 (pancreatic cancer-associated antigen) are used. For ovarian carcinoma, CA 125, CA 602, CA 54/61, and OVX1 are used. Other proteoglycans are CA 72-4, CA 130, ST-439 (serum tumor antigen 439), LASA (lipid-associated sialic acid), and sLeX (sialyl Lewis X blood group antigen). CEA (carcinoembryonic antigen) is a weak cell-cell adhesion molecule. Shedding of membrane-bound proteins is described for sE-cad (soluble E-cadherin), a cell-cell adhesion molecule, but also for the tyrosine kinase receptor c-erbB2 (human epidermal growth factor-like receptor (HER2)), PLAP (placental alkaline phophatase), and sE-LAM-1 (endothelial leucocyte adhesion molecule). *Secreted molecules*: Smaller proteins and polypeptides are secreted by cancer cells and can act as hormones in a paracrine way; examples are: AFP (alfa-fetoprotein), β-hCG (human chorionic gonadotropin), NSE (neuron-specific endolase), CGA (chromogranin A), IGF-I, and IGF-II (Insulin-like growth factor). Polypeptides that have an endocrine effect are serotonin, 5-HIAA (5-hydroxyindole acetic acid), catecholamines, calcitonin, and inhibin. Polypeptides with an unknown destination are α-subunits (alpha-subunits of peptidehormones), thyroglobuline, CK-BB (creatin kinase BB), GAT (galactosyl transferase), and TK (thymidine kinase). Cancer cells also secrete proteases such as PSA (prostate-specific antigen) and fPSA (free prostate specific antigen), CAT-D (cathepsin D), but also the protease inhibitors TATI (tumor–associated trypsin inhibitor) and SSC (squamous cell carcinoma antigen). Cancer cells communicate with immunological cells through sIL-2R (soluble interleukin-2 receptor), BTA (bladder tumor antigen) and β2-m (beta2-microglobulin). Angiogenic signals as VEGF (vascular endothelial growth factor), FGF2 (fibroblast growth factor), and IL6 (interleukin-6) are used for follow-up. Fibroblasts secrete HGF/SF (hepatocyt growth factor/scatter factor) and M-CSF (macrophage colony-stimulating factor) that affect the cancer cells, and osteoblasts release high amounts of bone AF (alkaline phosphatase). Breakdown product of the extracellular matrix (ECM) can be detected in the blood circulation; for example, laminin from the basement membrane (BM). In myeloma, free light chains can act as highly specific markers.

are extremely large.[41] Their counterparts in gastrointestinal carcinomas (CA 19-9, CA 50, DUPAN-2) are somewhat smaller, but they are heavily glycosylated.[42] In contrast to the breast carcinoma antigens that are assayed as proteins, the gastrointestinal cancer antigens are immunologically measured as carbohydrate structures. These moieties belong to the sialyl Lewis antigen family, are expressed by metastasizing cancer cells, and are recognized by the endothelial cells when the former extravasate. So, the circulating cancer antigens from gastrointestinal tumors can be considered as metastasis markers. Proteoglycans typical for ovarium cancer (CA 125, OVX1) are invasion markers that are poorly glycosylated.[43] CEA (carcinoembryonic antigen) takes a unique position under the membrane-bound markers: In normal epithelia, it serves as a cell-cell adhesion molecule, and it contributes to the composition of the intestinal flora through selective adhesion of bacteria.[44] In addition to the already mentioned membrane-bound markers, enzymatic ectodomain shedding from cell-cell adhesion molecules (E-cadherin)[45,46] or from peptide receptors (c-erbB2/HER2) can lead to diffusion of these extracellular fragments into the circulation.

Secreted molecules

Many proteins and small polypeptides are secreted by cancer cells and have proven to be useful markers after diffusion into the circulation. Some of these molecules (IGF-I, IGF-II, inhibin, AFP, NSE, β-hCG) act as hormones, and affect growth and invasion through endocrine, paracrine, or autocrine receptor triggering. Other secreted markers are proteases (PSA, cathepsin D) or protease inhibitors (TATI, SSC) and are believed to be involved in invasion of the cancer cells. Another group of circulating markers are direct cross-talk molecules between the cancer cells and the host cells. Cancer cells send signals (sIL-2R ectodomains, BTA, β2-m) toward immunological cells, and B-lymphocytes sometimes react by producing antibodies against mutated cancer cell proteins (anti-p53). Angiogenic signals from the cancer cells (VEGF, bFGF, IL-6) toward endothelial cells generate useful markers for the follow-up of patients treated with inhibitors of angiogenesis. Fibroblasts can affect the cancer cells through the secretion of colony-forming factors, while activated osteoblasts release high amounts of bone alkaline phosphatase (Ostase©). The latter is a useful marker to follow bone metastasis, especially in patients who develop osteogenic metastases from prostate carcinoma.

CONCLUSIONS

Invasion and metastasis are the hallmarks of malignancy. Metastasis can be explained by the seed and soil theory, wherein angiogenesis seems to be an important soil element. The cross-talk between cancer cells and host cells is a target for therapy, and future revelation of this cross-talk at the molecular level is likely to open new diagnostic marker possibilities and new treatment avenues.

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Part II Cadherins

2.1. Introduction to cadherins

Cadherins, Ca-dependent adhesion, are transmembrane glycoproteins. The cadherin super family consists already of more than 80 members (Tepass 2000); we will focus in this part on the classical cadherins. The extracellular part of a classical cadherin consists of 5 cadherin domains (CD, ±110 amino acids) and in the first cadherin domain, an HAV-sequence is present. When cadherins form dimers, there is an exchange or swapping of a β strand between partner cadherin EC1 domains (Boggon *et al*.2002). This swapping interaction is anchored by the insertion of the side chain of the conserved Trp2 residue into a complementary hydrophobic pocket in the partner molecule. This interface has been proposed to mediate binding between cadherins presented from opposing cells. The symmetry of the interaction ensures that each cadherin-cadherin interface buries two Trp2 side chains, one of each cadherin.

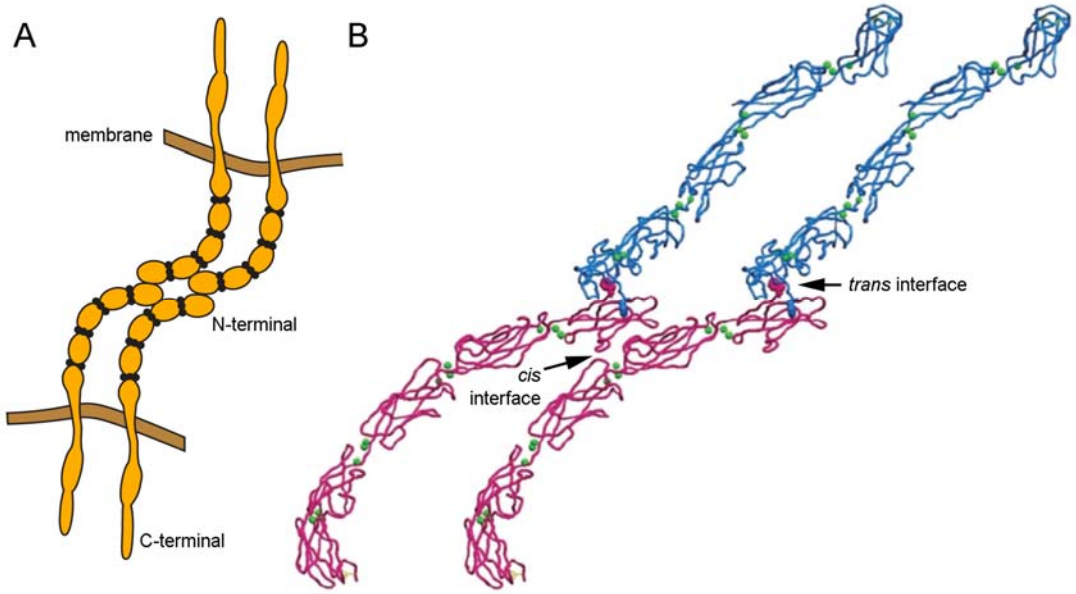

Figure 2.1.: (A) Schematic presentation of the formation of classical cadherin dimers from the same and opposing cell. (B) Presentation of the C-cadherin ectodomains, joined by cis and trans interfaces, observed in a crystal lattice. Molecules from either putative cell surface are shown in blue or pink. Trp 2 side chain are shown in CPK representation, green spheres are calcium ions and yellow present disulfide bonds (adapted from Boggon *et al.* 2002).

The strand exchange observed in cadherins exemplifies a more general domain-swapping strategy, which may enable homophilic interactions with low affinity and high specificity (Chen *et al.* 2005; Patel *et al.* 2006). The highly conserved cytoplasmic part directly interacts

with the catenins in a non-covalent way (Kemler 1993). p120 catenin binds to the juxtamembrane domain of cadherins and was originally identified as a substrate of Src (Anastasiadis *et al.* 2000). β-catenin and γ-catenin mutually bind to the same part of the cytoplasmic domain and are able to form the link with the actin cytoskeleton via α-catenin. Catenins can also be phosphorylated and by this activate different signalling pathways (Daniel and Reynolds 1997, Gumbiner 2005).

Epithelial (E)-cadherin and neural (N)-cadherin are two examples, which belong to the group of the classical cadherins. Both play an important role during embryogenesis: at the morula stage, all cells express E-cadherin, but during gastrulation E-cadherin is downregulated in the primitive streak as cells undergo epithelial-mesenchymal transition (EMT) and concomitantly express N-cadherin in the mesoderm (Hatta and Takeichi 1986). N-cadherin's function during gastrulation is required for a proper left-right axis (Garcia-Castro *et al.* 2000). N-cadherin is also implicated in cardiac development (Garcia-Castro *et al.* 2000), skeletal muscle development (George-Weinstein *et al.* 1997), neural crest migration (Nieto 2001), development of the early hematopoietic cells and the retention of these cells in the bone marrow (Puch *et al.* 2001) and cartilage formation (Panda *et al.* 2001). So, N-cadherin is expressed at different time points and tissues in the embryo. In the adult, N-cadherin is normally present in myocytes, lens cells, mesothelial cells (Hatta and Takeichi 1986), osteoblasts (Cheng *et al.* 1998), Sertoli cells (Chung *et al.* 1999), (myo)fibroblasts (Van Hoorde *et al.* 1999), oocytes and spermatozoa (Goodwin *et al.* 2000) and limb cartilage (Packer *et al.* 1997).

The best-studied cadherin implicated in cancer is Epithelial (E)-cadherin, a 120 kD protein. The E-cadherin gene was identified as an invasion suppressor gene. Transfection of E-cadherin cDNA in cell culture reverses undifferentiated, invasive cancer to the differentiated non-invasive phenotype (Vleminckxs *et al.* 1991; Takeichi *et al.* 1993). However, abrogation of E-cadherin-mediated cell-cell adhesion by functionally blocking antibodies, silencing of E-cadherin or by anti-sense constructs restores the invasive capacity (Behrens *et al.* 1989, Vleminckx *et al.* 1991). Loss of E-cadherin in cancer cells can be associated with a gain of expression of another cadherin, like N- (Hazan *et al.* 2004), placental (P) (Paredes *et al.* 2005)- or osteoblast (OB)-cadherin (Shibata *et al.* 1996; Tomita *et al.* 2000) leading to an invasive, fibroblastic phenotype. We have focussed on the role of N-cadherin known to stimulate motility and invasion of cancer cells in the same way as was seen during embryogenesis. Most epithelial cancers that express N-cadherin have lost E-cadherin, however some cells express still both. In squamous carcinoma cells, E- and N-cadherin are

not expressed at the same time. When N-cadherin was transfected, E-cadherin expression decreased and when an antisense construct for N-cadherin was introduced E-cadherin expression was upregulated (Islam *et al.* 1996). Furthermore, transfection of N-cadherin in the E-cadherin positive breast carcinoma cells, MCF-7, resulted in a co-expression of both cadherin molecules, and by this cells became metastatic. N-cadherin increased the adhesion to endothelial cells and accelerated cell migration and invasion *in vitro* and *in vivo* (Hazan *et al.* 2000). FGF-2 synergistically increased migration in these cells by activation of the mitogen activated protein kinase (MAPK) pathway leading to MMP9 gene transcription and invasion (Suyama *et al.* 2002). However, the mammary gland of transgenic mice expressing N-cadherin in the mammary epithelium appeared normal. To investigate the role of N-cadherin in mammary tumours, neu was overexpressed through breeding, and there were no histological differences observed between the -/ neu and the N-cadherin/neu mice (Knudsen *et al.* 2005). In many human tumours, N-cadherin expression was found, and we divided them in four groups (reviewed by Derycke and Bracke 2004 and table 2.1). The “De novo expression” group consists of most epithelial carcinomas, like those from the breast or prostate, where N-cadherin was only found in the cancer cells. A second group called “Re-expression” has melanoma and leukaemia as its members: the embryonic cells were positive for N-cadherin but during adult phase N-cadherin was lost but came back in the cancer cells. Mesothelioma we classified in a third group named “Upregulation”, N-cadherin levels found in the cancer cells where higher than in the adult cells. In the last group “Downregulation”, we collected cancers where the expression remained the same or was downregulated, for example in osteosarcoma. Some new examples are presented in table 2.1.

There is an important role for the tissue stroma in cancer cell invasion. N-cadherin is expressed by the invasive cancer cells but also by the host cells such as myofibroblast, neurons, smooth muscle cells, and endothelial cells. N-cadherin-dependent contacts may mediate matrix invasion, perineural invasion, muscular invasion and transendothelial migration (De Wever and Mareel 2003). Two examples of molecules regulating N-cadherin and influencing the cancer-stroma interaction are TGFβ and the non receptor tyrosine kinase Src. TGFβ upregulates N-cadherin in the cancer cells (Maeda *et al.* 2005), increases their motility and induces transdifferentiation of fibroblast cells to myofibroblasts (De Wever *et al.* 2004). The Src family kinase is also implicated in the transendothelial migration of melanoma cells. Src becomes activated at the heterotypic contact and tyrosine phosphorylates N-cadherin, as a result β-catenin then dissociates from the complex and translocates to the

nucleus of the transmigrating melanoma cells (Qi *et al.* 2006). Another kinase named Fer kinase physically associates with N-cadherin (Arregui *et al.* 2000, Xu *et al.* 2004) and regulates the mobility and clustering of N-cadherin. Fer does this by phosphorylation of N-cadherin-associated cortactin (El Sayegh *et al.* 2005). Cortactin was originally discovered as a prominent substrate of the Src family tyrosine kinases (Wu *et al.* 1991) and is an organizer of cortical actin (Weaver *et al.* 2001). Not only kinases but also proteases can influence the strengthening and mobility of the cadherin/catenin complex. Proteases such as matrix metalloproteinases (MMP) are degrading the extracellular matrix, they do that by cleaving large insoluble ECM components and ECM associated molecules, liberate bioactive fragments and growth factors and by this change the extracellular matrix (Mott and Werb 2004). For example, MMP's are able to cleave cadherin protein and shed an extracellular fragment (see also part 3). These cadherin ectodomain are able to stimulate invasion (Noë *et al.* 2001, Ryniers *et al.* 2002), neurite outgrowth (Utton *et al.* 2001) and angiogenesis (part 4) and by this influencing the behaviour of cadherin expressing cancer cells.

Table 2.1: Expression of N-cadherin in human cancer cell lines and biopsies and correlation with the expression in embryo and adult

Tumour type	Embryo	Adult	Cell line or biopsy	% positiviy	Observation	Reference
			DE NOVO EXPRESSION			
Breast carcinoma	-	-	Biopsies	76	Invasive micropapillary CA	Nagi *et al.* 2005
Prostate carcinoma	-	-	Biopsies	45	Correlates with increasing Gleason score	Jaggi *et al.* 2006
Bladder carcinoma	-	-	Biopsies	pT1: 14% pT2-3: 60%	Correlation with invasive status of tumour	Lascombe *et al.* 2006
Colon carcinoma	-	-	Cell lines and biopsies	44	No correlation with Twist	Rosivatz *et al.* 2004
Pancreatic carcinoma	-	-	Biopsies	50	Correlation with neuronal invasion	Nakajima *et al.* 2004
Thyroid adenoma	-	-	Biopsies		N-cadherin strongly upregulated and more interaction between thyrocytes and endothelial cells	Wattel *et al.* 2005
			RE-EXPRESSION			
Gastric carcinoma	+	-	Cell lines			Wang *et al.* 2006
Melanoma	+	-	Biopsies		Low expression in tumours but high expression in Spitz nevi	Krengel *et al.* 2004
Chordoma	+	-	Biopsies	36	Diminished recurrence free survival	Triana *et al.* 2005
			DOWNREGULATION			
Renal cell carcinoma	+	±	Biopsies		Associated with malignancy	Shimazui *et al.* 2006
Ovarian carcinoma	+	+	Biopsies		Only positivity in serous adenomas, carcinoma correlated with histological grade	Marques *et al.* 2004

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2.2. N-cadherin in the spotlight of cell adhesion, differentiation, embryogenesis, invasion and signalling L. Derycke and M. Bracke (2004) International Journal of Developmental Biology, 48: 463-476.

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N-cadherin in the spotlight of cell-cell adhesion, differentiation, embryogenesis, invasion and signalling

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ABSTRACT Cell migration is a process which is essential during embryonic development, throughout adult life and in some pathological conditions. Cadherins, and more specifically the neural cell adhesion molecule N-cadherin, play an important role in migration. In embryogenesis, N-cadherin is the key molecule during gastrulation and neural crest development. N-cadherin mediated contacts activate several pathways like Rho GTPases and function in tyrosine kinase signalling (for example via the fibroblast growth factor receptor). In cancer, cadherins control the balance between suppression and promotion of invasion. E-cadherin functions as an invasion suppressor and is downregulated in most carcinomas, while N-cadherin, as an invasion promoter, is frequently upregulated. Expression of N-cadherin in epithelial cells induces changes in morphology to a fibroblastic phenotype, rendering the cells more motile and invasive. However in some cancers, like osteosarcoma, N-cadherin may behave as a tumour suppressor. N-cadherin can have multiple functions: promoting adhesion or induction of migration dependent on the cellular context.

KEY WORDS: *N-cadherin, cancer, embryogenesis, invasion, signalling*

Migration and invasion

Cell migration is a process that is essential during embryonic development and throughout further life. In the adult, cell migration is crucial for homeostatic processes, such as effective immune responses and repair of injured tissues. To migrate, the individual cell body must modify its shape to interact with the surrounding tissue structures. The extracellular matrix (ECM) forms a substrate, as well as a barrier for the advancing cell body. Cell migration through tissues results from a continuous cycle of interdependent steps. In general, there are five steps involved in cell migration in the ECM. First comes the protrusion of the leading edge, where growing actin filaments connect to adapter proteins and push the cell membrane in an outward direction. In a second step cell-matrix interactions and focal contacts are formed. After that, surface proteases such as matrix metalloproteinases (MMP) are recruited and focal proteolysis takes place. Then the cell contracts by actomyosin activation, and finally the tail of the cell is detached from its substrate (Friedl and Wolf, 2003).

Border cells of the *Drosophila melanogaster* ovary are nowadays used as a model for migration. There are three recently discovered signalling pathways that control distinct aspects of migration: a global steroid-hormone signal defines the timing of migration, a highly localised cytokine signal that activates the Janus kinase-signal transducer and activator of transcription is both necessary and sufficient to induce migration, and finally, a growth factor that is analogous to platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) contributes to guiding the cells to their destination (Montell, 2003).

In embryonic morphogenesis two types of collective cell movement can be observed. The first one involves mass migration whereby a tissue moves in a coordinated manner. Gastrulation is an example of mass migration. In the blastocyst large groups of cells migrate collectively as sheets to form the three layers that will eventually form the embryo. Cells within these layers migrate to target locations and form various tissues and organs. The second type of movement requires loss of cell-cell contacts for the migration of individual cells or small groups of cells through the ECM, as seen in neural crest migration. Cells delaminate from the ectodermal layer and acquire migratory properties as they undergo the process of epithelial to mesenchymal transition (EMT). Another example is the migration of muscle precursor cells from the somites to the limbs (Locascio and Nieto, 2001; Horwitz and Webb, 2003).

The failure of cells to migrate to their appropriate locations can result in developmental abnormalities and also in pathological

Abbreviations used in this paper: ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; MMP, matrix metalloproteinase; N-cadherin, Neural cadherin.

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processes, including vascular and inflammatory diseases, and tumour invasion and metastasis (Lauffenberger and Horwitz, 1996). Aberrant cell migration may play a role in cancer. Cancer is one of the prime causes of human morbidity and mortality, and most of the cancer deaths arise from metastases. Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis. A cell becomes cancerous because of essential alterations in its physiology: limitless replicative potential due to self-sufficiency in growth signals, insensitivity to growth inhibitory signals or escape from programmed cell death, induction of angiogenesis and acquisition of invasive and metastasising potential (Hanahan and Weinberg, 2000). Of all the processes involved in tumour progression, local invasion and the formation of tumour metastases are clinically the most relevant ones, but the least well understood at the molecular level. They represent one of the great challenges in experimental cancer research.

During the progression of cancer, primary tumour cells move out, invade into adjacent tissues and travel to distant sites. Most important, these processes allow cancer cells to enter the lymphatic and blood vessels for dissemination into the circulation. Invasion is resumed when extravasation occurs in distant organs, and when the secondary tumour contributes to the metastatic cascade. Cancer cells use diverse patterns of migration. They can disseminate as individual cells or expand as solid strands, sheets, files or clusters. Leukemia, lymphoma and most sarcomas disseminate as single cells, while epithelial cells commonly use collective migration. In principle, the lower the differentiation state, the higher the tendency of the tumour to disperse via individual cells (Thiery, 2002; Friedl and Wolf, 2003).

Similarities between the three signalling pathways described for the ovarian border cell migration, and the pathways that are deregulated in human cancer cells indicate that signals that contribute to aberrant proliferation and survival of the tumour cells, can also promote motility, and hence invasion (Montell, 2003).

We will discuss in this review the impact of E- and N-cadherin on migration in embryogenesis and tumour invasion. Epithelial or E-cadherin plays a role in collective migration of epithelial cells. E-cadherin is also an invasion suppressor molecule, and in tumours this molecule can be downregulated in different ways (Mareel and Leroy, 2003). Downregulation of E-cadherin is often correlated with upregulation of neural or N-cadherin, an invasion promoter molecule (Tomita *et al.*, 2000; Li and Herlyn, 2000). However, both the regulation of N-cadherin expression and its molecular contribution to invasion are incompletely understood.

Cadherins

In humans there are more than 80 members of the cadherin superfamily. Sequencing the genome of *C. elegans* and *Drosophila* revealed the existence of 14 and 16 different genes, respectively. Cadherins are composed of an extracellular part, that mediates calcium-dependent homophilic interactions between cadherin molecules, a transmembrane and a cytoplasmic part. The extracellular part consists of several cadherin repeats (EC) of ±110 amino-acids, which are characterised by a number of conserved amino acid sequences such as PE, LDRE, DXNDN and DXD. These motifs can bind 3 calcium ions at each interdomain boundary in a cooperative manner. Classification of cadherins into subfamilies is based on domain layout of individual cadherins, which include the number and sequence of EC repeats, and the presence of other conserved domains and sequence motifs, like tyrosine kinase and EGF domains. There are four cadherin subfamilies conserved between *C. elegans, Drosophila* and humans: classic cadherins, fat-like cadherins, seven-pass transmembrane cadherins and a new subfamily of cadherins that is related to *Drosophila* Cad 102F. Classic cadherins consist of four subgroups: vertebrate type I classic cadherins like E-, placental (P)-, N- and retinal (R)-cadherin, with an HAV sequence in the first cadherin repeat, vertebrate type II classic cadherins which have no HAV in the first repeat, for example vascular endothelial (VE)-cadherin, ascidian classic cadherins and the non-chordate classic cadherins for example D (*Drosophila*) E- and D (*Drosophila*) N-cadherin (Tepass *et al.*, 2000). The molecular mechanism of type I cadherin interaction has recently been unravelled. The model was proposed after elucidation of the crystal structure of the C-cadherin ectodomain: the trans-interaction is formed by a strand dimer (EC1-EC1) where association is found between the side chain of Tryptophan 2 (Trp^2) in one molecule and a pocket in the hydrophobic core of another molecule. The cis interaction occurs between EC1 of one molecule and EC2 of another molecule, resulting in the formation of a lattice of a supramolecular complex (Boggon *et al.*, 2002).

It is now known that alterations in the expression and function of cell-cell and cell-matrix adhesion molecules correlate with progression to malignancy. E-cadherin, a homotypic cell-cell adhesion molecule is expressed on most epithelial cells and is an invasion suppressor. E-cadherin expression or function is lost in most of the carcinomas. This may be by mutational inactivation of the E-cadherin gene, hypermethylation of the promoter, transcriptional repression by SIP1 or snail, loss of transactivators like RB, Myc and WT1, transactivation of other cadherins, phosphorylation of Armadillo proteins by tyrosine kinases, sterical hindrance by mucin 1 (MUC-1) or by ectodomain shedding of E-cadherin by matrix metalloproteinases (MMP) (Van Aken *et al.,* 2001). The proof of principle that the loss of E-cadherin is involved in the progression of tumour malignancy came from a transgenic mouse model of pancreatic β cell carcinogenesis (Rip1Tag2). In these mice, the SV40 large T antigen was expressed under the control of the rat insulin promoter, thus inducing neoplastic transformation from differentiated adenoma to invasive carcinoma selectively in the β cells of the islets of Langerhans. In these tumours E-cadherin was downregulated. Forced expression of E-cadherin in the β cell tumours resulted in an arrest in tumour development at the adenoma stage. Conversely, expression of a dominant-negative form of E-cadherin resulted in early invasion and metastasis (Perl *et al.*, 1998). These results show that E-cadherin suppresses tumour invasion, and that loss of E-cadherin can actively participate in the induction of tumour invasion (Cavallaro and Christofori, 2001).

N-cadherin

N-cadherin was first identified in 1982 (Grunwald *et al.*, 1982) as a 130 kD molecule in the chick neural retina that was protected by calcium from proteolysis, and in 1984 A-CAM was identified (now called N-cadherin) as a molecule that was localised at the adherens junctions (Volk and Geiger, 1984). The N-cadherin gene in mice was located on chromosome 18 (Miyatani *et al.*, 1989). Via Yeast Artificial Chromosome (YAC) analysis the structure of the human N-cadherin gene was determined. The entire N-cadherin gene was

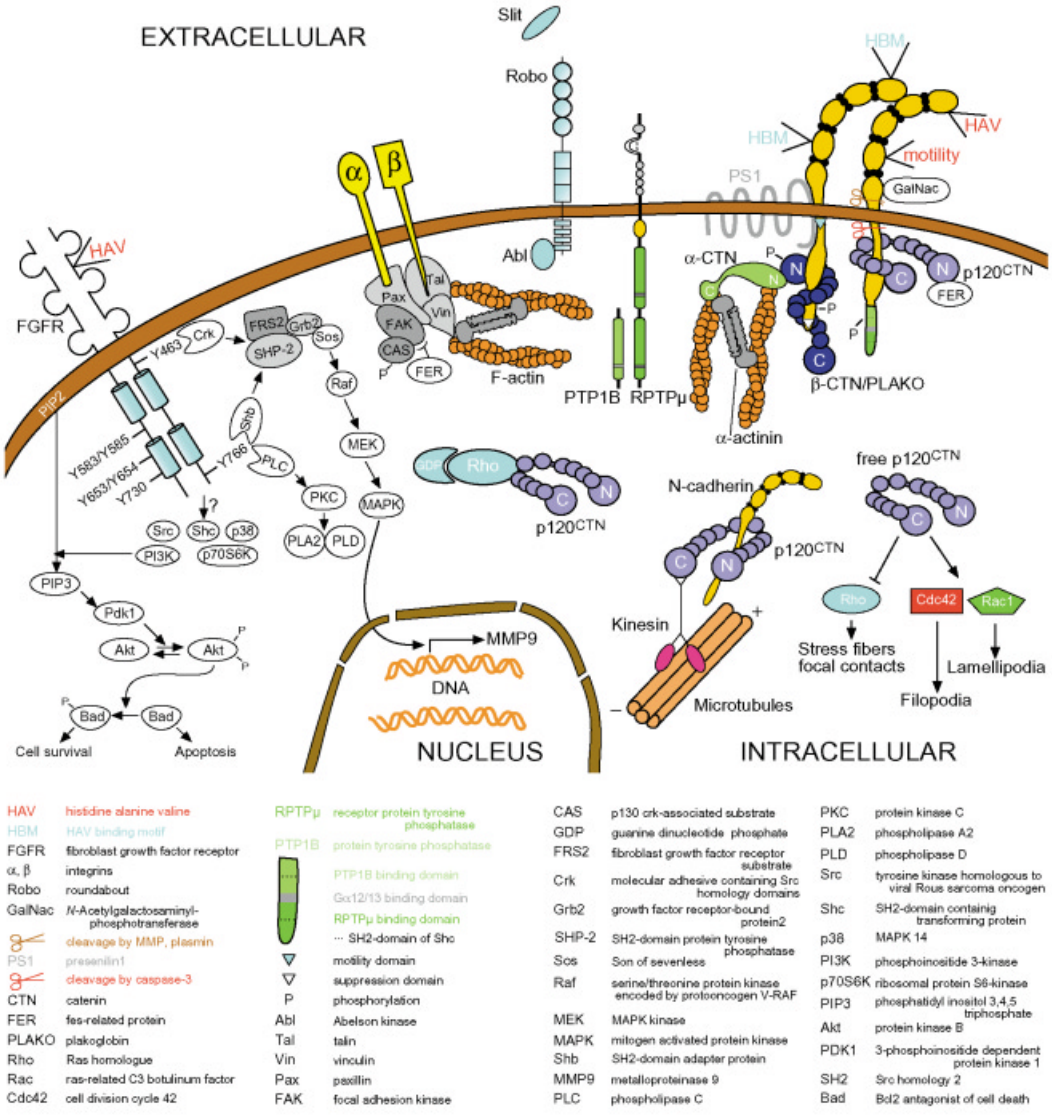

Fig. 1. Schematic overview of the N-cadherin/catenin complex and the multiple proteins which associate and influence the complex. *N-cadherin associates via its HBM motif with the HAV sequence of the FGFR. N-cadherin also has a HAV sequence in the first extracellular domain (EC). Activation of FGFR can lead to activation of MAPK and transcription of MMP9, inducing invasion and metastasis. In EC4, a domain is present which is responsible for N-cadherin's pro-migratory behaviour. However, in the cytoplasmic part there are also domains which can stimulate or inhibit migration. N-cadherin mediates also survival of cells via the PI3K-AKT pathway. Many molecules associate directly with the complex, such as GalNacPtase, p120^{ctn}, β-catenin, PTP1B, RPTPμ and Gα12, or indirectly like FER (implicated in the cadherin/catenin complex, but also in the integrin complex) and α-catenin. Many molecules can change the complex like proteases (MMP, caspase-3, PS 1) or the Robo-Abl kinase. When free cytoplasmic p120^{ctn} is present, it changes the morphology of the cell by blocking Rho in the GDP state or activating Rac1 and Cdc42. N-cadherin is already associated with the catenins in the ER and a microtubular kinesin-driven mechanism is involved in the trafficking to the plasma membrane (Adapted from Van Aken* et al., *2001 and Anastasiadis* et al., *2001).*

mapped to a 250-kb region on chromosome 18q11.2. The gene is composed of 16 exons, and homology was found not only between human and mouse, but also between N-cadherin and other cadherins (Wallis *et al.*, 1994). The protein exists of five extracellular cadherin repeats (EC1 to EC5), a transmembrane and a cytoplasmic part that are encoded by exons 4 to 13, 13 and 14, and 14 to 16, respectively. Eight sequence polymorphisms were identified in a Japanese population: three CCT or GCC-type trinucleotide repeat polymorphisms adjacent to the initiation codon and five other novel single-nucleotide polymorphisms in the coding region (Harada *et al.*, 2002). The promoter of N-cadherin does not contain CCATT or TATA boxes, but showed a high overall GC content, high CpG dinucleotide content, and several consensus Sp1 and Ap2 binding sequences (Li *et al.*, 1997).

N-cadherin domains and associated proteins

In this part we will discuss the molecules associated with the extracellular and intracellular part of N-cadherin, their influence on N-cadherin function and the induction of signalling pathways (Fig. 1).

Although N-cadherin typically forms homotypic homophilic interactions, also heterotypic homophilic and heterophilic interactions have been described. Examples include the interaction between N-cadherin molecules of Sertoli cells and spermatides, and between N- and R-cadherin in transfected L cells and in neurons at certain neural synapses (Shan *et al.*, 2000).

The fibroblast growth factor receptor (FGFR) is implicated in N-cadherin function. In the nervous system, N-cadherin function is involved in a number of key events that range from the control of axonal growth and guidance to synapse formation to synaptic plasticity (Doherty and Walsh, 1996). Neurite outgrowth stimulated by N-cadherin is inhibited by a wide variety of agents that block the FGFR function, including the expression of a dominant negative FGFR (Williams *et al.*, 1994). In addition, N-cadherin can promote contact-dependent survival of ovarian granulosa cells in an FGFR-dependent manner (Trolice *et al.*, 1997). More recently it has been illustrated that both N-cadherin and the FGFR are necessary to increase cell motility and induce metastasising capacity (Suyama *et al.*, 2002). N-cadherin and the FGFR interact directly: the HAV sequence present in the FGFR associates with the IDPVNGQ sequence present in EC4 of N-cadherin (Williams *et al.*, 2001). This motif was already previously described as a candidate for interaction with FGFR, based on sequence homology of the motifs within N-cadherin (INPISGQ in EC1) and R-cadherin (IDPVSGR in EC1) that interact with the HAV region in N-cadherin (Doherty and Walsh, 1996; Williams *et al.*, 2000b). Peptides used to investigate N-cadherin function were found to have opposite effects on neurite outgrowth: whereas INP (Williams *et al.*, 2000b) and a cyclic HAV peptide (Williams *et al.,* 2000a) antagonize its function, the cyclic dimeric version of the HAV and the INPISG sequence have an agonistic effect on neurite outgrowth (Williams *et al.*, 2002). The latter peptides act by binding to and clustering N-cadherin in the cells, thereby activating the N-cadherin/FGFR signalling cascade. After stimulation with FGF 2, invasion of breast carcinoma cells was demonstrated in the same degree as in cells transfected with N-cadherin, suggesting that N-cadherin and FGFR synergize to generate signals that affect the invasive behaviour. As a consequence of N-cadherin binding, internalisation of the FGFR is inhibited. This is causing a sustained cell surface expression of FGFR, leading to a persistent MAPK-ERK (mitogen activated protein kinase-extracellular signal regulated kinase) activation, MMP-9 expression and tumour invasion (Suyama *et al.*, 2002). Thus, N-cadherin may be involved in both ligand-dependent and ligand-independent interactions with the FGFR (Wheelock and Johnson, 2003).

Transfection of epithelial cells with N-cadherin influences the morphology and the behaviour of these cells: it induces a “motile phenotype” (Islam *et al.*, 1996; Hazan *et al.*, 1997). By transfection of chimeras of E- and N-cadherin in squamous epithelial cells, a 69 amino acid portion of EC4 was identified that is necessary for epithelial to mesenchymal transition and an increase in motility by N-cadherin. The motile phenotype induced by N-cadherin is independent of cell-cell adhesion because an antibody, recognizing the 69 amino acid sequence, inhibited cell motility without inhibiting cell-cell aggregation, providing evidence that adhesion and motility can be two separate features (Kim *et al.*, 2000).

Recently, an S (suppression of movement) -domain (a C-terminal domain: AA699-710 of E-cadherin) was identified in both E- and N-cadherin, though N-cadherin lacked the capacity to suppress motility, presumably because its domain is masked or latent. This inability of N-cadherin to suppress movement required the presence of the modulation-of-movement-domain (M-domain), consisting of the juxtamembrane domain. The authors suggested several ways in which diversity in cadherin function might arise in different cell types. Variations could be expected if cells differ in expression of molecules that interact with the S and M-domain (Fedor-Chaiken *et al.,* 2003). For example, N-cadherin has no influence on the movement of MDA-MB-435, but the same molecule inhibits the migration of LM8 mouse osteosarcoma cells (Kashima *et al.*, 2003). So, the effect of cadherins can be cell type specific.

The cytoplasmic part of N-cadherin is complexed with a multitude of molecules, such as the catenins p120 catenin ($p120^{ctn}$), β-catenin and α-catenin, which are possible regulators of cadherin function. $p120^{ctn}$ binds to the juxtamembrane domain and is a key molecule in the regulation of the adhesive or motile phenotype. When $p120^{ctn}$ is phosphorylated, its binding to N-cadherin is increased, reducing the adhesive activity of the latter. Cadherin adhesive activity is also subject to regulation by Rho GTPases. Overexpression of $p120^{ctn}$ in fibroblasts or cadherin-deficient cells causes a branching phenotype, whereas in epithelial cells an increasing lamellipodia formation is observed. In fibroblast, this cytoplasmic $p120^{ctn}$ inhibits RhoA, resulting in an increase in cell motility and activation of Rac1 and Cdc42. In line with the direct binding of RhoA and $p120^{ctn}$ in *Drosophila* (Magie *et al.,* 2002), one hypothesis says that a direct interaction of $p120^{ctn}$ with RhoA keeps RhoA in the inactive GDP state. According to another hypothesis the association of $p120^{ctn}$ with vav2, a Rho-GEF (guanine nucleotide exchange factor) explains the activation of Rac1 and Cdc42 (Anastasiadis *et al.*, 2001).

Fer (fes –related protein; fes: feline sarcoma), a nonreceptor tyrosine kinase, interacts via its coiled-coil domain with the coiled-coil domain of $p120^{ctn}$. Fer is implicated in the regulation of adherens junctions and focal adhesions. Trojan peptides, recognizing the juxtamembrane domain of N-cadherin, caused Fer to dissociate from N-cadherin, rendering Fer available for complex formation with FAK (Arregui *et al.*, 2000). This correlated with disruption of focal adhesion and reduced tyrosine phosphorylation of the docking protein p130Cas. These observations indicate that Fer has a role in the regulation of cell adhesion and migration through effects on both adherens junctions and focal adhesions (Greer, 2002). Fer and Fyn kinase phosphorylate Tyrosine 142 of β-catenin, and this (unphosphorylated) tyrosine is necessary for the association of β-catenin with α-catenin. In contrast, phosphorylation of tyrosine residues of $p120^{ctn}$ increases the binding of the Fer/Fyn-p120ctn complex to cadherin (Piedra *et al.*, 2003).

$P120^{ctn}$ not only modulates the function of cadherins but is also important in the trafficking and maturation of the cadherin-catenin complex. Wahl *et al.,* have shown that $p120^{ctn}$ readily associates to the cytoplasmic part of N-cadherin in the endoplasmatic reticulum (ER). Later on, the cytoplasmic part is phosphorylated, leading to additional binding of β- and α-catenin. The proregion is then removed by furin protease and the complex is transported to the plasma membrane (Wahl *et al.*, 2003). N-cadherin trafficking is mediated by a microtubular kinesin-driven mechanism (Mary *et al.*, 2002) and recent papers elucidated that $p120^{ctn}$ is the link with the microtubule network by direct association of $p120^{ctn}$ with kinesin (Chen *et al.*, 2003; Yanagisawa *et al.*, 2003). Presenilin 1 (PS1),

playing a role in the pathogenesis of early onset familial Alzheimer disease, also binds to the juxtamembrane domain and modulates the adhesive capacity. When dominant negative PS1 is expressed, cell-cell contacts are suppressed, and N-cadherin is localised perinuclearly at the ER and Golgi apparatus. So, PS1 is essential for the trafficking of N-cadherin to the plasma membrane (Uemura *et al.*, 2003).

Another point where the cadherin/catenin complex can be regulated is at its interaction with β-catenin, which is responsible for association with α-catenin and hence for linking the complete complex to the actin network. The interaction of β-catenin with N-cadherin is regulated by multiple proteins (Lilien *et al.*, 2002). The proteoglycan neurocan can inhibit N-cadherin- and β1-integrin–mediated adhesion and neurite outgrowth. Neurocan interaction with its receptor GalNAcPTase leads to tyrosine hyperphosphorylation of β-catenin and uncoupling of β-catenin from the complex (Lilien *et al.*, 1999; Li *et al.*, 2000). Hyperphosphorylation of β-catenin has consistently been correlated with loss of adhesive function. The nonreceptor protein tyrosine phosphatase PTP1B regulates the phosphorylation of β-catenin (Balsamo *et al.*, 1998). PTP1B needs to be phosphorylated on tyrosine-152 for its association with N-cadherin (Rhee *et al.,* 2001). PTP1B binds to the cytoplasmic part, specifically to the amino acids 872-891 of N-cadherin, and this domain partially overlaps with the β-catenin binding domain. Despite the partial overlap of binding domains, β-catenin and PTP1B do not compete with each other for binding (Xu *et al.*, 2002). The interaction of N-cadherin with PTP1B is essential for its association with β-catenin, its stable expression at the cell surface, and consequently, its function. Gα12/13, a Gα subunit of the heterodimeric G proteins, associates with the cytoplasmic part of N-cadherin, overlapping the binding site of PTP1B, so binding of Gα may displace PTP1B and vice versa (Kaplan *et al.*, 2001). The phosphatase PTPμ directly interacts with the carboxy-terminal domain of the cadherins, potentially dephosphorylating these. The absence of PTPμ is correlated with increased phosphorylation of the cadherin itself, but not of β-catenin (Brady-Kalnay *et al.*, 1998). On its turn, increased tyrosine phosphorylation of N-cadherin has been associated with increased turnover of N-cadherin, releasing a 90 kD extracellular fragment (Lee *et al.*, 1997). N-cadherin phosphorylated by Src on tyrosine 851 and 883, associates with the SH2 domain of the adapter protein Shc (Xu and Carpenter, 1999), opening the door to different signalling pathways.

N-cadherin function and signalling

N-cadherin promotes survival in melanoma and prostate carcinoma cells. N-cadherin ligation recruits phosphatidylinositol 3-kinase (PI3K) which activates Akt, resulting in inactivation of the pro-apoptotic molecule Bad (Bcl2 antagonist of cell death, Bcl2 is the acronym for B cell lymphoma) (Li *et al.*, 2001; Tran *et al.*, 2002). However, N-cadherin can also have an inhibitory effect on cell proliferation. Overexpression of N-cadherin in cells suppresses cell proliferation by prolonging the G2/M phase and inducing β-catenin dependent expression of p21 (inhibitor of cyclin dependent kinase, cdk) which inhibit Cdc2 activity (Kamei *et al.*, 2003). P27, another cdk inhibitor is involved in N-cadherin mediated contact inhibition of cell growth and cell cycle arrest in the G1 phase (Levenberg *et al.*, 1999).

N-cadherin stimulates migration and invasion of cells. Different groups demonstrated that aberrant expression of N-cadherin in cancer cells makes the cells more motile and invasive. Our laboratory has demonstrated that retinal pigment epithelial cells (RPE) are invasive in collagen type I. RPE cells have a polarised epithelial phenotype *in vivo* but become rapidly fibroblastic and invasive when explanted *in vitro*. In these conditions they undergo a switch from E- to N-cadherin expression. Such a switch was already seen in the epiblast cells of the chick embryo when the cells where treated with hepatocyte growth factor (HGF) (Deluca *et al.*, 1999). We found indications for an autocrine HGF/c-Met loop stimulating RPE cell invasion via focal adhesion kinase (FAK). N-cadherin activates FAK in invasive RPE cells (Van Aken *et al.*, 2003).

In order to mimic and control the formation of cadherin mediated cell-cell contacts, N-cad-Fc chimera, comprising the N-cadherin ectodomain linked to an IgG Fc fragment, have been used. These chimera form dimers by inter-chain disulfide bridges of the Fc domains. Chimera-loaded beads bound specifically to various cells expressing N-cadherin, inducing a rapid recruitment of cadherin/catenin complexes, followed by a strong anchorage of actin filaments, leading to cytoskeletal reorganisation and activation of intracellular signalling pathways (Lambert *et al.*, 2000). Rac1 is required for the anchoring of the cadherin/catenin complex to the actin filaments in the myogenic C2 cells (Lambert *et al.*, 2002). Further studies demonstrated that for the formation of lamellipodia a $p120^{ctn}$-PI3K-Rac1 pathway is triggered, while for the organisation of the cadherin complex and the actin cytoskeleton only $p120^{ctn}$ and Rac1 are needed (Gavard *et al.*, 2004). In addition, N-cadherin also controls crucial steps in myogenic differentiation, and addition of N-cad-Fc beads triggered myogenesis in isolated myoblasts. Here, inactivation of Rac1 and Cdc42 was observed, while RhoA was activated. The RhoA GTPase activity is important for myogenic differentiation since it controls the expression and the activity of the transcription factor SRF (serum response factor) which binds to motifs present in the promoter of muscle-specific genes. As a result the promoter of muscle-determining factor MyoD is stimulated by N-cadherin-dependent contact formation (Charasse *et al.*, 2002). A balance between Rac1-Cdc42 and RhoA activity determines the cellular phenotype and biological behaviour of various cell systems: actin cytoskeleton organisation, formation of focal adhesions, neurite extension and myogenesis. In fibroblasts the activation of RhoA leads to assembly of stress fibers and focal contacts, which mediate adhesion to ECM. Activation of Rac1 and Cdc42, however, results in the formation of filopodia and lamellipodia. In mouse fibroblasts, Rac1 signalling is able to antagonize Rho activity. Activation of Rac1 by the GEF Tiam1 in these cells induces an epithelial-like morphology with functional cadherin-based adhesion and inhibition of migration (Sander *et al.*, 1999; Yap and Kovacs, 2003).

Full length N-cadherin and its 90 kD N-terminal fragment have been shown to promote cell-matrix adhesion and neurite outgrowth when presented as a substratum (Paradies and Grunwald, 1993; Bixby *et al.*, 1994). Soluble N-cad-Fc can also stimulate FGFR dependent neurite outgrowth (Utton *et al.*, 2001).

N-cadherin is expressed in human endothelial cells, but its function in angiogenesis is not fully elucidated. Literature data demonstrated that N-cadherin is expressed during early neuroectoderm vascularization where it probably establishes interactions

between neuroectoderm and endothelium, followed by a downregulation of N-cadherin in endothelia when the cells differentiate to a blood-retina and blood-brain barrier (Gerhardt *et al.*, 1999). N-cadherin has also been indicated as an angiogenic factor in non-small-cell lung cancer because biopsies positive for N-cadherin were hypervascular (Nakashima *et al.*, 2003). In our laboratory we could find that plasmin cleaved a 90 kD ectodomain fragment from N-cadherin, coined soluble N-cadherin. Soluble N-cadherin induced angiogenesis in the chick chorioallantoic membrane and the rabbit cornea. The 10-mer HAV peptide (LRAHAVDING) had the same pro-angiogenic effect as soluble N-cadherin (our unpublished data).

N-cadherin up- and downregulation

The N-cadherin/catenin functions are influenced by multiple intracellular and extracellular factors (Table 1). We will discuss a few factors more into detail. The upregulation of N-cadherin at the transcription level has been explored. In *Drosophila* development the transcription factor twist initiates DN-cadherin expression during early mesoderm formation. Another transcription factor, snail, is required for an increase in the level of N-cadherin (Oda *et al.*, 1998). In biopsies of gastric carcinoma, a correlation was demonstrated between the expression of N-cadherin and twist (Rosivatz *et al.*, 2002). Growth factors as EGF and HGF are able to induce a switch from E- to N-cadherin. An example is found in breast carcinoma cells co-expressing E- and N-cadherin. When treated with EGF they undergo epithelial-mesenchymal transition-like changes, including upregulation of vimentin, downregulation of E-cadherin and upregulation of N-cadherin (Ackland *et al.*, 2003).

P120^{ctn} is an important regulator of the turnover of cadherins. Upon p120^{ctn} knockdown with siRNA (small interfering RNA), the cadherins are rapidly degraded, probably via ubiquitination (Davis *et al.*, 2003). Also, proteases like MMP (Paradies and Grunwald, 1993), caspase-3 (Hunter *et al.*, 2001) and presenilin (Marambaud *et al.*, 2003) may cleave N-cadherin, giving rise to different fragments. MMPs shed a 90 kD ectodomain fragment, soluble N-cadherin, that is still functional while the role and the fate of the

TABLE 1

MECHANISMS OF REGULATION OF THE N-CADHERIN/CATENIN COMPLEX

Factor	Context	Properties	Reference
		UPREGULATION	
twist and snail	*Drosophila*	correlation between twist and N-cadherin expression	Oda *et al.*, 1998
	gastric cancer		Rosivatz *et al.*, 2002
GATA-4	heart	binding to N-cadherin promoter	Zang *et al.*, 2003
SOX9	chondrocytes	enhancing N-cadherin promoter activity	Panda *et al.*, 2001
Pax6	Lens placode	induction of N-cadherin expression	Van Raamsdonk *et al.*, 2000
HOXD3	Lung cancer cells	induction of N-cadherin expression	Hamada *et al.*, 2001
HGF	epiblast cells	when cells ingress the primitive streak	Deluca *et al.*, 1999
phorbol ester	osteoblasts	PKC dependent	Delannoy *et al.*, 2001
EGF	breast carcinoma cells	induction of EMT	Ackland *et al.*, 2003
gonadal steroids	hippocampus	mRNA levels increased	Monks *et al.*, 2001
	testis		Pötter *et al.*, 1999
	Sertoli cells	mRNA levels increased	MacCalman *et al.*, 1997
	Sertoli cells	protein levels increased	Perryman *et al.*, 1996
	granulosa cells		Blaschuk and Farookhi, 1989
	ovary	mRNA levels increased	MacCalman *et al.,* 1995
		DOWNREGULATION/ FUNCTIONAL INHIBITION	
IL-6	melanoma	mRNA and protein level decreased	Gil *et al.*, 2002
dexamethasone	osteoblasts	inhibition of expression	Lecanda *et al.,* 2000
caspase 3	osteoblasts	Proteolysis at the juxtamembrane domain	Hunter *et al.*, 2001
plasmin		producing a 90 kD ectodomain fragment	Our unpublished data
MMP	retina	producing a 90 kD ectodomain fragment	Paradies and Grunwald, 1993
presenilin	neurons	ε-cleavage produces an intracellular domain peptide CBP	Marambaud *et al.*, 2003
Porphyromonas gingivalis	epithelial cells	loss of cell-cell adhesion and apoptosis	Chen *et al.*, 2001
Bismuth/ cadmium	proximal tubule epithelium	nephrotoxicity	Leussink *et al.*, 2001
			Prozialeck *et al.*, 2003
siRNA of p120^{CTN}		rapid turnover of cadherin by proteasome/lysosome	Davis *et al.*, 2003
thalidomide		binds to N-terminal domain mimicking a tryptophan residue	Thiele *et al.*, 2000
Robo	axons	activation of the receptor by Slit: complex formation of Robo/Abl/N-cadherin resulting in β-catenin phosphoryaltion	Rhee *et al.*, 2002
Chlamydia trachomatis	cervical epithelial cells	Breakdown of the N-cadherin/β-catenin complex	Prozialeck *et al.*, 2002
N-acetylglucosaminyl	neural retina cells	loss of cell-cell adhesion and uncoupling of the N-cadherin	Balsamo and Lilien, 1990
transferase V		/transferase complex from actin	Balsamo *et al.*, 1991
			Balsamo *et al.*, 1995
			Guo *et al.*, 2003

Abbreviations used: GATA-4, zinc finger transcription factor recognizes the consensus motif (A/T)GATA(A/G); SOX9, DNA binding SRY box found in SOX family member; Pax6, paired box protein 6; HOXD3, Homeobox D3; HGF, hepatocyte growth factor; EGF, epidermal growth factor; IL-6, interleukin 6; siRNA, small interfering RNA; TF, transcription factor; PKC, protein kinase C; CBP, CREB binding protein; CREB, cyclic AMP response element binding protein.

residual transmembrane/ intracellular part is not clear. Only for the intracellular peptide fragment of N-cadherin, produced after PS1 cleavage, a role is described. It forms a complex with transcriptional coactivator CBP (CREB binding protein) in the cytoplasm and promotes the proteasomal degradation of CBP, via the ubiquitin-proteasome pathway. N-cadherin has an important role during embryogenesis. Thalidomide, a drug that causes teratogenicity, affects mostly organs originating from neural crest cells. Thalidomide was found to bind at the N-terminal domain of N-cadherin, mimicking a tryptophan residue which is critical for its homodimerization, and thus functionally inhibiting homodimerisation (Thiele *et al.*, 2000). In axon trajectories, the Robo transmembrane receptor forms a complex with N-cadherin. After activation with Slit, a complex between Robo, Abl and N-cadherin is formed, followed by tyrosine phosphorylation of β-catenin and resulting in loss of the critical N-cadherin-actin connection (Rhee *et al.*, 2002).

N-cadherin expression from embryo to adult

Members of the cadherin superfamily have distinct expression patterns during embryonic development and in the adult. Changes in cadherin expression are often associated with changes in cellular morphology and tissue architecture. During gastrulation, E-cadherin is downregulated in the primitive streak as cells undergo an epithelial-mesenchymal transition and concomitantly express N-cadherin in the mesoderm (Hatta and Takeichi, 1986). This expression of N-cadherin is initiated by the transcription factor twist in *Drosophila* (Oda *et al.*, 1998). During neurulation, a similar change in expression occurs in the developing neuroepithelium. Different groups analysed the role of N-cadherin in embryogenesis by using knockouts or an artifical system of cytodifferentiation, in which either teratomas or cultured embryoid bodies from genetically manipulated embryonic stem (ES) cells are generated and analysed. When N-cadherin was constitutively expressed in the E-cadherin negative ES cells, the resulting teratomas formed neuroepithelia and cartilage (Larue *et al.*, 1996). N-cadherin knockout mice die at day 10 of gestation. The embryos display major heart defects and malformed neural tubes and somites (Radice *et al.*, 1997). However, all tissues expected to be formed at this stage are apparently present and seem to be normally differentiated. Re-expression of N-cadherin using muscle-specific promoters (α- or β-myosin heavy chain) partially rescues N-cadherin null embryos. These embryos exhibit an increased number of somites, branchial arches and the presence of forelimb buds, however, brain development is still impaired (Luo *et al.*, 2001).

N-cadherin is implicated in several aspects of cardiac development including sorting out of the precardiac mesoderm, establishment of left-right asymmetry, cardiac looping morphogenesis and trabeculation of the myocardial wall. N-cadherin is one of the earliest proteins to be asymmetrically expressed in the chicken embryo and its activity is required during gastrulation for a proper establisment of the left-right axis (Garcia-Castro *et al.*, 2000). In the early embryo N-cadherin is found in the mesoderm and the notochord, while in the late embryo it is present in neural tissue, lens and some other epithelial tissues, cardiac and skeletal muscles, nephric primordial, some mesenchymal tissue, mesothelium and primordial germ cells (Hatta *et al.*, 1987; Takeichi, 1988).

N-cadherin is expressed in early hematopoietic cells ($CD34^+CD19^+$) and is involved in the development and retention of early hematopoietic cells in the bone marrow (Puch *et al.*, 2001). Cartilage formation in the developing vertebrate embryonic limb consists of highly coordinated and orchestrated series of events involving the commitment, condensation and chondrogenic differentiation of mesenchymal cells and the production of cartilaginous matrix. Here, N-cadherin has a role in the cellular condensation (Tuan, 2003), being a direct target of SOX9, a transcription factor that is essential for chondrocyte differentiation and cartilage formation (Panda *et al.,*2001). Misexpression of wnt7a (wingless/int, a chondro-inhibitor *in vitro*) in mesenchymal chondrogenic cultures directly led to prolonged expression of N-cadherin, stabilisation of N-cadherin mediated cell-cell adhesion and eventual inhibition of chondrogenesis (Tufan and Tuan, 2001; Tufan *et al.*, 2002). N-cadherin mRNA levels increase during osteogenic and myogenic differentiation and decrease during adipogenic differentiation. N-cadherin is expressed in all stages of osteoblast bone formation: mRNA levels for example increase at the stages of nodule formation and mineralisation, and *in vitro* N-cadherin levels increase concomitantly with osteoblast differentiation (Ferrari *et al.*, 2000). A lot of factors regulate the expression of N-cadherin in osteoblasts: BMP-2, FGF-2 and phorbol ester increase the level of N-cadherin in a PKC-dependent way, while TNFα and IL-1 are responsible for a decrease in expression. However, N-cadherin expression is decreased in primary and metastatic osteosarcoma (see also below) (Marie, 2002).

N-cadherin plays also an important role in skeletal muscle differentiation. Cells with the potential to undergo skeletal myogenesis are present in the epiblast layer. All cells express the skeletal muscle-specific transcription factor MyoD but only the epiblast cells that express N-cadherin but not E-cadherin will differentiate into skeletal muscle (George-Weinstein *et al.*, 1997). So, N-cadherin is involved in myoblast migration and homing as well as in muscle differentiation (Brand-Saberi *et al.*, 1996).

Migratory cells play an important role in embryonic development and disease. A migratory cell population known as neural crest can be defined as a pluripotent population of cells that arise from the dorsal part of the neural tube during or just before closure. After an epithelial-mesenchymal transition (EMT), they migrate over long distances along distinct pathways to many different regions of the embryo and contribute to a diverse array of tissues and cell types, such as the peripheral nervous system, melanocytes, some endocrine cells, craniofacial cartilage and bone. The transcription factor Slug is involved in both the formation of the neural crest precursors and in neural crest migration. Slug downregulates cadherins, leading to a loss of cell-cell contacts and allowing the cells to migrate. Indeed, when neural crest cells are still associated with the neural tube, they express N-cadherin but once they start migrating N-cadherin is downregulated. At the end of the dorso-ventral migration N-cadherin is re-expressed in aggregating cells, just before the formation of the dorsal root and sympathic ganglia. After the dorso-lateral migration only the dermal melanocytes express N-cadherin and establish contacts with the fibroblasts in the dermis (Nieto, 2001; Pla *et al.*, 2001).

As is evident from the above, N-cadherin is expressed at different time points and tissues in the embryo. In the adult, N-cadherin is restricted to neural tissue, retina, endothelial cells, fibroblasts, osteoblasts, mesothelium, myocytes, limb cartilage, oocytes, spermatids and Sertoli cells.

TABLE 2

EXPRESSION OF N-CADHERIN IN HUMAN CANCER CELL LINES AND BIOPSIES AND CORRELATION WITH THE EXPRESSION FOUND IN EMBRYO AND ADULT

tumour type	embryo	adult	cell line or biopsy	% positivity	observation /properties	reference
DE NOVO EXPRESSION						
Breast carcinoma	-	-	BT549, MDA-MB-436, HS578T, HS578N		invasive, fibroblastic, metastatic	Hazan *et al.*, 1997
			SUM159PT		motile, invasive	Nieman *et al.*, 1999
			Biopsies		+ in sarcomatoid metaplastic carcinoma	Han *et al.*, 1999
			Biopsies	48	no correlation with survival	Peralta Soler *et al.,* 1999
			Ectopic expression in MCF-7 cells		motile, invasive	Hazan *et al.*, 2000
			Biopsies of ductal carcinoma *in situ*	12.3	no correlation with grade	Paredes *et al.*, 2002
			Biopsies	30	+ in invasive carcinomas	Kovacs *et al.*, 2003
Prostate carcinoma	-	-	PC3N and JCA1		induction of epithelial-mesenchymal interactions	Tran *et al.*, 1999
			TSU-pr1, PPC-1, ALVA-31, PC3, JCA-1		invasive, metastatic	Bussemakers *et al.*, 2000
			Biopsies	60	when Gleason score above 7	Tomita *et al.*, 2000
Bladder carcinoma	-	-	5637, Wmcub2, SW-780, SW-800,			
			SW-1710, J82, T24		fibroblastic	Giroldi *et al.*, 1999
			T24, RT112, TCCSUP		epithelioid/ fibroblastic	Mialhe *et al.*, 2000
			Biopsies	39	+ in invasive tumours	Rieger-Christ *et al.*, 2001
Thyroid carcinoma	-	-	HTh7, C643, SW1736, HTh74		fibroblastic	Husmark *et al.*, 1999
Squamous cell carcinoma	-	-	SCC1, UM-SSC-11A, UM-SCC-11B SCC9		fibroblastic	Islam *et al.*, 1996
						Li *et al.*, 1998
RE-EXPRESSION						
Melanoma	+	-	Biopsies / cell lines	75/ 90		Hsu *et al.*, 1996
			MeWo, A375		stronger adhesion, invasive, metastatic	Matsuyoshi *et al.*, 1997
			Biopsies		+ in metastases	Sanders *et al.*, 1999
			Biopsies	56		Laskin and Miettinen, 2002
Leukemia	+	-	Oh13T, F6T, K3T, Molt-4F, CEM, Jurkat		ATL and T-cell leukemia +	Tsutsui *et al.*, 1996
			Hut102	50	ATL cell lines	Matsuyoshi *et al.*, 1998
			Oh13T, F6T, K3T		aggregation and co-aggregation with mesenchymal cells	Kawamura-Kodoma *et al.*, 1999
Gastric carcinoma	+	-	Biopsies of AFP producing carcinoma	100		Yanagimoto *et al.*, 2001
			Biopsies	21	correlation with twist	Rosivatz *et al.*, 2002
Chordomas	+	-	Biopsies	100		Laskin and Miettinen, 2002
			Biopsies	50		Horiguchi *et al.*, 2004
Rhabdomyosarcoma	+	-	RD, HS729		no correlation	Soler *et al.*, 1993
UPREGULATION						
Leiomyoma	+	+	Cells		grow irregular compared to normal	Kobayashi *et al.*, 1996
			Biopsies		overexpression	Tai *et al.,* 2003
Mesothelioma	+	+	Biopsies		+ in pleural mesothelia	
					– in lung adenocarcinoma	Han *et al.*, 1997
			Biopsies	70		Laskin and Miettinen, 2002
			Biopsies	70 to 100		Ordonèz, 2003
Adrenal tumours	+	+	Biopsies		up in pheochromocytomas	
					down in adrenocortical carcinoma	Khorram-Manesh *et al.*, 2002
DOWNREGULATION						
Osteosarcoma	+	+	Biopsies		– in metastasis	Kashima *et al.*, 1999
			Dunn and LM8		migration and metastasis inhibited	Kashima *et al.,* 2003
Ovarian carcinoma	+	+	Biopsies		+ in benign and borderline tumours	
					not in ovarian cancer	Daraï *et al.*,1997
			Biopsies		– in mucinous cystadenoma	Peralta Soler *et al.*, 1997
			Biopsies		+ in normal and metaplastic ovarian	Wong *et al.*, 1999
			Biopsies		aberrant P-cadherin expression	Patel *et al.*, 2003
Gliobastoma	+	+	Biopsies		no differences	Shinoura *et al.*, 1995
			Biopsies		down at time of recurrence	Asano *et al.*, 2000
			Biopsies		correlation with histological grade	Utsuki *et al.*, 2002
Renal cell carcinoma	+	+/-	Caki-1, Caki-2, ACHN,A498		– in oncocytomas	Tani *et al.*, 1995
					+ in renal cell carcinoma	
OTHERS NOT CLASSIFIED						
Small cell carcinoma in cervix			Biopsies	0	no expression compared with 65 % in other small cell carcinoma	Zarka *et al.*, 2003
Merkel cell carcinoma			Biopsies (neuroendocrine)	63		Han *et al.*, 2000

Abbreviations used: '+', expression of N-cadherin; '-', no expression of N-cadherin; up, upregulation; down, downregulation; AFP, alpha-foetoprotein; ATL, adult T-cell leukemia; T cell leukemia, human thymus derived cell line.

N-cadherin and cancer

The process of EMT not only occurs under physiological conditions during normal embryonic development, it also takes place in pathological situations, such as the acquisition of an invasive phenotype in tumour cell lines of epithelial origin. This goes together with the first steps of the metastatic process. The EMT associated with tumour progression frequently involves downregulation of E-cadherin expression and the acquisition of migratory properties. Snail is a strong and direct repressor of E-cadherin (Cano *et al.*, 2000), and influencing the levels of N-cadherin expression, a pro-migratory factor. Indeed, in a number of human cancer types which have lost E-cadherin, *de novo* expression of N-cadherin is observed (Tomita *et al.*, 2000).

The cadherins have been investigated in different areas of tumour biology. In early neoplasia cadherins play a role in the transformation of cells to an abnormal proliferative phenotype. E and N-cadherin are normally involved in inducing cell cycle arrest. However, N-cadherin also promotes survival in normal granulosa cells (Makrigiannakis *et al.*, 1999) and in melanoma cells (Tran *et al.*, 2002) by distinct mechanisms. In epithelial carcinomas E-cadherin is downregulated in most cases, sometimes accompanied by the upregulation of another cadherin, for example N-cadherin, P-cadherin or cadherin –11. Here, we will focus on the expression of N-cadherin in cancer. We reviewed the literature and present an overview of N-cadherin expression in cancer cells and looked whether this was also the case in their embryonic and adult normal counterparts (Tabled 2). The table is divided into 4 groups: in the first one (including breast, prostate, bladder, thyroid and squamous cell carcinoma) N-cadherin is 'DE NOVO EXPRESSED' (Table 2) in the cancer cell and N-cadherin is never expressed in the corresponding precursor or adult normal cells. In 1996 the aberrant expression of N-cadherin in squamous cell carcinoma was described. The inappropriate expression of N-cadherin in these cells correlated with a scattered fibroblastic phenotype along with decreased expression of E- and P-cadherin. Transfection with antisense N-cadherin resulted in reversion to a normal appearing squamous epithelial cell morphology, and increased expression of E- and P-cadherin. In addition, transfection of a normal squamous epithelial cell line with N-cadherin induced the scattered fibroblastic phenotype (Islam *et al.*, 1996). Aberrant N-cadherin expression was also found in breast carcinoma cells and biopsies. Breast carcinoma cells expressing N-cadherin are more motile and invasive (Hazan *et al.*, 1997 and 2000). In biopsies N-cadherin was mostly found in invasive carcinoma, but no correlation could be found with grade (Paredes *et al.*, 2002) or patient survival (Peralta Soler *et al.*, 1999). *De novo* expression of N-cadherin was found most frequently in prostate carcinoma: in one series, 60% was positive in carcinomas with a Gleason score above 7 (Tomita *et al.*, 2000). *In vitro* studies show that the expression of N-cadherin mediates an epithelial-mesenchymal transformation, possibly improving the physical interaction with the surrounding stromal fibroblasts and facilitating metastasis (Tran *et al.*, 1999).

In the group 'RE-EXPRESSION' (Table 2) we classified tumours that had embryonic precursor cells expressing N-cadherin. One of the best examples are melanoma cells: melanocytes are derived from neural crest cells, which are N-cadherin positive before they start migrating. N-cadherin was found back in metastasising melanomas (Matsuyoshi *et al.*, 1997; Sanders *et al.*, 1999). In gastric carcinoma N-cadherin was found in all α-foetoprotein producing tumours (Yanagimoto *et al.*, 2001) and a correlation was found between the expression of twist and N-cadherin expression (Rosivatz *et al.*, 2002). During early development N-cadherin is found in some basal granulated epithelial cells of the stomach, duodenum and jejunum (Gaidar *et al.*, 1998). Another example is the expression of N-cadherin in T-cell leukemia cell lines. Here, N-cadherin is functionally active because it stimulates the co-aggregation and adhesion with mesenchymal cells, which presumably facilitates invasion in mesenchymal tissues of the skin and the central nervous system (Kawamura-Kodama *et al.*, 1999).

A third group, 'UPREGULATION' (Table 2), shows that cells already expressing N-cadherin in embryonic and adult stages can still increase their levels of expression in neoplastic stages. One example is pleural mesothelioma, where a high and homogeneous expression is characteristic (Han *et al.*, 1997; Ordónez, 2003).

In the last group we collected cancers where N-cadherin levels remain unaltered or are 'DOWNREGULATED' (Table 2). In osteosarcoma, N-cadherin inhibits cell migration and the formation of metastasis (Kashima *et al.*, 1999 and 2003). In gliobastoma no differences were found in N-cadherin expression but at the time of recurrence, decreased N-cadherin expression correlates with dissemination in malignant astrocytic tumours (Asano *et al.*, 2000). In ovarian carcinoma, N-cadherin is expressed in the different stages but one report mentioned that mucinous cystadenomas were N-cadherin negative (Peralta Soler *et al.*, 1997). Recently it was shown that probably P-cadherin is the important aberrantly expressed cadherin in ovarian cancer (Patel *et al.*, 2003).

In summary, multiple *in vitro* and *in vivo* studies showed that aberrant N-cadherin (re-) expression correlates in most cases with a morphological change towards a more fibroblastic phenotype, with cells becoming more motile, invasive and metastatic. There are, however, invasive tumours where N-cadherin is downregulated and where it may play the role of a tumour suppressor molecule.

Nowadays, loss of immunohistochemical E-cadherin expression is sometimes used in surgical pathology to characterize gastric and breast carcinomas. It may be worthwhile to explore also the cases where N-cadherin is aberrantly expressed, and challenge N-cadherin as a candidate prognostic marker. Another ongoing project in our laboratory is the use of circulating soluble N-cadherin, the 90 kD fragment that is released after MMP cleavage, as a potential tumour marker of invasion. Soluble E-cadherin, a 80 kD ectodomain fragment, in the serum or urine of patients with urothelial carcinoma (Griffiths *et al.*, 1996), ovarian carcinoma (Gadducci *et al.*, 1999) and gastric carcinoma (Gofuku *et al.*, 1998) has already been launched as a circulating tumour marker. Yet, we believe that soluble N-cadherin has better chances as a potential circulating tumour marker than soluble E-cadherin, because in general N-cadherin expression is upregulated in invasive tumours.

Conclusion

N-cadherin is associated with a lot of molecules that regulate its function. It is involved in a lot of processes like cell-cell adhesion, differentiation, embryogenesis, migration, invasion and signal transduction. In embryogenesis, during gastrulation, cells undergo an epithelial-mesenchymal transition leading to the expression of N-cadherin and the downregulation of E-cadherin in the mesoderm. This switch is regulated by multiple growth and transcription

factors. A similar situation appears in carcinomas where loss of E-cadherin is correlated with an upregulation of N-cadherin. The aberrant expression (*de novo* or re-expression) of N-cadherin attributes a more fibroblastic phenotype to the cancer cells, and they become more motile and invasive. One of the transcription factors responsible for upregulation is twist. Further research on other possible factors that affect the N-cadherin switch, on the signalling pathways initiated in N-cadherin mediated invasion and on the perspective of N-cadherin as a potential marker of invasion is needed.

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Part III Cadherins as circulating tumour markers

3.1. Molecular Markers for Cancer

The progression from preneoplasia to cancer is accompanied by genetic alterations. These lead to altered expression patterns and modifications in protein structure and function. Changes that occur exclusively or commonly in cancer cells are detectable in biopsies or body fluids and used as molecular markers for cancer. These markers are useful in detecting cancer at early stages, selection of therapy, monitoring disease progression and determining response to therapy (Sidransky *et al.* 2002). Molecular markers can add information to the traditional TNM (Tumour size, Lymph node spread, Metastases) staging system, because patients with a cancer of apparently equivalent type, stage and grade can give different outcomes. Those differences in outcome may relate in part to the time at which a single cancer cell happens to undergo all the steps necessary for successful metastasis or can relate to molecular factors that can be reasonably well understood at a deterministic level. The emerging use of molecular markers may herald an era in which physicians no longer make treatment choices that are based on population based statistics but rather on the specific characteristics of individual patients and their tumour (Dalton and Friend 2006). An example is the presence or not of the estrogen receptor (ER) and neu in breast carcinoma, they determine the prognosis and therapy.

There is a whole range of markers nowadays, and some examples are DNA-based markers, RNA-based markers and protein markers. The DNA-based markers include single-nucleotide polymorphism, chromosomal aberrations, changes in DNA copy number, micro-satellite instability and differential promoter-region methylation. RNA-based markers are overexpressed or underexpressed transcripts, and regulatory RNA's (microRNA). The protein markers include cell surface receptors such as CD20, tumour antigens such as prostate-specific antigen (PSA), phosphorylation states, carbohydrate determination, and peptides released by tumours in serum, urine, sputum, nipple aspirates or other body fluids (Ludwig and Weinstein 2005). However, the lack of availability of biomarkers with high specificity and sensitivity limits the ability to screen for most cancers. Sensitivity refers to the percentage of individuals with disease who are marker positive, whereas specificity refers to the likelihood that a given marker will be elevated only in individuals with the disease. PSA for example is a very sensitive marker but has a low specificity for prostate cancer (Chatterjee and Zetter 2005). Therefore, new markers are still required for all cancers. Recently,

advancements in biomarker development using microarrays and proteomics have facilitated the discovery of new markers, and examples are survivin (Shariat *et al.* 2004) and calreticulin (Kageyama *et al.* 2004) for bladder cancer. However, the major difficulty in utilizing circulating tumour makers is that very small tumours, which need to be detected and removed prior to metastasis to other organs, may not produce sufficient amounts of the marker for detection in serum or urine.

Cancer involves the transformation and proliferation of altered cell types that produce high levels of specific proteins and enzymes such as proteases. These proteinases not only modify the array of existing serum proteins (Anderson and Anderson 2002) but also their metabolic peptide products. The serum contains thousands of proteolytically derived peptides (Richter *et al.* 1999). Villanueva *et al.* (2006) reported that by correlating the proteolytic patterns with disease groups and controls, they could show that exoprotease activities superimposed on the *ex vivo* degradation pathways contribute to generation of not only cancer-specific but also cancer type-specific serum peptides. Their study provides a direct link between peptide marker profiles of disease and differential protease activity. In several tumours elevated levels of protease where observed, Riddick *et al.* (2005) detected significant more MMP15 and MMP26 in prostate cancer and the levels correlated with the Gleason score, whereas TIMP3 and TIMP4 inversely correlated with the Gleason score. In addition, in intestinal carcinoma significant changes in the expression of matrix metalloproteinases were identified (Martinez *et al.* 2005).

3.2. Soluble cadherins

Damsky *et al.* (1983) reported for the first time that an 80 kD fragment is found in the conditioned medium of MCF-7 cells and this fragment had adhesion disrupting activities. This fragment was a cleaved form of human E-cadherin (Wheelock *et al.* 1987). Many proteases like matrilysin and stromelysin (Noë *et al.*, 2001, Davies *et al.* 2001), plasmin (Ryniers *et al.* 2002, Hayashido *et al.* 2005) and ADAM10 (Maretzky *et al.* 2005) can cleave E-cadherin *in vitro* and shed the E-cadherin ectodomain (sE-CAD) (see figure 3.2.). Other enzymes like caspases (Steinhusen *et al.* 2001) and calpain (Rashid *et al.* 2001, Rios-Dora *et al.* 2002) cleave E-cadherin in its cytoplasmic part. E-cadherin, which is present on all epithelial cells and in some cancer cells in human can also be cleaved by present proteases. Many articles describe the detection of sE-CAD in different biological fluids (blood, urine

and cyst fluid) of healthy persons, cancer patients and patients suffering form other diseases.

Table 3.2.: Overview of the literature detecting sE-CAD in human biological fluids

Biological fluid	**Disease**	**Concentration (µg/ml) H: healthy C: cancer B: benign**	**Properties**	**Reference**
Serum	Cancer patients	H 1,99 C 3,80 Diabetes 2,33	53,7% of the 54 cancer patients elevated sE-CAD	Katayama *et al.* 1994
Serum	Gastric carcinoma	H 3,53 C 3,19	No significant difference	Velikova *et al.* 1997
Serum	Gastric carcinoma	H 2,515 C 4,735	67% abnormal high sE-CAD, other tumour markers like CEA 4,4% and CA19.9 13.3%. but no correlation with the clinopathology	Gofuku *et al.* 1998
Serum	Gastric carcinoma	H 5,128 C 7,079	Prognostic marker, high concentrations predict T4 invasion	Chan *et al.* 2001
Serum	Gastric carcinoma	Pretherapeutic 9,159	sE-CAD is independent factor predicting long term survival	Chan *et al.* 2003
Serum	Gastric carcinoma	± 14	More in the intestinal type compared to diffuse type	Juhasz *et al.* 2003
Serum	Gastric carcinoma		Cut-off of 10 µg/ml, the sensitivity to predict disease recurrence was at 3 months 47% and at 6 months 59%.	Chan *et al.* 2005
Serum	Bladder carcinoma	H 1,013 C 3,955	No correlation between high sE-CAD and abnormal E-cad expression in tumour	Griffiths *et al.* 1996
Serum	Bladder carcinoma	G2/3 ± 14,5 G1 9,4	Higher levels of sE-CAD correlate with tumour grade but not with the clinopathology	Durkan *et al.* 1999
Plasma	Radical cystectomy Bladder cancer	H: 2,718 C: 4,301	High preoperative sE-CAD identify patients with metastasis to regional an distant lymph nodes	Matsumoto *et al.* 2003
Serum	Prostate carcinoma		Associated with metastatic progression Strongly elevated in metastasis, correlation with elevated MMP secretion	Kuefer *et al.* 2003
Serum	Prostate carcinoma		Patients with sE-CAD higher than 7,9 µg/ml at time of diagnosis have an increased risk for recurrence of the cancer	Kuefer *et al.* 2005
Serum	Ovarian carcinoma	C 0,66 B 0,55	FIGO stage 3 and 4 significant difference	Gadducci et a1999
Serum	Colorectal cancer		Correlation with T, but not with N and M, correlation with CEA if liver metastases present	Wilmanss *et al.* 2004
Serum	Cutaneous disease		Psoriasis patients correlation with PASI score	Matsuyoshi, *et al.* 1995
Serum	Melanoma	0,879	No significant difference but a few patients with Paget and melanoma with multiple metastasis in various organs had abnormal high levels	Shirahama *et al.* 1996
Serum	Melanoma		Correlating with increased S100 tumour marker	Billion *et al.* 2006
Serum	Lung cancer		Use as prospective tumour marker	Cioffi *et al.* 1999
Serum	Non small cell lung cancer		Significant elevated levels compared to control, and an association with the development of distant metastasis	Charalabopoulus *et al.* 2006
Serum	Hodgkin			Syrigos *et al.* 2004
Serum	Multiple myeloma		sE-CAD correlated with LDH levels and poor prognosis, independent prognostic factor of survival	Syrigos *et al.* 2004
Serum	Cholecystectomy		Reduced sE-CAD level	Karayiannakis *et al.* 2002
Serum	Endometriosis		sE-CAD do not vary during menstrual cycle, but higher levels were found in people suffering from endometriosis	Fu *et al.* 2002
Serum	Sjögren syndrome	0,06195	SS: autoimmune exocrinopathy	Jonsson *et al.* 2005
Plasma	Systemic inflamma respons- multiorgan dysfunction	H 3,21 Sepsis: 6,00	Concentration of sE-CAD tended to increase with the severity of the organ failure	Pittard *et al.* 1996
Urine	Bladder carcinoma		Cannot pass through glomerular filter => derived from urinary tract epithelium	Banks *et al.* 1995
Urine	Bladder carcinoma	H 0,582 C 1,2725	Two bands 65 and 80 kD	Protheroe *et al.* 1999
Urine	Bladder carcinoma	H: 0,904 C 1,606	levels associated with positive cytology assay results and muscle bladder invasion	Shariat *et al.* 2005
cyst fluid	Ovarian carcinoma		More in cystadenocarcinoma and borderline tumours	Sundfeldt *et al.* 2001

An overview of the literature is given in table 3.2. If we scroll through the data, we can conclude that:

1) In serum of healthy persons ± 2,3 µg/ml of sE-CAD is present, while in serum of cancer patients ± 6,8 µg/ml of sE-CAD is present.

2) No correlation is found with TNM staging. Only in gastric cancer sE-CAD is used as a marker for disease recurrence (Chan *et al.* 2005)

3) Not only in cancer patients, elevated levels were observed also in patients suffering from any type of inflammatory process elevated levels of sE-CAD were found (Pittard *et al.* 1996).

The P-cadherin ectodomain is also detected in several biological fluids: serum (Knudsen *et al.*2000), milk of lactating women (Soler *et al.* 2002) and semen (De Paul *et al.* 2005). VE-cadherin can also be cleaved by MMP and caspases, to release a 90 kD fragment. sVE-CAD was found in blood of atherosclerosis patients (Soeki *et al.* 2004), but elevated levels were also found in sera of patients with colorectal cancer (Sulkowska *et al.* 2006).

N-cadherin is cleaved by several enzymes: plasmin (see 4.2), ADAM10 (Reiss *et al.* 2005), MT1-MMP (Covington *et al.* 2006), MT5-MMP (Monea *et al.* 2006) which shed a 90 kD fragment in the medium and by Presenilin 1 (PS1)/γ-secretase which can cleave N-cadherin in the cytoplasmic part (see figure 3.2.). Recently ADAM10 and PS1 cleavage sites were characterised: ADAM10 cleaves between R^{714} and I^{715} in the extracellular domain and PS1 between K^{747} and R^{748} located at the membrane-cytosol interface (Uemura *et al.* 2006). Up to now, sN-CAD was only detected in the embryonic vitreous humor. We could detect sN-CAD in serum of healthy persons and significant higher levels in serum of cancer patients.

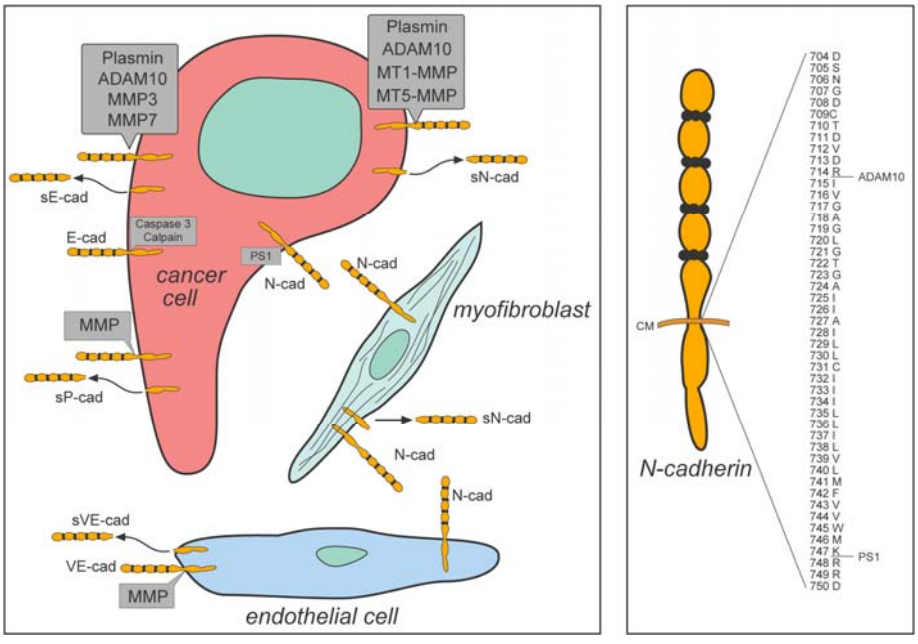

Figure 3.2. Sources of soluble-CAD in the tumour micro ecosystem

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Soluble N-cadherin in human biological fluids

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Classical cadherins such as E-, P- and N-cadherin are transmembrane proteins that mediate cell-cell adhesion, and are important in embryogenesis, maintenance of tissue integrity and cancer. Proteolytic shedding of the extracellular domain results in the generation of soluble E-, P- or N-cadherin ectodomains. Circulating soluble E- and P-cadherin have been described in the serum, and elevated levels were detected in cancer patients as compared to healthy persons. Here we report the presence of soluble N-cadherin, a 90 kD protein fragment, in the serum of both healthy persons and cancer patients, using a direct ELISA and immunoprecipitation. A correlation was found between prostate specific antigen and soluble N-cadherin, and significantly elevated levels were detected in prostate cancer follow-up patients. The N-cadherin protein is neo-expressed by carcinomas of the prostate, and is responsible for epithelial to fibroblastic transition. This is reflected in the higher concentrations of soluble N-cadherin in prostate cancer patients than in healthy persons.

Keywords: soluble N-cadherin, serum and cancer

Cell-cell adhesion molecules play an important role during embryogenesis, tumor invasion and metastasis. The cadherins, Ca^{2+}-dependent cell-cell adhesion molecules, are essential for intercellular connections. E (epithelial)-cadherin is involved in normal epithelial cell-cell interactions but is often downregulated in epithelioid cancer cells. These cancer cells can switch cadherin expression from E- to N- (neural)[1], E- to P- (placental)[2] or E- to OB- (osteoblast)-cadherin.[3] This coincides with the transition from an epithelioid to a fibroblastic phenotype, and is often correlated with invasiveness.[4]

Proteolysis can contribute to the impairment of the cadherin function. Several enzymes have been shown to be responsible for the shedding of the extracellular domain. Cleavage of E-cadherin and shedding of an 80 kD fragment can be mediated by matrilysin, stromelysin-1[5,6]; plasmin[7] and a disintegrin and metalloproteinase 10 (ADAM10).[8] For N-cadherin, a 90 kD fragment can be released by the enzymes matrix metalloproteinase (MMP)[9], plasmin[10], ADAM10,[11] Membrane Type 1 –MMP (MT1-MMP)[12], MT5-MMP.[13]

Soluble E-cadherin (sE-CAD) has been described in the circulation of cancer patients.[14] In that study, the mean sE-CAD level, detected by Enzyme Linked ImmunoSorbent Assay (ELISA), in serum was significantly higher in the studied cancer patients (3.8 ± 2.36 µg/ml) compared with the healthy controls (1.99 ± 0.5 µg/ml). In addition, sE-CAD was detected in serum of patients with diabetes or hepatitis, but these values were not significantly different from the healthy controls. As a result, sE-CAD is now propagated as a marker for early prediction of tumor recurrence of gastric cancer.[15] sE-CAD is also found in urine[16] and the mean levels in the urine of patients with bladder cancer, are significantly higher than in the urine of healthy persons (1.6 versus 0.9 µg/ml).[17] High levels of sE-CAD appear to be associated with positive cytology results and with bladder muscle invasion of transitional cell carcinoma.[18]

Soluble P-cadherin (sP-CAD) is found in serum[19] of healthy persons as well as in patients with breast cancer, but no significant difference was found between these two populations. Remarkably, the sP-CAD levels are 20-fold lower than the sE-CAD levels in serum. In addition, sP-CAD is also detected in milk[20] of lactating women (in the range 100-400 µg/ml, while sE-CAD was not found in milk) and in semen of fertile and non-fertile men.[21]

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Three major observations made us speculate that soluble N-cadherin (sN-CAD) might be present in extracellular fluids of cancer patients: its upregulation in epithelioid cancers, for example in breast, prostate and bladder carcinoma[1,3,22,23], its reactive upregulation in stromal cells of malignant tumors[24] and the presence of multiple extracellular proteases, like MMP[26] that are reported to cleave the extracellular part of cadherins. Apart from its presence in the embryonic vitreous humor[9], no reports of sN-CAD in biological fluids are available. Here we report the presence of sN-CAD in biological fluids. Moreover, we developed an ELISA method to quantify sN-CAD levels. We demonstrate the presence of sN-CAD in serum and found significantly higher levels in cancer patients compared with individuals without evidence of disease. sN-CAD was also present in seminal fluid, and urine but was undetectable in synovial fluid or spinal fluid.

Material and methods

Cell lines and preparation of medium containing soluble N-cadherin

S180-N-cadherin (ARM), mouse sarcoma cells (a gift from R.M. Mège, INSERM, Paris, France)[27] were grown in D-MEM (Invitrogen, Merelbeke, Belgium) supplemented with 10% foetal bovine serum and penicillin, streptomycin and Fungizone (Invitrogen). The S180-NCAD cells, used as a source of sN-CAD, are S180 cells transfected with chicken cDNA encoding for N-cadherin. The cells were incubated in a 100% water-saturated atmosphere of 10% CO_2. All cells were routinely tested for mycoplasma contamination by staining with 4',6'-diamidino-2-phenylindole (DAPI) and were found to be negative.
Subconfluent monolayers were washed 3 times with phosphate buffered saline (PBS) and were incubated with serum free medium for 24 hours, washed another 3 times with PBS and incubated for 48 hours with serum free medium. The medium containing sN-CAD was harvested and centrifuged for 5 minutes at 250 g and for 15 minutes at 2,000 g. The supernatant was filtered through a 0.22 µm filter and was concentrated 10 times (Amicon Ultra 50 kD, Millipore Corp., Bedford, MA) before use in ELISA or Western blot.

Reagents and antibodies

Recombinant human N-cadherin/Fc chimera was purchased from R&D Systems (Abingdon, UK) and used to set up a standard curve with the ELISA method. Mouse GC-4 antibody (Sigma, St. Louis, Missouri, USA) against the extracellular part of N-cadherin was used for ELISA, immunoprecipitation and immunostaining of the Western blot. The secondary antibodies were goat anti-mouse linked to alkaline phosphatase (Sigma) for ELISA and anti-mouse linked to horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden) for Western blot.

Collection of human biological fluids

Serum samples were collected from 193 healthy subjects (with no evidence of disease, N.E.D., and having C-reactive protein values below 0.6 mg/dl) and 179 patients during follow-up of cancer. The selection of the cancer patients was based on the presence of high serum levels of an established tumor marker: prostate specific antigen (PSA) was above 50 ng/ml (prostate carcinoma, n = 54), episialin (CA15-3) above 50 U/ml (breast carcinoma, n = 71), carcino embryonic antigen (CEA) above 100 ng/ml (various carcinoma, n = 15), CA 125 above 50 U/ml (ovarium carcinoma, n = 12) or CA19-9 above 1000 U/ml (gastro-intestinal carcinoma, n = 12). Also 8 sera samples from patients with Chronic Lymphocytic Leukemia (CLL) were included. Venous blood samples were collected and centrifuged (after clotting) at 1500 g for 10 minutes. All sera were stored at -20 °C until sN-CAD assessment. Sera were residual samples with an approval from the ethical committee of the Ghent University Hospital (nr. 2001/333). Other human biological fluids collected were urine (n = 33), seminal fluid (n = 15), synovial fluid (n = 2) and spinal fluid (n = 18). After reception of these samples, they were centrifuged to remove cells and stored at -20 °C.

Immunoenzymometric assay for soluble N-cadherin

sN-CAD levels were measured with a home-made ELISA. A dilution series of recombinant N-cadherin from 1 to 1000 ng/ml was prepared. All serum samples were diluted 5 times in PBS containing 0.1% Bovine Serum Albumin (BSA). A 96-well immunoplate (Nunc) was coated with 75 µl of the diluted sample overnight at 4°C. The wells were washed with PBS/0.05% Tween-20 and quenched at 37°C with PBS/1% BSA for 1 hour. Next, the plates were washed again (4 times) and incubated with the primary antibody (GC-4, 1/200 in PBS/0.1% BSA) at 37°C for 2 hours. The plates were washed (4 times) and subsequently incubated with a mouse secondary antibody linked to alkaline phosphatase (Sigma, 1/3000). The substrate p-nitrophenylphosphate (Sigma, St. Louis, Missouri, USA) was added to the plates and after 30 minutes the optical density of each well was determined with a microplate reader (Molecular Devices, Wokingham, UK) at 405 nm. In control experiments we omitted the primary antibody. Measurements were done at least in duplicate for each sample, and the mean value was calculated.

Immunoprecipitation and Western blotting

Serum samples were depleted from albumin and immunoglobulins using the Aurum™ Serum Protein Mini kit (Bio-RAD, Hercules, USA). The eluted fractions were used to start the immunoprecipitations. For the immunoprecipitation, equal amounts of serum samples were incubated with Dynabeads Protein G (Dynal Biotech, Oslon, Norway) for 30 minutes. After discarding the beads, the supernatant was incubated with primary antibody for 3 hours at 4°C, followed by incubation with Dynabeads Protein G for 1 hour. Next, sample buffer (Laemmli) with 5% 2-mercaptoethanol and 0.012% bromophenol blue was Serum samples were depleted from albumin and immunoglobulins using the Aurum™ Serum Protein Mini kit (Bio-RAD, Hercules, USA). The eluted fractions were used to start the immunoprecipitations. For the immunoprecipitation, equal amounts of serum samples were incubated with Dynabeads Protein G (Dynal Biotech, Oslon, Norway) for 30 minutes. After discarding the beads, the supernatant was incubated with primary antibody for 3 hours at 4°C, followed by incubation with Dynabeads Protein added and supernatant was separated on 8% SDS-PAGE and blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech). Quenching and immunostaining were done in 5% non-fat dry milk in PBS containing 0.5% Tween-20. The membranes were quenched for 30 minutes, incubated with primary antibody (1/1000) for 1 hour, washed four times for 10 minutes, incubated with horseradish peroxidase-conjugated secondary antibody (1/3000) for 45 minutes, and washed 6 times for 5 minutes. Detection was carried out using enhanced chemiluminescence reagent (ECL) (Amersham Pharmacia Biotech) as a substrate.

Statistical Analysis

Data were collected and analysed with SPSS (SPSS Inc, Chicago, USA). Comparisons were performed with the non-parametric Mann-Whitney *U*-test because the populations were not normally distributed. Differences were considered significant at p values < 0.001. Spearman correlation coefficients were used to examine the correlation between sN-CAD and the tumor markers like PSA, CA15-3 and CEA.

Results

ELISA for the detection of soluble N-cadherin in serum samples and other biological fluids

A recombinant N-cadherin chimera (RECN), which consists of amino acids 1 to 724 of human N-cadherin fused to the carboxyterminal end of the Fc region of human IgG1, migrated at 135 kD in SDS-PAGE, and was reactive with the GC-4 antibody in ELISA and immunostaining of Western blot. This recombinant protein was used as an assay standard for quantification of antigen levels in several biological fluids. A dilution series of RECN was made from 1 to 1000 ng/ml and 75 µl of each dilution was incubated at 4°C overnight in an immunoplate in order to coat the wells. On the next day the immunoplate was quenched with 1% BSA, followed by incubation at 37°C with the primary antibody GC-4 for 2 hours. Next, the immunoplate was incubated with the goat anti-mouse antibody linked to alkaline phosphatase, and finally the chromogen p-nitrophenylphosphate was added to the plate. After 30 minutes the optical density was read at 405 nm (Fig. 1).

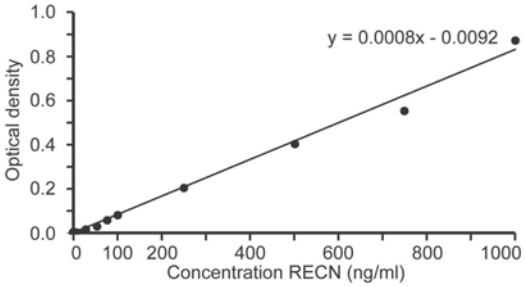


Figure 1: Standard curve used to measure the sN-CAD concentrations

A dilutions series from 1 to 1000 ng/ml of recombinant human N-cadherin/Fc chimera was prepared with 0.1% BSA in PBS, and coated on a immunoplate overnight at 4°C. After washing with PBS/ 0.05% Tween-20 followed by an incubation with 1% BSA during 1 hour, plates were washed and incubated with GC-4 antibody for 2 hours at 37°C. Plates were washed again and incubated with an anti-mouse antibody linked to alkaline phophatase. After 1 hour the substrate p-nitrophenylphospate was added for 30 minutes, and the optical density of each well was read at 405 nm.

Dilution curves from two serum samples were linear, suggesting that the same immuno-reactive substance is measured at the different dilutions (Fig. 2). We found that a dilution factor of 5 of all samples gave the best read out compared with the standard curve. The precision of the ELISA was tested assaying 8 times serum samples from 5 healthy donors, with a mean sN-CAD value between 50 and 300 ng/ml. Intra-assay and inter-assay coefficients of variation were 6% and 9% respectively.

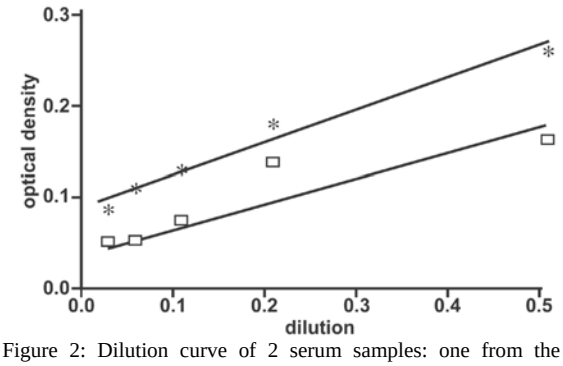
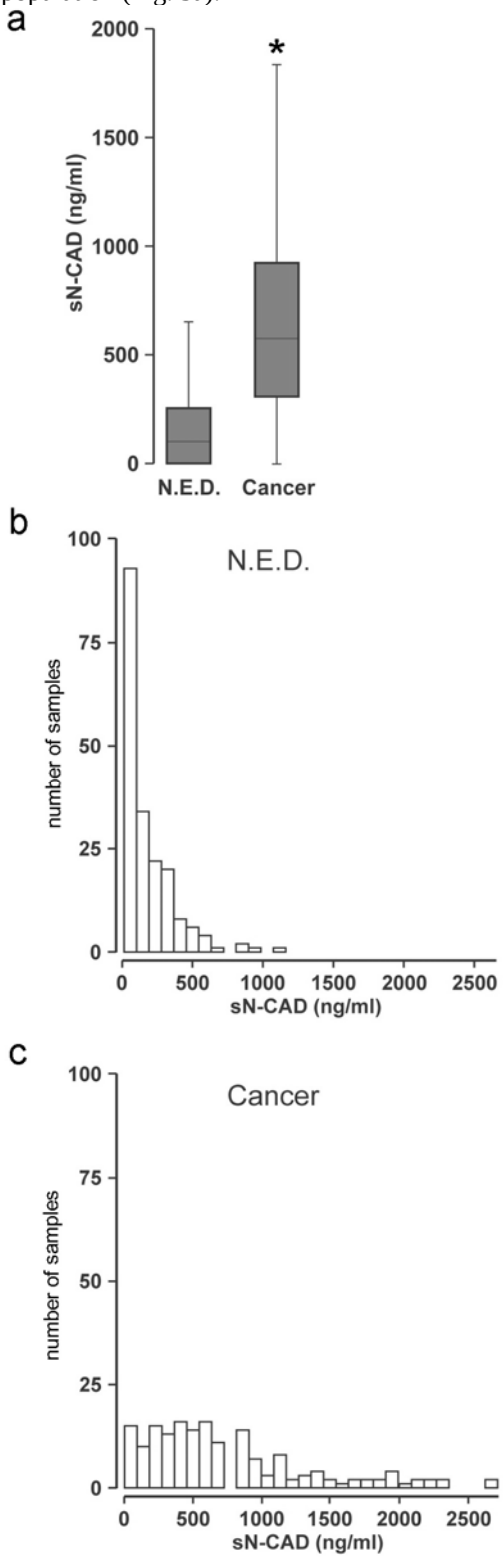


Figure 2: Dilution curve of 2 serum samples: one from the N.E.D. group (squares) and one from the cancer follow-up patients group (stars).

Serum samples from 193 people with no evidence of disease (N.E.D.) were collected and tested in the ELISA. sN-CAD concentrations had a median value of 99 ng/ml (range 0-1130 ng/ml). 179 serum samples from cancer follow-up patients were selected on the basis of elevated tumor maker concentration of CA15-3, CEA, PSA, CA19-9 and CA 125 and CLL patients. The results are shown in a box plot, and compared with the N.E.D population (Fig. 3a).



Significantly elevated levels were observed in the cancer patient group with a median value of 584 ng/ml (range 0-2713 ng/ml) (Mann Witney *U*-test, $p < 0.001$). Neither the frequency distribution curve of the N.E.D population (Fig. 3b) nor that of the cancer patients group (Fig. 3c) showed a normal distribution as assessed by the Kolmogorov – Smirnov test.

Figure 3: (a) Box plots representing the levels of sN-CAD in sera of 193 people with no evidence of disease (N.E.D.) and 179 follow-up cancer patients. *Significant difference was determined using the Man-Whitney *U*-test ($p < 0.001$). Serum N-cadherin levels were determined by a direct ELISA (b-c) Histograms representing the distribution of the sN-CAD concentrations in the N.E.D. and cancer population

Using the nonparametric Spearman correlation coefficient, the sN-CAD serum concentrations showed no correlation with the tumor markers CA15-3 ($r = -0.086$) and CEA ($r = -0.273$). A significant, but weak correlation was found between PSA and sN-CAD levels ($r = 0.254$, $p < 0.05$) (Fig. 4a). The PSA positive population ($n = 54$) had significant higher sN-CAD values (range 0-1445 ng/ml, median 675 ng/ml) compared with the N.E.D. population (range 0-1130 ng/ml, median 99 ng/ml) ($p < 0.001$) (Fig. 4b).

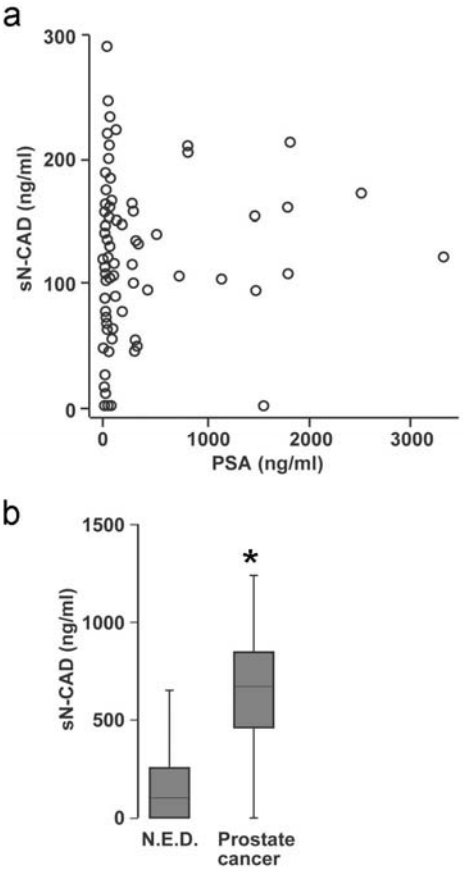


Figure 4: (a) Significant correlation ($p < 0.05$) between PSA and sN-CAD in sera from prostate cancer patients (PSA > 3,6 ng/ml) (b) Box plots representing the sN-CAD values found in the N.E.D. population compared to those in sera from prostate cancer patients (* Man Whitney *U*-test, $p<0.001$).

Other biological fluids where also tested for the presence of sN-CAD: we found sN-CAD in seminal fluid samples (range 1063-2553 ng/ml) and in urine (range 0-655 ng/ml), but none in synovial fluid or spinal fluid (data not shown).

Immunoblot analysis of serum soluble N-cadherin

In immunoblot analysis after 8% SDS-PAGE, we separated the immuno-isolated proteins, reactive with the GC-4 antibody, from serum samples of 3 subjects with no evidence of disease and 4 cancer patients (2 breast cancer patients and 2 prostate cancer patients). Serum samples were first depleted for albumin and immuno-globulins. As positive control we used medium containing sN-CAD, which was harvested from S180 cells transfected with cDNA of N-cadherin (S180-NCAD). Various amounts of the 90 kD N-cadherin fragment (sN-CAD) were detected in the serum samples by the GC-4 antibody (Fig. 5). Moreover, these could be correlated with the concentrations measured by ELISA. The molecular weight of the soluble N-cadherin was in all samples identical. The upper band in lane 6 is an aspecific band, recognised by the secondary antibody (anti-mouse linked to HRP) alone.

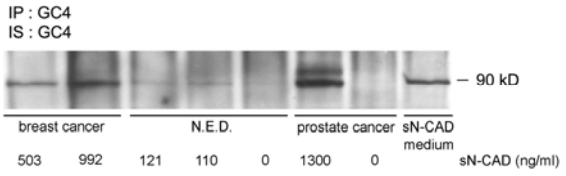


Figure 5: Immunoblot of a 90 kD fragment (sN-CAD) present in serum. Serum samples from 3 healthy individuals and 4 cancer patients are shown after depletion for albumin and immuno-globulins. Serum samples were immunoprecipitated with the GC-4 antibody and loaded on a SDS-PAGE, transferred onto nitrocellulose membrane and immunostaining with the same antibody. As a positive control we used medium containing sN-CAD harvested from S180-NCAD cells. sN-CAD concentrations of each serum sample tested, was also measured by ELISA (result mentioned under each lane). The upper band in lane 6 is immuno-reactive with the secondary antibody (anti-mouse linked to HRP).

Stability of the soluble N-cadherin in serum

To investigate the role of calcium in the stabilization of sN-CAD, S180-NCAD cells were treated with EDTA (0.125 to 3 mM) for 24 hours. The release of soluble N-cadherin remained the same as with untreated cells up to a concentration of 0.5 mM of EDTA in the culture medium (Fig. 6a). Treatment with higher concentrations of EDTA made sN-CAD less detectable in the culture medium. Concentrations of EDTA higher than 0.75 mM are known to chelate all divalent cations (for example Ca^{2+}) in the medium. Adding EDTA to serum samples at a final concentration of 1 mM dramatically reduced the amount of sN-CAD to 50%. So, this shows that we detect the cadherin protein. This effect was also observed when blood from the same individual was collected in tubes containing (EDTA K2 or EDTA K3, 8%) or citrate (3.2% sodium citrate). The plasma concentrations of sN-CAD were reduced to 30% and 45% respectively (Fig. 6b) compared with serum concentrations. For this reason we used serum from clotted blood to measure sN-CAD.

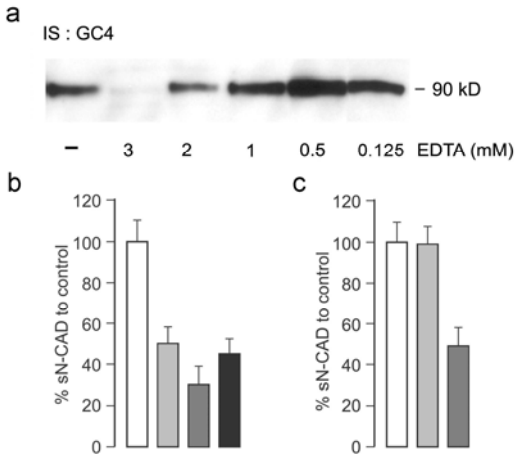


Figure 6: (a) Detection of sN-CAD in the culture medium of S180-NCAD cells in presence of several concentrations of EDTA. Subconfluent monolayers of S180-NCAD cells were serum starved and followed by a treatment with serum free DMEM with or without EDTA at several final concentrations (0.125 to 3 mM). Culture medium was harvested after 24 hours and centrifuged to remove cells. The medium containing sN-CAD was concentrated 10 times and loaded on SDS-PAGE. Immunoblot was stained with GC-4. (b) Detection of sN-CAD in serum (white), serum plus 1mmol/l EDTA added (grey), plasma from blood collected in a tube containing 8% EDTA (dark grey) or plasma from blood collected in a tube containing 3.2% sodium citrate (black) using the direct ELISA. Results were compared with the amount of sN-CAD in serum from the same vena puncture. (c) Stability of sN-CAD at different conditions tested with the ELISA: serum (white), serum 5 times frozen and thawed (grey) and serum left at room temperature for 2 days (dark grey).

Serum with a known concentration of sN-CAD was mixed (1:1) with serum containing high concentrations of hemoglobin (± 15 g/dl), bilirubin (± 10 mg/dl) or triglycerides (± 1500 mg/l), affecting the aspect of the serum. These factors did not interfere with the detection of sN-CAD (data not shown). Repeated freezing and thawing (5 times) of the biological fluids did not affect the stability of sN-CAD in serum samples. Storage of the samples at 4 °C did not change the amount of sN-CAD. However, storage of the samples for 2 days at room temperature diminished the concentration of sN-CAD (49% to control) (Fig. 6c).

Discussion

Tumor invasion is characterised in part by the ability of cancer cells to downregulate cell-cell adhesion and to invade into the surrounding tissues. In this respect the progression of a carcinoma is

often characterised by a switch from E- to N-cadherin. This switch in cadherin type expression has been observed in different malignant tissues, like breast, prostate, bladder, skin and colon carcinoma.[1,3,22,23] This switch also coincides with the transition of the cells from an epithelial to a fibroblastic phenotype, and invasiveness of the cancer cells. By extension in the tumor micro-environment, N-cadherin expression is not exclusively found on the cancer cells, but is also reactively overexpressed on the surface of surrounding fibroblasts and endothelial cells.[24] During cancer progression a multitude of extracellular proteinases are present and are now known as the cancer degradome.[25,28] These proteinases are responsible for the shedding of several transmembrane protein ectodomains, including cadherins and tyrosine kinase receptors.[29]
In the eighties Damsky *et al*.[30] reported the cleavage of E-cadherin in cell culture experiments, with the release of an 80 kD extracellular fragment (sE-CAD). Later, this fragment was also found in different biological fluids, like serum[14] and urine[17], and elevated concentrations were described in the serum of gastric carcinoma patients.[14,31,32] In recent studies the serum sE-CAD concentration was considered as an independent predictive factor for long term survival[33] and can predict tumor recurrency after cancer resection.[15]
sP-CAD, also an 80 kD fragment, was measurable in different biological fluids like semen, milk and serum. The concentrations observed in serum were approximately 20-fold lower than those of sE-CAD. However, no correlation was found with the sE-CAD levels or the occurrence of breast cancer.
Paradies and Grunwald[9] reported the finding of a 90 kD fragment of N-cadherin during retinal development of the chick, but until now no data were reported on sN-CAD in other biological fluids. Here we describe the presence of sN-CAD in serum by using a direct ELISA or via immunoprecipitation and Western blot. sN-CAD is a relatively stable molecule, and repeated freezing and thawing of the samples does not affect the levels. However, EDTA at concentrations higher then 0.5 mM reduces the serum concentration of sN-CAD dramatically, which confirms the cadherin nature of the measured protein. Measuring sN-CAD in a population with no evidence of disease resulted in a median value of 99 ng/ml which is approximately 10 times lower than the levels of sE-CAD and in the same range as the sP-CAD value reported for a similar population.[19] This could be explained by the number of cells expressing either E-, N- or P-cadherin, E-cadherin being the more abundant protein. It is no surprise that low levels of soluble cadherins are found in the control population, as the normal turn over of the cells will be responsible for the shedding of these soluble cadherins. However when people are suffering from a disease, like diabetes or liver cirrhosis, higher amounts of sN-CAD are measured (data not shown), probably because more protease is shed. The same has been observed for sE-CAD.[34]
In a population of cancer follow-up patients selected for elevated serum concentration of different tumor markers, we could find an almost 6-fold higher median concentration. In our population various types of cancers at different stages were included. In the future a more stratified study along tumor types, grades and stages is mandatory to elucidate the clinical performance of sN-CAD as a potential tumor marker. Therefore, we have started collecting documented serum samples from cancer patients. Prostate cancer patients are of particular interest, because we found a correlation between PSA and sN-CAD, and a recent report demonstrated that N-cadherin expression occurs in high grade prostate cancer and correlates significantly with increasing Gleason patterns.[1]
It is currently not clear whether the circulating sN-CAD possesses a biological function. We found recently that sN-CAD stimulates angiogenesis in the chorioallantoic membrane assay and in the rabbit cornea assay.[10] Moreover, it exerts a motogenic action on cancer cells *in vitro*, and we found evidence that the fibroblast growth factor receptor is involved in both signalling processes.
Our results indicate that sN-CAD is present in human serum, and that significantly higher amounts are present in cancer patients than in persons with no evidence of disease. Further clinical studies are needed to evaluate whether this finding can be the basis for the development of potentially useful tumor marker for cancer follow-up.

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Part IV Cadherins and Angiogenesis

4.1. Introduction to angiogenesis

Blood vessels form a complex network of tubes that carry oxygen and nutrients throughout our organism. Vasculogenesis is the assembly of vessels through in situ differentiation from endothelial precursors in an embryo, while angiogenesis is the expansion of this network by sprouting from pre-existing vessels (Carmeliet and Jain 2000). Angiogenesis is regulated by a very sensitive interplay of growth factors and inhibitors, and their imbalance can lead to disease. In cancer, diabetic eye disease and rheumatoid arthritis, excessive angiogenesis feeds diseased tissue and destroys normal tissue. Conversely, insufficient angiogenesis underlies conditions such as coronary heart disease, stroke and delayed wound healing, where inadequate blood vessel growth leads to poor circulation and tissue death.

We could demonstrate a novel proangiogenic molecule, named soluble N-cadherin (article: "soluble N-cadherin promotes angiogenesis"), by using *in vivo* assays as a model for angiogenesis. The chorioallantoic membrane (CAM)-assay was used as a first screening test (Jain *et al.* 1997). The CAM is a critical component of the oxygen exchange system of the developing chick embryo. The membrane is normally highly vascular and transparent, allowing any alteration in blood vessel growth to be easily seen. The second test we used was the rabbit corneal micropocket assay. The accessibility and the transparency of the cornea make it an ideal site for the study of experimental neovascularization (Gimbrone *et al.* 1974).

The role of other adhesion molecules during angiogenesis are well described, like PECAM1 or CD31 (Wright *et al.* 2002) and Vascular Endothelial (VE)- cadherin (Carmeliet 1999, Cavallaro *et al.* 2006). Endothelial cells express both cadherins, N- and VE-cadherin, but have a differential localisation: VE-cadherin is localised at the cell contacts whereas N-cadherin is dispersed over the whole cell membrane (Dejana 2004). VE-cadherin promotes the homotypic interaction between endothelial cells while N-cadherin may be responsible for the anchorage of the endothelium with the surrounding cell types expressing N-cadherin such as vascular smooth muscle or pericytes (Navarro *et al.*. 1998; Gerhardt *et al.*. 2000). These mural cells are important for the vascular stabilisation. The stabilisation is regulated by the activation of the sphingosine 1-phosphate receptor, which regulates the trafficking of N-cadherin and strengthening of N-cadherin dependent cell-cell adhesion with mural cells (Paik

et al., 2004). The platelet-derived growth factor (PDGF) – PDGF-receptor pathway plays a critical role in the recruitment of pericytes to newly formed vessels. The endothelial cells secrete PDGF, which signals through PDGF-receptor expressed by the mural cells, resulting in proliferation and migration of the pericytes during vessel maturation (Armulik *et al.* 2005). The matrix metalloproteinases are also involved in the recruitment of pericytes by direct activation of pericyte invasion, stimulation of proliferation and releasing growth factors from the extracellular environment (Chantrain *et al.* 2006).

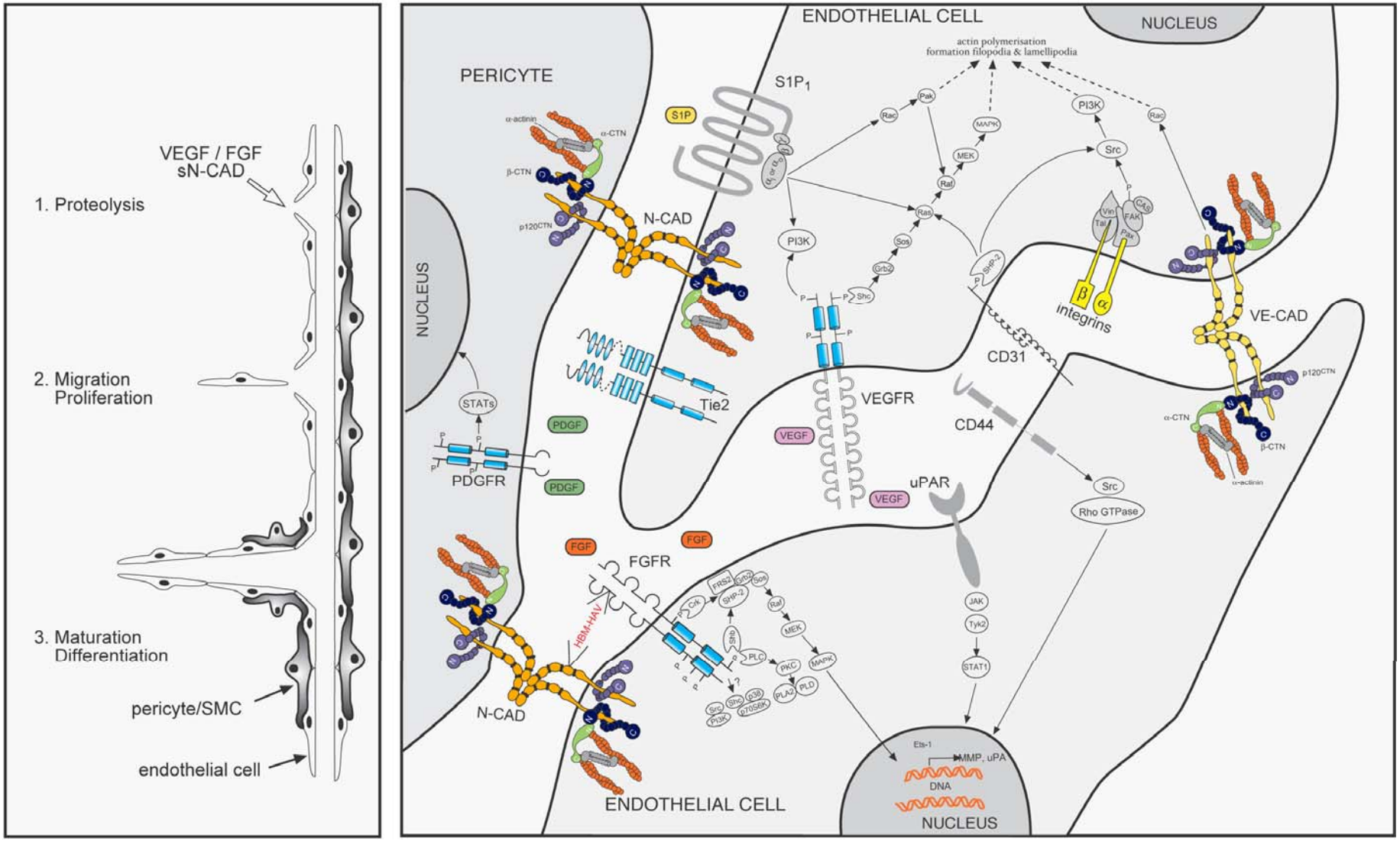
Recent work from the Luo and Radice describes knockdown experiments of N-cadherin in the endothelial cells *in vivo* and *in vitro*. Loss of N-cadherin caused a significant decrease in VE-cadherin and p120ctn, and N-cadherin seemed to be important for the proliferation and motility of the endothelial cells (Luo and Radice 2005). This opens new perspectives for the role of N-cadherin in regulating angiogenesis. Cyclic N-cadherin peptides are known to work as antagonists and induce apoptosis in endothelial cells (Erez *et al.* 2004), while the dimeric N-cadherin-peptides, agonists, are able to stimulate neurite outgrowth (Utton *et al*. 2001). Both events are FGF-receptor dependent. Interestingly, the FGF-receptor is known to interact with N-cadherin via the HAV binding region in extracellular domain 4 (Williams *et al.* 2001), by this, the FGF-receptor is not internalized and maintains on the membrane, and induces a state of continuous cell activation (Suyama *et al.* 2002). We could speculate that N-cadherin, which is present at sites where endothelial cells meet pericytes, engagement to FGF-receptor would promote endothelial motility and vessel elongation.

In the process of angiogenesis, the FGF/FGF-receptor system plays an important role (Gerwins *et al.* 2000). The process of angiogenesis exists out of 3 steps (figure 4.1), namely proteolysis, migration/proliferation and maturation and differentiation in new vascular tubes. External signals involved in these processes are mainly secreted paracrine factors. Tyrosine kinase, G-coupled receptor and serine-threonine receptors are able to evoke an angiogenic signal. In this part, some signalling pathways involved in the degradation of the extracellular matrix and migration of the endothelial cells are described (see figure 4.1). FGF induces proliferation, extracellular matrix degradation, endothelial cell migration and modulation of the integrins. Extracellular matrix degradation represents an important step during the first phases of the angiogenic process: the plasmin-plasminogen system and matrix metalloproteinases cooperate in the degradation of the matrix. FGF upregulates uPA and MMPs production in endothelial cells and regulates the expression of the uPA receptor (uPAR) on the endothelial cell surface, thus allowing the localisation of the proteolytic activity at the migration front (Presta *et al.* 2005). Association of uPAR with Jak1 and Tyk2,

transforms uPAR in a tyrosine kinase receptor and transduce signals via the STAT proteins to the nucleus of the endothelial cells (Dumler *et al.* 1999). Also, the zinc finger transcription factor Ets-1 seems to play a key role in the activation of the proteolytic system (Chen et. al 1997). This transcription factor is upregulated by activation of the VEGF-receptor 2 and FGF-receptor (Sato *et al.* 2000). CD44 might also be involved in the upregulation of proteolytic enzymes. CD44 lacks kinase activity but associates with Rho GTPases and members of the Src family, which leads to the upregulation of uPA and uPAR (Thorne *et al.* 2004). Multiple signalling pathways also stimulate migration: the integrins (for example $\alpha_5\beta_1$ or α_v integrins) can be activated when they bind to specific substrates like fibronectin (Friedlander *et al.* 1995, Urbich *et al.*, 2002). Angiopoietin, a ligand of the Tie2 receptor is substrate for the α_5 integrins and playing by this a role in endothelial cell migration (Carlson *et al.* 2001). Binding of VEGF to VEGF-receptor 2, induces the phosphorylation of MAPK which modulates actin polymerisation (Landry and Huot 1999), but VEGF-receptor 2 can also activate FAK, and this non-receptor tyrosine kinase regulates the organisation of the actin cytoskeleton via the MAPK pathway or via the PI3K pathway (Abedi and Zachary 1997). The phospholipid sphingosine-1-phosphate stimulates the G-coupled receptor EDG1 (endothelial differentiation gene 1, also called S1P1) leading to activation of PI3K and can activate Rac. In this way, S1P1 is involved in the assembly of the actin cortex and in endothelial chemotaxis (Morales-Ruiz *et al.* 2001). PECAM1 or CD31 was recently being found involved in mechanosignal transduction, and tyrosine phosphorylation of PECAM1 leads to activation MAPK signalling cascade (Fujiwara 2006). Other important signalling molecules involved in angiogenesis are wnt, Frizzled, β-catenin, TGFβ, NFκB, G-coupled receptors like Endothelin B receptor, IL-8 and CXCR1, hypoxia and ROS (Munoz-Chápuli *et al.* 2004).

Legend figure 4.1: Angiogenesis and its signalling pathways
A. Schematic outline of angiogenesis. Angiogenesis can be divided into a series of temporally regulated responses, including protease induction, migration, proliferation and differentiation. VEGF, FGF and sN-CAD are important factors involved in angiogenesis (adapted from Gerwins *et al.* 2000).
B. Schematic overview of factors and signalling pathways involved in migration and matrix degradation by endothelial cells, and recruitment and interaction with the surrounding pericytes. The VEGF-VEGFR, FGF-FGFR, PDGF-PDGFR, S1P-$S1P_1$ pathway are involved in the different processes of angiogenesis. VE-cadherin and CD31 (or PECAM1) are responsible for the adhesion between endothelial cells, while N-cadherin mediate the interaction of the endothelial cell with the pericytes. The integrins mediate the interaction with the substrate and has important signalling function during migration. The uPA receptor and the CD44 receptor contribute to the upregulation of proteases necessary for matrix degradation.

ANGIOGENESIS



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4.2. Soluble N-cadherin promotes angiogenesis L. Derycke, L. Morbidelli, M. Ziche , O. De Wever, M. Bracke and E. Van Aken Clinical and Experimental Metastasis, In Press

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Soluble N-cadherin fragment promotes angiogenesis

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Abstract Endothelial cells express two dependent intercellular adhesion molecules: VE-cadherin, specific for endothelial cells, and N-cadherin, also present in neuronal, lens, skeletal and heart muscle cells, osteoblasts, pericytes and fibroblasts. While there exists a vast amount of evidence that VE-cadherin promotes angiogenesis, the role of N-cadherin still remains to be elucidated. We found that a soluble 90 kD fragment N-cadherin promotes angiogenesis in the rabbit cornea assay and in the chorioallantoic assay when cleaved enzymatically from the extracellular domain of N-cadherin. Soluble N-cadherin stimulates migration of endothelial cells in the wound healing assay and stimulates phosphorylation of extracellular regulated kinase. *In vitro* experiments with PD173074 and knock-down of N-cadherin and fibroblast growth factor receptor, showed that the pro-angiogenic effect of soluble N-cadherin is N-cadherin and fibroblast growth factor receptor dependent. Our results suggest that soluble N-cadherin stimulates migration of endothelial cells through the fibroblast growth factor receptor.

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Key words:
angiogenesis – endothelial cells - Fibroblast Growth Factor Receptor – migration - N-cadherin

Introduction

Angiogenesis is the process of endothelial cells detaching from the vascular wall, invading the underlying tissues, and forming tubes that branch and organise into anastomotic networks [1]. Angiogenesis occurs in physiological (embryology, ovulation, wound healing) and in pathological situations (neoplasia, diabetic retinopathy, rheumatoid arthritis) [2]. The process is regulated by a balance between pro- and anti-angiogenic molecules, emanating from endothelial, stromal and epithelial cells [3], by comprising growth factors [4], proteinases [5] and their respective inhibitors [6], extracellular matrix molecules [7], and also by cell-cell and cell-substratum adhesion molecules [8].
In vivo angiogenesis in cornea or chorioallantoic membrane models, induced by fibroblast growth factor (FGF)-2 depends on $\alpha_v\beta_3$ integrin, whereas angiogenesis induced by Vascular Endothelial Growth Factor type A (VEGF-A) depends on $\alpha_v\beta_5$ integrin. Antibody to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptor for vitronectin blocked angiogenesis in the chick chorioallantoic membrane induced by FGF-2 and VEGF-A respectively [9, 10, 11]. Vitronectin not only binds to integrins, but also to urokinase plasminogen activator receptor (uPAR) [12], both localized at focal adhesion sites. uPAR can stimulate endothelial migration in two ways: first by plasmin formation and subsequent proteolytic degradation of the extracellular matrix [13], and second, by direct interaction with vitronectin. Vascular Endothelial (VE)-cadherin, a cell-cell adhesion molecule specific for endothelial cells serves interendothelial cell-cell adhesion and prevents endothelial apoptosis [2]. Neural (N)-cadherin is a cell adhesion molecule expressed by various cells, like neurons, fibroblasts, oocytes, spermatides, Sertoli cells, lens cells, osteoblasts and also by endothelial cells. Homophilic homotypic N-cadherin interaction in non-endothelial cells, like in cardiomyocytes serves stabilisation of the adherens junction [14]. Heterotypic N-cadherin interactions on the other hand stimulate migration. Various cancer cells invade the surrounding stroma by expressing N-cadherin aberrantly [15], and neural cells migrate on oligodendrocytes, expressing N-cadherin as well [16]. The function of N-cadherin in

angiogenesis remains to be elucidated. Homophilic heterotypic interactions exists between endothelial and stromal cells [17], specifically between endothelial cells and pericytes [18, 19]. Also homotypic N-cadherin interactions were found at the intercellular junctions in endothelial cells. Luo and Radice [20] concluded that N-cadherin controls vasculogenesis upstream of VE-cadherin, because loss of N-cadherin in endothelial cells results in embryonic lethality at mid-gestation due to severe vascular defects. The knock-down of N-cadherin caused a significant decrease in VE-cadherin expression.

Our laboratory has provided evidence that an 80 kD Epithelial (E)-cadherin fragment (sE-CAD), released by plasmin, matrilysin or stromelysin-1, affects epithelial tissue integrity, causing loss of cell-cell adhesion and gain of invasion [21, 22]. Similar soluble fragments are released from cells expressing N-cadherin (sN-CAD), and they also exert regulatory functions, such as during neurite outgrowth in the retina of the chick embryo [23]. The aim of the present study was to identify a possible effect of sN-CAD on angiogenesis. We found that sN-CAD mediated the invasion of endothelial cells into the extracellular matrix during angiogenesis, like sE-CAD mediated the invasion of cancer cells. Therefore, we used N-cadherin expressing mouse sarcoma cells as a source of sN-CAD. We found that sN-CAD stimulated angiogenesis *in vivo*. *In vitro* studies revealed that sN-CAD is able to stimulate migration of endothelial cells through activation of the fibroblast growth factor (FGF)-receptor.

Material and Methods

Cell lines

Human endothelial cells, PSV1, derived from umbilical veins (a gift from Véronique Fafeur, Institut Pasteur de Lille) [24]. These cells were checked after thawing for endothelial cell markers VE-cadherin, N-cadherin and factor VIII. All experiments were done with cells between passage 3 and 10. Cells were grown on 0,1% gelatin-coated dishes in RPMI 1640 (Invitrogen, Merelbeke, Belgium) supplemented with 20% foetal bovine serum, 100 IU/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen) and 2.5 µg/ml Fungizone® (Bristol-Meyers Squibb, Brussels, Belgium). S180-NCAD and S180 cells, mouse sarcoma cells (a gift from R.M. Mège, INSERM, Paris, France) [25] were grown in D-MEM (Invitrogen) supplemented with 10% foetal bovine serum and penicillin, streptomycin and Fungizone® (see above). The S180-NCAD cells, used as a source of sN-CAD, are S180 cells transfected with chicken cDNA encoding for N-cadherin. The cells were incubated in an 100% water-saturated atmosphere of 5% CO_2 for PSV1 and 10% CO_2 for S180-NCAD and S180 cells. All cells were routinely tested for mycoplasma contamination by staining with 4',6-diamidino-2-phenylindole (DAPI) and found negative.

Reagents and antibodies

A 10-mer histidine-alanine-valine (HAV)-comprising peptide, identical to amino acids 235-244 in the first extracellular domain of N-cadherin (hu N-CAD[10], LRAHAVDING) [26] served as a peptidomimetic for the first extracellular domain (ECD1) of N-cadherin. As a control, a scrambled 10-mer peptide (scrambled N-CAD[10], LHDANVGRIA) (Eurogentec, Seraing, Belgium) was included. Recombinant human N-cadherin/Fc chimera was purchased from R&D Systems (Abingdon, UK). Recombinant human basic Fibroblast Growth factor (FGF-2) (Sigma, St. Louis, Missouri, USA) and recombinant human Vascular Edothelial Growth Factor (VEGF-A) (R&D Systems, Abingdon, UK) were used as positive control. Cortisone Acetate (Sigma) was used to block the process of inflammation in the chorioallantoic assay. PD173074, a selective inhibitor of the fibroblast growth factor receptor [27] was a gift from P. Doherty (King's College London, UK).

The antibodies used for immunoprecipitation were rabbit polyclonal anti-human β-catenin (Sigma), rabbit polyclonal anti-human FGF receptor (FGFR1) (Santa Cruz Biotechnology, California, USA), mouse monoclonal anti-pan cadherin (CH19) (Sigma) and mouse IgG isotype control (eBiosciences). The rat monoclonal antibody NCD-2 (a gift from C. Redies, University Hospital Essen, Germany) [28] was used for immunodepletion of sN-CAD out of the medium containing sN-CAD. The primary antibodies for Western blot were: mouse monoclonal antibody GC-4, CH-19 (Sigma) and 13A9 (kindly provided by M.J. Wheelock, Department of Biology, University of Toledo, Toledo, USA) against N-cadherin, mouse monoclonal antibody anti-α-tubulin (Sigma), rabbit polyclonal antibody anti-phospho-ERK kinase and rabbit polyclonal antibody anti-ERK kinase (Cell Signaling technology, Beverly, USA). The secondary antibodies were anti-mouse or anti-rabbit antibody linked to horseradish peroxidase (Amersham Pharmacia Biotech).

Electroporation

PSV1 cells were seeded and, at 70-80% confluency, were trypsinized and collected in a Nucleofector™ certified cuvette (Amaxa GmBH, Cologne, Germany). A mixture of 100 µl Nucleofector Solution and 3 µg of short interference RNA (siRNA) was added. The cells were electroporated in the Nucleofector electroporator with the A34 specific Nucleofector program. siRNAs targeting N-cadherin (GenBank/EMBL/DDBJ accession number NM-001792) were designed by Qiagen (Leusden, The Netherlands). Inhibition of N-cadherin expression was achieved by RNA interference using the following double-stranded oligoribo-nucleotides: siN-cad2 5'-AGUGGCAAGUGGCAGUA AA-3' and siN-cad3 5'-GGAGUCAGCAGAAGUUG AA-3' [29]. siRNA targeting FGFR1 (GenBank/EMBL

/DDBJ accession number NM-000604) were designed by DHARMACOM (Lafayette, Co). The FGFR1 siRNA represented mixtures of four distinct RNA duplexes (SmartPool). To verify specificity of the knock-down effect, we used an oligonucleotide sequence with no known mammalian target (con 5'-UUCUCCGAACGUGUCACGU) as a control.

Preparation of medium containing sN-CAD

Confluent monolayers were washed 3 times with phosphate buffered saline (PBS) and incubated for 2 hours at 37°C with serum free DMEM to which plasmin 1 μg/ml (Sigma) was added. Our recombinant N-cadherin could also be cleaved by plasmin and releasing a 90kD fragment. sN-CAD was also spontaneously released by the cells: for this subconfluent monolayers were washed 3 times with PBS and put on serum free medium for 24 hours, washed another 3 times with PBS and incubated for 48 hours with serum free medium. The medium containing sN-CAD was harvested, centrifuged at 250 g for 5 minutes followed by a centrifugation step at 2,000 g for 15 minutes and filtered through a 0.22 μm filter. The medium containing sN-cad after plasmin treatment or the medium after 48 hours contact with the cells was always checked by western blot for the presence of the 90 kD N-cadherin fragment before use in functional assays (see also Figure 2). sN-CAD was removed from the medium by immunoadsorption as follows. Medium containing sN-CAD was incubated four times for 1 hour with protein G Sepharose 4 fast flow beads (Amersham, Pharmacia Biotech) coated with NCD-2 antibody against N-cadherin. Beads and supernatants were separated and the remaining supernatant was finally filtered through a 0.22 μm filter. Medium without sN-CAD (serum-free DMEM) was also incubated for 2 hours at 37°C with 1 μg/ml plasmin for evaluation of the effect of plasmin on angiogenesis. Media with or without sN-CAD were concentrated twice (Amicon Ultra 50 kD, Millipore Corp., Bedford, MA) before use in angiogenesis assays *in vivo* and *in vitro* assays.

Rabbit cornea assay

In vivo angiogenesis was studied in the cornea of New Zealand rabbits (Charles River, Calco, Como, Italy) since this is an avascular and transparent tissue, where inflammatory reactions and growing capillaries can be easily monitored and changes quantitated by stereomicroscopic examination. Slow-release pellets were prepared under sterile conditions, incorporating the test substances into a casting solution of an ethynil-vinyl copolymer (Elvax-40, DuPont-De Nemours, Wilmington, DE). Rabbits were anaesthetized by sodium pentothal (30 mg/kg) and in the lower half of the eyes, one or two micropockets were surgically made using an iris spatula. The pellets were implanted in the micropockets. Pellets impregnated with recombinant growth factor FGF-2 (R&D Systems) were used as positive control [30]. Subsequent daily observations of the implants were made with a slit lamp stereomicroscope by two independent operators. Angiogenic activity is indicated by the number of implants exhibiting neovascularization over the total implants studied, and by the angiogenic score. The angiogenic score was considered positive when budding of vessels from the limbal plexus occurred after 3-4 days and capillaries progressed to reach the implanted pellet. The angiogenic score is calculated by the number of newly formed vessels and by their growth rate (number of vessels x distance from the limbus) [31]. A density value of 1 corresponded to 0-25 vessels per cornea, 2 from 25-50, 3 from 50-75, 4 from 75-100 and 5 for > 100 vessels. The distance from the limbus was graded with the aid of an ocular grid. The Student's *t*-test for unpaired data was used for statistics ($p < 0.05$).

Chorioallantoic membrane (CAM) assay

The CAM assay was performed as described by Maragoudakis et al.[32] with some modifications. Briefly, fertilized eggs were incubated for 3 days at 37°C. On day 3, albumen was removed to detach the shell from the developing CAM. On day 4, a window was made in the eggshell, exposing the CAM, and covered with cellophane tape. The eggs were returned to the incubator until day 9, prior to application of the test compounds. Test compounds and control compound (PBS) were poured onto separate sterile discs (11 mm diameter), which were allowed to dry under sterile conditions. A solution of cortisone acetate (100 μg/disc) was poured onto all discs to prevent an inflammatory response. Test discs probed with the 165 amino acid isoform of VEGF-A served as positive control. On each CAM, the disc containing control compound and the disc containing test compound were placed at a distance of 1 cm. The windows were covered and the eggs were incubated until day 11, before assessment of angiogenesis. Therefore, the eggs were flooded with 10% buffered formalin, the discs were removed, and the eggs were kept at room temperature for at least 2 hours. A large area around the discs was cut out and placed on a glass slide, and the vascular density index was measured by the method of Harris-Hooker et al. [33]. Briefly, a grid containing three concentric circles of 6-, 8- and 10-mm diameter was positioned on the surface of the CAM previously covered by the disc. All vessels intersecting the circles were counted. The angiogenic index = (t-c)/c, with t the number of intersections in the area covered by the test disc and c the number of intersections in the area covered by the control disc in the same egg. All experiments were performed at least twice, and the Mann Whitney *U*-test was used for statistics ($p < 0.05$).

Wound healing migration assay

Cells were grown in 6-well tissue culture dishes until confluent. Medium was removed and the monolayers were wounded with a plastic tip. Wounded monolayers

were washed 3 times with Ca^{2+}- and Mg^{2+}-containing PBS pH 7.4 to remove dead cells. Cell migration occurred in 1 ml serum free medium. Wounds were marked and measured at time points zero and 16 hours with an inverted microscope. The migration distance of the untreated cells was put at 100% and compared to the treated cultures. The Student's *t*-test was used for statistics ($p < 0.05$).

F-actin staining

Cells were grown on glass coverslips in 24 well culture dishes until islands of cells were formed. Cells were washed with PBS and serum-starved overnight, followed by treatment or left untreated for 6 hours in serum free medium. Cells were fixed in 3% paraformaldehyde, blocked in 50 mM NH_4Cl in PBS, permeabilized in 0.2% Triton-X-100 in PBS and stained with Phalloidin–FITC (Sigma).

Immunoprecipitation and Western blotting

All cell lysates were made from cell cultures at approximately 70% confluence. All cells were washed three times with PBS, serum-starved overnight, washed again three times and treated for the indicated times. Cells were lysed with PBS containing 1% Triton X-100 and 1% Nonidet P-40 and the following protease- and phosphatase inhibitors: aprotinin (10 µg/ml), leupeptin (10 µg/ml), phenylmethylsulphonyl fluoride (1.72 mM), NaF (10 mM), $NaVO_3$ (1 mM) and $Na_4P_2O_7$ (1 mM) (Sigma). For the co-immunoprecipitation of FGF-receptor and N-cadherin the following lysis buffer was used: 50 mM Tris-HCl pH of 7.5, 150 mM NaCl, 1% Nonidet P-40 and the same protease- and phosphatase inhibitors as described above. The protein concentration was measured using Rc Dc protein assay (Bio-Rad), and samples were prepared at equal protein concentrations. For immunoprecipitation, equal amounts of proteins were first incubated with protein A or G Sepharose beads (Amersham Pharmacia Biotech) for 30 minutes. After discarding the beads, the supernatant was incubated with primary antibody for 3 hours at 4 °C, followed by incubation with protein A or G-Sepharose beads for 1 hour. Sample buffer (Laemmli) with 5% 2-mercaptoethanol and 0.012% Bromophenolblue was added, followed by boiling for 5 minutes and separated on 8% SDS-PAGE and transferred on a nitrocellulose membrane (Amersham Pharmacia Biotech.). Quenching and immunostaining were done in 5% non-fat dry milk in PBS containing 0.5% Tween 20, except for anti-phospho-ERK antibody, where 4% bovine serum albumin in PBS containing 0.2% Tween 20 was used instead. The membranes were quenched for 1 hour, incubated with primary antibody for 1 hour, washed four times for 10 minutes, incubated with horseradish peroxidase-conjugated secondary antibody for 45 minutes, and washed six times for 10 minutes. Detection was carried out using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) as a substrate. To control for equal loading of total lysates, immunostaining with anti-tubulin antibody was performed. Quantification of the bands was done using Quantity-One software (Bio-Rad).

Results

HAV-comprising N-CAD^{10} peptide promotes angiogenesis in vivo

The effect of hu N-CAD^{10} peptide, comprising the HAV motif of the first extracellular domain of N-cadherin, was examined in the CAM (chorioallantoic assay) (Figure 1a).VEGF-A (1µg/ml), serum free medium without and with hu N-CAD^{10} peptide(2 µg/µl) or with scrambled peptide (2 µg/µl) were tested. VEGF-A and hu N-CAD^{10} peptide induced angiogenesis, 45% and 27,7% respectively, with angiogenic indices that were statistically different from the angiogenic index of the scrambled peptide. Serum free medium with or without scrambled peptide did not induce angiogenesis.

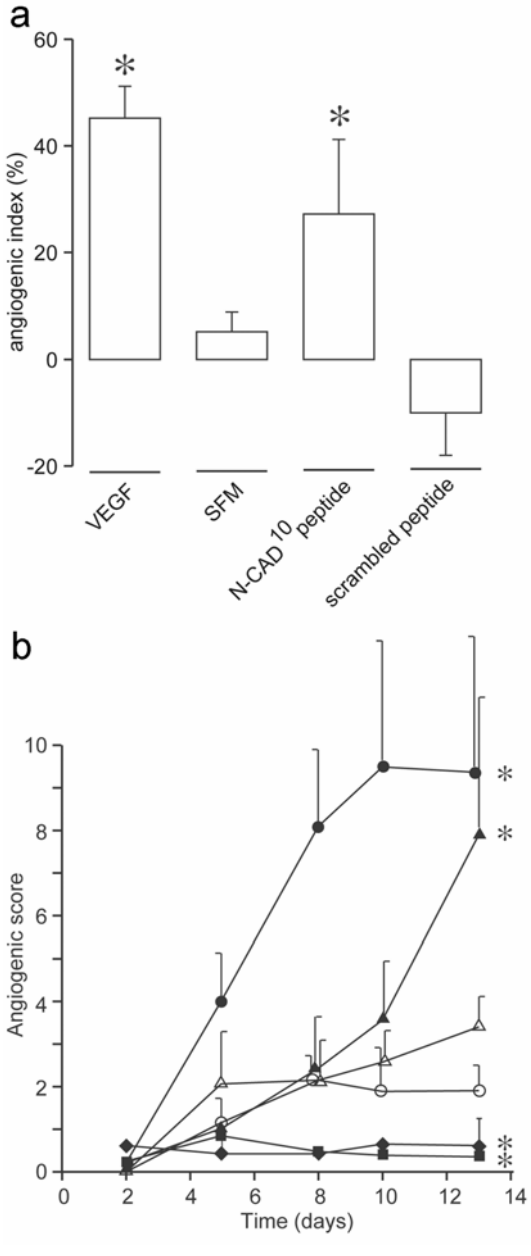


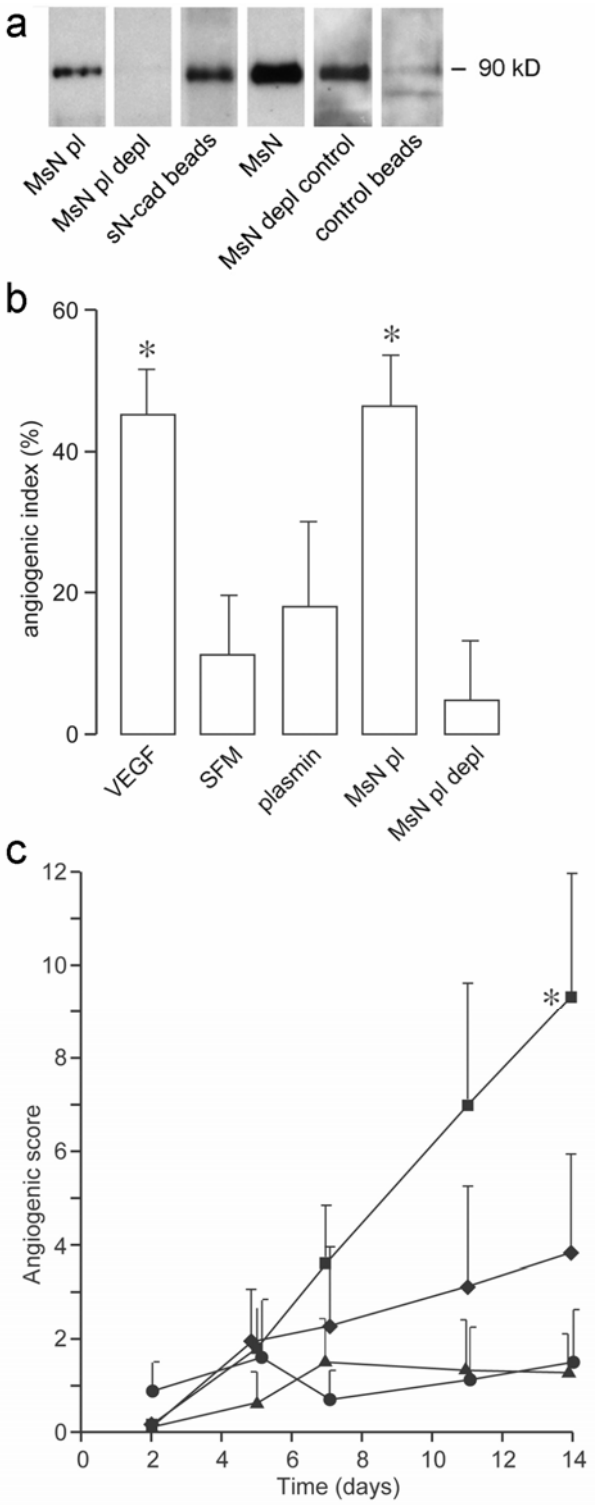
Fig.1 Hu N-CAD[10] peptide induces angiogenesis. (a) hu N-CAD[10] peptide induces angiogenesis in the CAM. Bars indicate angiogenic indices of CAM probed with VEGF-A (1 μg/ml), serum free medium without or with hu N-CAD[10] peptide 2 μg/μl, or with a scrambled N-CAD[10] peptide 2 μg/μl. Each value (mean + standard deviation) is the result of three experiments. In each experiment, 5 eggs were tested per condition. * Statistically different from the mean angiogenic index of CAMs probed with scrambled peptide (Mann-Whitney *U*-test, $p < 0.05$). (b) Hu N-CAD[10] peptide induces angiogenesis in the rabbit cornea. Angiogenic scores of rabbit corneas probed with pellets impregnated with hu N-CAD[10] peptide 200 ng (closed diamonds, n=4), hu N-CAD[10] peptide 500 ng (closed squares, n=4), hu N-CAD[10] peptide 10 μg (closed triangles, n=3), hu N-CAD[10] peptide 50 μg (closed circles, n=3), scrambled N-CAD[10] peptide 10 μg (open triangles, n=3), and scrambled N-CAD[10] peptide 50 μg (open circles, n=3). Symbols represent the mean + standard deviation of angiogenic scores of n number of rabbit corneas tested. *Statistically different from the mean angiogenic score of rabbit corneas probed with scrambled peptide 10 μg (Student's *t*-test, $p < 0.05$).

Different concentrations of hu N-CAD[10] peptide and scrambled peptide were tested in the rabbit cornea assay (Figure 1b). Mean angiogenic scores of hu N-CAD[10] peptide were concentration dependent. Low doses of hu N-CAD[10] peptide (200 ng/pellet and 500 ng/pellet) were devoid of any angiogenic capacity (0/4 rabbit corneas were positive for both concentrations). Pellets impregnated with 10 μg or with 50 μg hu N-CAD[10] peptide induced angiogenesis in respectively 2/3 and 3/3 rabbit corneas. Pellets impregnated with 10 μg or 50 μg scrambled N-CAD[10] peptide induced angiogenesis in only 1 rabbit cornea (1/3 rabbit corneas positive for both concentrations). The mean angiogenic score of rabbitcorneas probed with pellets impregnated with hu N- CAD[10] peptide in any concentrations was statistically different from the angiogenic score of rabbit corneas probed with pellets impregnated with scrambled peptide. No inflammatory effect was microscopically observed at any of the concentrations tested.
In these *in vivo* experiments we could prove that the HAV comprising N-cadherin peptide induced angiogenesis.

sN-CAD promotes angiogenesis in vivo

To approach the physiological situation, we used for all further experiments soluble N-cadherin, which is released from the mature N-cadherin after enzymatical cleavage. Different enzymes, like MMP, plasmin, ADAM10, are able to shed the 90 kD N-cadherin fragment. sN-CAD is also present in different body fluids of the patients, like the serum [34].

Fig. 2 Detection of sN-CAD and induction of angiogenesis. (a) Immunodepletion of medium containing sN-CAD is performed by immunoadsorption with NCD-2 antibody-coated beads removing sN-CAD. Lanes represent medium containing sN-CAD, immunodepleted medium, and sN-CAD linked to NCD-2 antibody-coated beads used for immunodepletion of medium containing sN-CAD. As a control for sN-CAD containing medium, spontaneously released sN-CAD was used. For this purpose, S180-NCAD cells were incubated with serum-free medium and medium was harvested after 48 hours. As control for the sN-CAD depletion we used an isotype control antibody. Media and beads were separately dissolved in sample buffer, and proteins were separated by SDS-PAGE, blotted and immunostained with NCD-2 antibody. (b) Bars indicate angiogenic indices of CAMs probed with



VEGF-A 1 μg/ml, serum free medium, serum free medium with plasmin1 μg/ml, medium containing sN-CAD or sN-CAD immunodepleted medium. Each value (mean + standard deviation) is the result of three experiments. In each experiment, 5 eggs were tested per condition. *Statistically different from the mean angiogenic index of CAMs probed with serum free medium (Mann-Whitney *U*-test, $p < 0.05$). (c) Angiogenic scores of rabbit corneas, probed with pellets impregnated with serum free medium (diamonds, n=4), serum free medium with plasmin (triangles, n=5), medium containing sN-CAD (squares, n=6), or sN-CAD immunodepleted medium (circles, n=3). Symbols represent the mean of angiogenic scores of n number of rabbit corneas tested. Flags represent standard deviations. * Statistically different from the mean angiogenic score of rabbit corneas probed with pellets impregnated with serum free medium (Student's *t*-test, $p < 0,05$).

Medium from S180-NCAD cells treated with 1 µg/ml of plasmin was collected after 2 hours (MsN pl). This medium was used in the CAM assay because we presumed it contained less growth factors than the 48 hours medium containing sN-CAD. Medium harvested from S180-NCAD cells after a 48 hours incubation-period with serum-free medium contained spontaneously released sN-CAD (MsN) (Figure 2a). The immunosignal for sN-CAD was present at 90 kD in medium containing sN-CAD, but not when sN-CAD was immunodepleted by immunoadsorption. NCD-2 antibody-treated beads, used for immunoadsorption of sN-CAD, also showed an immunosignal for sN-CAD. When the medium containing sN-CAD was immunodepleted with an isotype control antibody, there was no change in MsN and no immunosignal appeared in isotype antibody-treated beads.

The effect of sN-CAD on angiogenesis was examined in the chorioallantoic membrane assay (CAM) (Figure 2b). VEGF-A, serum free medium, plasmin, medium containing sN-CAD and sN-CAD immunodepleted medium were tested. Only VEGF-A and medium containing sN-CAD induced angiogenesis statistically different from serum free medium ($p < 0.05$). Results with plasmin alone were not statistically different from those with serum free medium. sN-CAD immunodepleted medium did not induce angiogenesis compared to serum free medium while sN-CAD medium depleted with isotype control antibody gave the same angiogenesis response as the non-depleted medium.

Medium containing sN-CAD, sN-CAD immunodepleted medium, and serum free medium with and without plasmin were also tested in the rabbit cornea assay (Figure 2c). The mean angiogenic score of rabbit corneas probed with pellets impregnated with medium containing sN-CAD, was 7 and statistically different from the mean angiogenic score of rabbit corneas probed with pellets impregnated with serum free medium. 6/6 rabbit corneas probed with pellets impregnated with medium containing sN-CAD were positive. Pellets impregnated with sN-CAD immuno-depleted medium or with serum free medium with plasmin induced angiogenesis in respectively 1/3 and 1/5 rabbit corneas. The mean angiogenic score of rabbit corneas probed with pellets impregnated with sN-CAD immunodepleted medium or with serum free medium with plasmin did not differ statistically from the mean angiogenic score of rabbit corneas probed with pellets impregnated with serum free medium.

sN-CAD induced angiogenesis the CAM and in the rabbit cornea assay.

sN-CAD-stimulated migration in vitro is N-cadherin dependent

Since angiogenesis is dependent on migration of endothelial cells we were interested whether sN-CAD modulates the migration of endothelial cells in the wound healing migration assay *in vitro*. Confluent PSV1 cultures were serum-starved for a minimum of 24 hours to establish quiescence such that the presence of cells in the wounded area was owed to cell motility rather than cell proliferation. After wounding the monolayer, PSV1 cells were treated with different concentrations (0,5 to 2 mg/ml) of the hu N-CAD^{10} peptide (Figure 3a). Endothelial cells migrated perpendicularly to the wound in a irregular shaped front and there was a statistically difference in migration between hu N-CAD^{10} peptide treated cells (144%) and serum free treated cells (100%)

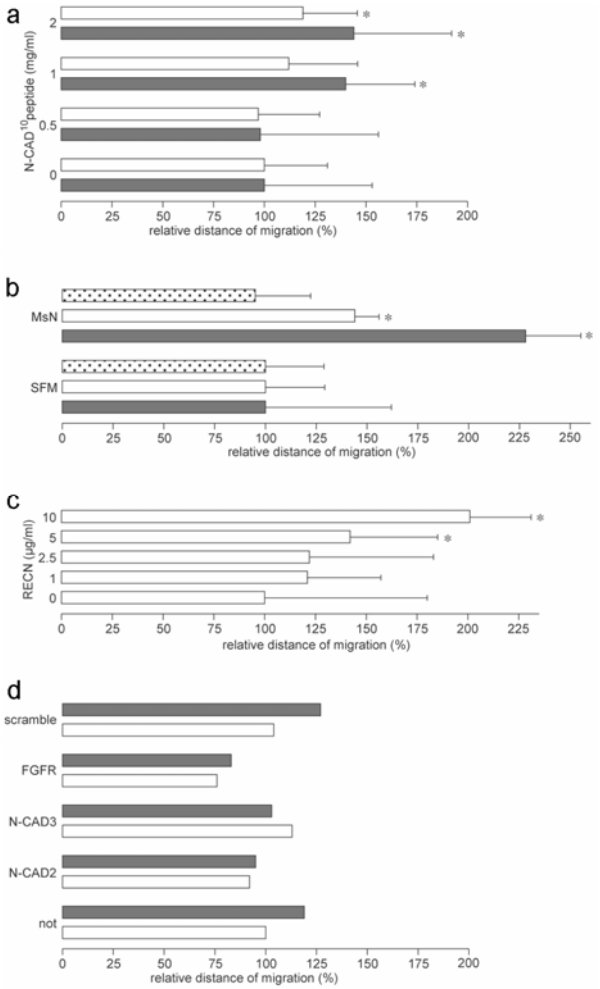


Fig. 3. sN-CAD stimulates migration (a) Confluent monolayers of S180-NCAD (white bars) and PSV1 (grey bars) cells were wounded with a plastic tip, and treated with hu NCAD^{10} peptide in a concentration range of 0,5 to 2 mg/ml or with serum free medium (SFM). The distance of migration of SFM treated cultures was set at 100%. Each value (mean + standard deviation) is the result of two experiments. * Statistically different from the mean relative distance of SFM treated cultures (Student's *t*-test, $p < 0.05$) (b) Confluent monolayers of S180 (dotted bars), S180-NCAD (white bars) and PSV1 (grey bars) cells were wounded with a plastic tip, and treated with medium containing sN-CAD (MsN) or serum free medium (SFM). Each value (mean + standard deviation) is the result of three experiments. * Statistically different from the mean relative distance of SFM treated cultures (Student's *t*-test, $p < 0.05$) (c) Confluent monolayers of PSV1 cells were wounded with a plastic tip, and treated with different concentrations of recombinant N-cadherin (RECN, 1 to 10 µg/ml). Wounds were marked and measured at time points zero and after 16 hours. Bars represent mean values of at least three independent experiments and flags indicate standard deviation. * Statistically different from the mean migration of SFM treated cultures (Student's *t*-test, $p < 0.05$). (d) Confluent monolayers of siRNA transfected PSV1 cells were wounded after 48 hours with a plastic tip and subsequently treated with serum free medium (SFM) (white bars) or RECN (5 µg/ml) (grey bars). Wounds were marked and scored at time zero and after 16 hours. Bars represent mean value of one experiment performed in triplet.

Next, we treated wounded PSV1 monolayers with serum free medium containing sN-CAD (MsN) or with serum free medium (SFM) (Figure 3b). Medium containing sN-CAD induced migration that was significantly faster (228%) than cells treated with SFM (100%). Medium from S180, not expressing N-cadherin, was also tested in the wound healing assay but had no stimulatory effect on the PSV1, S180-NCAD or S180 cells. Moreover, recombinant N-cadherin (RECN), which consist of the extracellular domain of N-cadherin linked to the Fc fragment of human IgG1, stimulated migration of PSV1 cells in a dose response manner (0 to 10 µg/ml) (Figure 3c). Untreated PSV1 cells migrated slower compared to PSV1 cells treated with 10 µg/ml of RECN (100% versus 201%). Other recombinant cadherins like E- or P-cadherin did not stimulate the migration of the endothelial cells. For all migration assays, knock-down and immunocytochemistry experiments we used RECN at a concentration of 5 µg/ml. Furthermore, we use this *in vitro* assay to analyse the molecular mechanism of sN-CAD stimulated migration.

We found the presence of full-length N-cadherin to be a prerequisite for the stimulatory effect of sN-CAD. Migration of S180-NCAD cells, transfected with full-length N-cadherin, and their parental S180 cells devoid of N-cadherin, were compared (Figure 3b). S180-NCAD cells treated with their own medium containing sN-CAD (MsN) migrated significantly faster then S180-NCAD cells treated with SFM (144% and 100% respectively). Migration of S180 cells (100%) was not stimulated by adding MsN (95%). To confirm the role of N-cadherin in sN-CAD-stimulated migration, we used the siRNA knock-down approach. N-cadherin expression was silenced using siRNA. For this, endothelial cells were electroporated with two double stranded oligonucleotides derived from different regions of N-cadherin cDNA. PSV1 cells electroporated either with control oligonucleotide or without were used as controls. As revealed by Western blot analysis 72 hours after transfection, siN-cadherin supresses N-cadherin protein expression by 93% (see Figure 5). After 48 hours, confluent monolayers of electroporated PSV1 cells were wounded and treated with or without RECN (5 µg/ml) (Figure 3d). Control cells were stimulated by RECN (5 µg/ml) in the wound healing assay (not: SFM: 100 and RECN 119% and scramble: SFM 104% and RECN 127%). RECN-stimulated migration of N-cadherin silenced cells was hampered in comparison with not or control transfected cells (siNCAD2 SFM 92% and RECN 95% and siNCAD3 SFM 113% and RECN 103%).

The promigratory effect of sN-cad on PSV1 cells in the wound healing assay was dependent on N-cadherin.

sN-CAD-stimulated migration in vitro is FGF-receptor dependent

We investigated a possible association between full-length N-cadherin and the FGF-receptor (Figure 4a). S180-NCAD cells of approximately 70% confluency were lysed and co-immunoprecipitation was performed using an antibody against the C-terminus of N-cadherin or the C-terminus of the FGF-receptor. After gel electrophoresis, proteins were blotted and immuno-stained using an antibody against N-cadherin and the FGF-receptor. N-cadherin co-immunoprecipitated with the FGF-receptor in both cell lines, suggesting a direct or indirect interaction between both molecules (as has been demonstrated before in cell lines by Suyama et al. [35]).

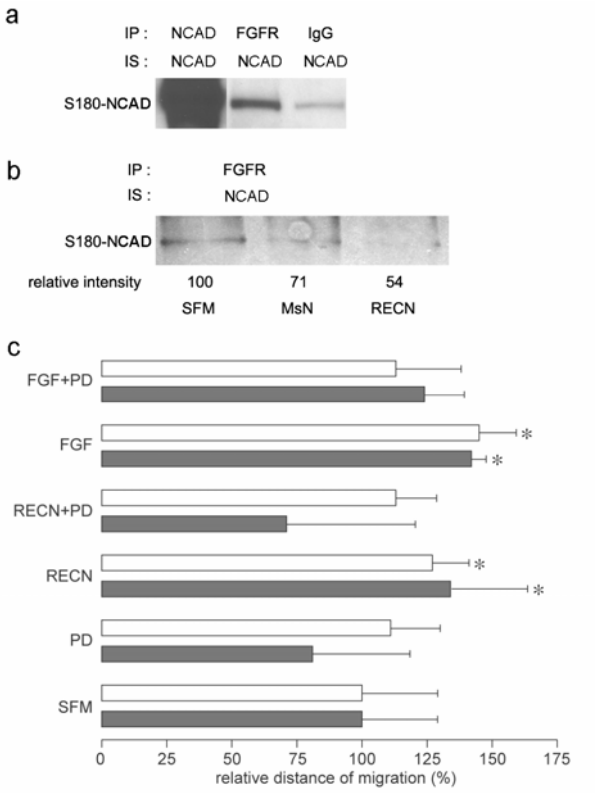


Fig. 4 sN-cad activates the FGF-receptor. (a) Subconfluent monolayers of S180-NCAD were solubilized in low detergent lysis buffer and immunoprecipitation was performed with an antibody against N-cadherin or the FGF-receptor. The precipitated proteins were resolved by SDS-PAGE, and immunoblots were stained with antibody against N-cadherin. (b) sN-CAD dissociates the N-cadherin/FGF-receptor interaction. Serum starved S180-NCAD cells were treated 30 minutes with serum free medium (without sN-CAD), medium containing sN-CAD (MsN) or RECN (5 µg/ml), followed by solubilisation in low detergent lysis buffer. Equal amounts of protein were immunoprecipitated with an antibody against the FGF-receptor, the precipitated proteins were resolved by SDS-PAGE. Immunoblots were stained for N-cadherin. The bands in the immunoblot stained with anti-FGFR remained the same. All imunoblots were quantified with the Quantity One Software and the relative intensity of the N-cadherin / FGFR bands are showed in the figure and this figure is representative for at least 3 independent experiments performed. (c) Confluent monolayers of S180-NCAD (white bars) and PSV1 (grey bars) cells were wounded with a plastic tip and subsequently treated with serum free medium (SFM), PD173074 (PD, 500nM), RECN (5 µg/ml), RECN with PD, FGF-2 (12.5 ng/ml) or FGF-2 with PD. Wounds were marked and measured at time points zero and after 16 hours. Bars represent mean values of at least three independent experiments and flags indicate standard deviation. * Statistically different from the mean migration of SFM treated cultures (Student's *t*-test, $p < 0.05$).

We then examined the effect of sN-CAD on the N-cadherin/FGF-receptor complex. S180-NCAD cells

were used at 70% confluency, followed by serum starvation overnight. Cells were treated for 30 minutes with serum free medium (SFM), medium containing sN-CAD (MsN) or RECN. Cells were lysed and co-immunoprecipitation was performed. In SFM treated cells N-cadherin still interacted with the FGF-receptor. In sN-CAD treated cells however, N-cadherin was dissociated from the FGF-receptor (MsN: 71% and RECN 50%)(Figure 4b).

We then examined the effect of PD173074, a specific FGF-receptor inhibitor (Mohammadi et al.,1998 [27]), on the migration of PSV1 and S180N-CAD cells. Wounded monolayers were stimulated to migrate by treatment with RECN or FGF-2 (12.5 ng/ml +heparin 5 µg/ml) (Figure 4c). PSV1 and S180-N-CAD cells that were treated with SFM migrated slower then treatment with RECN stimulated migration up to 134% and 127%. Addition of 500nM PD173074 to RECN-treated cultures counteracted the pro-migratory effect of sN-CAD both in PSV1 and in S180-NCAD cells. By contrast, PD173074 alone had no effect on migration of cells.

Next, the FGF-receptor was knocked-down in PSV1 cells using siRNA. For this, endothelial cells were electroporated with a pool of 4 double stranded oligonucleotides derived from different regions of FGF-receptor cDNA. PSV1 cells electroporated either with control oligonucleotide or without were used as controls. As revealed by Western blot analysis 72 hours after transfection, siFGF-receptor supresses FGF-receptor protein expression by 74% (Figure 5b). After 48 hours, confluent monolayers of electroporated PSV1 cells were wounded and treated with or without RECN (5 µg/ml) (Figure 3d). Control cells were stimulated by RECN (5 µg/ml) in the wound healing assay (not: SFM: 100% and RECN 199% and scramble: SFM 104% and 127%). RECN-stimulated migration of FGF-receptor silenced cells was hampered in comparison with control (siFGFR SFM 76% and RECN 83%). We conclude that FGF-receptor expression and activity is necessary to observe the pro-migratory effect of sN-CAD in the wound healing assay.

sN-CAD phosphorylates ERK

The FGF-receptor signals through the ERK pathway to stimulate migration (Presta et al., 2005 [11]). We therefore examined the possible contribution of ERK as signalling component in sN-CAD-stimulated migration of endothelial cells. As shown in figure 5a, PSV1 and S180-NCAD cells were grown until 70% confluency, followed by serum starvation overnight. Cells were treated for 30 minutes with SFM, medium containing sN-CAD (MsN), RECN (5 µg/ml) or FGF-2 (12.5 ng/ml), as a positive control, in the presence or absence of PD173074. ERK was hardly phosphorylated when cultures of the three cell lines were treated with serum free medium, but ERK was strongly phosphorylated in PSV1 and S180-NCAD cell lines treated with MsN, RECN or FGF-2 (Figure 5a). Staining with the anti-ERK1/2 antibody and anti-tubulin was used as control and remained the same in all conditions. The relative intensity was calculated by measuring the intensity of the p-ERK bands compared to intensity of the total ERK bands.

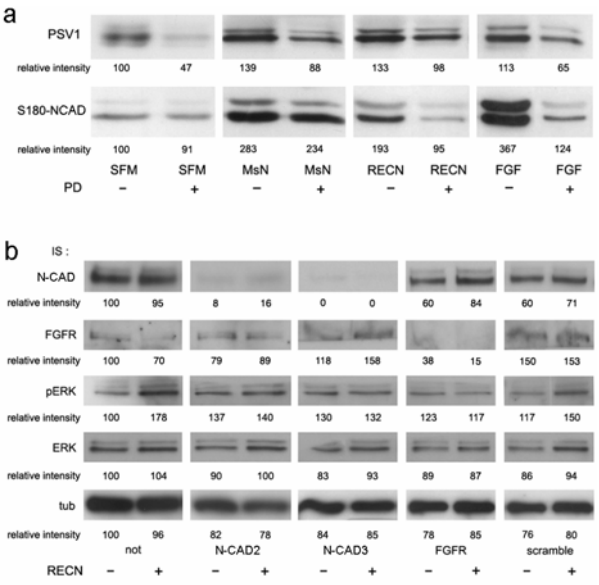


Fig. 5 sN-CAD stimulates ERK phosphorylation (a) sN-CAD induces ERK phosphorylation. PSV1 and S180-NCAD were serum starved and treated for 30 minutes with serum free medium (SFM), medium containing sN-CAD (MsN), RECN (5 µg/ml) or FGF-2 (12.5 ng/ml) without or with PD173074 (PD). Equal amounts of protein were loaded on SDS-PAGE and stained with an antibody against phospho-ERK and with an antibody for total ERK. Immunoblots from phospho-ERK and total ERK were quantified with a Quantity One Software and the relative intensity is the value of pERK corrected for the amount of total ERK present. This result is representative for at least 3 independent experiments performed. (b) PSV1 cells, knocked-down with oligonucleotides against N-cadherin, the FGF-receptor or a non-mammalian target, were serum starved after 60 hours and after 72 hours treated with serum free medium (SFM) or RECN (5 µg/ml) during 30 minutes. Cells were lysed and equal amounts of protein were loaded on SDS-PAGE. Western blot was stained for N-cadherin, the FGF-receptor, phospho-ERK and total ERK and tubulin. All imunoblots were quantified with the Quantity One Software. This experiment was done twice and gave the same result.

PSV1 cells were knocked-down for N-cadherin and FGFR, using oligonucleotides, and were serum starved after 60 hours and treated for 30 minutes in absence or presence of RECN. As revealed by Western blot analysis, siRNA efficiently reduced N-cadherin and FGF-receptor expression (Figure 5b). Tubulin expression was used as control for equal protein loading. Again, phosphorylation of ERK was checked. (Figure 5b). By knocking-down the expression of N-cadherin and FGF-receptor phosphorylation of ERK was strongly diminished in RECN-treated cell cultures. As control these cells were also treated with FGF-2, in the FGF- receptor silenced cells ERK could not be phosphorylated (data not shown).

Our experiments suggest that sN-CAD-stimulated ERK activation is dependent on expression of N-cadherin and FGF-receptor, and FGF-receptor activity.

sN-CAD induces cytoskeleton reorganisation

PSV1 and S180-NCAD were seeded at low density on glass coverslips. Cells were put on serum free medium and cells were treated further with serum free medium, or with medium containing sN-CAD (MsN), RECN, RECN with PD173074 or PD173074 alone for 6 hours (Figure 6). MsN and RECN treatment of PSV1 and S180-NCAD cells induced cytoskeleton reorganisation of cells: loss of stress fibers, and more filopodia-like extensions were formed and cells were more elongated, compared to cells treated with SFM. Cytoskeleton reorganisation induced by sN-CAD was counteracted by adding PD173074.

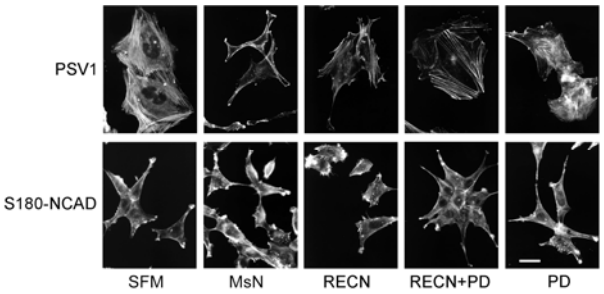


Fig 6 sN-CAD induces filopodia formation. PSV1 and S180-NCAD were sparsely seeded on glass coverslips and grown until islands of cells were formed. Cells were put on serum-free medium overnight and treated with serum free medium, medium containing sN-CAD (MsN), RECN (5 µg/ml), RECN with PD173074 or PD173074 (500 nM) alone for 6 hours. Actin filaments were visualised with FITC-phalloidin. Scale bar, 50µm.

Discussion

We present here evidence that a hu HAV N-CAD[10] peptide (LRAHAVDING) induces angiogenesis in the chorioallantoic membrane and in the rabbit cornea assay dose-dependently. Several experiments have been published with substratum bound N-cadherin peptides containing HAV-sequence dimeric versions of the N-CAD peptides promote neuronal cell survival and neurite outgrowth, while cyclic peptides containing the HAV-sequence of extracellular domain 1 induce FGF-receptor–mediated apoptosis in endothelial cells [36] and inhibit neurite outgrowth [37]. When presented as soluble molecules, dimeric peptides stimulate neurite outgrowth in a manner similar to native N-cadherin [38, 39].

As a better approach of the physiological situation we tested the complete ectodomain of N-cadherin (90 kD extracellular N-cadherin fragment, sN-CAD) in two angiogenesis models *in vivo*, where sN-CAD stimulated angiogenesis in both assays. Different proteases have already been described which are able to cleave N-cadherin extracellularly, like MMP (matrix metallo-proteinases) [23] and ADAM10 (protein with a disintegrin and a metalloprotease domain) [40], giving rise to a 90 kD sN-CAD fragment. Other proteases like presenilin/γ-secretase [41] and caspase-3 [42] are able to cleave N-cadherin intracellularly. In tumours sN-cad can originate from different cell types, like endothelial, fibroblast, cancer cells,…because N-cadherin on the cell membrane can be cleaved by multiple proteases present in the micro-environment We used plasmin, a serine protease, to cleave N-cadherin in its extracellular domain, in order to release a 90 kD sN-CAD in culture medium of the cells.

To elucidate the possible working mechanism we tested both the medium containing sN-CAD and the dimeric recombinant N-cadherin/Fc chimera (termed both as sN-CAD) *in vitro*, which consist of the extracellular part of N-cadherin linked to the Fc fragment of the human IgG1 antibody. N-cadherin is known as a cell-cell adhesion molecule, but it is also a pro-migratory factor, since transfection of epithelial cells with N-cadherin induces the motile phenotype [43, 44]. The domain implicated in migration was restricted to 69 amino acids in extracellular domain 4 [45]. Although the role of N-cadherin in endothelial cells is not yet completely clear, it is important for its interaction with surrounding pericytes in the micro-environment [19]. Recent knockdown experiments of N-cadherin in endothelial cells showed a role of N-cadherin during vasculogenesis [20]. Furthermore, recom-binant N-cadherin/Fc chimera was shown to stimulate neurite outgrowth in an FGF-receptor dependent manner [46], but nothing has been reported to date about its function in migration and invasion of endothelial cells. We found that sN-CAD stimulates the migration of endothelial cells and this event requires the presence of N-cadherin on the acceptor cells, because silencing of N-cadherin by siRNA in the endothelial cells strongly reduced the sN-CAD promigratory effect and S180 (N-cadherin negative, parent) cells cannot be stimulated by sN-CAD containing medium nor by recombinant N-cadherin. We observed no differences in the cell-cell adhesion of endothelial cells when they were treated with sN-CAD or HAV N-CAD10 peptide (data not shown). So, the sN-CAD mediated migration is not due to alterations in cell-cell adhesion.

We also investigated the role of the FGF-receptor in the pro-migratory effect of sN-CAD, because we could demonstrate that N-cadherin co-immunoprecipitates with the FGF-receptor. It was shown in literature that in neuronal cells N-cadherin interacts directly with the FGF-receptor via the HAV-binding region present in extracellular domain 4 of N-cadherin [48], and by this prolongs the activation of the FGF-receptor by stabilisation of the receptor on the membrane [35]. In pancreatic tumour cells as well as in neurons N-cadherin can trigger FGF-receptor signalling independently from FGF [48, 49]. Indeed we were able to reduce sN-CAD mediated migration of endothelial cells in two ways. First, by using a specific inhibitor PD173074, which binds to the ATP pocket of the FGF-receptor [27], and second by knocking down the FGF-receptor by siRNA. FGF stimulated chemotaxis and/or chemokinesis in endothelial cells requires the activation of the ERK signalling pathway [11]. We confirmed that both sN-CAD containing medium and recombinant N-cadherin stimulated phosphorylation of ERK, and this was abolished by addition of the FGF-receptor inhibitor, PD173074. However, the stimulated phosphorylation of

ERK induced by sN-CAD containing medium could not be blocked by the PD173074, presumably because of the presence of other growth factors.

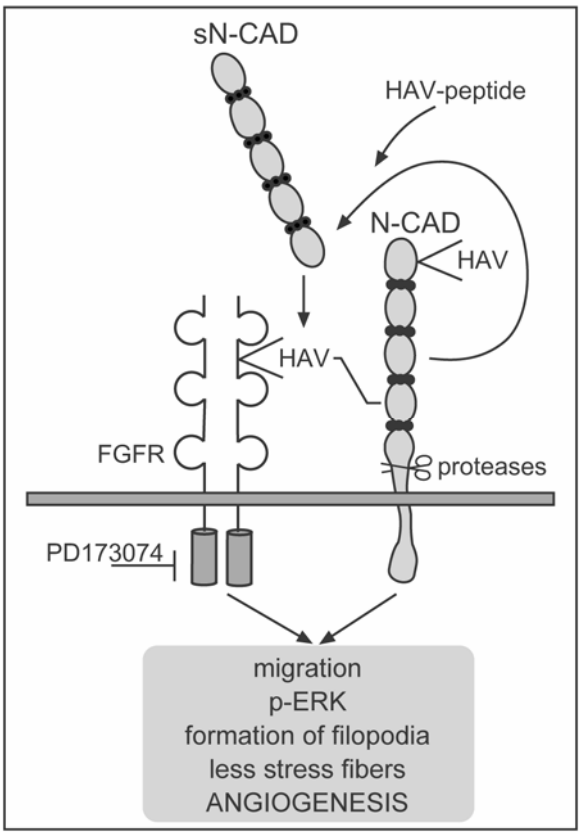


Fig. 7. Hypothetical model of the sN-cadherin-mediated angiogenesis pathway. N-cadherin (N-CAD) contains an HAV (Histidine-Alanine-Valine)-sequence in its first extracellular domain and an HAV-binding motif in extracellular domain 4. In quiescent endothelial cells, N-cadherin is directly linked to the HAV-sequence present on the FGF-receptor (FGFR). In the presence of proteases, endothelial cells are activated by soluble N-cadherin (sN-CAD). sN-CAD, a 90 kD fragment, is directly released by proteases. sN-CAD can directly or indirectly interact with the FGF-receptor. sN-CAD stimulates the migration of the endothelial cells, sN-CAD phosphorylates extracellular regulated kinase (p-ERK), which can be blocked by adding PD173074, it stimulates the formation of filopodia and activates Cdc42. All these cell activities promote sN-CAD or the 10-mer HAV peptide (LRAHAVDING) mediated angiogenesis.

Cytoskeletal reorganisation is essential for migration of endothelial cells and therefore the formation of new vessels. sN-CAD stimulates the loss of stress fibers and the formation of filopodia and cells become elongated. Again these effects are N-cadherin- and FGF-receptor-dependent as evidenced by using siRNA in endothelial cells and N-cadherin deficient S180 cells. It is noteworthy that sN-CAD stimulates the activation of Cdc42 which usually held responsible for the formation of filopodia, and diminishes the activation of RhoA (data not shown).

Comparable results were published with another neural cell adhesion molecule, L1. Plasmin is responsible for the posttranslational cleavage of L1 in fibronectin domain 3 of the molecule with the release of a 150 kD fragment in the medium [51], and ADAM10 can also cleave L1 extracellularly with the shedding of a 200 kD fragment. Both L1 extracellular fragments can stimulate cell migration [52]. Furthermore, pro-migratory effects were observed also with other soluble cadherins, like sE-cadherin [22].

In "quiescent" endothelial cells N-cadherin is responsible for the adhesion with other endothelial cells and with stromal cells like pericytes (Figure 7). However in the micro-environment of tumours and in inflammatory processes, numerous proteases activate endothelial cells to form new blood vessels. We hypothesize that sN-CAD plays an important role in this process. Proteases cleave the extracellular fragment of N-cadherin from stromal cells, endothelial cells or N-cadherin expressing tumour cells. sN-CAD will on its turn interact with the N-cadherin/FGF-receptor complex present on endothelial cells and stimulate the migration of endothelial cells in an FGF-receptor-dependent manner. sN-CAD activates the ERK pathway, leading to upregulation of protease expression, like plasmin and MMP, via the zinc-finger transcription factor Ets-1 [52], which has indeed been shown to induce angiogenesis [53]. By this an autocrine loop is formed: newly expressed proteases on their turn are responsible for the formation of sN-CAD which again induces migration of the endothelial cells.

Our results indicate that sN-CAD stimulates angiogenesis *in vivo* and migration of endothelial cells *in vitro* through an N-cadherin/ FGF-receptor complex.

Acknowledgements

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Part V Cadherins as targets for (anti-) invasive agents

5.1. Overview of factors influencing the cadherins

E-cadherin can be (down) regulated at various levels: several inactivating mutations were already found in lobular breast carcinoma (Berx *et al.* 1996), gastric cancer (Suriano *et al.* 2003a and b; Suriano *et al.* 2005), thyroid carcinoma (Rocha *et al.* 2004) and gynaecologic cancer (Risinger *et al.* 1994). Not only genomic mutations but also abnormal epigenetic methylation can significantly contribute to gene silencing and carcinogenesis. Methylation is particularly often observed in the CpG islands of the promoter regions. However, there are considerable differences between genes in the incidence of methylation. Examples of methylated tumour suppressor genes are APC, Rb and E-cadherin (Curtis and Goggins 2005, Graff *et al.* 1995). Promoter methylation of the E-cadherin gene is related to EMT transition in breast carcinoma cell lines, while the E-cadherin inactivating mutation is not involved (Lombaerts *et al.* 2006). Another way to deregulate the E-cadherin expression is at the level of transcription. In the promoter of E-cadherin, specific regulatory elements are present: CCAAT box, GC Box and CANNTG (E-) box, where different repressors bind. Some examples of transcriptional repressors are SNAIL (Battle *et al.* 2000), SIP1 (Comijn *et al.* 2001), SLUG (Hajra *et al.* 2002), E12/E47 (Perez-Moreno *et al.* 2001), Twist1 (Yang *et al.* 2004) and DeltaEF1 (Eger *et al.* 2005). Others, like p300, AML1, SP1, HNF3 (Liu *et al.* 2005) and SMAD4 (Muller *et al.* 2002) are positive regulators of E-cadherin. Furthermore, the transactivation of other cadherins like N-, P- or OB-cadherin can also deregulate the E-cadherin/catenin complex. The function of the E-cadherin/catenin complex can also be influenced by phosphorylation of the associated catenins via cytokines like EGF (Shiozaki *et al.* 1995), IGF-1 (André *et al.* 1999) or heregulinβ1 (Stove *et al.* 2005, Stove *et al.*) or by proteolysis and ectodomain shedding via for example MMP's (Noë *et al.* 2001), ADAM10 (Maretzsky et la 2005) or plasmin (Ryniers *et al.* 2002). Environmental factors also influence the E-cadherin/catenin complex: xanthohumol, present in hop, is able to stimulate the function of the complex (Vanhoecke *et al.* 2005, see article 5.3), hypotonic stress (Kippenberger *et al.* 2005) and other factors are extensively reviewed by Van Aken *et al.*.

Not only E-cadherin but also P-cadherin is regulated at different levels: two mutations are described, one missense mutation (R503H) is altering the Ca-binding domain (Indelman *et al.* 2002) and the other one is a nonsense mutation (Y615X) resulting in blocking of the P-

cadherin translation (Indelman *et al.* 2005). Both mutations cause hypothrichosis with juvenile macular dystrophy in human. P-cadherin is expressed aberrantly in different carcinomas, for example, P-cadherin is expressed in high-grade ductal carcinoma in situ which lacks the estrogen receptor (Paredes *et al.* 2002). When the antiestrogen, ICI182780, was added to P-cadherin negative and ER positive breast carcinoma cells this resulted in upregulation of P-cadherin (Paredes *et al.* 2004, see article 5.4). Not only the estrogen receptor has seemed to play role in the aberrant expression of P-cadherin but also the methylation status of the promoter. The P-cadherin expression in breast cancer might be regulated by promoter hypomethylation (Paredes *et al.* 2005).

Furthermore, N-cadherin is also influenced by multiple intracellular and extracellular factors (see section 2.2 and Table 5.1., Derycke and Bracke 2004). So far, there were no data about any mutation in N-cadherin. At the epigenetic level, the transcription factor NFκB is enhancing the N-cadherin promoter activity in melanoma cells (Kuphal and Bosserhof 2006). Furthermore, the growth factor TGFβ (Maeda *et al.* 2005) and the extracellular matrix molecule collagen type 1 (Shintani *et al.* 2006) induce N-cadherin expression at the mRNA and the protein level, and this results in EMT. While the nuclear protein HMGN1 (high-mobility group nucleosome binding domain 1) downregulates the N-cadherin expression (Rubinstein *et al.* 2005). Finally, the protease ADAM10 downregulates the function of the N-cadherin/catenin complex by ectodomain shedding and alters by this the N-cadherin mediated cell-cell adhesion (Reiss *et al.* 2005).

Table 5.1.: Mechanisms of regulation of the N-cadherin/catenin complex

Factor	Context	Properties	Reference
	UPREGULATION		
Twist and snail	*Drosophila* gastric cancer	Correlation between twist and N-cadherin expression	Oda *et al.* 1998 Rosivatz *et al.* 2002 Kang and Massague 2004 Alexander *et al.* 2006
Notch1	Melanoma	Enhancing cell-cell adhesion	Liu *et al.* 2006
NFκB	Melanoma	Enhancing N-cadherin promoter activity	Kuphal and Bosserhoff 2006
Gelsolin	Mammary cells	Knockdown of gelsolin, induction of EMT	Tanaka *et al.* 2006
Genistein	Teratocarcinoma cells	Induction of neuronal differentiation	Hung *et al.* 2005
Endothelin -1	Ovarian cancer	Induction of EMT	Rosano *et al.* 2006
Myeloid zinc finger -1	Osteoblasts	Acts as basal regulatory element of the promoter	Le Mée *et al.* 2005
Activin A	Esophageal cancer	Protein level increased	Yoshinaga *et al.* 2004
IFNβ1	endothelial cells	Protein level increased	Harzheim *et al.* 2004
TGFβ	Mammary epithelial cells	Upregulation N-cadherin and increased motility	Maeda *et al.* 2005
Ki-RAS	Pancreatic adenocarcinoma	Upregulation of N-cadherin	Deramaudt *et al.* 2006
Collagen type 1	NMuMG cells	mRNA and protein level	Shintani *et al.* 2006
	DOWNREGULATION		
HMGN1	Embryonic fibroblast	Downregulation of N-cadherin expression	Rubinstein *et al.* 2005
IFNγ	Endothelial cells	Protein levels decreased	Harzheim *et al.* 2004
ADAM10	fibroblast	Ectodomain shedding	Reiss *et al.* 2005

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5.2. The heregulin/human epidermal growth factor receptor as a new growth factor system in melanoma with multiple ways of deregulation. C. Stove, V. Stove, L. Derycke, V. Van Marck, M. Mareel, M. Bracke.(2003) Journal of Investigative Dermatology 121(4):802-12.

ORIGINAL ARTICLE
See related Commentary on page xi

The Heregulin/Human Epidermal Growth Factor Receptor as a New Growth Factor System in Melanoma with Multiple Ways of Deregulation

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In a screening for new growth factors released by melanoma cells, we found that the p185-phosphorylating capacity of a medium conditioned by a melanoma cell line was due to the secretion of heregulin, a ligand for the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases. Expression of heregulin, including a new isoform, and secretion of functionally active protein was found in several cell lines. Receptor activation by heregulin, either autocrine or paracrine, resulted in a potent growth stimulation of both melanocytes and melanoma cells. Heregulin receptor HER3 and coreceptor HER2 were the main receptors expressed by these cells. Nevertheless, none of the cell lines in our panel overexpressed HER2 or HER3. In contrast, loss of HER3 was found in two cell lines, whereas one cell line showed loss of functional HER2, both types of deregulations resulting in unresponsiveness to heregulin. This implies the heregulin/HER system as a possible important physiologic growth regulatory system in melanocytes in which multiple deregulations may occur during progression toward melanoma, all resulting in, or indicating, growth factor independence. ***Key words: heregulin-neuregulin-1/autocrine-paracrine communication/receptor tyrosine kinases.*** ***J Invest Dermatol 121:802–812, 2003***

Growth of melanocytes and their malignant counterparts is regulated by a variety of cytokines and other polypeptides (Lázár-Molnár *et al*, 2000; Payne and Cornelius, 2002). Under physiologic conditions, melanocytes depend for their survival on paracrine stimulatory factors provided by the surrounding keratinocytes (Meier *et al*, 1998). Transformed melanocytes have a decreased dependence on paracrine stimulation, which facilitates their survival outside their natural environment, the epidermis. Changes in several growth factor systems contribute to this decreased dependence. Whereas overexpression of receptor tyrosine kinases (RTK) may lead to increased growth factor sensitivity and constitutive signaling, loss of expression may result in insensitivity to inhibitory factors or indicate growth factor independence (Easty and Bennett, 2000). Also, the profile of growth factors secreted by melanoma cells is frequently altered, compared to melanocytes. Whereas *de novo* expression of some growth factors by melanoma cells may stimulate proliferation of these cells in an autocrine loop, these factors may act on the surrounding cells as well, stimulating or inhibiting these cells in a paracrine way (Halaban, 2000; Lázár-Molnár *et al*, 2000; Ruiter *et al*, 2002).

The human epidermal growth factor receptor (HER) family of RTK consists of four members, epidermal growth factor receptor (EGFR)/erbB1/HER1, neu/erbB2/HER2, erbB3/HER3, and erbB4/HER4 (Olayioye *et al*, 2000; Yarden and Sliwkowski, 2001). Although constitutive activation of these receptors, owing to overexpression, frequently occurs in various types of cancers (Révillion *et al*, 1998), this does not seem to be common in melanoma (Natali *et al*, 1994; Korabiowska *et al*, 1996; Persons *et al*, 2000; Fink-Puches *et al*, 2001). Constitutive RTK signaling may also be the result of truncation, mutation, association with other cell-surface proteins, transactivation via other receptors, or the presence of autocrine loops (Blume-Jensen and Hunter, 2001; Gullick, 2001). The latter may result from the aberrant expression of HER ligands.

Neuregulin-1 is the term for a family of proteins derived by alternative splicing from a single gene, functioning as ligand for HER3 and HER4 (Holmes *et al*, 1992; Yarden and Sliwkowski, 2001). At present, at least 24 splice variants have been identified in different species, of which 10 were found in humans. Alternative splicing at the N-terminus results in three types of proteins: heregulins (HRG, type I) (Holmes *et al*, 1992), glial growth factors (type II) (Marchionni *et al*, 1993), and sensory- and motor-neuron-derived factors (type III) (Ho *et al*, 1995). Further alternative splicing of HRG at the EGF-like domain (α or β), the C-terminal part of the EGF-like domain (1, 2, or 3) (**Fig 1**), and/or at the intracellular tail (a, b, or c) gives rise to closely related proteins, differing in size and cellular localization and having distinct receptor activation potentials and functions (Wen *et al*, 1994; Pinkas-Kramarski *et al*, 1996; Meyer *et al*, 1997). Transmembrane

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Abbreviations: bFGF, basic fibroblast growth factor; CM, conditioned medium; EGF, epidermal growth factor; HER, human EGF receptor; HRG, heregulin; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; rHRG-β1, 7-kDa recombinant EGF-like domain of heregulin isoform b1; RTK, receptor tyrosine kinase.

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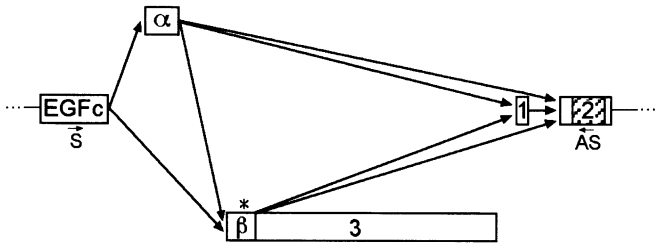

Possible exon combinations	Isoform	Expected PCR product length
(EGFc) - (α) - (2)	α2	202 bp
(EGFc) - (α) - (1) - (2)	α1	226 bp
(EGFc) - (β) - (2)	β2	193 bp
(EGFc) - (β) - (1) - (2)	β1	217 bp
(EGFc) - (α) - (β) - (2)	?	261 bp
(EGFc) - (α) - (β) - (1) - (2)	α4	285 bp
(EGFc) - (α) - (β) - (3)	α3	NA
(EGFc) - (β) - (3)	β3	NA

Figure 1. Neuregulin-1 splicing variation in the EGF-like domain. The scheme depicts the genomic organization of the exons encoding the region surrounding the variable part of the EGF-like domain of the NRG-1 gene. The locations of the sequences that are complementary to the primers used for RT-PCR are indicated with *S* and *AS* for the sense and antisense primers, respectively. The table indicates all possible splicing variants within this domain with their expected amplification product lengths in base pairs (bp), when using the indicated primer pair. *NA,* not amplified; *EGFc,* sequence common in the EGF-like domain of all HRG; *hatched,* coding sequence for the HRG transmembrane domain; *asterisk,* location of the stop codon in case of the (α)–(β) combination; *?,* putative isoform not yet characterized.

HRG typically function as precursor molecules that are subject to the action of metalloproteinases. This results in the release of the extracellular domain that may subsequently bind to nearby receptors (autocrine/paracrine action) (Montero *et al*, 2000; Shirakabe *et al*, 2001). Necessary and sufficient for receptor binding is the EGF-like domain; the roles of the various other domains have not been fully elucidated yet. Some regulation may be exerted by the cytoplasmic tail (Liu *et al*, 1998a, b; Han and Fischbach, 1999) or by interaction of the N-terminal heparin-binding motif with other molecules such as cell surface heparan sulfate proteoglycans (Li and Loeb, 2001). A recently proposed model for ligand-mediated HER activation proposes receptor conformational changes as the driving force for receptor activation (Cho and Leahy, 2002; Garrett *et al*, 2002; Ogiso *et al*, 2002). For HRG, this would mean that binding of its EGF-like domain to HER3 or HER4 leads to an altered receptor conformation, thus promoting dimerization with another HER, preferentially HER2. Hetero- or homodimerization of the receptors leads to *trans*- and autophosphorylation, creating specific docking sites for signal transduction molecules (Dankort *et al*, 2001; Hellyer *et al*, 2001) and initiating further downstream signaling. When only HER2 and HER3 are present, this model of HRG-induced receptor activation implies that HER3, which lacks catalytic activity (Guy *et al*, 1994; Sierke *et al*, 1997), can only become phosphorylated *in trans* by heterodimerization with HER2 (Kim *et al*, 1998). Conversely, HER2, for which no direct ligand has been identified yet, only becomes activated after ligand binding to HER3.

Based on the initial observation that conditioned medium (CM) from a melanoma cell line induced a strong phosphorylation of HERs in MCF-7 mammary cancer cells, we decided to dissect the role of this putative ligand–receptor system in melanocytes and a panel of melanoma cell lines. Here, in 4 of a panel of 13 melanoma cell lines, we describe a number of deregulations in the HRG/HER system. Production and release of functionally active HRG in the medium were found in three cell lines and resulted in an autocrine loop in one case. Whereas exogenous HRG-stimulated growth of the majority of melanoma cell lines and melanocytes, three cell lines did not respond to HRG, owing to the absence of HER3 or owing to a functionally incompetent HER2.

MATERIALS AND METHODS

Cell lines The cell lines were obtained as follows: 530 and BLM melanoma cell lines from L. Van Kempen (University of Nijmegen, the Netherlands); A375 melanoma cell line from J. Hilkens (NKI, the Netherlands); Bowes melanoma from G. Opdenakker (Rega Institute, Belgium); DX3 and DX3azaLT5.1 melanoma cell lines from J. Ormerod (Imperial Cancer Research Fund, UK); FM3/D, FM3/p, FM45, and FM87 melanoma cell lines from J. Zeuthen (Danish Cancer Society, Denmark); HMB2, MeWo, and MJM melanoma cell lines from D. Rutherford (Rayne Institute, St Thomas Hospital, UK); MCF-7/6 mammary carcinoma cell line (further called MCF-7) from H. Rochefort (University of Montpellier, France); COLO-16 squamous skin carcinoma cell line and SK-BR-3 mammary carcinoma cell line from C. De Potter (Ghent University Hospital, Belgium); and MDA-MB-231 breast cancer cell line from American Type Culture Collection (Manassas, VA). Cell lines were routinely maintained in the following media (Gibco BRL, Belgium): RPMI 1640 (FM and COLO-16 cell lines), L15 (MDA-MB-231), 50% Dulbecco's modified Eagle's medium/50% Ham's F12 (MCF-7), or Dulbecco's modified Eagle's medium (all other cell lines). All media for routine culture contained 10% heat-inactivated fetal bovine serum (Greiner Bio-One, Belgium), 100 IU per mL penicillin, 100 μg per mL streptomycin, and 2.5 μg per mL amphotericin B. Epidermal melanocyte primary cultures were obtained from neonatal foreskins and established in M199 medium (Gibco BRL), supplemented with 2% fetal bovine serum, 10^{-9} M cholera toxin, 10 ng per mL basic fibroblast growth factor (bFGF), 10 μg per mL insulin, 1.4 μM hydrocortisone, and 10 μg per mL transferrin (all from Sigma, Belgium). Postprimary cultures were maintained in low-calcium (0.03 mM) M199 medium, supplemented with the same factors and 10% fetal bovine serum. The melanocytic origin of all melanoma cell lines was checked by immunocytochemistry using two antibodies against melanoma-specific proteins, HMB45 (Enzo Diagnostics, Farmingdale, NY) and NKI/C3 (Biogenex, San Ramon, CA). All melanoma cell lines were positive for at least one of these markers (data not shown). Because most of the experiments were carried out with Bowes melanoma cells, which were only positive for NKI/C3, additional electron microscopy was performed to confirm the presence of premelanosome-like structures in this nonpigmented cell line (data not shown).

Antibodies and reagents Primary antibodies used were: rabbit polyclonal anti-HER1, -2, -3, and -4 and anti-HRG precursor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antitubulin (Sigma), mouse antiphospho-mitogen-activated protein kinase (MAPK; Westburg, the Netherlands), and antiphosphotyrosine antibody RC20 conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY). Goat anti-HRG-α and recombinant HRG-β1, consisting of the EGF-like domain of HRG (rHRG-β1, used at 10 ng/mL unless indicated otherwise), was purchased from R & D Systems (Abingdon, UK). Full-length recombinant HRG-β1 was obtained from Laboratory Vision (Fremont, CA), whereas heparin, PD168393 (used at 2 μM, unless indicated otherwise), and PD98059 were from Calbiochem (Darmstadt, Germany).

Preparation of CM Subconfluent monolayers were washed three times with phosphate-buffered saline (PBS), incubated for 24 h with serum-free medium, and washed again three times with PBS, followed by a 48-h incubation with serum-free medium. The latter medium was cleared from cells by 5 min centrifugation at 250 × *g*. The resulting supernatant was centrifuged for an additional 20 min at 2000 × *g* to remove cell debris, filtered through a 0.2-μm filter, and stored at –20°C until use. To isolate the heparin-binding fraction from the CM, the latter was depleted from heparin-binding factors by triple precipitations with heparin beads (Bio-Rad, Hercules, CA). Elution of the heparin-binding fraction was done with 1 M NaCl, followed by desalting and dilution in fresh serum-free medium.

Western blotting and (immuno)precipitation All lysates were made of cells of approximately 90% confluence. For phosphorylation experiments, cells were washed three times with PBS, serum-starved overnight, washed again three times with PBS, and treated with serum-free medium for the indicated times. Before making all lysates, the cells were washed three times with PBS. Cells were lyzed with PBS containing 1% Triton X-100, 1% Nonidet P-40 (Sigma), and the following protease inhibitors: aprotinin (10 μg/mL), leupeptin (10 μg/mL) (ICN Biomedicals, Costa Mesa, CA), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 μM), $NaVO_3$ (500 μM), and $Na_4P_2O_7$ (500 μg/mL) (Sigma). After clearing the lysates, protein concentration was determined using Rc Dc protein assay (Bio-Rad), and samples were prepared such that equal amounts of protein were to be loaded. For immunoprecipitation, equal amounts of protein were first incubated with protein A–Sepharose (Amersham Pharmacia Biotech, UK) for 30 min. After discarding the beads, the supernatant was incubated with primary antibody for 3 h at 4°C, followed by incubation with the added protein A–Sepharose beads for 1 h. For heparin and streptavidin precipitations, cell lysates were incubated with heparin beads (Bio-Rad) or streptavidin beads (Sigma). Sample buffer (Laemmli) with 5% 2-mercaptoethanol and 0.012% bromophenol blue was added, followed by boiling for 5 min and separation of proteins by gel electrophoresis on a 8 or 12% polyacrylamide precast gel (Invitrogen, San Diego, CA) and transfer onto a nitrocellulose membrane (Amersham Pharmacia Biotech). Quenching and immunostaining of the blots were done in 5% nonfat dry milk in PBS containing 0.5% Tween 20, except for RC-20 and antiphospho-MAPK antibodies, where 4% bovine serum albumin in PBS containing 0.2% Tween 20 was used instead. The membranes were quenched for 1 h, incubated with primary antibody for 1 h, washed four times for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody for 45 min, and washed six times for 10 min. Detection was done using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) as a substrate. To control for equal loading of total lysates, immunostaining with antitubulin antibody was performed routinely (not shown). Quantification of bands was done using Quantity-One software (Bio-Rad).

RT-PCR, cloning, and sequencing Total RNA was extracted from approximately 5×10^6 cells using the Qiagen RNeasy kit (Qiagen, Chatsworth, CA). One microgram of total RNA was reverse transcribed with oligo(dT) primers using the Qiagen RT kit (Qiagen) according to the manufacturer's instructions. HRG cDNA encoding all transmembrane isoforms was amplified using the sense primer 5′-CTGTGTGAATGGAG-GGGAGTGC-3′ (complementary to a sequence encoding a conserved part of the EGF-like domain) and the antisense primer 5′-GACCACAAG-GAGGGCGATGC (complementary to a sequence encoding part of the transmembrane domain) (**Fig 1**). As a control (not shown), β2-microglobulin cDNA was amplified using the sense primer 5′-CATCCAGCGTACTC-CAAAGA-3′ and the antisense primer 5′-GACAAGTCTGAATGCTC-CAC-3′ to generate a 165-bp product. PCR was performed on 250 ng template cDNA using the Qiagen *Taq* PCR kit (Qiagen) according to the manufacturer's instructions. Reactions were done in a Minicycler (Biozym, the Netherlands) with an initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 50 s (denaturation), 61°C for 50 s (annealing), and 72°C for 1 min (elongation), followed by a final extension at 72°C for 10 min. For cloning of the HRG amplification products, the HRG sense and antisense primers were extended at the 5′ end with GCCGGATCCG, creating a *Bam*HI restriction site, and with TCCGAATTC, creating a *Eco*RI restriction site, respectively. The resulting amplification products were either separated by agarose gel electrophoresis, followed by gel extraction using Qiagen gel extraction kit (Qiagen), or used directly for digestion with *Bam*HI and *Eco*RI restriction enzymes (Roche Diagnostics, Germany). Digested products were ligated into dephosphorylated *Bam*HI/*Eco*RI-digested pIRES2-EGFP vector (Clontech, Palo Alto, CA). After transformation of competent DH5α bacteria with the ligated product, the kanamycin-resistant clones were screened by PCR using primers complementary to sequences of the pIRES2-EGFP vector surrounding the insert. This resulted in PCR products of different lengths, corresponding to different HRG isoforms, which were subjected to sequencing (Applied Biosystems, Foster City, CA). The sequence of the α4-isoform was submitted to GenBank (Accession Number AY207002).

Scattering assay MCF-7 cells were seeded until small islands were formed. The cells were washed three times with PBS and were serum-starved overnight. The following day, the cells were washed again three times with PBS, after which the treatments (all in serum-free medium) were applied for 2 h. Pictures were taken with an Axiovert 200M microscope (Carl Zeiss Vision, Germany) on living cultures or after the cultures had been fixed with crystal violet (0.5% in 4% formaldehyde, 30% ethanol and 0.17% NaCl) for 15 min.

Cell proliferation assays A total of 12,500 melanocytes were seeded in the wells of a 96-well plate in 100 μL of Dulbecco's modified Eagle's medium/Ham's F12 medium containing 10% fetal bovine serum. After attachment, 100 μL of medium, supplemented with growth factors as indicated, was added. After 5 days, metabolic activity was measured with a colorimetric assay. Briefly, 100 μL of medium was taken off, followed by the addition of 20 μL of 5 mg per mL 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) in PBS. After a 2-h incubation and removal of all fluid, the colored formazan formed was dissolved in 100 μL of dimethyl sulfoxide and absorption was measured with an ELISA reader at 490 nm. Proliferation of melanoma and MCF-7 cells was measured with the sulforhodamine B colorimetric assay. A total of 5000 cells were seeded in 96-well plates, allowed to attach, and treated as indicated. After 5 days, 50 μL of 50% trichloroacetic acid was added to the culture medium, followed by an incubation of 1 h at 4°C. The wells were rinsed with water, dried, incubated with 100 μL of sulforhodamine B (0.4% in 1% acetic acid) for 30 min, rinsed with 1% glacial acetic acid, and dried again. Bound dye was dissolved in 200 μL of 10 mM Tris buffer, pH 10.5, and absorption was measured with an ELISA reader at 490 nm.

Annexin V staining Melanocytes were seeded on a collagen type I gel for 4 days. After taking photographs, adherent cells were detached with a swab and brought together with floating cells. Annexin V staining was performed with Annexin V–PE (Becton Dickinson Biosciences, Mountain View, CA), according to the manufacturer's instructions. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) with an argon–ion laser tuned at 488 nm and a helium–neon diode laser at 635 nm. Forward light scattering, orthogonal scattering, and two fluorescence signals were stored in list-mode data files. Data acquisition and analysis were done using the CellQuest software (Becton Dickinson). Additional propidium iodide (PI) staining was performed to rule out cells that were necrotic (Annexin V^+ and PI^+).

Statistics Differences between means were considered significant when the p value was less than 0.01, using Student's *t* test.

RESULTS

Expression of HERs in a panel of melanoma cell lines Expression of HERs by melanocytes and melanoma cell lines was examined by Western blotting and immunostaining with anti-HER1, -2, -3, and -4 antibodies. Neither melanocytes nor any of the melanoma cell lines showed expression of full-length HER1 or HER4, compared with the respective positive controls A431 and T47D (data not shown). Nevertheless, this does not necessarily mean that these receptors are completely absent. All melanoma cell lines, as well as melanocytes, expressed HER2 (**Fig 2**). HER2 levels in melanocytes and in all

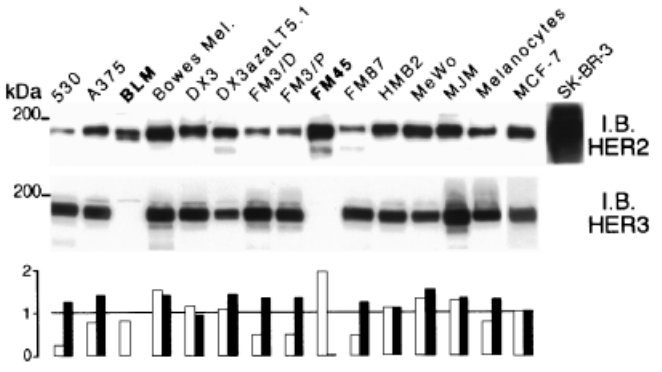

Figure 2. Analysis of HER2/HER3 expression in melanocytes and melanoma cell lines. Whole-cell lysates were analyzed by immunoblotting (*I.B.*) with anti-HER2 and anti-HER3 antibodies, as described under Materials and Methods. Quantification of the resulting bands was done relatively to the level of HERs in MCF-7 mammary carcinoma cells, set at 1. *Open and filled bars,* HER2 and HER3 expression, respectively.

melanoma cell lines were far below the level found in the HER2-overexpressing SK-BR-3 mammary carcinoma cell line (Press *et al*, 1993). Moreover, quantification showed that none of the melanoma cell lines had HER2 levels that were more than two times higher than that seen in MCF-7 mammary carcinoma cells, often used as a control for normal expression (Press *et al*, 1993; Aguilar *et al*, 1999). Comparable levels of HER3 were expressed by melanocytes and 11 of the melanoma cell lines. Two melanoma cell lines (BLM and FM45) did not show expression of HER3 protein (**Fig 2**). The absence of HER3 protein was due to a strongly reduced level of HER3 mRNA in these cell lines, compared with the other cell lines (data not shown).

Expression of HRG by melanoma cell lines Western blotting, followed by immunostaining of total lysates using an anti-HRG antibody directed against a cytoplasmic sequence conserved in all transmembrane HRG isoforms, revealed the presence of a ± 105-kDa band in Bowes melanoma, BLM, and MJM, the first two having the stronger expression (**Fig 3*A***, *top panel*). This band was also present in the positive controls MDA-MB-231 (although very faint) and COLO-16, previously described to secrete HRG (Holmes *et al*, 1992) or HRG-like activity (De Corte *et al*, 1994), respectively, and was not found in the MCF-7-negative control (Aguilar *et al*, 1999; Aguilar and Slamon, 2001). The size of this band indicates that it corresponds to the full-length HRG precursor (Burgess *et al*, 1995; Aguilar and Slamon, 2001). The 50-kDa band, also seen by others using this antibody (Aguilar and Slamon, 2001), probably represents an artifact, because it could also be found in the MCF-7 HRG-negative cells. Also two other bands (at ± 85 and 75 kDa), seen in some melanoma cell lines, are likely due to cross-reactivity of the antibody with other proteins. Because these bands were not consistently found in cell lines expressing HRG mRNA, and CM of these cells had no HRG-like activity (see below), they are unlikely to represent cleavage products of transmembrane HRG. The localization of the 105-kDa immunoreactive band at the plasma membrane was confirmed for Bowes melanoma cells by biotinylation (**Fig 3*B***, *lane 4*) and by precipitation using heparin beads (**Fig 3*B***, *lane 3*), which is consistent with the presence of a heparin-binding motif at the extracellular N-terminus of HRG.

RT-PCR analysis was carried out to verify the results obtained by western blotting and to detect which isoforms were expressed by the HRG-positive melanoma cell lines. Because alternative splicing of the HRG-encoding gene leads to multiple isoforms, with most variation in the EGF-like domain, primers were chosen so that different lengths of amplification products were obtained, depending on the isoform expressed. The sense and antisense primers chosen were complementary to the mRNA encoding a conserved part of the EGF-like domain and a sequence conserved in all transmembrane isoforms, respectively (**Fig 1**). RT-PCR using this primer set, with MDA-MB-231 and COLO-16 as positive controls and MCF-7 as a negative control, confirmed the results obtained by western blotting for Bowes melanoma, BLM, and MJM cells (**Fig 3*A***, *bottom panel*). In addition, lower levels of mRNA were found in some other melanoma cell lines, possibly resulting in HRG protein levels that were below the detection level in western blotting. Melanocytes had amounts of mRNA that were comparable to those found in MJM cells. Because we initially did not detect HRG protein in these cells (**Fig 3*A***), we loaded more protein and overexposed the film, which eventually resulted in the appearance of a weak band at ± 105 kDa (**Fig 3*C***). The pattern of PCR amplification products that was obtained from Bowes melanoma suggested the presence of multiple isoforms. Cloning and sequencing of these products revealed that α2, β1, and β2 isoforms were the most abundant transmembrane isoforms in this cell line (**Fig 3*D***). In addition, a new isoform, designated α_4, was identified. This isoform combines the sequences from

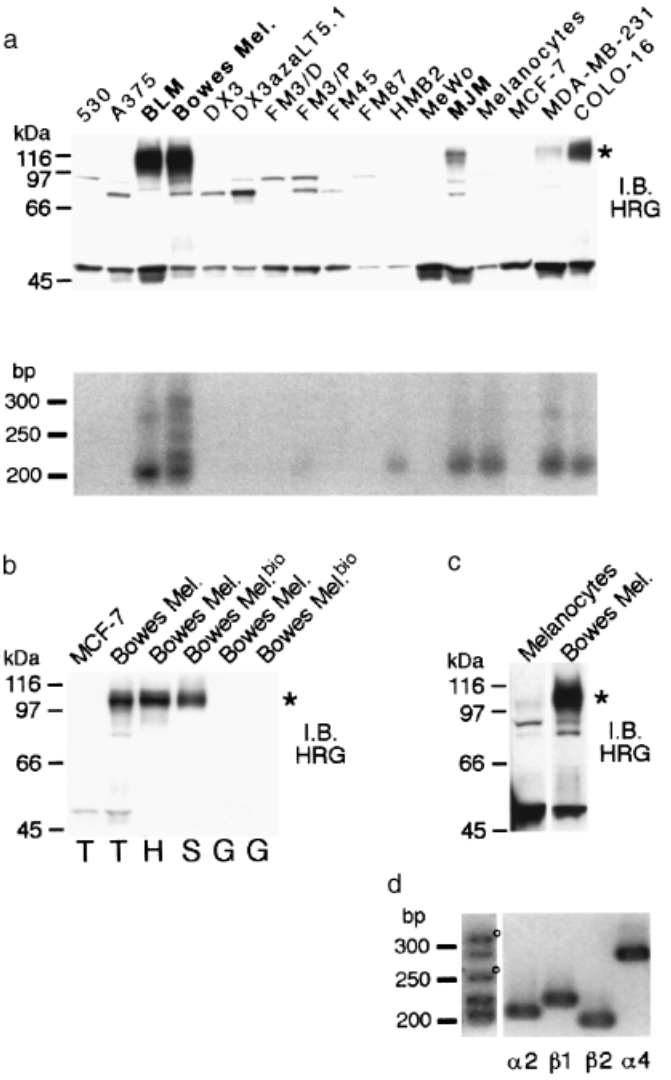

Figure 3. Presence of HRG protein and mRNA in melanocytes and melanoma cell lines. (*a, top panel; c*) HRG precursor expression was analyzed by immunoblotting (*I.B.*) of whole-cell lysates with an anti-HRG antibody. (*b*) Total lysates of MCF-7 and Bowes melanoma (*T*), a heparin precipitate (*H*), streptavidin precipitate (*S*), or control protein G–Sepharose precipitates of Bowes melanoma cells or biotinylated Bowes melanoma cells (*bio*) were analyzed by immunoblotting with an anti-HRG antibody. (*c*) Prolonged exposure after immunoblotting of total lysates of melanocytes and Bowes melanoma cells reveals a weak band in the former. *Asterisk,* the band of full-length HRG at 105 kDa. (*a, bottom panel*) RT-PCR analysis of HRG mRNA in the indicated cell lines using the primer panel indicated in the legend to **Fig 1**. (*d*) The *first lane* depicts HRG mRNA expression in Bowes melanoma as assessed by RT-PCR, using the primer panel indicated in the legend to **Fig 1**. *Lanes 2–5,* PCR analyses of clones derived from Bowes melanoma, representing the indicated HRG isoforms. *Open circle,* bands corresponding to PCR products formed by cross-annealing of two related isoforms and thus considered as aspecific.

both the exon leading to the α isoforms and the exons leading to the β_1 isoform (**Figs 1, 3*D***). Two bands (indicated with an *open circle*) did not correspond to a specific isoform because they were the result from cross-annealing of PCR products coming from β1–β2 or α2–β2 isoforms, presumably resulting in an imperfect double strand with slower migration on agarose gel (**Fig 3*D***).

Melanoma cells release functionally active HRG in the culture medium Following cleavage in the juxtamembrane

extracellular region, HRG are released into the culture medium as 40- to 45-kDa proteins, depending on the isoform and glycosylation level (Holmes *et al*, 1992; Lu *et al*, 1995a, b). Western blotting of 50 × concentrated CM, using an antibody directed against the HRG extracellular domain, revealed the presence of a broad band at the expected molecular weight (**Fig 4*A***, *lane 1*). This band was absent upon depletion of the concentrated CM from heparin-binding factors (**Fig 4*A***, *lane 2*).

To test whether the released HRG was functional, we verified whether CM from the melanoma cell lines was capable of activating HERs in MCF-7 cells. Prominent tyrosine phosphorylation of a 185-kDa protein was evident upon treatment with the positive controls recombinant HRG-β1 (rHRG-β1) and COLO-16 CM and was further restricted to CM from HRG-positive melanoma cell lines (**Fig 4*B***). This phosphorylation could be blocked by pretreating the MCF-7 cells for 30 min with the HER-specific irreversible inhibitor PD168393 (Fry *et al*, 1998) or by adding heparin to the CM (**Fig 4*C,D***). Heparin treatment did not interfere as such with the capability of HERs in MCF-7 cells to become activated, because the combination with rHRG-β1 (lacking a heparin-binding domain) still resulted in full phosphorylation of HERs in these cells (data not shown). To quantify the phosphorylating capacity of the Bowes melanoma CM, we made a comparison with the phosphorylation of MCF-7 cells that had been treated with different concentrations of rHRG-β1. As also shown by others (Aguilar and Slamon, 2001), phosphorylation of a 185-kDa protein could readily be detected using 0.5 ng per mL rHRG-β1 (**Fig 4*E***). Treatment with CM of Bowes melanoma cells resulted in a phosphorylation at 185-kDa equivalent to ±5 ng per mL (700 pM) rHRG-β1, correlating with a concentration of ±100 pM 45-kDa HRG in the CM. Also the kinetics of this phosphorylation were similar, with phosphorylation occurring already after 1 min of treatment, suggesting a similar mechanism of direct receptor activation (**Fig 4*F***).

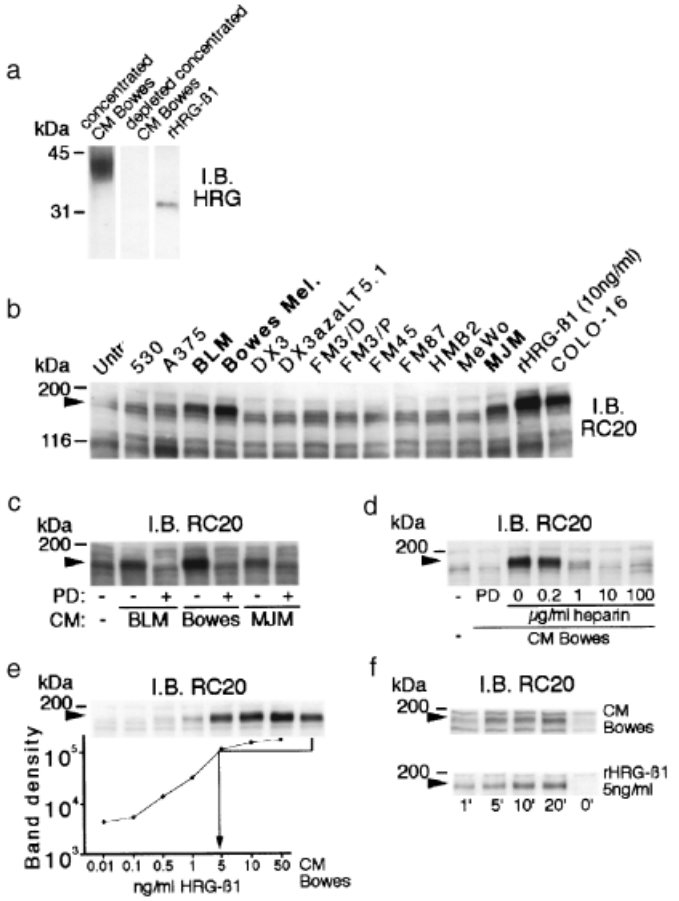

Figure 4. Melanoma cells release receptor activating HRG in the medium. (*a*) Immunoblotting (*I.B.*), using an anti-HRG antibody directed against the EGF-like domain, of concentrated CM of Bowes melanoma, before (*lane 1*) and after (*lane 2*) depletion of heparin-binding factors reveals the presence of a 45-kDa protein only in *lane 1.* Full-length recombinant HRG-β1 (*rHRG-β1*), produced in *Escherichia coli* (*lane 3*) migrates at 33 kDa owing to differences in glycosylation. (*b–e*) Analysis of tyrosine-phosphorylated proteins in serum-starved MCF-7 cells. (*b*) Cells treated for 30 min with serum-free medium (*Untr*), with rHRG-β1, or with CM from the indicated cell lines. (*c*) Cells pretreated or not for 30 min with 2 μM PD168393 (*PD*) and treated for an additional 30 min with serum-free medium or CM from the indicated cell lines. (*d*) Cells pretreated or not for 30 min with 2 μM PD168393 (*PD*) and treated with serum-free medium or with CM of Bowes melanoma to which heparin was added in the indicated concentrations. (*e*) Quantification of tyrosine phosphorylation, induced by treating MCF-7 cells with Bowes melanoma CM or by treating these cells with increasing concentrations of rHRG-β1. The *arrow* indicates that, by extrapolation, the phosphorylating capacity of Bowes melanoma CM is equivalent to that of ± 5 ng per mL rHRG-β1. (*f*) MCF-7 cells treated with 5 ng per mL rHRG-β1 or CM Bowes melanoma for the indicated periods of time. *Arrowheads,* ±185-kDa tyrosine-phosphorylated bands.

Based on the fact that HRG contains an extracellular heparin-binding domain, we performed precipitations using heparin beads on 50 × concentrated CM. Three consecutive precipitations completely abolished the ability of the CM to induce phosphorylation (**Fig 5*B***, *lane 4*) or spreading/scattering of epithelial MCF-7 islands (**Fig 5*C***). In contrast, after eluting the heparin-binding factors from the heparin beads, desalting, and dilution of these factors in serum-free medium, used for treating MCF-7 cells, the phosphorylation of a 185-kDa band (**Fig 5*B***, *lane 3*) as well as the induction of spreading/scattering (**Fig 5*C***) were evident. Both effects could be blocked by pretreating the cells for 30 min with PD168393 (**Fig 5*C,B***, *lane 6*).

An autocrine loop in Bowes melanoma cells leads to constitutive HER phosphorylation, MAPK activation, and increased growth Total lysates from different melanoma cell lines were immunostained for tyrosine-phosphorylated proteins. This revealed the presence of a highly phosphorylated protein at 185 kDa, possibly reflecting activated HER2 and HER3, in Bowes melanoma cells, but not in the other melanoma cell lines tested (**Fig 6*A***). This band was also found in the HRG-positive COLO-16 cells, but not in the weaker HRG-positive MDA-MB-231 cells. By precipitating HER2 and HER3 from Bowes melanoma cells and staining for tyrosine phosphorylated proteins, we could show constitutive phosphorylation of HER2 and HER3 (**Fig 6*B***, *middle panel*). Treating the cells for 30 min with PD168393 resulted in a complete block of this phosphorylation (**Fig 6*B***, *left and middle panels*). This was not due to alterations in receptor levels, as immunostaining for HER2 and HER3 showed no differences between untreated cells and cells treated with PD168393 (**Fig 6*B***, *right panel*). In line with this, when tested in a 5-day growth assay, PD168393 gave a significant ($p < 0.01$) and concentration-dependent growth inhibition of Bowes melanoma cells (**Fig 6*C***). This effect was not due to general cytotoxicity because virtually no growth inhibition was seen of MCF-7 cells (HER2/3-positive and HRG-negative) or BLM cells (HER2/HRG-positive and HER3-negative).

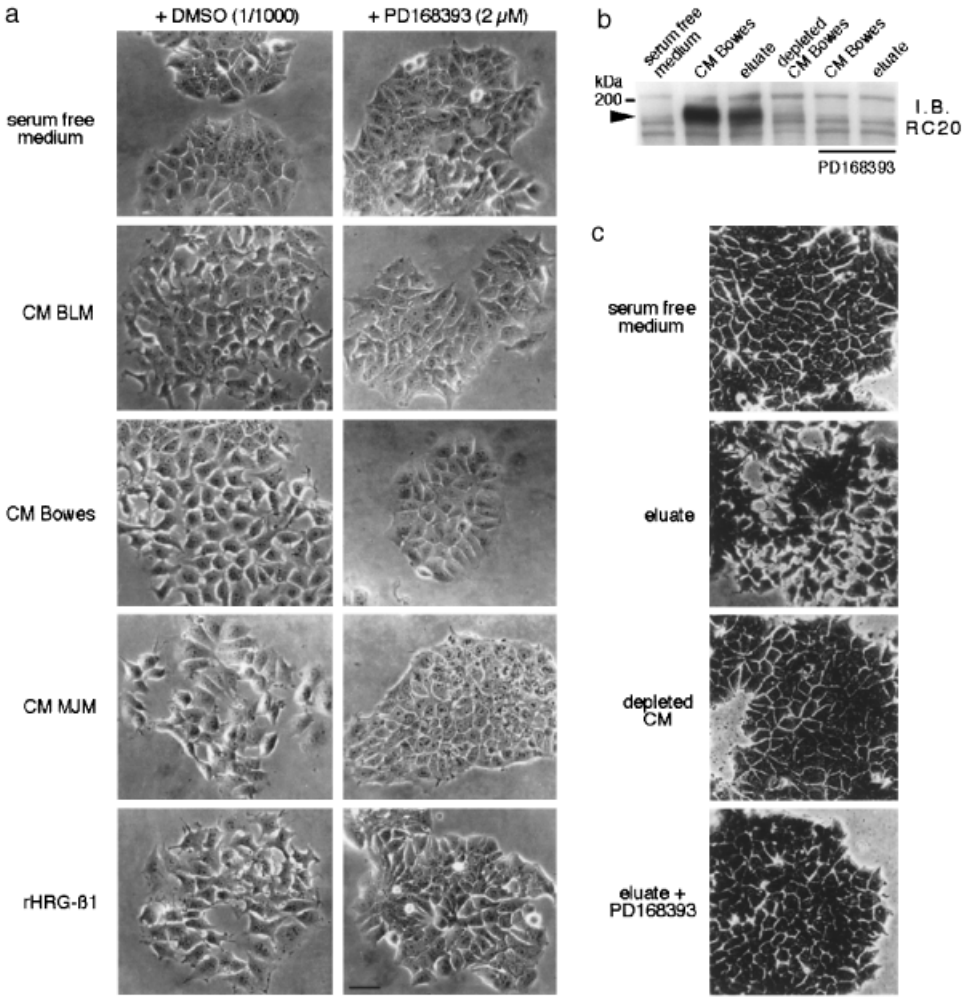

Figure 5. Scattering of MCF-7 epithelial islands by rHRG-β1 and by CM of HRG-positive cell lines. Serum-starved MCF-7 cells pretreated or not for 30 min with 2 μM PD168393, followed by an additional 30-min treatment and preparation of lysates (*b*) or followed by additional 2-h treatments before taking pictures of living cultures (*a*) or crystal-violet-fixed cultures (*c*). (*b*) Antiphosphotyrosine immunoblotting (*I.B.*) of whole-cell lysates of MCF-7 cells that were treated for 30 min with serum-free medium, CM of Bowes melanoma (*CM Bowes*), the heparin-binding fraction from CM Bowes (*eluate*) or CM Bowes depleted from heparin-binding factors. *Arrowhead,* ± 185-kDa phosphorylated band. (*c*) Indicated conditions as in (*b*). *Bars,* 50 μm.

Constitutive receptor activation in Bowes melanoma cells could be inhibited not only by directly blocking its kinase activity, but also the interference with ligand binding resulted in this effect. This was evident from the rapid, concentration-dependent inhibition of HER phosphorylation and the consequent growth inhibition seen upon treatment with heparin (**Fig 6*D,E***). The MAPK pathway is a major pathway implicated in uncontrolled growth of melanomas (Govindarajan *et al*, 2003; Satyamoorthy *et al*, 2003). Because it is also a well known signaling pathway activated by HRG (Pinkas-Kramarski *et al*, 1998), we checked its activation status in Bowes melanoma cells by western blotting using a phospho-MAPK-specific antibody. Constitutive HER phosphorylation of Bowes melanoma cells was accompanied by a constitutively active MAPK pathway (**Fig 6*F***, *lane 1*). Blocking HER phosphorylation with PD168393 rapidly led to a block of MAPK activation (**Fig 6*F***, *lanes 2,3*), showing that continuous HER activation is the main cause of the constitutively activated MAPK pathway in these cells. The importance of the continuous activation of this pathway for the growth of Bowes melanoma cells was shown in experiments in which we used PD98059, a MAPK inhibitor. Bowes melanoma cells were particularly sensitive to this inhibitor and showed significant growth inhibition at concentrations that did not have any effect on growth of control BLM or MCF-7 cells (**Fig 6*G***). In conclusion, constitutive HER activation by autocrine HRG supports growth of Bowes melanoma cells via continuous MAPK activation.

Exogenous HRG stimulates growth of melanoma cells and melanocytes but does not protect melanocytes against apoptosis Melanocytes depend for their survival *in vitro* strongly upon the addition of extracellular stimuli. A prominent growth factor promoting growth and survival of these cells is bFGF (Halaban, 2000). To test whether HRG could have similar effects, we treated melanocytes with different concentrations of rHRG-β1, bFGF, or the combination of both. As is evident from **Fig 7*A***, rHRG-β1 stimulated HER phosphorylation of melanocytes and a variety of melanoma cell lines. rHRG-β1 concentration-dependently stimulated growth of melanocytes and could even provide an additive stimulus over bFGF (**Fig 7*B***). A significant growth stimulation was also seen when, e.g., MCF-7, MeWo, and A375 cells were treated with rHRG-β1 (data not shown). Because bFGF is also a potent antiapoptotic factor for melanocytes (Alanko *et al*, 1999), we next tested whether HRG might have a similar effect. Upon seeding of melanocytes on a collagen gel, these cells undergo apoptosis, round up, and become annexin V-positive owing to the exposure of phosphatidylserine at the outer surface of the cells. This can be inhibited by adding bFGF to the medium (Alanko *et al*, 1999) (**Fig 7*C,D***). Although a small decrease in the percentage of apoptotic cells was reproducibly seen upon treatment with rHRG-β1, this effect was negligible compared to the antiapoptotic effect of bFGF (**Fig 7*C,D***). Overall, the results from these assays show that HRG potently stimulates growth of melanoma cells and melanocytes, but does not protect melanocytes against collagen-induced apoptosis.

Defective HRG/HER system in various melanoma cell lines As shown in **Fig 8*A***, phosphorylation in Bowes melanoma cells was already maximal, because treatment with

rHRG-β1 did not result in an increase of phosphorylation. Consistent with this, no additional growth stimulation could be seen upon treatment with rHRG-β1 (data not shown). The observation that the HRG-positive BLM cells lack constitutive receptor activation (**Fig 6*A***) and cannot be activated upon addition of rHRG-β1 (**Fig 8*A***) is in agreement with the absence of the HRG-receptor HER3 (**Fig 2*A***) in these cells. The lack of HER3 also accounts for the unresponsiveness of FM45 cells to rHRG-β1 (**Fig 8*A***). MJM cells seem to have a defective HER-system as well: although HER2 and HER3 were present (**Fig 2*A***), treatment with rHRG-β1 led only to a minor phosphorylation, compared with treated MCF-7 cells (**Fig 8*A***). This minor phosphorylation was due to a small increase in phosphorylation of HER3, but not of HER2 (**Fig 8*B***, *lane 2*). To check whether HER2 can be activated in MJM cells, we treated them for 10 min with pervanadate, a phosphatase inhibitor. This led to phosphorylation of multiple proteins, including HER2 and HER3 (**Fig 8*B***, *lane 3*). Cotreatment with rHRG-β1 and pervanadate led to an additional increase in phosphorylation of only HER3 (**Fig 8*B***, *lane 4*), compared to treatment with pervanadate only. To verify whether mutations in HER2 could be responsible for the lack of activation in response to signaling from outside the cell, we sequenced all exons of the *HER2* gene. Apart from described polymorphisms in the sequences encoding the transmembrane domain (Ile^{655} to Val^{655}) (Ehsani *et al*, 1993) and the C-terminal tail (Pro to Ala), no mutations were found. Furthermore, biotinylation revealed that full-length HER2 was present at the plasma membrane of MJM cells (data not shown). So, despite the lack of mutations of HER2 and its localization at the plasma membrane in MJM cells, this receptor lacks the potential to become activated via stimulation with ligands.

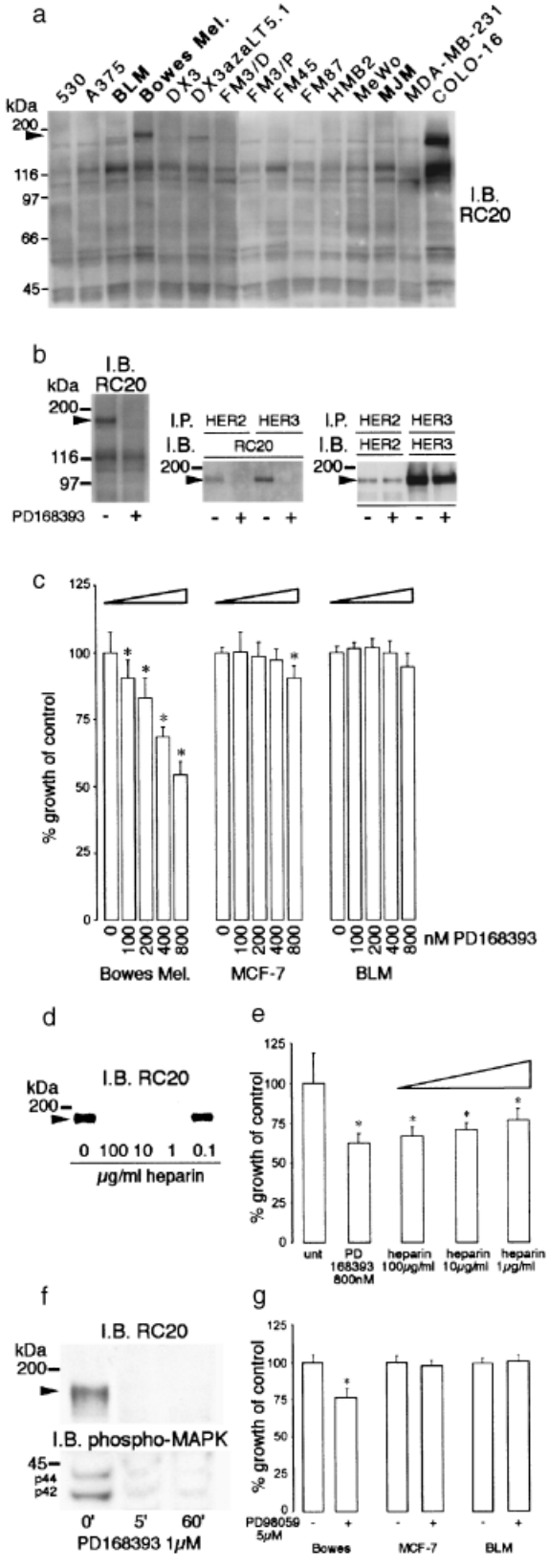

Figure 6. Correlation between constitutive HER activation in Bowes melanoma, continuous MAPK activation, and increased growth. (*a*) Whole lysates from 48-h serum-starved cell lines analyzed by immunoblotting (*I.B.*) using an antiphosphotyrosine antibody. (*b,d*) Immunoblotting of tyrosine-phosphorylated proteins in serum-starved Bowes melanoma cells, treated or not with 2 μM PD168393 for 30 min (*b*) or with different concentrations of heparin for 10 min before making cell lysates (*d*). (*b*) The *left panel* indicates whole-cell lysates. *Middle and right panels,* equal amounts of protein were immunoprecipitated (*I.p.*) using anti-HER2 or anti-HER3 antibodies, before immunoblotting with anti-phosphotyrosine, anti-HER2, or anti-HER-3 antibodies. (*f*) Bowes melanoma cells treated with 1 μM PD168393 for different periods of time. Whole-cell lysates were analyzed by immunoblotting using antiphosphotyrosine (*top panel*) or antiphospho-MAPK antibodies (*bottom panel*). *Arrowheads,* ±185-kDa phosphorylated bands. (*c,e,g*) Growth, relative to vehicle-treated cells, as measured by sulforhodamine B assay. Cells were treated for 5 days with the indicated concentrations of PD168393 (*c,e*) or heparin (*e*) or with 5 μM PD98059 (*g*). *Asterisks,* differs significantly ($p < 0.01$) from controls.

DISCUSSION

In this report, we investigated the expression and function of the HRG/HER ligand–receptor system in 13 melanoma cell lines, compared to normal melanocytes. HER2 and HER3 were found to be the main members of the EGFR family expressed in these cells. Nevertheless, these receptors were not overexpressed, which is consistent with the analysis of both nevi and melanoma tumor material by others (Natali *et al*, 1994; Korabiowska *et al*, 1996; Persons *et al*, 2000; Fink-Puches *et al*, 2001). Similar amounts of HER3 protein were present in melanocytes and in 11 of the 13 cell lines. The fact that two melanoma cell lines (BLM and FM45) showed only low HER3 mRNA levels and even no detectable HER3 protein is a first example of how the HRG/HER system may be deregulated in melanoma (**Fig 9**). Loss of HER3 may imply that transformed melanocytes no longer depend on HRG, normally provided by the surrounding keratinocytes (Schelfhout *et al*, 2000), for their survival. Downregulation or loss of other melanocytic RTK (e.g., c-Kit, protein-tyrosine kinase 4, ephrin receptor EphA4) during melanoma progression has been

described before (Easty and Bennett, 2000). Their loss may uncouple the melanocytes from certain physiologic regulatory mechanisms or may indicate an independence from the growth factor system involved. In the latter case, other systems (autocrine/paracrine) and/or activating mutations of other (intracellular) proteins may have substituted for the loss. In this context, it is noteworthy that we found a mutated N-*ras* allele in BLM, and also in MJM cells (see further), resulting in a constitutively active N-ras (data not shown), whereas FM45 cells have a mutation in the tumor suppressor PTEN (Guldberg *et al*, 1997), resulting in constitutive phosphoinositide 3-kinase signaling. We have evidence (unpublished data) that the absence of HER3 in BLM and FM45 cells is accompanied by the loss of microphtalmia transcription factor M, a melanocyte-specific transcription factor necessary for melanocyte development. Interestingly, expression of both HER3 and microphtalmia transcription factor M has been described to be under the control of SOX-10, a transcription factor necessary for melanocyte development, thus making this protein a putative regulatory candidate (Verastegui *et al*, 2000; Britsch *et al*, 2001).

The MJM cell line represents a second example of a deregulated HRG/HER system in melanoma (**Fig 9**). Although it expresses HRG, HER2, and HER3, surprisingly, it cannot use the secreted HRG in an autocrine loop. Following treatment of MJM cells with exogenous HRG, HER2 is not activated at all, whereas HER3 becomes only weakly phosphorylated. The fact that a minute phosphorylation of HER3 still occurs is surprising, because HER3 lacks catalytic activity and needs HER2 for its phosphorylation in the absence of other HERs. We cannot exclude that HER1 or HER4, whose levels were below the detection limit of our western blotting experiments, account for this effect. We can exclude the possible (lack of) regulatory action of heparan sulfate proteoglycans to be responsible for the impaired response, because the used rHRG-β1 only consists of the EGF-like domain. Also the interference by circulating soluble HER2

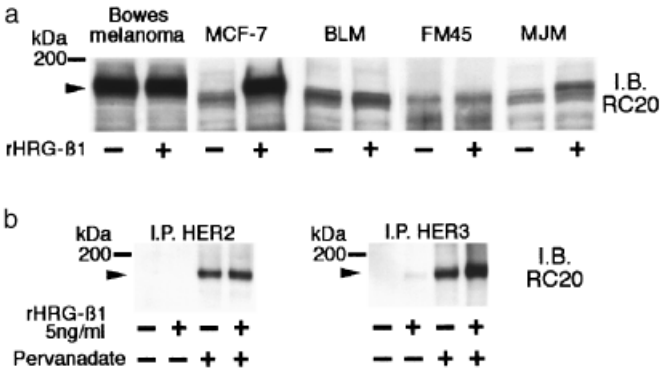

Figure 8. Deregulated HRG/HER system in several melanoma cell lines. (*a*) Serum-starved cells, treated with rHRG-β1 or not before the preparation of whole-cell lysates and analysis by immunoblotting (*I.B.*) using an antiphosphotyrosine antibody. (*b*) Equal amounts of protein from cell lysates of MJM cells that had been treated for 10 min with rHRG-β1 and/or pervanadate were subjected to immunoprecipitation (*I.p.*) with anti-HER2 or anti-HER3 antibodies before immunoblotting using an anti-phosphotyrosine antibody. *Arrowhead,* ±185-kDa phosphorylated band.

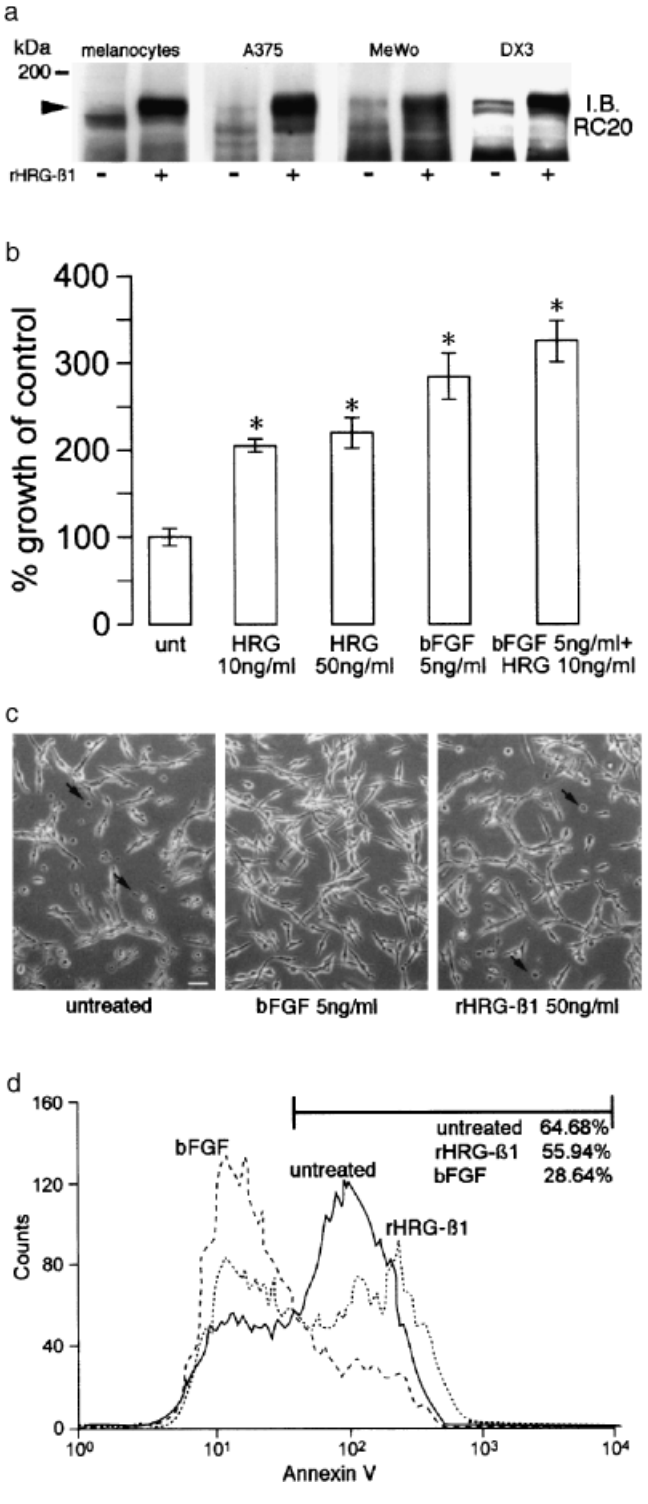

Figure 7. Receptor-activating and growth-promoting, but no anti-apoptotic effects of exogenous HRG on melanoma cells and melanocytes. (*a*) Immunoblotting (*I.B.*), using an antiphosphotyrosine antibody, of lysates from serum-starved cells, treated or not with rHRG-β1. *Arrowhead,* ±185-kDa phosphorylated band. (*b*) Growth, relative to untreated cells, as measured by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Melanocytes were treated for 5 days with the indicated concentrations of growth factors. *Asterisks,* differs significantly ($p < 0.01$) from control. (*c*) Phase contrast photographs of melanocytes cultured on a collagen gel for 4 days with the treatments indicated. *Arrows,* rounded cells in melanocyte cultures that were left untreated or were treated with rHRG-β1. These rounded cells were not found in bFGF-treated cultures. *Bar,* 50 μm (*d*) Histogram, showing the profile of annexin V positivity in melanocytes that were cultured on a collagen gel in the absence of added growth factors (*solid line*) or in the presence of 5 ng per mL bFGF (*broken line*) or 50 ng per mL rHRG-β1 (*dotted line*). The percentage of annexin V-positive cells is indicated.

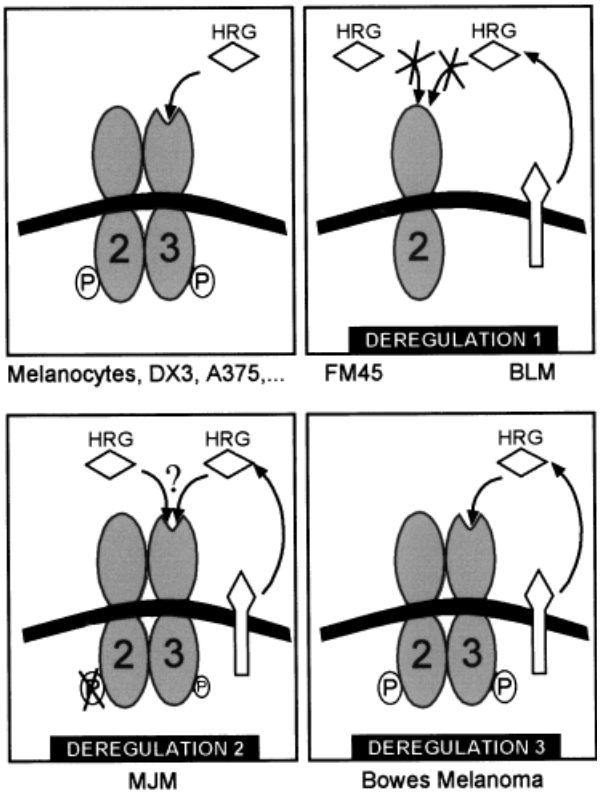

Figure 9. Schematic representation of the several deregulations of the HRG/HER system found in human melanoma cells. In melanocytes and the majority of melanoma cell lines, HERs can be activated by exogenous HRG. The absence of HER3 in BLM and FM45 cells or the presence of functionally inactive HER2 in MJM hampers HRG responsiveness. Bowes melanoma cells show constitutive HER activation, owing to the presence of an autocrine loop.

or HER3 ectodomains (Doherty *et al*, 1999; Aigner *et al*, 2001; Lee *et al*, 2001; Justman and Clinton, 2002) is unlikely, because CM from the HRG-positive MJM cells still induced activation of HERs in MCF-7 cells. Biotinylation experiments suggested a transmembrane localization of HER2 in MJM cells, whereas analysis of functional sequences failed to show mutations. Nevertheless, sequencing did reveal two polymorphisms, one of which (Ile to Val at position 655 in the HER2 transmembrane domain) was predicted to favor the formation of stable HER2 homodimers (Fleishman *et al*, 2002). Although it still needs to be shown whether the proposed model also holds for HER2 activation by heterodimerization (following ligand binding), it would mean that HER2 in MJM cells would be more easily activated than in, e.g., Bowes melanoma cells, which is in contrast to our findings. It thus seems unlikely that this polymorphism may provide an explanation for the observed lack of HER2 activation in MJM cells treated with HRG. A possible role for the other polymorphism we identified in MJM cells has not been established yet. Remaining explanations for the lack of HER2 activation following stimulation with HRG are the interference with ligand binding by sterical hindrance, the constitutive association or action of intracellular negative regulatory proteins or receptor mislocalization (although transmembrane).

Here, we have shown by western blotting, RT-PCR, cloning, and using functional assays the presence and function of HRG as new growth factors produced by human melanoma cells. In addition to three known HRG isoforms, we could identify a new isoform, designated α_4. This isoform combines the sequences that normally lead to either α- or β-isoforms. A similar combination was already described for the α_3-isoform, which differs from the α_4-isoform because the latter contains the coding sequence for the transmembrane domain (Wen *et al*, 1994) (**Fig 1**). Nevertheless, the resulting protein is the same, because this α–β combination leads to a frameshift, resulting in the generation of a stop codon upstream of the sequence encoding the transmembrane domain. This truncated protein is most likely cytosolic because the transmembrane domain functions as a signal peptide (Burgess *et al*, 1995). The presence of HRG in melanomas fits with the neuroectodermal origin of melanocytes and the fact that HRG are molecules typically expressed in neuroectodermal tissues (Marchionni *et al*, 1993; Meyer and Birchmeier, 1995). Although melanocytes showed HRG expression at the mRNA level, HRG could barely be detected at the protein level, suggesting the presence of (post)translational negative regulatory mechanisms in these cells. Activating mutations in H-*ras* have been shown to result in upregulation of HRG in mammary epithelial cells (Mincione *et al*, 1996). Although two of the HRG-producing cell lines (BLM and MJM) have an activating mutation in N-*ras*, transient transfections using dominant-negative and constitutively active N-ras constructs learned that this was unlikely to be the underlying cause of the increased HRG expression (data not shown). Thus, the molecular basis for the high HRG expression in some melanoma cell lines is not clear, yet. Although exogenous HRG did not exert a significant antiapoptotic effect, it potently stimulated growth of cultured melanocytes and melanoma cells and could even provide an additive growth stimulation over bFGF. Upregulation of HRG expression in melanomas may result in the generation of an autocrine loop and in the independence from HRG normally provided by the keratinocytes (Schelfhout *et al*, 2000). This decreased dependence from paracrine growth factors is one of the hallmarks of melanoma progression (Lázár-Molnár *et al*, 2000). Our data clearly show that in the Bowes melanoma cell line, in the absence of receptor overexpression, HER2 and HER3 are permanently activated, leading to continuous MAPK activation and stimulation of growth. This activation is due to continuous ligand–receptor interactions and not to, e.g., activating mutations. Arguments hereto are that the phosphorylation could be abolished by adding heparin to the culture medium and that refreshing of the culture medium led to a transient, gradual decrease in receptor phosphorylation, followed by a gradual recovery to the initial levels (data not shown). Thus, the Bowes melanoma cell line, with its autocrine loop, represents a third example of how the HRG/HER system may be deregulated in melanoma (**Fig 9**). Expression of the HRG/HER system was also described in various other types of cancers (e.g., breast, lung, endometrium, thyroid, head and neck, colon, ovarium) (Ethier *et al*, 1996; Fernandes *et al*, 1999; Srinivasan *et al*, 1999; Fluge *et al*, 2000; O-Charoenrat *et al*, 2000; Gilmour *et al*, 2002; Venkateswarlu *et al*, 2002). Although in only some of these studies constitutive receptor activation, owing to an autocrine loop, was looked at, it may play a role in the other cases as well, rendering it a possible target for future therapies. In line with this is the increased attention that is being given toward receptor activation status in certain cancers, rather than only taking into account receptor levels as a criterium of malignancy (Thor *et al*, 2000).

Overall, it is striking that two of the three HRG-positive cell lines, BLM and MJM, cannot use the secreted HRG in an autocrine loop, because of the absence of HER3 or because of an impaired HER2 activation, respectively. Still, in a physiologic situation, the HRG secreted by such cells may have prominent effects on the surrounding cells, directly or indirectly contributing to malignant progression. Direct effects may include an increased motility of the surrounding keratinocytes (Schelfhout *et al*, 2002), possibly rendering the environment in which the melanocytes reside less tight. Indirect effects may be the stimulation of angiogenesis owing to a HRG-mediated upregulation of vascular endothelial growth factor or increased expression of other growth factors by the target cells (O-Charoenrat *et al*, 2000; Talukder *et al*, 2000; Yen *et al*, 2000; Ruiter *et al*, 2002).

In summary, we have shown the presence of HRG, including one new isoform, as new factors produced and secreted by human melanoma cell lines. The HRG/HER system is functional in melanocytes and in the majority of melanoma cell lines, leading to growth stimulation. Nevertheless, multiple deregulations in this growth factor system may release the melanocytes from their natural dependence on keratinocyte-derived factors and thus represent a step toward melanoma progression. Lack of stimulation by HRG in some melanoma cell lines is due to the loss of expression of HER3 protein or to a severely impaired HER2 activation. In contrast, the aberrant expression and secretion of HRG by melanoma cells may serve as an autocrine and/or paracrine signal, promoting cell growth and/or migration. These distinct types of deregulation of the HRG/HER system may contribute to the malignant phenotype of melanoma cells. In the future, it will be important to verify whether these deregulations are present in tumor samples of melanoma patients and may become a therapeutic target for this disease with ever increasing incidence.

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5.3. Anti-invasive effect of xanthohumol, a prenylated chalcone present in hops (Humulus lupulus L.) and beer. B. Vanhoecke, L. Derycke, V. Van Marck, H. Depypere, D. De Keukeleire, M. Bracke (2005) .International Journal of Cancer;117(6):889-95

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Antiinvasive effect of xanthohumol, a prenylated chalcone present in hops (*Humulus lupulus* L.) and beer

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The female inflorescences of the hop plant (*Humulus lupulus* L.) are essential during brewing to add taste and flavor to beer and to stabilize beer foam. Xanthohumol, the main prenylated chalcone in hops, was investigated for its antiinvasive activity on human breast cancer cell lines (MCF-7 and T47-D) *in vitro*. Xanthohumol was able to inhibit the invasion of MCF-7/6 cells at 5 μM in the chick heart invasion assay and of T47-D cells in the collagen invasion assay. Xanthohumol inhibited growth of MCF-7/6 and T47-D cells, but not of chick heart cells. Moreover, it induced apoptosis of these tumor cells as demonstrated by the cleavage of nuclear PARP after 48 hr treatment. To probe the mechanism of the antiinvasive effect of xanthohumol, involvement of the E-cadherin/catenin invasion-suppressor complex was investigated. An aggregation assay demonstrated stimulation of aggregation of MCF-7/6 cells in the presence of 5 μM xanthohumol and this could be completely inhibited by an antibody against E-cadherin. Xanthohumol upregulates the function of the E-cadherin/catenin complex and inhibits invasion *in vitro*, indicating a possible role as an antiinvasive agent *in vivo* as well.

Key words: breast cancer; xanthohumol; invasion; E-cadherin

Beer has been brewed for thousands of years and has been an integral part of the diet in many cultures. In recent years, researchers have been looking for hard scientific evidence for the potential health benefits of beer and its flavoring component, hop. Xanthohumol is a prenylated flavonoid of the chalcone type, produced in the lupulin glands of the hop cones (hops). Recent studies reported a promising role for xanthohumol as a chemopreventive agent, as it can modulate the carcinogen metabolism and act by cytotoxic/static mechanisms.[1,2] However, the information on chemopreventive activities of xanthohumol is not conclusive.

Invasion is the hallmark of malignancy and the search for antiinvasive agents remains a challenge. Within the large family of flavonoids, interesting molecules of different classes have been shown to possess antiinvasive properties *in vitro*.[3–5] A few antiinvasive agents were detected among many hundreds of polyphenolics tested at our laboratory in the chick heart invasion assay.[6] We found that a number of prenylated chalcones with high homology to xanthohumol were active at low concentrations ($\leq$ 10 μM) and those displayed a selective cytotoxic effect on the cancer cells, but not on normal tissue. As xanthohumol is a naturally occurring structural congener from hops, we wondered whether it would inhibit invasion of human mammary adenocarcinoma cells in the same organotypic invasion assay. The effect of xanthohumol was tested on 3 cell lines derived from human mammary carcinomas (MCF-7/6, MCF-7/AZ and T47-D). These cell lines were selected because they have retained many morphologic and biochemical characteristics of their mammary origin, such as the expression of the estrogen receptor α.[7,8]

In addition, we investigated if xanthohumol could target specific activities implicated in breast cancer progression, namely, growth, cell-cell adhesion and invasion. We focused on a direct form of intercellular communication, namely, cell-cell adhesion via the E-cadherin/catenin complex. This complex is a powerful invasion suppressor and invasiveness has been correlated with its downregulation or functional inactivity at the cell surface.[9] We were interested in the possible invasion-inhibitory action of xanthohumol, examining its effect on cell-cell adhesion mediated by the E-cadherin/catenin complex of human mammary adenocarcinoma cells.

Material and methods

Cell lines and culture media

The mammary adenocarcinoma cell lines were obtained as follows: MCF-7/6 from Dr. H. Rochefort (Unité d'Endocrinologie Cellulaire et Moléculaire, Montpellier, France), MCF-7/AZ from Dr. P. Briand (Fibiger Institute, Copenhagen, Denmark) and T47-D from the American Type Culture Collection (Manassas, VA). Cell lines were maintained in the following media (Invitrogen, Merelbeke, Belgium): 50:50 D-MEM/HAMF12 (MCF-7/6 and MCF-7/AZ) and D-MEM (T47-D). All media contained 10% heat-inactivated fetal bovine serum (Invitrogen), 100 IU/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen) and 2.5 μg/mL amphotericin B (Bristol-Meyers Squibb, Brussels, Belgium). The cells were incubated in a 100% water-saturated atmosphere of 10% CO_2 in air at 37°C. Chick embryonic heart cells were obtained from precultured chick heart fragments (PHFs).[10]

Chemicals and antibodies

Xanthohumol was extracted and purified from hops using the procedure of De Keukeleire *et al.*[11] ^{1}H NMR spectral data were in agreement with those reported in the literature.[12] The compound was dissolved in ethanol as a stock solution of 0.1 M, from which further dilutions were made.

Affinity-purified MB2 (2 μg/mL) is a murine antihuman E-cadherin monoclonal antibody, blocking the cell-cell adhesive function of E-cadherin after binding to the extracellular 80 kD fragment.[13] 5D10,[14] obtained from Dr. L. Plessers (Willems Instituut, Diepenbeek, Belgium), is a murine monoclonal antibody against MCF-7 cells, binding to a plasma membrane epitope unrelated to E-cadherin. The primary antibodies for Western blotting were a monoclonal mouse antihuman poly(ADP)-ribose polymerase (PARP) antibody (Pharmingen, San Diego, CA) and monoclonal mouse anti-α-tubulin clone B-5-1-2 (Sigma, St. Louis, MO). The secondary antibody was an antimouse antibody linked to horseradish peroxidase (Amersham Biosciences Europe, Roosendaal, The Netherlands). Staurosporine (Sigma) was used as a positive control for cleavage of PARP.[15]

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Assays for invasion

Chick heart invasion assay. One assay for invasion was based on the *in vitro* confrontation between cancer cell aggregates and embryonic chick heart fragments in organ culture.[16] Precultured 9-day-old PHFs were selected for a diameter of 0.4 mm and confronted with aggregates of MCF-7/6, MCF-7/AZ, or T47-D cells with a diameter of 0.2 mm. After an overnight incubation on top of semisolid agar, the confronting pairs were cultured in suspension for 8 days. After fixation in Bouin-Hollande's solution, the cultures were imbedded in paraffin, serially sectioned and stained with hematoxylin and eosin. In alternating sections, the cancer cells were stained immunohistochemically with the 5D10 monoclonal antibody in order to evaluate the interaction between the cancer cells and PHF. Invasion was scored as follows: grade 0, only PHF is found and no confronting cells can be observed; grade 1, the confronting test cells are attached to the PHF and do not occupy the heart tissue; grade 2, occupation of the PHF is limited to the outer fibroblast-like and myoblast cell layers; grade 3, the confronting cells have occupied the PHF, but have left more than half of the original amount of heart tissue intact; grade 4, the confronting cells have occupied more than half of the original volume of the PHF.

Cultures were treated with xanthohumol at concentrations ranging from 10^{-9} and 10^{-4} M for 8 days. To avoid breakdown or isomerization of the compound, the medium was refreshed every 2 days according to Stevens *et al*.[17] Each group was tested 3 times. The number of cultures examined for each group was between 15 and 21.

Collagen type I invasion assay. Another assay for invasion was done with MCF-7/6, MCF-7/AZ and T47-D cells on a collagen type I gel.[18] Collagen type I is the main component of the extracellular matrix and, as such, a suitable substrate for *in vitro* invasion studies. Briefly, neutralized collagen type I (0.09%; Upstate, VA) was incubated for 1 hr at 37°C to allow gelification. Single-cell suspensions were prepared in corresponding medium mixed with different concentrations of xanthohumol, placed on top of the collagen gel and cultured at 37°C for 24 hr. The number of cells penetrating into the gel or remaining at the surface was counted using an inverted microscope controlled by a computer program. The invasion index, expressing the percentage of penetrating cells divided by the total number of cells, was calculated. All experiments were done 3 times.

Assays for aggregation

Slow aggregation assay (SAA). Confluent monolayers were detached by a standard trypsinization procedure. A total of 20,000 cells in 200 μL medium was seeded on solidified agar (Sigma) in a 96-well plate (Nunc, Roskilde, Denmark) and treated with test agents. Aggregate formation was evaluated with an inverted microscope after 24 hr incubation.[19] The experiment was done twice.

Fast aggregation assay (FAA). The assay was based on the preparation of a single-cell suspension in E-cadherin-saving conditions followed by quantification of cell aggregation in a Ca^{2+}-containing medium.[19] The suspension was further treated with varying xanthohumol concentrations for 30 min at 4°C and incubated at 37°C for 30 min under continuous shaking. Untreated cells were incubated with 0.1% EtOH. Cells were fixed in 2.5% paraformaldehyde at the start of the incubation (N_0) and after 30 min (N_{30}) and the particle size distribution was measured with a Coulter Particle Size Counter LS 200 (Coulter, Miami, FL). All experiments were done at least 3 times.

Western blotting

Lysates were made from cell cultures at approximately 70% confluency from aggregates of MCF-7/6 and from PHFs. Cells, aggregates and PHFs were washed 3 times with ice-cold PBS before lysis. Cells were lysed with lysis buffer containing 1% Triton X-100, 1% Nonidet P-40 and the following protease inhibitors: aprotinin (10 μg/mL), leupeptin (10 μg/mL; ICN Biomedicals, Asse-Relegem, Belgium), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 μM), $NaVO_3$ (500 μM) and $Na_4P_2O_7$ (500 μg/mL; Sigma). Samples containing equal amounts of protein were prepared by mixing lysates and sample buffer (Laemmli with 5% β-mercaptoethanol) in appropriate amounts and boiling for 5 min. Proteins were separated on a 8% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. Immunostaining of the blots was performed using the primary antibodies followed by the secondary antibody conjugated to horseradish peroxidase and detection by enhanced chemiluminescence reagent (Amersham Biosciences Europe). Quantification of the autoradiograms was done using the Quantity One software (Bio-Rad, Nazareth, Belgium). Experiments were performed at least *in duplo*.

Assays for growth

Measurement of growth in Erlenmeyer flasks. For the chick heart tissue, fragments with a diameter of 450.6 ± 50.4 μm; for MCF-7/6 cells, aggregates of 284.6 ± 19.2 μm; for MCF-7/AZ cells, aggregates of 217.8 ± 19.5 μm; and for T47-D cells, aggregates of 527.1 ± 31.4 μm were transferred to 6 ml Erlenmeyer flasks in 1.5 ml medium in the presence or the absence of varying xanthohumol concentrations and kept in suspension for 8 days on a Gyrotory shaker (72 rpm). Ethanol was used as a solvent control. To avoid breakdown or isomerization of the compound, the medium was refreshed every 2 days.[17] The larger (a) and the smaller (b) diameter of the cultures were measured individually using a macroscope. The volume (v) was calculated in accordance with the formula of Attia and Weiss[20] as follows: $v = 0.4 \times a \times b^2$. All confrontations were done twice.

Measurement of growth in 96-well plates. A total of 5,000 cells (MCF-7/6, MCF-7/AZ, T47-D and cultured embryonic chick heart cells) were seeded in wells of a 96-well plate. After 2 days of attachment and growth, cells were incubated with varying xanthohumol concentrations and the protein content was measured with a colorimetric assay.[21] Briefly, cells were fixed by adding 50 μL 50% trichloroacetic acid to the medium. After 1 hr incubation at 4°C, cells were rinsed with water, dried and stained with 100 μL sulforhodamine B (0.4% in 1% acetic acid) for 30 min. After 4 wash steps with 1% glacial acetic acid, cells were dried and the bound dye was dissolved in 200 μL 10 mM Tris buffer, pH 10.5. The optical density of the solubilized protein-bound stain was measured with an ELISA reader (Molecular Devices, Palo Alto, CA) at 490 nm. Six replicate wells were tested for each condition. Each condition was tested twice.

Morphologic measurement of apoptosis

The morphologic changes of cells during apoptosis were observed with a fluorescence microscope following staining with Hoechst 33258 (ICN). The cells were seeded out on coverslips. Before staining, cells were rinsed twice with PBS and fixed in ice-cold (−20°C) methanol for 10 min. After rinsing, cells were stained with 5 mg/L Hoechst 33258 for 10 min at 37°C in the dark and then visualized with a fluorescence microscope. Apoptotic cells were defined as cells showing nuclear and cytoplasmic shrinkage, chromatin condensation and apoptotic bodies.

Statistics

Statistical evaluation of the data was performed with the Student's *t*-test except for profile comparison in the FAA, where the Kolmogorov-Smirnov method was used.

Results

Xanthohumol inhibits invasion of MCF-7/6 cells into the precultured chick heart fragments in vitro

The invasive properties of 3 breast cancer cell lines (MCF-7/6, MCF-7/AZ and T47-D) were assessed in the chick heart invasion model. Histologic analysis of confronting cultures between MCF-

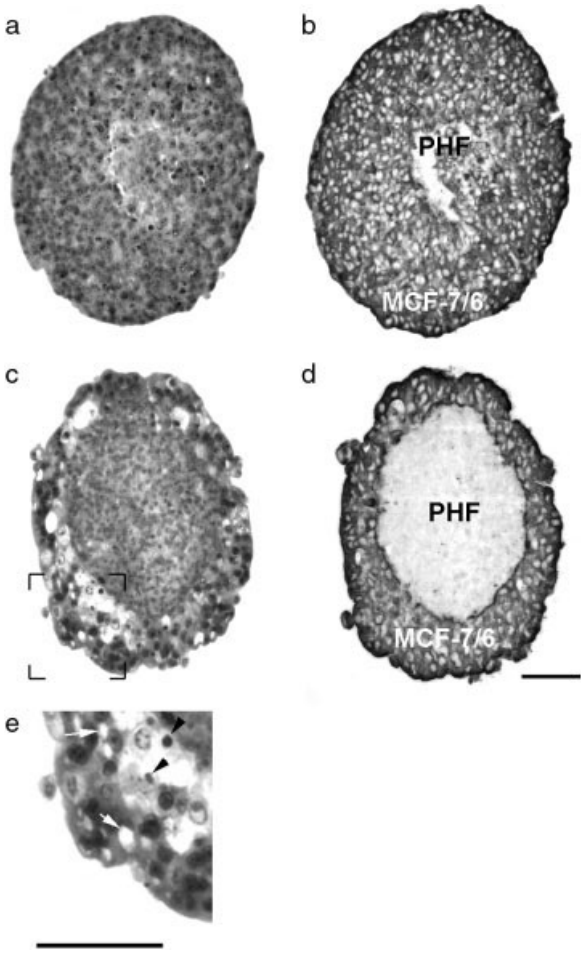

FIGURE 1 – Light micrographs of sections from 8-day-old confronting cultures of precultured heart fragments and MCF-7/6 cells. Untreated confrontations (*a* and *b*) are compared with confrontations treated with 5 μM xanthohumol (X; *c* and *d*). Sections on the left panels were stained with hematoxylin-eosin; in (*b*) and (*d*), MCF-7/6 cells were stained immunohistochemically with the monoclonal antibody 5D10 and appear dark. Scale bar = 50 μm. (*e*) Detailed morphologic analysis of the confronting cultures revealed a selective effect of X on the MCF-7/6 cells. Nuclear pyknosis (arrowheads, black) and vacuolization (arrows, white) could be observed in the MCF-7/6 cells but not in the heart cells. Scale bar = 50 μm.

7/6, MCF-7/AZ, or T47-D cell aggregates and PHF revealed striking differences in invasiveness. Earlier studies also demonstrated the existence of invasive and noninvasive variants of the MCF-7 cell population.[5] Indeed, MCF-7/6 cells did invade spontaneously into the chick heart tissue (Fig. 1*a* and *b*), whereas MCF-7/AZ and T47-D formed a multilayered epithelium covering the heart fragments.

The effect of xanthohumol on invasion of breast cancer cell lines into chick heart fragments was examined during 8 days of treatment in the chick heart invasion assay. Varying xanthohumol concentrations (0.1, 1, 5, 10, 20, 50 and 100 μM) were tested; 5 μM xanthohumol inhibited invasion of MCF-7/6 cells completely (Fig. 1*c*), and the effect could be described as grade 1. Immunohistochemical staining with 5D10, a specific antibody against the MCF-7 cells, confirmed that no cancer cells had invaded the chick heart (Fig. 1*d*), in contrast to the untreated cells (Fig. 1*b*). On the other hand, 1 μM xanthohumol partially inhibited invasion and could be scored as grade 3. Concentrations < 1 μM did not inhibit invasion, as the cultures did not differ from control

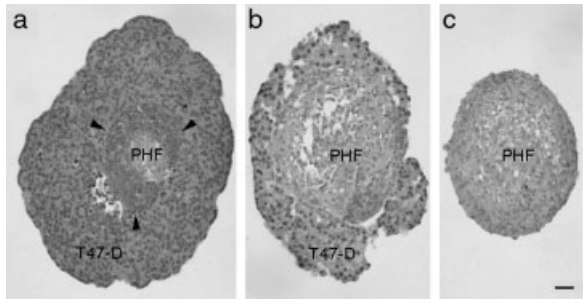

FIGURE 2 – Light micrographs of sections from 8-day-old confronting cultures of precultured heart fragments (arrowheads) and T47-D cells. Untreated confrontations (*a*) are compared with confrontations treated with 1 and 5 μM xanthohumol (X; *b* and *c*). Sections were stained with hematoxylin-eosin. Scale bar = 50 μm.

conditions (grade 4). Higher concentrations (10, 20, 50 and 100 μM) were toxic for both MCF-7/6 cells and chick heart tissue. We ruled out the possibility that the antiinvasive effect of 5 μM xanthohumol was due to irreversible cytotoxicity, since the effect was reversed on removal of xanthohumol from the medium after 8 days of treatment and further culturing the confrontation in xanthohumol-free medium. In addition, we investigated whether pretreatment of the heart tissue was sufficient to block invasion, but invasion did still occur. Thus, it is unlikely that xanthohumol increases resistance of the chick heart tissue against the invasive behavior of the MCF-7/6 cells on pretreatment. In parallel, pretreatment of the cancer cell aggregates alone was unsufficient to block their invasion; thus, treatment with xanthohumol during the confrontation was necessary for the antiinvasive effect.

Furthermore, a selective effect of 5 μM X on the MCF-7/6 cells could be observed: signs of nuclear pyknosis and vacuolization were present in the MCF-7/6 cells but not on the chick heart cells (Fig. 1*e*).

As proinvasive effects of xanthohumol could not be excluded, we investigated this possible effect using MCF-7/AZ and T47-D cells, which are noninvasive in the chick heart invasion assay. However, invasion could not be induced on treatment with xanthohumol. Interestingly, a reduction of T47-D cells could be observed starting from 1 μM while the heart cells stayed intact (Fig. 2).

A second assay for invasion was done to evaluate whether the antiinvasive effect of xanthohumol was restricted to MCF-7/6 cells. First of all, invasiveness of MCF-7/6, MCF-7/AZ and T47-D cells was assessed in the collagen invasion assay. MCF-7/6 and MCF-7/AZ cells were considered noninvasive (1–2%), whereas T47-D invaded strongly the collagen gel (10–15%). Consequently, T47-D cells were chosen to test varying xanthohumol concentrations (5, 10 and 30 μM). A significant concentration-dependent antiinvasive effect could be observed after 24 hr ($p \leq 0.001$; Fig. 3). Trypan blue staining of the cells was done to screen cytotoxicity of the compound. However, no toxicity could be observed after 24 hr of incubation.

To summarize, xanthohumol inhibits the invasive potential of a panel of human breast cancer cells *in vitro*.

Xanthohumol inhibits growth of human breast cancer cells in vitro

To examine if the antiinvasive effect of xanthohumol could be at least partially attributed to a selective growth-inhibitory effect on the cancer cells, we performed different growth assays. Solitary cultures of PHF, MCF-7/6, MCF-7/AZ and T47-D aggregates were brought into suspension separately in the presence of 5 μM xanthohumol or solvent (0.1% EtOH). Volume measurements were done every 2 days at the occasion of refreshment of the medium. After 8 days, the experiment was stopped and cultures were processed for histologic analysis. After 8 days, PHF control

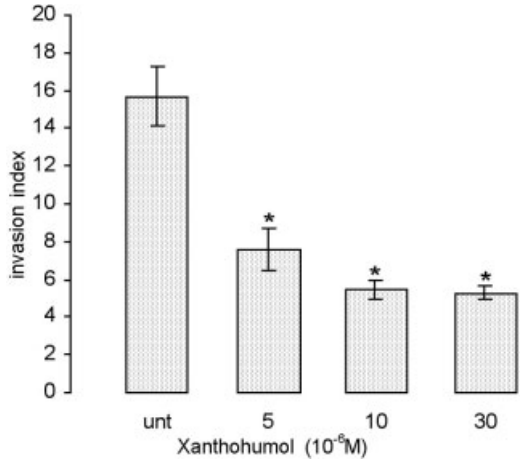

FIGURE 3 – Effect of xanthohumol on invasion of T47-D cells into collagen type I gel. Cells were treated with varying concentrations (5, 10 and 30 μM) during 24 hr. Results are presented as percentage (%) of invasion (percentage of penetrating cells divided by the total number of cells; mean ± standard deviation). The % of invasion of xanthohumol-treated T47-D cells is compared with the % of invasion of solvent-treated cells (unt). Asterisks, $p \leq 0.001$.

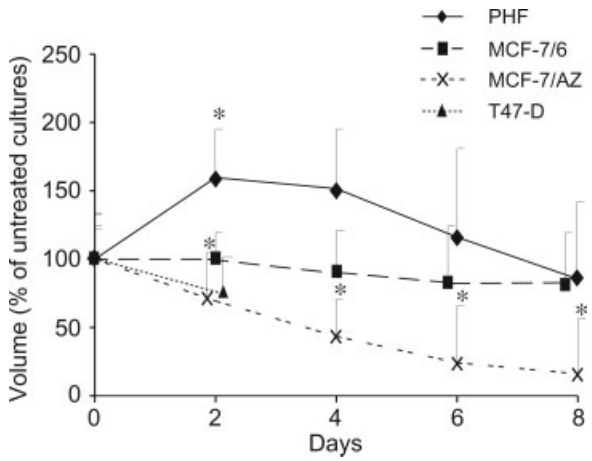

FIGURE 4 – Effect of 5 μM xanthohumol on the growth of PHFs and MCF-7/6, MCF-7/AZ and T47-D aggregates. The volumes of cultures treated for 8 days are presented as a percentage of the volumes of untreated cultures (mean ± standard deviation). The volume of xanthohumol-treated cultures is compared with the volume of solvent-treated cultures corrected for their initial main volumes. Asterisks, $p \leq 0.01$.

cultures had decreased their initial volume by ± 30%. The volume of solvent-treated cancer cell aggregates increased during 8 days by ± 100% (MCF-7/6), ± 400% (MCF-7/AZ) and ± 450% (T47-D; data not shown). Xanthohumol-treated PHF showed a significant increase in volume during the first 2 days of incubation compared to control cultures ($p \leq 0.01$); however, after 8 days the volume did not differ from the solvent-treated PHF cultures (Fig. 4). The effect of 5 μM xanthohumol on the volume of cancer cell aggregates was dependent on the cell type. The xanthohumol-treated MCF-7/6 aggregates did not differ from the untreated cultures, in contrast to MCF-7/AZ and T47-D aggregates, where a strong decrease could be observed in function of time. Volumes of MCF-7/AZ aggregates were reduced 4 times compared to untreated cultures. T47-D aggregates appeared to be even more sensitive than MCF-7/AZ as after 2 days of incubation with 5 μM xanthohumol, aggregates completely fell apart into single cells ($p \leq 0.01$).

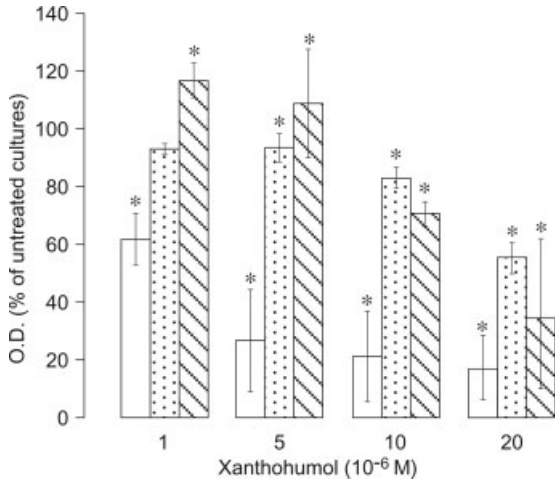

FIGURE 5 – Effect of xanthohumol on sulforhodamine B-staining of T47-D (white bars), MCF-7/6 (dotted bars) and embryonic chick heart cells (dashed bars). The cells were treated for 8 days with 0, 1, 5, 10 and 20 μM X. The effects are presented as a percentage of optical density (O.D.) of untreated cells (mean ± standard deviation). The protein content of xanthohumol-treated cells is compared with the protein content of the solvent-treated cells. $p \leq 0.01$.

Histologic staining of 2- and 8-day-old cultures of PHF showed no morphologic changes of the xanthohumol-treated PHF tissue when compared to the control cultures (data not shown). In contrast, nuclear pyknosis and large vacuoles were present in the xanthohumol-treated MCF-7/6 aggregates, although the volume did not change during the treatment. This is in agreement with the observations in the chick heart invasion assay (Fig. 1*e*).

A second growth assay was performed on MCF-7/6, T47-D and chick embryonic heart cells cultured in a 96-well plate and stained with sulforhodamine B. After 8 days of treatment with varying xanthohumol concentrations (1, 5, 10 and 20 μM), a significant increased signal from the chick heart cultures was noted when incubated with 1 and 5 μM xanthohumol ($p \leq 0.01$) compared to solvent-treated cultures (Fig. 5). This effect was already observed after 24 hr (data not shown). However, higher concentrations dramatically decreased the growth of the heart cells. In contrast to a growth-stimulatory effect on chick heart cultures, a growth-inhibiting effect of xanthohumol was evident on both breast cancer cells. T47-D cells were, again, more sensitive in all conditions than MCF-7/6, as treatment with concentrations as low as 1 μM already reduced the proliferation with ± 40%.

To summarize, xanthohumol inhibited the growth of the breast cancer cells, while the effects on chick heart were depending on the assay type and the treatment dose.

Xanthohumol stimulates apoptosis of human breast cancer cells in vitro

Reduced growth is the net result of reduced proliferation and/or increased cell death. The chick heart invasion assay revealed a strong selective effect on the morphology of the MCF-7/6 cells, whereas chick heart cells remained intact. Nuclear pyknosis and formation of vacuoles suggested signs of cell death (Fig. 1*e*). Therefore, the effect of xanthohumol on apoptosis of a panel of breast cancer cells was investigated. First, a Hoechst 33258 staining revealed the appearance of nuclear condensation in xanthohumol-treated cultures (Fig. 6*a* and *b*). We additionally examined cleavage of the nuclear protein, PARP, which is an indicator of caspase-dependent apoptosis. MCF-7/6 and T47-D cells were treated for 48 hr in the presence or the absence of varying xanthohumol concentrations. Staurosporine (1 μM) was used as a positive control for PARP cleavage.[15] Cell lysates were used for Western blotting and blots were stained with an antibody against

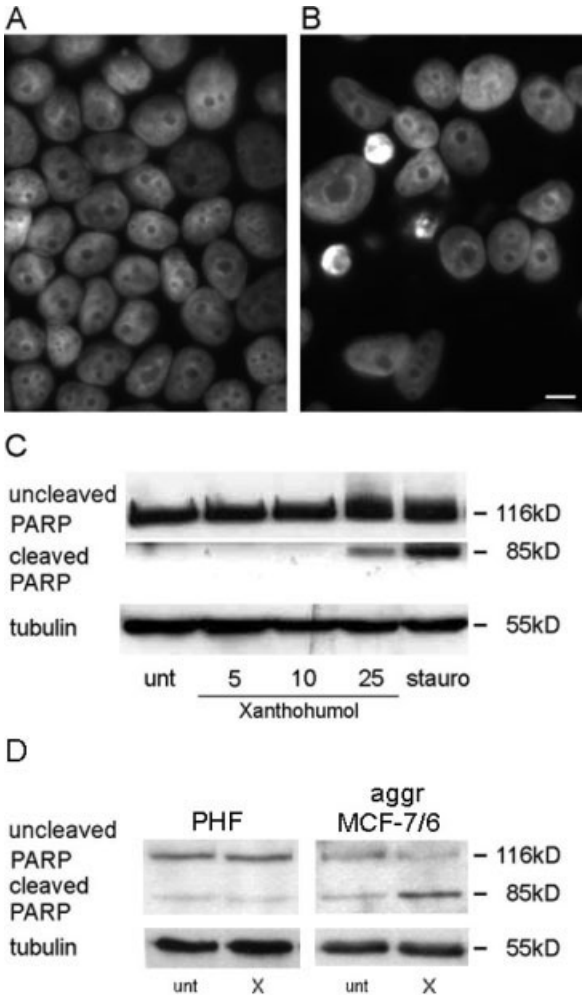

FIGURE 6 – Hoechst 33258 staining of the nuclei of MCF-7/6 cells: (*a*) demonstrates the solvent-treated culture (EtOH) and (*b*) the 10 μM xanthohumol-treated cultures. The appearance of nuclear condensation could be observed after 96 hr treatment with xanthohumol. (*c*) Immunodetection of uncleaved and cleaved PARP in lysates of T47-D cells. The cells were incubated with varying xanthohumol concentrations (5, 10 and 25 μM) for 48 hr. 0.1% EtOH-treated cells were used as control (unt). 1 μM staurosporine (stauro) was used as a positive control for PARP cleavage. Western blotting and immunostaining were performed. Note that the antibody recognizes both uncleaved PARP (116 kD) and the larger cleavage fragment (85 kD). α-tubulin-staining was used as a control for loading. (*d*) Immunodetection of uncleaved and cleaved PARP in lysates of PHFs and MCF-7/6 aggregates (aggr MCF-7/6). The PHFs and cancer cell aggregates were brought into suspension and incubated with 0.1% EtOH (unt) or 25 μM xanthohumol (X) for 48 hr. Western blotting and immunostaining was performed. α-tubulin-staining was used as a control for loading.

human PARP. On treatment with xanthohumol, both cell lines showed sensitivity to PARP cleavage, as illustrated for T47-D cells (Fig. 6*c*). In parallel, PARP cleavage could be observed in MCF-7/6 cells, although they are caspase-3-deficient (data not shown).

In addition, we examined the effect of xanthohumol on the proteolysis of PARP in cancer cell aggregates (MCF-7/6) and PHFs. The aggregates and PHFs were brought into suspension in the presence of 25 μM xanthohumol or solvent (0.1% EtOH). After 48 hr, cell lysates were made and Western blotting was performed. Treatment of PHFs did not increase the cleavage of PARP compared to solvent-treated PHFs, where some spontaneous cleavage could be observed after 48-hr culturing (Fig. 6*d*, PHF). In contrast,

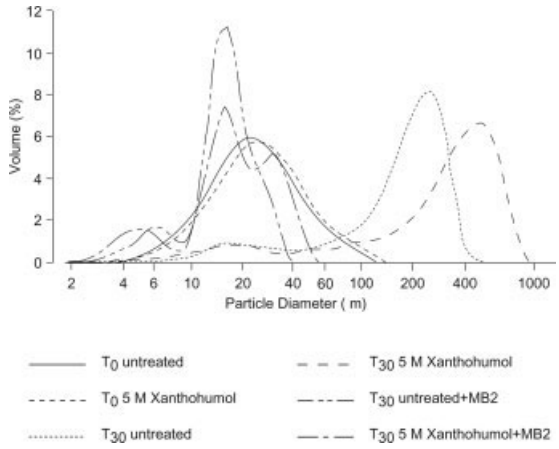

FIGURE 7 – Effect of xanthohumol (X) on cell-cell adhesion in the FAA. The volume percentage distribution is presented plotted against the particle diameter of MCF-7/6 aggregates at time 0 in the presence (T_0 5 μM Xanthohumol) and the absence (T_0 untreated) of X and after 30 min in the presence (T_{30} 5 μM Xanthohumol) and the absence (T_{30} untreated) of X. In addition, the effect of MB2, an antibody against the extracellular part of E-cadherin, on cell-cell adhesion is evaluated in the assay. The particle diameter of the MB2-treated MCF-7/6 aggregates after 30 min in the presence (T_{30} 5 μM xanthohumol + MB2) and the absence (T_{30} untreated + MB2) of X is presented.

a strong cleavage fragment of 85 kD was detected in the xanthohumol-treated MCF-7/6 aggregates compared to the control aggregates (Fig. 6*d*, Aggr MCF-7/6).

To summarize, xanthohumol appeared to induce apoptosis of human breast cancer cells *in vitro*.

Xanthohumol stimulates E-cadherin-mediated cell-cell adhesion of human breast cancer cells in vitro

In SAA, numerous irregular and small aggregates were formed in the untreated cultures of MCF-7/6, whereas MCF-7/AZ and T47-D cells aggregated spontaneously and showed compaction. Treatment with varying xanthohumol concentrations did not change the aggregation pattern, *i.e.*, no stimulation of aggregation of MCF-7/6 could be observed, nor inhibition of aggregation of MCF-7/AZ and T47-D cells.

Consequently, the effect of xanthohumol on cell-cell aggregation was tested in FAA (Fig. 7). Cells were pretreated for 24 hr with varying xanthohumol concentrations followed by trypsinization in E-cadherin-saving conditions to preserve E-cadherin-mediated cell-cell adhesion during the assay. In the presence of a Ca^{2+}-containing aggregation buffer, cells were allowed to aggregate for 30 min in the presence or the absence of varying xanthohumol concentrations. The initial mean particle diameter of the MCF-7/6 cells at time 0 was 27.65 μm (standard deviation = 19.03) for solvent-treated cells (Fig. 7, T_0 untreated) and 30.27 μm (standard deviation = 21.68) for the 5 μM xanthohumol-treated cells (Fig. 7, T_0 5 μM Xanthohumol), indicating suspensions of single cells or cell doublets in both conditions. After 30 min, the mean particle diameter shifted toward 177.4 μm (standard deviation = 97.58) for solvent-treated cells, indicating aggregates of around 6 cells (Fig. 7, T_{30} untreated). However, for xanthohumol-treated cells, aggregates of around 11 cells were formed and the mean particle diameter shifted toward 328.2 μm (standard deviation = 211.5; Fig. 7, T_{30} 5 μM Xanthohumol). Lower concentrations were not active. These increases were statistically significant as shown by the Kolmogorov-Smirnov statistics ($p < 0.05$). A 24-hr pretreatment with xanthohumol was necessary to stimulate aggregation.

The implication of E-cadherin was indicated by a neutralizing monoclonal antibody, MB2, which functionally blocks the mole-

cule. Cotreatment with xanthohumol and MB2 (Fig. 7, T_{30} 5 μM xantohumol + MB2) during aggregation completely abrogated the effect of 5 μM xanthohumol (Fig. 7, T_{30} 5 μM Xanthohumol).

To summarize, xanthohumol appeared to stimulate E-cadherin-mediated cell-cell adhesion of human breast cancer cells *in vitro*.

Discussion

Using the chick heart invasion assay, we have investigated the action of xanthohumol on invasion in organotypic confrontations. This differs from a cell culture by the fact that the cells are not adherent to an artificial substrate, but present in a 3-dimensional architecture. Our model involves fragments of normal chick heart tissue, mainly composed of cardiomyocytes, fibroblasts, endothelial cells and extracellular matrix, to which the cancer cells adhere to proliferate and invade. Factors produced by both the cancer cells and the host cells, and also those present in the culture medium, may affect different activities of both cancer and host cells: proliferation, apoptosis, angiogenesis, proteolysis, cell-matrix adhesion, cell-cell adhesion and motility. Our results showed that, after 8 days of confrontation, the cancer cells had already invaded extensively into the heart tissue of untreated cultures, while in the xanthohumol-treated culture no cancer cell invasion could be observed. Moreover, in contrast to the heart that looked healthy, morphologic analysis of the cancer cells revealed signs of pyknosis accompanied by intense vacuolization. We concluded that xanthohumol inhibits invasion presumably by exerting a selective growth-inhibiting effect on the cancer cells. Earlier studies performed in our laboratory have shown a similar selectivity, *i.e.*, when B16-BL6 mouse melanoma cells are cocultured with mouse PHF and treated with a combination of TNF-α and INF-γ.[22] This treatment selectively killed the melanoma cells and left the heart tissue intact.

Interestingly, we already described antiinvasive activity of structural analogs of xanthohumol.[6,23,24] Looking for alkaloids and polyphenolics that could possibly affect invasiveness, compounds belonging to various classes such as methoxyflavones, chalcones and coumarins were evaluated by the chick heart invasion assay. The antiinvasive activity was frequently found among chalcones having a prenyl group. Our results add xanthohumol to the list of prenylated chalcones with antiinvasive activity.

The disposal of invasive (MCF-7/6) and noninvasive variants (MCF-7/AZ and T47-D) allowed us to investigate both inhibitory and stimulatory effects on invasion by xanthohumol. A proinvasive effect on the tested breast cancer cell lines in the *in vitro* invasion assays was absent. The noninvasive variants did not become invasive (MCF-7/AZ and T47-D in the chick heart invasion assay; MCF-7/6 and MCF-7/AZ in the collagen invasion assay). This differs from drugs such as retinoic acid[25] and alkyllysophospholipid,[26] which have been evaluated in the same models.

In vitro, many activities of cancer cells can be downregulated by flavonoids. Effects on growth and on the mechanisms underlying this activity are being intensively studied. In particular, the antiproliferative activity of prenylated chalcones on different cancer cell lines has been reported by different groups.[1,2] To assess the antiproliferative potential of xanthohumol on human breast cancer cells, Gerhauser *et al.*[1] investigated its influence on DNA synthesis of MDA-MB-435 cells in an *in vitro* system and found that xanthohumol inhibited the activity of human DNA polymerase α with an IC_{50} of 23.0 ± 3.5 μM. In addition, they found an accumulation of the MDA-MB-435 cells in the S-phase of the cell cycle and induction of terminal differentiation of HL-60 human promyelocytic leukemia cells. Our growth studies and histologic analyses confirmed this antiproliferative effect on a panel of breast cancer cells.

In addition, we found that xanthohumol induced apoptosis of breast cancer cells and aggregates, as evidenced by the cleavage of PARP. PARP (116 kD), a nuclear substrate of caspases, is cleaved into 2 fragments, an N-terminal DNA-binding domain (24 kD) and a C-terminal catalytic domain (85 kD). As MCF-7/6 cells are deficient in caspase-3, one of the key executioners of the intrinsic apoptotic pathway, PARP must be the target of other caspases such as caspases-7 and -9 or of cathepsins on treatment with xanthohumol.[15,27,28] Additional tests are needed to unravel the mechanism of this proapoptotic effect.

As epithelial cells are critically dependent on cell-matrix and cell-cell adhesion for growth and survival, the effect of xanthohumol on adhesion of breast cancer cells was also a subject for investigation. We examined the cell-cell adhesion of the human MCF-7/6 breast cancer cells with a functionally defective E-cadherin/catenin complex. Xanthohumol was able to stimulate aggregation of MCF-7/6 cells in suspension, restoring the function of the complex. These effects could be inhibited by MB2, a monoclonal antibody against the extracellular domain of E-cadherin. Previous experiments have shown that IGF-I, tamoxifen and retinoic acid can upregulate the function of this complex in the same aggregation assay[25,29,30] and are inhibitors of invasion. In addition, phytoestrogens such as genistein, daidzein and equol (data not shown) and 8-prenylnaringenin from hops[5] were found to stimulate cell-cell adhesion *in vitro*. However, these flavonoids were unable to inhibit invasion in chick heart fragments. In this respect, they mimicked the effects of 17β-estradiol, which is a promotor of cell-cell adhesion[5] and growth of MCF-7/6 cells, but is unable to block invasion. Other published inhibitory effects of xanthohumol, such as on cyclooxygenase 1 and 2 activity and on estrogen reactivity,[1] may contribute to its antiinvasive activity. Many reports have pointed toward the stimulating role of prostaglandins on mammary cancer invasion,[31–33] and inhibition of cyclooxygenases has been associated with inhibition of tumor invasion. The role of estrogens/antiestrogens in mammary cancer invasion, however, is a matter of debate, but recent data indicate an antiinvasive, rather than proinvasive, effect of estrogens on mammary cancer cells.[34,35]

In conclusion, xanthohumol has multiple effects on human breast cancer cells *in vitro*. It elicits an indirect effect on invasion through a decrease in the number of invasive cells. However, although growth and invasion are part of the invasion program of tumors,[36] growth inhibition is not *per se* linked to inhibition of invasion. Although xanthohumol is present in beer, these effects cannot be achieved by drinking beer as during the brewing process, xanthohumol is mainly converted to its flavanone isomer, isoxanthohumol, resulting in low xanthohumol concentrations in beer (5–800 μg/L).[17] However, agents that inhibit cancer cell invasion by selective killing of cancer cells, inhibiting their growth, or stimulating cell-cell adhesion between cancer cells should be considered as potential therapeutic drugs in cancer.

Acknowledgements

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P-Cadherin Is Up-Regulated by the Antiestrogen ICI 182,780 and Promotes Invasion of Human Breast Cancer Cells

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ABSTRACT

P-cadherin expression in breast carcinomas has been associated with tumors of high histologic grade and lacking estrogen receptor-α, suggesting a link between these proteins. In the MCF-7/AZ breast cancer cell line, blocking estrogen receptor-α signaling with the antiestrogen ICI 182,780 induced an increase of P-cadherin, which coincided with induction of *in vitro* invasion. Retroviral transduction of MCF-7/AZ cells, as well as HEK 293T cells, showed the proinvasive activity of P-cadherin, which requires the juxtamembrane domain of its cytoplasmic tail. This study establishes a direct link between P-cadherin expression and the lack of estrogen receptor-α signaling in breast cancer cells and suggests a role for P-cadherin in invasion, through its interaction with proteins bound to the juxtamembrane domain.

INTRODUCTION

Classical cadherins are a superfamily of transmembrane glycoproteins responsible for calcium-dependent cell–cell adhesion, mediating homophilic protein interactions (1). These are modulated by their conserved cytoplasmic juxtamembrane domain and catenin-binding domain, linking them to the actin cytoskeleton. β-, γ-, p120-, and α-Catenins are the best-documented interaction partners (2). β-Catenin (and perhaps also γ-catenin) is a signaling molecule, implicated in tissue patterning, of which the functions are regulated by binding to the catenin-binding domain of cadherins and by interactions with receptor tyrosine kinases and transcription factors of the lymphocyte enhancer factor/T-cell factor family (2). P120-catenin was identified as a substrate for *Src* and several receptor tyrosine kinases and interacts directly with the juxtamembrane domain of cadherins, modulating cadherin clustering and cell motility in a cell-type and phosphorylation state-dependent way (3). The cadherin/catenin junctional complex is linked to the actin cytoskeleton via α-catenin, thus strengthening its adhesive force (1).

Reduced expression of E-cadherin is associated with tumor progression in many different cancers, including breast cancer (4), and may result from mutations, loss of heterozygosity, promoter hypermethylation, or up-regulation of transcriptional repressors, as SIP1, Snail, Slug, or Twist (1). Moreover, the invasion suppressor function of normally expressed E-cadherin may be overcome by the aberrant expression of N-cadherin (5) or cadherin-11 (6), which have been associated with progression of breast carcinoma through interference with E-cadherin function (7).

P-cadherin, another classical cadherin, is expressed in ectodermal tissues, more specifically in the basal layers of stratified epithelia (8, 9) and in myoepithelial cells of the breast (10). P-cadherin is implicated in growth and differentiation, as evidenced by knockout mice displaying precocious differentiation of the mammary gland (11), and is aberrantly expressed in mammary carcinomas of high histologic grade and with a poor prognosis (12–16), as well as in other types of carcinomas and proliferative inflammatory lesions (17–19). It has been suggested that suppression of the P-cadherin gene is lost during carcinogenesis (9), but the nature of this mechanism and the biological role of the newly acquired P-cadherin remain to be investigated.

Because aberrant expression of P-cadherin identified a subgroup of estrogen receptor-α-negative breast carcinomas (16), we raised the hypothesis that the expression of P-cadherin in mammary epithelial cells is hormonally regulated, as described for E-cadherin (20), N-cadherin (21), and cadherin-11 (22).

In mammary epithelial cells, estrogen receptor-α is a key regulator of proliferation and differentiation and a crucial prognostic indicator and therapeutic target in breast cancer. Estrogen receptor-α is a ligand-dependent transcription factor acting through direct transcriptional target activation (23). Estradiol acts as a potent mitogen for many breast cancer cell lines, and ~70% of breast carcinomas are estrogen receptor-α positive. This mitogenic effect is blocked by estrogen antagonists. Pure antiestrogens (like ICI 182,780) and selective estrogen receptor modulators (like tamoxifen; ref. 24) are used for the treatment of osteoporosis, breast cancer, and other diseases. Continuous exposure of steroid–hormone-responsive breast cancer cell lines to ICI 182,780 leads to resistant sublines, with signaling pathways alternative to estrogen receptor-α (25). Similarly, in breast cancer, a high number of patients eventually develop antiestrogen resistance for unknown reasons.

Using the antiestrogen ICI 182,780, we investigated a putative molecular and functional link between the absence of estrogen receptor-α signaling and P-cadherin expression in breast cancer cells. To understand the relationship between P-cadherin and the aggressive breast cancer phenotype, we studied the effect of wild-type P-cadherin and several mutants on cell aggregation and invasion. We report that aberrant expression of P-cadherin may result from a lack of estrogen receptor-α signaling and may induce cell invasion in a juxtamembrane domain-dependent manner.

MATERIALS AND METHODS

Plasmids and cDNA Constructs. The hP-cad/pBR322–23-b expression vector, containing the 3.2kb cDNA encoding full-length human P-cadherin (8), was kindly provided by Prof. Keith R. Johnson (Department of Oral Biology, College of Dentistry and the Eppley Cancer Center, Nebraska Medical Center, Omaha, NE), with the permission from Prof. Yukata Shimoyama (Department of Surgery, International Catholic Hospital, Nakaochiai, Shinjuku, Tokyo, Japan). The cDNA encoding full-length mouse E-cadherin was kindly provided by Jolanda van Hengel (Department of Molecular Biomedical Research, VIB-Ghent University, Ghent, Belgium). Both cDNAs (PC-WT and mEC-WT) were transferred to the expression vector pIRES2-EGFP (Clontech, Palo Alto, CA), allowing easy evaluation of transfection efficiencies due to co-

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Note: J. Paredes and C. Stove contributed equally to this article and should be considered as first authors. Supplementary data for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org).

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expression of enhanced green fluorescent protein (EGFP). To generate P-cadherin deletion mutants, P-cadherin was *Eco*RI subcloned into pBluescript (Promega, Madison, WI) and *Nde*I/*Sal*I digested to remove the region encoding its COOH-terminal tail. PCR fragments corresponding to different lengths of the removed tail, flanked by *Nde*I/*Sal*I restriction enzyme digest sites at the 5′ and 3′ ends, respectively, were obtained always using the same sense primer (5′-AAGCAGGATACATATGACGTG-3′) and different antisense primers for the following constructs: PC-CT682: 5′-CGTGCCGTCGACCTACTTCCG-CTTCTT-3′; PC-CT702: 5′-CGTGCCGTCGACCTAGCCATAGTAGAA-3′; PC-CT711: 5′-CGTGCCGTCGACCTACTGGTCCTCTTC-3′; PC-CT719: 5′-CGTGCCGTCGACCTAGTGGAGCTGGGT-3′; PC-CT727: 5′-CGTGC-CGTCGACCTACTCCGGCCTGGC-3′; and PC-CT762: 5′-CGTGCCGTC-GACCTACAGGTTCTCAAT-3′. After *Nde*I/*Sal*I digestion, these products were ligated into *Nde*I/*Sal*I digested pP-cad-Bluescript, and the resulting construct was *Eco*RI/*Sal*I transferred to pIRES2-EGFP. Additionally, a mutant with a small deletion in the P120-catenin-binding sequence (lacking the nucleotides coding EEGGG) and retaining the intact catenin-binding domain was created (PC-Δ703–707). Therefore, pP-cad-IRES2-EGFP was *Xho*I/*Sma*I digested, and the removed fragment was cut with *Ear*I. After removal of the small fragment between the two *Ear*I restriction sites (encoding EEGGG), the two remaining fragments (*Xho*I/*Ear*I and *Ear*I/*Sma*I) were ligated into *Xho*I/*Sma*I digested pP-cad-IRES2-EGFP. To create the P-cadherin point mutant (PC-R503H), a PCR product, encompassing the point mutation, was obtained, using the following primers: a sense primer (5′-GGCACCC*TCGAC***CAT**-GAGGATGAG-3′), with the *Taq*I restriction site in italics and the point mutation in bold; and the antisense primer used for generating PC-CT762. This product was *Taq*I/*Nde*I digested and used in a three-point ligation with a *Bam*HI/*Taq*I and a *Bam*HI/*Nde*I fragment of pP-cad-Bluescript, followed by *Eco*RI transfer of the construct to pIRES2-EGFP. Direct sequencing (ABI, Perkin-Elmer, Foster City, CA) was performed for all of the constructs to confirm their integrity.

Restriction Enzymes, Antibodies, and Chemical Reagents. All of the restriction enzymes were purchased from New England BioLabs (Beverly, MA). Antihuman primary mouse monoclonal antibodies used were against P-cadherin (clone 56) and P120-catenin (clone 98; BD Transduction Laboratories, Lexington, KY), N-cadherin (CH-19 and GC-4), α-tubulin (B-5–1-2; Sigma-Aldrich, Bornem, Belgium), E-cadherin (HECD-1; Takara Biochemicals, Kyoto, Japan), and estrogen receptor-α (NCL-L-ER-6F11; Novocastra, Newcastle, United Kingdom). 17β-Estradiol was purchased from Sigma-Aldrich Química (Sintra, Portugal) and ICI 182,780 was kindly provided by AstraZeneca (Barcarena, Portugal). Both drugs were dissolved in 100% EtOH and added to the culture media. The concentrations used were 10 nmol/L for 17β-estradiol and 100 nmol/L for ICI 182,780, unless mentioned otherwise. Cycloheximide was obtained from Sigma and used at 25 μg/mL. For the control situations, cells were treated only with 100% EtOH.

Cells and Transient Transfection. Human cancer cell lines were obtained as described: BT-20 from Peter Coopman (Laboratory of Molecular Biology, Ghent University, Belgium), MCF-7/AZ (MCF7) from Per Briand (The Fibiger Institute, Copenhagen, Denmark), ZR-75.1 and T47D from American Type Culture Collection (Manassas, VA), and HEK 293T (HEK) cells from Veerle De Corte (Department of Biochemistry, Faculty of Medicine and Health Sciences, VIB-Ghent University, Belgium). Cell lines were routinely maintained at 37°C, 10% CO_2, in the following media (Invitrogen, Merelbeke, Belgium): 50% DMEM/50% HamF12 (MCF7), DMEM (BT-20, T47D, HEK), or RPMI 1640 (ZR-75.1). All of the media for routine culture contained 10% heat-inactivated fetal bovine serum (Greiner bio-one, Wemmel, Belgium), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (Invitrogen). To obtain transient transfectants, appropriate expression vectors (2.5 μg) were introduced into HEK cells (2×10^5) with Fugene (Roche Molecular Biochemicals, Mannheim, Germany), and transfection efficiencies were evaluated by measuring EGFP expression by flow cytometry.

Retroviral Transduction. The P-cadherin coding sequence was *Eco*RI digested from pIRES2-EGFP and *Eco*RI subcloned into the LZRS-IRES-EGFP vector to generate the LZRS-P-cad-IRES-EGFP vector. The LZRS-IRES-EGFP retroviral vector, encoding only EGFP, was used as a control. For the production of retroviral supernatant, the Phoenix-Amphotropic packaging cell line (a kind gift from Dr. Garry P. Nolan, Stanford University School of Medicine, Stanford, CA) was transfected with the LZRS-IRES-EGFP and the LZRS-P-cad-IRES-EGFP plasmids using calcium-phosphate precipitation (Invitrogen) to generate both retroviruses. The viral supernatant was spun (10 minutes at 350 × *g*), and aliquots were stored at −70°C until use. Transduction of cell lines was performed as described before (26).

Flow Cytometry Staining and Cell Sorting. For analysis of E- and N-cadherin surface expression, cells were detached under cadherin saving procedures (27), and $\sim 1 \times 10^5$ cells were used for staining. Cells were washed with cold PBS containing bovine serum albumen (BSA) and incubated for 30 minutes with the anti-E-cadherin HECD-1 or anti-N-cadherin GC-4 antibodies. This was followed by two washes with PBS/BSA, 30 minutes incubation with biotinylated rabbit antimouse monoclonal antibody, two washes with PBS/BSA, 20 minutes incubation with streptavidin-phycoerythrin, and a final wash with PBS/BSA. For routine analysis of EGFP expression, cells were detached with trypsin/EDTA. Flow cytometric analysis and/or cell sorting were performed as described before (26).

Biotinylation, Immunoprecipitation, and Immunoblotting. Immunoprecipitation and immunoblotting experiments and quantification of bands, were performed as published before (28). For biotinylation, the cells were washed three times with cold PBS and incubated with 0.5 mg/mL of Biotinylation Reagent (EZ-Link Sulfo-NHS-LC-Biotin, Pierce) during 30 minutes at 4°C, followed by four washes with cold PBS before cell lysis. To control for equal loading of total lysates, immunostaining with anti-α-tubulin was performed routinely (not always shown). Each immunoblot was done at least three times, and the ones that were selected to show are representative experiments.

Reverse Transcription-PCR. Reverse transcription-PCR (RT-PCR) experiments were done as described previously (28). Primers specific for P-cadherin cDNA included the following: sense 5′-ACGAAGACACAA-GAGAGATTGG-3′ and antisense 5′-CGATGATGGAGATGTTCATGG-3′, to generate a 287-bp product. PCRs were done in a Minicycler (Biozym, Landgraaf, the Netherlands) with an annealing temperature of 55°C.

Slow Aggregation Assays. For semi-solid substratum, 2×10^4 cells in 200 μL medium were seeded on solidified agar in a 96-well plate (27). Aggregate formation was evaluated under an inverted microscope after 24, 48, and 72 hours. In suspension, 6×10^5 cells were added to 50 mL-Erlenmeyer flasks in 6 mL of medium. The flasks were incubated on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, NJ) at 72 rpm and continuously gassed with humidified 10% CO_2 in air. The particle size distribution of the aggregates was measured with a Coulter Particle Size Counter (LS2000, Coulter Company, Miami, FL). The diameter of the particles can be considered as a measure for aggregate formation. Statistical analysis of differences between the particle size distribution curves was done with the Kolmogorov-Smirnov method.

Invasion Assays. For collagen type I invasion assay (29), six-well plates were filled with 1.3 mL of neutralized collagen type I (0.09% w/v, Upstate Biotechnology, Inc., Lake Placid, NY) and incubated for at least 1 hour at 37°C to allow gelification. 1×10^5 cells of a single-cell suspension were seeded on top of the gel, and cultures were incubated at 37°C for 24 hours. Using an inverted microscope controlled by a computer program, invasive and superficial cells were scored blind-coded in 12 fields of 0.157 mm^2. The invasion index expresses the percentage of cells invading into the gel over the total number of cells counted. For Matrigel invasion assay, transwell chambers with polycarbonate membrane filters (6.5 mm diameter, 8 μm pore size, Costar, Corning, NY) were coated with 20 μL of a Matrigel solution (Becton Dickinson). 1×10^5 cells were added to the upper compartment of the chamber. In the lower compartment, conditioned cell culture medium of the MRC-5 human embryonic lung fibroblast cell line was added as a chemoattractant. After 24 hours of incubation at 37°C, the upper surface of the filter was cleared from nonmigratory cells with a cotton swab and washes with serum-free DMEM. The remaining (invasive) cells at the lower surface of the filter were fixed with cold methanol and stained with 4′, 6-diamidino-2-phenylindole (Sigma, 0.4 mg/mL). Invasive cells were scored by counting 50 fields per filter with a fluorescence microscope, at ×250 of magnification. Rat myofibroblast DHD-FIB cells were routinely included as a positive control for invasion in both assays. Each experiment was repeated at least three times. For collagen invasion assay, data are expressed as mean ±SD; for Matrigel invasion assay, a representative experiment is shown, with the SD for the number of cells scored on the 50 microscopic fields. Statistical significance was determined by *t* test, and differences between groups were analyzed using the ANOVA; $P < 0.05$ was considered significant.

RESULTS

The Antiestrogen ICI 182,780 Up-Regulates P-Cadherin in Estrogen Receptor-α–Positive Breast Cancer Cell Lines. To test the hypothesis that estrogen receptor-α negatively regulates P-cadherin, we examined the expression of estrogen receptor-α and cadherins in breast cancer cell lines by Western blot (Fig. 1*A*). Interestingly, higher levels of P-cadherin were found in estrogen receptor-α–negative BT-20 cells.

A 24-hour treatment with the antiestrogen ICI 182,780 (10^{-7} mol/L) increased P-cadherin protein levels in MCF7 and ZR-75.1 cells but not in BT-20 cells (Fig. 1*B*). There were no significant changes in P-cadherin levels observed in T47D cells, bearing already higher pretreatment levels of P-cadherin and lower levels of estrogen receptor-α than the responsive cell types. ICI 182,780-induced increase of P-cadherin was associated with a decline of estrogen receptor-α levels (Fig. 1*B*).

For additional investigation, we chose the MCF7 cell line, because it is estrogen receptor-α positive, highly responsive to estrogen, and extensively investigated as a model of breast cancer. In these cells, ICI 182,780 induced, respectively, up- and down-regulation of P-cadherin and estrogen receptor-α in a time- and dose-dependent way (Fig. 2, *A* and *B*). A decrease of estrogen receptor-α levels was already observed after 6 hours of treatment, whereas P-cadherin levels nearly doubled after 12 hours. After 24 hours of exposure to ICI 182,780, higher P-cadherin and lower estrogen receptor-α levels persisted for several days, with normalization 96 hours after ICI 182,780 withdrawal (Fig. 2*C*). To examine whether or not the effect of ICI 182,780 on P-cadherin expression was mediated via estrogen receptor-α, we did a competition experiment. As already described (30), 17β-estradiol readily decreased estrogen receptor-α levels, although to a lesser extent than ICI 182,780 (Fig. 2*D*). Importantly, 17β-estradiol counteracted the ICI 182,780-induced up-regulation of P-cadherin (Fig. 2*D*) and accelerated normalization of P-cadherin levels in cells treated for 24 hours with ICI 182,780 (Fig. 2*E*). Together, these results suggest that not the decrease in estrogen receptor-α, but the lack of estrogen receptor-α signaling is responsible for the increase of P-cadherin by ICI 182,780.

RT-PCR revealed an increase of P-cadherin mRNA after ICI 182,780 treatment, suggesting that the higher P-cadherin protein expression results from an up-regulation of P-cadherin transcripts (Fig. 2*F*). This was confirmed by a micro-array study performed on 17β-estradiol– or ICI 182,780-treated MCF7 cells, in which 17β-estradiol did not alter P-cadherin mRNA levels, whereas ICI 182,780 induced an 8-fold increase. Finally, it remained to be determined whether induction of the P-cadherin gene (*CDH3*) was a direct effect of ICI 182,780 or required prior induction of other genes. We addressed this question by blocking protein synthesis in cells, because the induction of primary target proteins or immediate early genes should not be sensitive, whereas secondary targets should be blocked. The treatment of MCF7 cells with cycloheximide, a *de novo* protein synthesis inhibitor, largely blocked P-cadherin up-regulation by ICI 182,780 (Fig. 2*G*), which is consistent with a requirement for newly synthesized proteins, probably induced by ICI 182,780, before *CDH3* activation. In contrast, as expected, this drug did not block estrogen receptor-α down-regulation mediated by ICI 182,780 (Fig. 2*G*).

ICI 182,780 Decreases Cell–Cell Adhesion and Increases Invasiveness of MCF-7/AZ Cells. MCF7 cells formed compact aggregates on top of soft agar or when incubated in Erlenmeyer flasks under continuous shaking (Fig. 3*A, panel i,* and Fig. 3*B*). In presence of ICI 182,780, this effect was counteracted (Fig. 3*A, panel ii,* and 3*B*). Even a 24-hour pretreatment with ICI 182,780, followed by testing these cells in the absence of ICI 182,780, was sufficient to prevent the formation of large aggregates (Fig. 3*A, panel iii*). On plastic substratum, no changes in morphology or migrating behavior (as measured by a wound healing assay) could be observed upon treatment with ICI 182,780 (data not shown).

Whereas MCF7 cells failed to invade in collagen type I and Matrigel invasion assays, a 24-hour pretreatment with ICI 182,780 was sufficient to induce invasion of these cells in both assays (Fig. 3, *C* and *D*). These proinvasive effects of ICI 182,780 were counteracted by 17β-estradiol (Fig. 3, *C* and *D*), indicating that they are mediated by interference with estrogen receptor-α signaling.

Aggregation and invasion of MCF7 cells, in the presence of ICI 182,780, mimics the behavior of the poorly aggregating and invasive estrogen receptor-α–negative and P-cadherin–positive BT-20 cells (Fig. 1*A*), which remained unchanged upon treatment with ICI 182,780 (Fig. 3, *E* and *F*).

P-Cadherin Expression Increases Invasiveness but Does Not Alter Cell–Cell Adhesion of MCF-7/AZ cells. Cells, retrovirally transduced to encode only EGFP (MCF7.LIE) or both P-cadherin and EGFP (MCF7.P-cad), were sorted to >90% EGFP positivity (Fig. 4*A*). P-cadherin levels were higher at the cell surface in P-cadherin–transduced cells (Fig. 4*B*). The levels of cell-surface E-cadherin were the same in P-cadherin–transduced cells, as in vector-transduced cells (Fig. 4, *A* and *B*), excluding an effect of the exogenous cadherin on the levels of the major endogenous cadherin.

On plastic substratum, P-cadherin–transduced MCF7 cells, like their parental or vector-transduced cells, formed epithelioid islands, showing no morphotype differences (data not shown). Transduction with P-cadherin did not interfere with E-cadherin–mediated cell–cell adhesion (Fig. 4, *C* and *D*). However, in a wound healing migration assay, P-cadherin–transduced cells migrated faster (data not shown) and, in contrast to parental or vector-transduced (LZRS-IRES-EGFP) controls, invaded into collagen type I and Matrigel (Fig. 4, *E* and *F*).

P-Cadherin-Induced Invasion Is Not Breast Cancer Cell or Endogenous Cadherin-Specific. P-cadherin retroviral transduction was also done on HEK cells, expressing at their surface low and

A

	Type	E-cad[a,b]	P-cad[a,b]	N-cad[a,b]	ERα[a,b]	Invasion in Collagen Type I[c,d]
MCF-7/AZ	Breast	+++	+	-	+++	-
ZR-75.1	Breast	+++	+	+	+++	-
T47D	Breast	+++	++	-	++	+
BT-20	Breast	+++	+++	-	-	+
HEK 293T	Kidney	-	-	+++	-	-

[a], determined by Western blot analysis

[b], -, negative; +, low; ++, moderate; +++, strong

[c], determined by Collagen Type I Invasion Assay

[d], -, not invasive; +, invasive

B

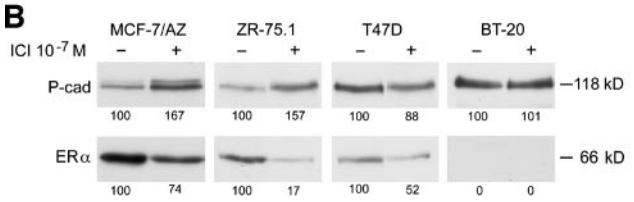

Fig. 1. *A,* analysis of E-cad, P-cad, N-cad, and ERα expression, and *in vitro* invasion into collagen type I, of the human cell lines used in this study. *B,* effect of ICI on P-cad and ERα expression in breast cancer cell lines. Immunoblotting, for P-cad and ERα analysis, of cell lysates derived from MCF7, ZR-75.1, T47D (50 μg of protein loaded), and BT-20 (30 μg) breast cancer cell lines, after a 24-hour treatment with ICI. Band quantification was done relative to the expression levels in control cells. An increase of P-cad expression in MCF7 and ZR-75.1 cells was observed, whereas the levels in ERα-negative BT-20 cells were not altered. P-cad levels were not changed in T47D cells, although ERα levels declined in all positive cell lines. (cad, cadherin; ER, estrogen receptor; ICI, ICI 182,780)

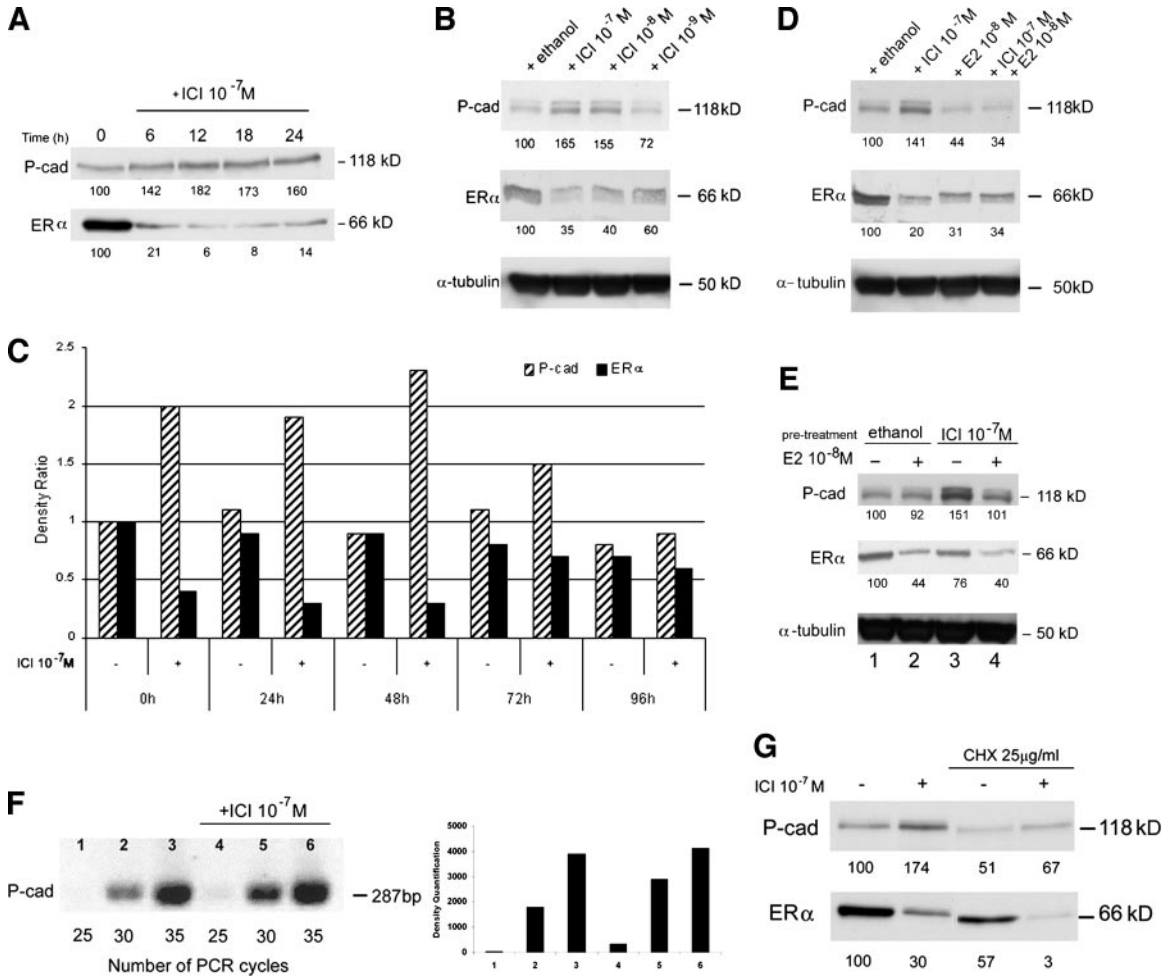

Fig. 2. Regulation of P-cad expression by an ERα-dependent signaling pathway in MCF7 breast cancer cells. Immunoblotting, for P-cad and ERα analysis, of cell lysates from MCF7 cells that had been treated with the indicated concentrations of ICI for the indicated time points. Band quantification was done relative to the expression levels in untreated cells. Immunostaining for anti-α–tubulin was done to control for equal loading. *A,* ICI induces up-regulation of P-cad and down-regulation of ERα levels in a time-dependent manner, being maximal after 12 hours of treatment. *B,* a 24-hour treatment with ICI induces up-regulation of P-cad and down-regulation of ERα levels in a dose-dependent way, the higher concentrations leading to a more pronounced effect. *C,* MCF7 cells were grown in the presence of ICI for 24 hours. At time 0 hours, ICI was withdrawn, and cell lysates were prepared at the indicated time points. Immunoblotting was performed to analyze P-cad (▨) and ERα (■) expression. The levels of both proteins start to normalize again 96 hours after ICI withdrawal, showing the reversibility of the effect. *D.* Cells were treated with ICI, E2, or a combination of both, for 24 hours. Although both ICI and E2 decreased ERα levels, the ICI-induced up-regulation of P-cad was counteracted by estradiol. *E.* MCF7 cells were grown in the presence of solvent control (*lanes 1* and *2*) or ICI (*lanes 3* and *4*) for 24 hours. After that, ICI was withdrawn, all media were refreshed, and cells were treated (*lanes 2* and *4*) or not (*lanes 1* and *3*) with E2 for additional 24 hours. E2 accelerated the reversion of P-cad expression to control levels in cells that had been treated with ICI. *F,* RT-PCR analysis of P-cad mRNA levels after ICI treatment of MCF7 cells for 24 hours. The analysis was done after the indicated number of cycles of PCR amplification. P-cad mRNA increased in the presence of ICI (more evident at the 30-cycle point, in the exponential phase). Band quantification is presented in the graph shown. *G,* immunoblotting, using anti-P-cad and anti-ERα antibodies, of lysates from cells treated with CHX during 24 hours, alone or in combination with ICI. Band quantification was done relative to the expression levels in untreated cells. CHX blocked P-cad up-regulation induced by ICI, suggesting the involvement of *de novo* protein synthesis. (cad, cadherin; ER, estrogen receptor; ICI, ICI 182,780; E2, 17β-estradiol; CHX, cycloheximide)

high levels of E- and N-cadherin, respectively (Fig. 1*A* and Fig. 5*A*), and being invasive neither into collagen type I nor into Matrigel. Sorting of vector- or P-cadherin–transduced cells resulted in populations having either moderate or high EGFP expression (HEK.LIE.Med, HEK.LIE.High, HEK.P-cad.Med, and HEK.P-cad.High; Fig. 5, *A* and *B*). As for MCF7 cells, no differences in morphotype or aggregation were observed between parental and transduced cells (Fig. 5, *C* and *D*). Although there was a down-regulation of superficial N-cadherin in the highest P-cadherin–expressing cells (Fig. 5*B*), this did not result in a significant decrease in total levels of N-cadherin (Fig. 5*A*). P-cadherin–transduced cells were significantly more invasive into collagen type I or Matrigel than vector-transduced cells, with higher invasiveness of the cells expressing more P-cadherin (Fig. 5, *E* and *F*). In both assays, the control cells with higher LZRS-IRES-EGFP expression levels showed an increased invasion index when compared with the ones with moderate levels of expression. This may be due to the insertion of viral promoters into the host genome, leading to the aberrant activation of host genes. However, although this observation highlights the care that should be taken when using these systems, it does not influence the interpretation of our results as such: the values of the P-cadherin–transduced cells remain significantly different from those of the respective vector-transduced cells.

P-Cadherin Mediates Invasion of HEK 293T Cells via Its Juxtamembrane Domain. To identify the P-cadherin domain(s) necessary for its proinvasive effects, we used several P-cadherin constructs (Fig. 6*A*) for transient transfection of the HEK cell line. Biotinylation and immunoblotting confirmed expression of all of the constructs at the plasma membrane (Fig. 6*B*). Transient transfection with P-cadherin induced invasion into collagen type I, as observed with stably transduced HEK cells (Fig. 6*C*).

The P-cadherin point mutant, PC-R503H (Fig. 6*A*), representing the missense mutation in *CDH3*, found in hypotrichosis with juvenile macular dystrophy (31), failed to support strong cell–cell adhesion

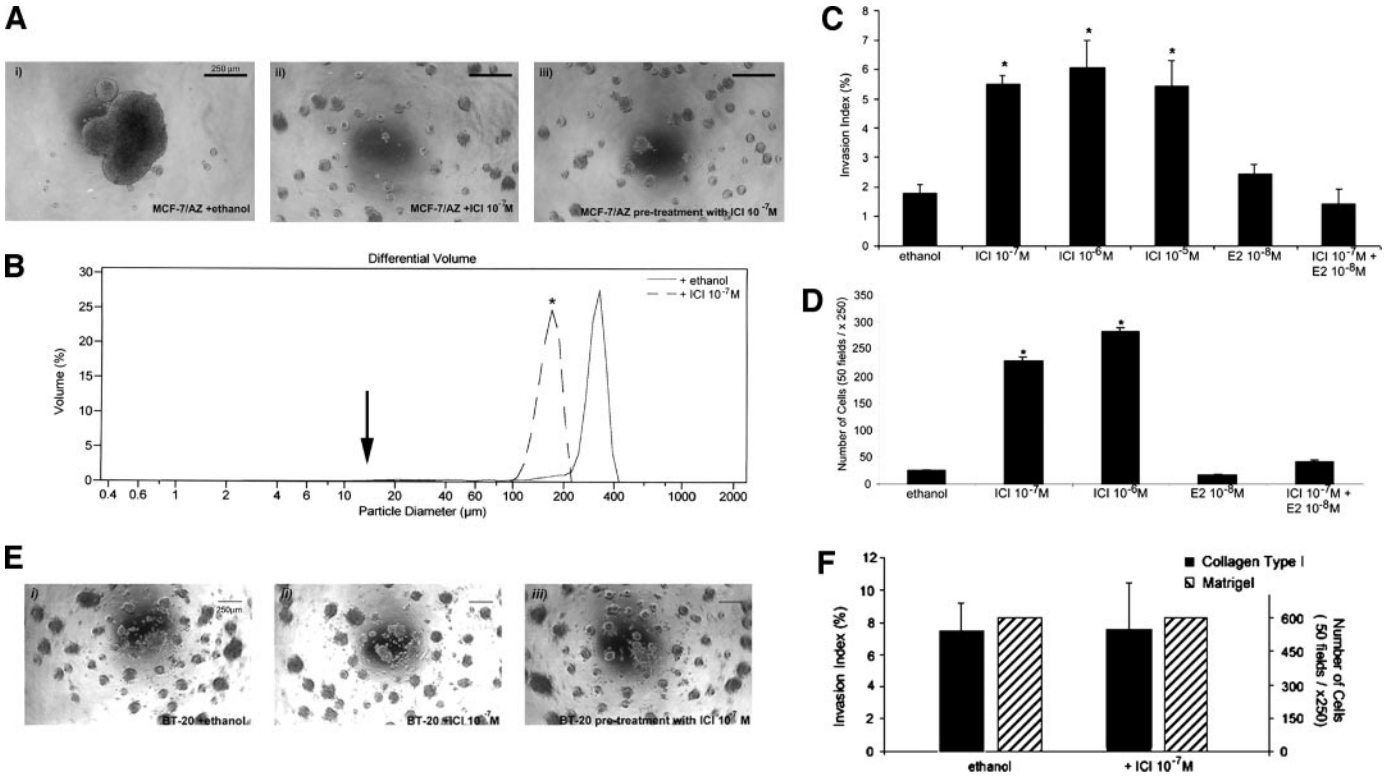

Fig. 3. Effect of ICI on aggregation and on *in vitro* invasion of MCF7 and BT-20 cells. *A,* pictures, after 72 hours, of the slow aggregation assay on semi-solid substratum of MCF7 cells. *Panel i,* cells that were left untreated form compact aggregates, which are inhibited in cells cultured in presence of ICI (*panel ii*) and in cells that have been pretreated with ICI before seeding on top of agar (*panel iii*). *B,* slow aggregation assay in suspension of MCF7 cells: the cells were pretreated with ICI for 24 hours before incubation in Erlenmeyer flasks. A particle size distribution curve was generated using a particle size counter. Aggregates formed by ICI-treated cells were significantly smaller (∗, $P = 0.002$) than control aggregates. The *arrow* indicates the measurement of a single cell suspension at the beginning of the experiment. *C* and *D,* MCF7 cells that had been pretreated for 24 hours with the indicated concentrations of ICI, E2, or the combination of both, were seeded as a single cell suspension on top of collagen type I gels (*C*) or on Matrigel-coated filters (*D*). In collagen type I invasion assay, ICI-treated cells were significantly more invasive than control cells [∗, $P = 0.0081$, $P = 0.02$, and $P = 0.03$ for ICI (10^{-7} mol/L, 10^{-6} mol/L, and 10^{-5} mol/L), respectively]. In Matrigel, the differences were also statistically significant [∗, $P < 0.001$ for ICI (10^{-7} mol/L and 10^{-6} mol/L)]. *E,* pictures, after 72 hours, of the slow aggregation assay on semi-solid substratum of BT-20 cells. *Panel i,* cells that were left untreated do not form compact aggregates; no alterations (*panel ii*) in cells cultured in presence of ICI and (*panel iii*) in cells that have been pretreated with ICI before seeding on top of agar, in the absence of ICI. *F.* BT-20 cells that had been pretreated for 24 hours with ICI were seeded as a single cell suspension on top of collagen type I gels (■) and on Matrigel (▨). ICI-treated cells are as invasive as control cells; *bars,* ±SD. (ICI, ICI 182,780; E2, 17β-estradiol)

unlike wild-type P-cadherin.[6] Most likely, the reason for this failure is the disruption of the strongly conserved LD*R*E Ca^{2+}-binding motif in the fourth extracellular domain of P-cadherin. Nevertheless, PC-R503H still induced invasion (Fig. 6*C*).

Mutants of the P-cadherin cytoplasmic tail were also generated (Fig. 6*A*). Transfection into HEK cells showed that PC-CT762, retaining the intact P-cadherin juxtamembrane domain, induced invasion-like wild-type P-cadherin (Fig. 6*C*). Because this mutant is truncated just before the catenin-binding domain, we assume that β-catenin, γ-catenin, or any other protein that binds to this region are not needed for P-cadherin–mediated invasion.

With mutants within the juxtamembrane domain (Fig. 6*A*), statistically significant invasion into collagen was seen only with the truncation mutants that still retained the intact juxtamembrane domain (PC-CT719 and PC-CT727; Fig. 6*C*). The somewhat decreased ability of PC-CT719 to induce invasion (Fig. 6*C*) might be due to its lower expression levels (Fig. 6*B*). In line with the results obtained with the truncation mutants and confirming that the catenin-binding domain is not involved in the proinvasive effects, the PC-Δ703–707 mutant (lacking EEGGG in the P120-catenin binding site), with impaired P120-catenin binding (Fig. 6*D*), was not able to induce invasion of HEK cells into collagen type I (Fig. 6*C*). In conclusion, P-cadherin needs its intact juxtamembrane domain to induce invasion of HEK cells into collagen type I.

To exclude that the gain of any exogenous cadherin, retaining its juxtamembrane domain, would be sufficient for a proinvasive effect, we demonstrated that HEK cells transfected with mouse wild-type E-cadherin cDNA (Fig. 6*A*) failed to invade into collagen type I (Fig. 6*C*). In conclusion, the juxtamembrane domain of P-cadherin confers to this molecule the specific ability to induce invasion of HEK cells, in the presence of the endogenously expressed cadherin.

DISCUSSION

We demonstrated that the antiestrogen ICI 182,780 increased time- and dose-dependently P-cadherin expression in estrogen receptor-α–positive breast cancer cells. This increase could be completely reverted by 17β-estradiol, categorizing *CDH3* as an estrogen-repressed gene and pointing to 17β-estradiol as a key regulator of this cadherin. In addition to competing for binding to estrogen receptor-α, ICI 182,780 also increases its breakdown (24). As a result, ICI 182,780 abrogates estrogen receptor-α signaling and the subsequent regulation of 17β-estradiol responsive genes. Because the human P-cadherin promoter (GI: 2950171) does not contain the consensus sequence 5′-GGTCAnnnTGACC-3′ of the estrogen-responsive elements (32), 17β-estradiol is unlikely to have a direct inhibitory effect on transcription of the *CDH3* gene. Instead, the increase of P-cadherin by ICI 182,780, some hours after the decrease of estrogen receptor-α, and its

[6] V. Van Marck, C. Stove, V. Stove, J. Paredes, M. Bracke. P-cadherin promotes cell-cell adhesion and counteracts invasion in human melanoma, manuscript in preparation.

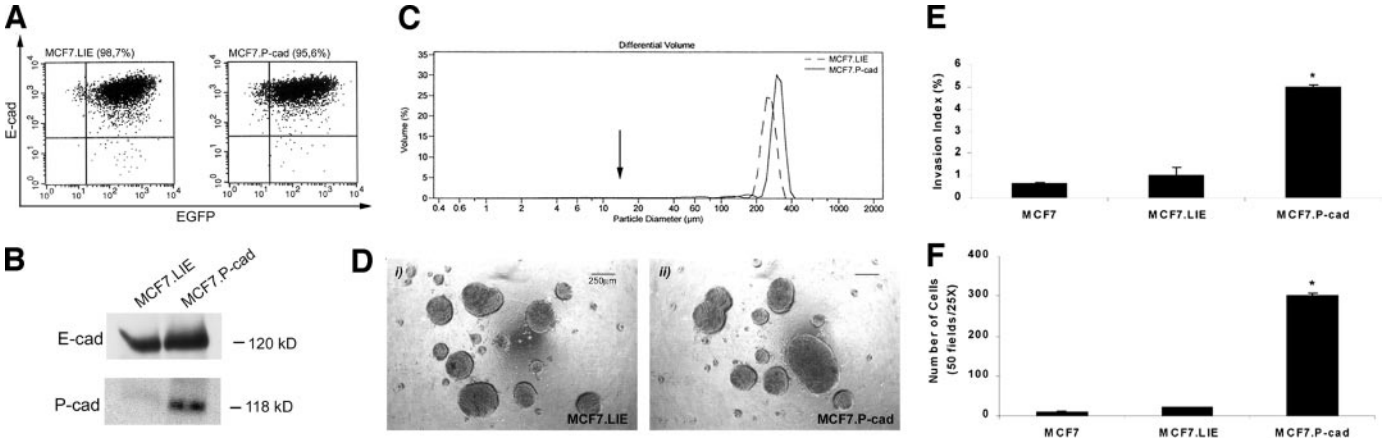

Fig. 4. Cell aggregation and *in vitro* invasion of stably transduced MCF7 cells with P-cad cDNA. *A,* flow cytometric evaluation of EGFP expression (*X* axis) and E-cad expression (*Y* axis) in MCF7.LIE and MCF7.P-cad cells. P-cad expression did not induce alterations in E-cad expression levels (*right plot*). The percentage of EGFP positivity is indicated for each cell line. *B,* immunoblotting, using anti-E-cad and anti-P-cad antibodies, of lysates from biotinylated MCF7.LIE and MCF7.P-cad cells. *C.* In the slow aggregation assay in suspension, both MCF7.LIE and MCF7.P-cad cells form similar compact aggregates after 48 hours. The *arrow* indicates the measurement of a single cell suspension at the beginning of the experiment. *D,* pictures, after 48 hours, of the slow aggregation assay on semi-solid substratum. MCF7.LIE (*panel i*) and MCF7.P-cad (*panel ii*) cells form round and compact aggregates, with no differences observed. *E.* In the collagen type I invasion assay, MCF7.P-cad cells invade significantly more (∗, $P = 0.0024$). *F,* representative experiment of Matrigel invasion assay, where MCF7.P-cad cells invade significantly more than empty-vector transduced cells (∗, $P < 0.001$). (cad, cadherin)

inhibition by cycloheximide, pleads for the existence of a *CDH3*-regulating transcription factor. In the absence of estrogen receptor-α signaling (as in estrogen receptor-α–positive cells treated with ICI 182,780 or in estrogen receptor-α–negative cells), this 17β-estradiol–regulated factor might account for the high P-cadherin levels in some breast cancer cell lines and for the inverse correlation between estrogen receptor-α and P-cadherin expression in mammary tumors.

In MCF7 breast cancer cells, ICI 182,780 treatment led to a decreased cell–cell adhesion and promotion of invasion *in vitro*. This is in line with the finding that 17β-estradiol (33) and even the unliganded receptor (34) may decrease *in vitro* invasiveness and motility of breast cancer cells, suggesting that some estrogen-regulated genes negatively control invasion. Because this control is lost in cells treated with high concentrations of ICI 182,780, which up-regulate P-cadherin, the effect of the latter was additionally investigated on *in vitro* aggregation and invasion of cells retrovirally transduced with P-cadherin. Surprisingly, retroviral transduction of MCF7 and HEK cells with P-cadherin had no detectable influence on cell–cell adhesion. This result suggests that P-cadherin does not shift the aggregation balance established by the other cadherins in these systems. By contrast, such balance may well be changed for invasion, as demonstrated with P-cadherin–transduced cells. It should be noted that this

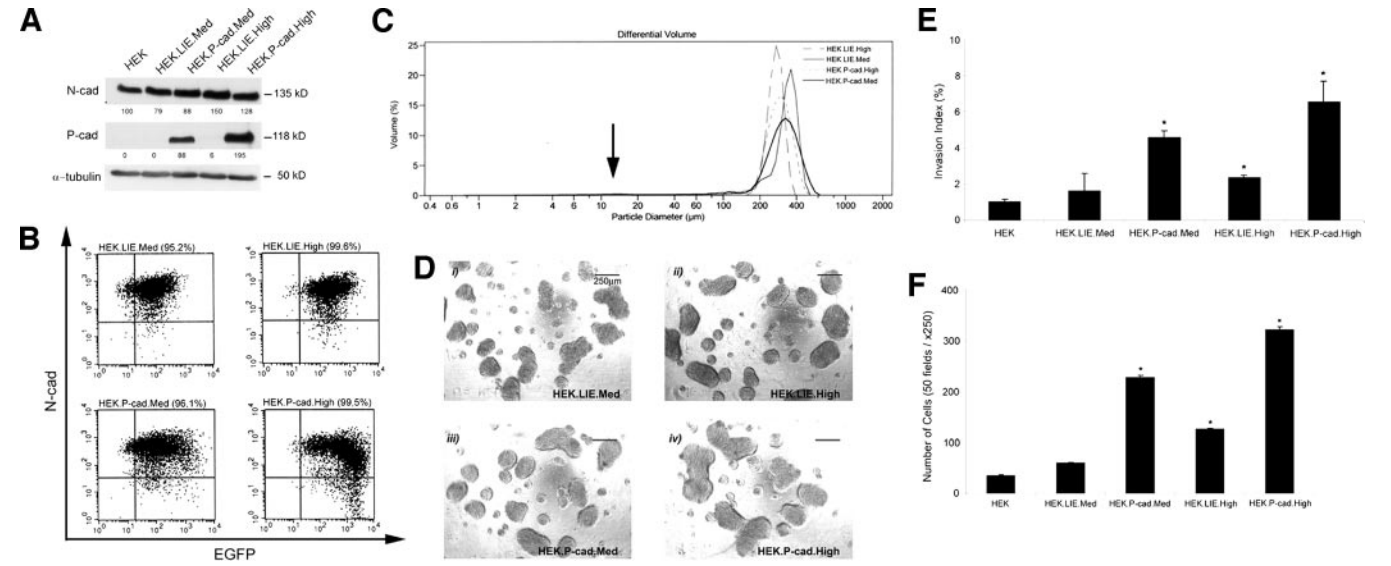

Fig. 5. Cell aggregation and *in vitro* invasion of stably transduced HEK cells with P-cad cDNA. *A,* immunoblotting, using anti-N-cad and anti-P-cad antibodies, of lysates from biotinylated HEK, HEK.LIE.Med, HEK.P-cad.Med, HEK.LIE.High, and HEK.P-cad.High cells. Band quantification was done relatively to the expression levels in control cells. To control for equal loading, immunostaining with anti-α-tubulin was done. *B,* flow cytometric analysis of EGFP expression (*X* axis) and N-cad expression (*Y* axis) in the indicated cell lines. High levels of P-cad in the HEK.P-cad.High cell line induced down-regulation of endogenous N-cad. The percentage of EGFP positivity is indicated for each cell line. *C.* In the slow aggregation assay in suspension, HEK.LIE.Med, HEK.P-cad.Med, HEK.LIE.High, and HEK.P-cad.High cell lines form similar compact aggregates after 48 hours. The *arrow* indicates the measurement of a single cell suspension at the beginning of the experiment. *D,* pictures, after 48 hours, of the slow aggregation assay on semi-solid substratum. HEK.LIE.Med (*panel i*), HEK.LIE.High (*panel ii*), HEK.P-cad.Med (*panel iii*), and HEK.P-cad.High (*panel iv*) cells form round and compact aggregates, with no differences observed. *E.* A single cell suspension of these cell lines was seeded on top of collagen type I gels. After 24 hours of incubation, HEK.P-cad.Med and HEK.P-cad.High cells invaded significantly more into the collagen type I matrix (∗, $P = 0.005$ and $P = 0.02$, respectively). Although HEK.LIE.High was statistically different from nontransduced cells (∗, $P = 0.003$), the comparison with HEK.P-cad.High still shows the significant effect of P-cad ($P = 0.03$). *F,* representative experiment of Matrigel invasion assay. HEK.P-cad.Med and HEK.P-cad.High cells invaded significantly more than empty vector-transduced cells (∗, $P < 0.001$). Also here, although HEK.LIE.High differed significantly from control, the comparison with HEK.P-cad.High still shows the significant effect of P-cad on invasion ($P < 0.001$). (cad, cadherin)

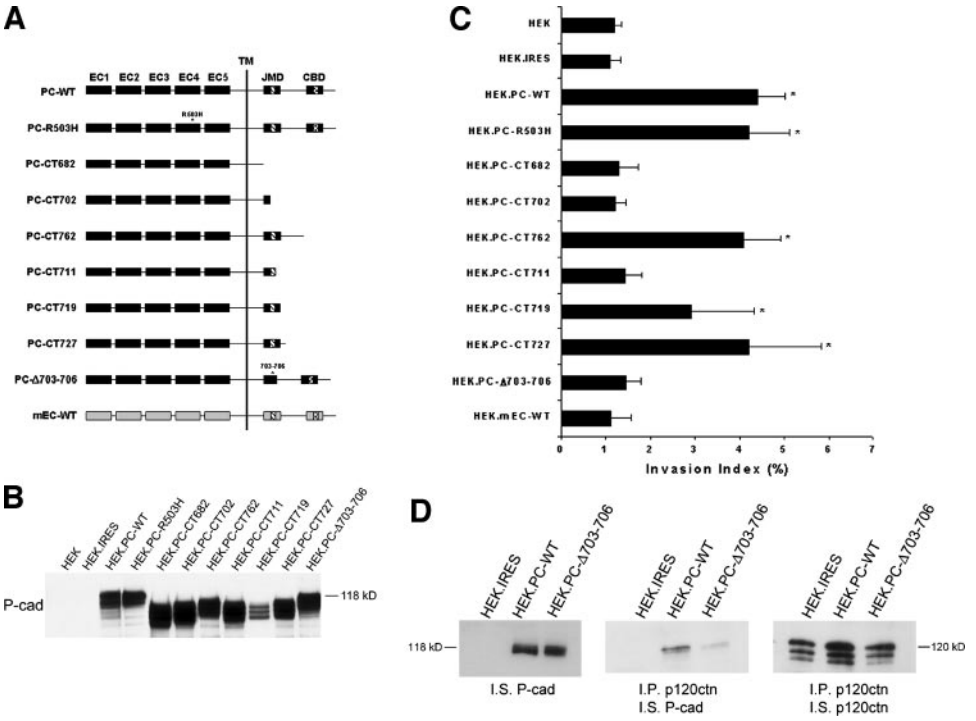

Fig. 6. *In vitro* invasion of HEK cells transiently transfected with P-cad constructs. *A,* schematic diagram of the various constructs used for transient transfection of HEK cells. *B,* immunoblotting, using anti-P-cad antibody, of streptavidin precipitations of biotinylated HEK cells, transiently transfected with P-cad constructs. *C.* A single cell suspension of HEK cells, transiently transfected with the indicated constructs, was seeded on top of collagen type I gels. After 24 hours, invasive cells were scored. Besides HEK.PC-WT cells, also HEK.PC-R503H, HEK.PC-CT762, HEK.PC-CT719, and HEK.PC-CT727 invaded significantly more than empty-vector transfected cells (*HEK-.IRES*) into the collagen (*, $P < 0.001$ for HEK.PC-WT and HEK.PC-R503H, $P = 0.003$ for HEK.PC-CT762, $P = 0.015$ for HEK.PC-CT719, and $P = 0.023$ for HEK.PC-727). *D,* I.P., using anti-p120ctn antibody, of total lysates of HEK cells transiently transfected with the empty vector (IRES), with PC-WT and with PC-Δ703–706 constructs. I.S. of these blots with anti-P-cad and anti-p120ctn antibodies showed that PC-WT strongly precipitates with p120ctn, as compared with PC-Δ703–706, where there is interference with this binding (*right two blots*). The staining of total lysates with P-cad indicates a similar expression of the transfected P-cad constructs (*left blot*); *bars,* ±SD. (I.P., immunoprecipitation; I.S., immunostaining; cad, cadherin; p120ctn, P120-catenin)

does not allow us to draw conclusions about the necessity of P-cadherin up-regulation for ICI 182,780-induced invasion of MCF7 cells. In contrast to P-cadherin–transduced cells, which migrated faster than controls in a wound healing migration assay, ICI 182,780-treated cells did not. This might be due to the fact that the extent by which P-cadherin is up-regulated by ICI 182,780 may not be sufficient to promote motility as such or, alternatively, the growth-inhibitory effect of ICI 182,780 nullified the promigratory effect of P-cadherin in this assay. Furthermore, ICI 182,780 up-regulated additional pro-invasive genes in MCF7 cells, such as MMP-2 and -9, of which the expression was not influenced by P-cadherin (data not shown). Hence, whereas high levels of P-cadherin may be sufficient for induction of invasion, ICI 182,780-induced invasion might require the synergistic action of multiple genes. This hypothesis, in which a critical level of P-cadherin seems to be needed for its proinvasive activity, is supported by the comparison between the invasive and highly P-cadherin–positive BT-20 and T47D cells and the noninvasive and weakly P-cadherin–positive MCF7 and ZR-75.1 cells (Fig. 1*A*). In contrast to its proinvasive activity in our cells, transfection of other cell lines with P-cadherin inhibited invasion (35, 36),[6] suggesting that P-cadherin may act both as an invasion promoter and suppressor, depending on the cell type and its invasive status. Transgenic mice expressing high levels of P-cadherin in the normal mammary epithelium (37) contributed little to this issue, because they did not produce tumors, and because *neu* oncogene-induced mammary tumors in P-cadherin transgenic mice were always P-cadherin negative.

In the present study, the proinvasive action of P-cadherin is unlikely to be the result of alterations in cell–cell adhesion, because the assays score invasion of single cells into or through a matrix, the retroviral transduction of MCF7 and HEK cells with P-cadherin did not change aggregation, and the point mutant PC-R503H, incapable of supporting strong P-cadherin mediated adhesion, still induced invasion. We presume that the proinvasive activity of P-cadherin is due to changes in signaling pathways.

Recently, Wong and Gumbiner (38) attributed the anti-invasive activity of wild-type E-cadherin to its interaction with β-catenin. An E-cadherin mutant, retaining the catenin-binding domain but with a point mutation that abolishes P120-catenin binding, was still able to suppress invasion. By contrast, in P-cadherin, maintenance of the juxtamembrane domain is crucial for the induction of invasion, irrespective of the catenin-binding domain. Although the juxtamembrane domain is highly conserved between cadherins, its function is very context-dependent, being implicated in both positive and negative regulation of cadherin activity. Cells expressing mutated E-cadherin juxtamembrane domain are weakly adherent (39), more motile, but still epithelioid. Upon formation of adhesive contacts, the juxtamembrane domain recruits and activates Rac, regulating the actin cytoskeleton (40). In another context, the juxtamembrane domain may inhibit aggregation mediated by classical cadherins and induce cell motility (41, 42) or, alternatively, exclude another cadherin from junctions and regulate cell proliferation (43). Via its binding to P120-catenin, this domain has been implicated recently in maintenance of the stability of endogenous cadherins (44, 45). Thus, a possible mechanism for the induction of invasion by P-cadherin might be its competition with the endogenous cadherin for the available P120-catenin, leading to the destabilization of pre-existing anti-invasive cadherin/catenin complexes. Yet, we consider this possibility less likely. Although the down-regulation of N-cadherin in HEK cells by high levels of the several P-cadherin constructs coincided with stimulation of invasion (Fig. 6*C* and Supplementary Data), moderate P-cadherin expression levels, leaving the endogenous cadherin unchanged, were sufficient to induce invasion. Furthermore, transfection of HEK cells with E-cadherin did not induce invasion (Fig. 6*C*), despite decreased endogenous cadherin in highly expressing cells (Supplementary Data) and expected competition for cadherin-binding proteins.

Alternatively, P-cadherin may generate a specific proinvasive signal via its juxtamembrane domain. In this hypothesis, the binding of proteins to the P-cadherin juxtamembrane domain may differ from their binding to E- or N-cadherin by strength, conformation, or recruitment of other members of the complex. This, in turn, may result in the activation of pathways that overcome the suppressive signals mediated by the endogenous cadherins.

Although binding of proteins to the juxtamembrane domain of P-cadherin has just been documented for P120-catenin (46), other molecules, like Hakai and presenilin-1 (PS-1), have been reported to bind to the juxtamembrane domain of classical cadherins as well, to a sequence adjacent to or overlapping the P120-catenin–binding domain, thereby competing with P120-catenin for binding (47, 48). Although the significance of these interactions is not well known, we cannot exclude the possibility that disruption of the P120-catenin–binding sequence introduces conformational changes and/or uncouples the interaction of these or other proteins, which could be responsible for our observations. Striking examples of this were shown for E-cadherin, where functional differences have been noted between larger and minimal deletions of the juxtamembrane domain, with even the minimal changes disrupting binding of multiple molecules (47).

Data about the role of P120-catenin in normal and cancer cells are conflicting. Positive and negative regulation of cell–cell adhesion and motility possibly reflect differences in cell type, cadherins, P120-catenin isoforms, and shuttling between cadherin-bound and cytoplasmic pools (3). When overexpressed in the cytoplasm, P120-catenin may regulate the actin cytoskeleton and cell motility, through Rho GTPases (49). Similar to the differences seen between E-and N-cadherin in terms of strength (3) and preference (50) of binding to distinct P120-catenin isoforms, P120-catenin binding to P-cadherin may be unique. This unique interaction may influence its impact on the activity of the Rho GTPases, possibly making the cells more prone to invade. Alternatively, the panel of molecules recruited by P120-catenin may differ depending on the isoform or on the cadherin it is bound to.

In conclusion, our study establishes an as yet unknown role for P-cadherin in cancer: (1) P-cadherin expression is regulated through estrogen receptor-α signaling, suggesting that the inverse *in vivo* correlation between these molecules stems from a causal relationship; (2) P-cadherin induces invasion, in the context of endogenous E- or N-cadherin expression; because P-cadherin expression in breast cancer is far more frequent than aberrant expression of N-cadherin, its physiologic relevance is more likely to be higher (15); (3) except from the presently demonstrated induction of invasion, no regulatory functions have been described for the P-cadherin juxtamembrane domain. This establishes a novel role for this domain and distinguishes P-cadherin–mediated invasion from invasion induced by N-cadherin, which depends on a physical interaction of its extracellular domain with the fibroblast growth factor receptor (51). Remarkably, although the P-cadherin juxtamembrane domain differs in only few amino acids from the corresponding E-cadherin domain, it exerts an opposite function: whereas the E-cadherin juxtamembrane domain suppresses motility (52), the P-cadherin juxtamembrane domain is necessary for induction of invasion. To understand why such related domains can have opposite functions, it will be crucial to identify new interaction partners and/or to study if the interaction of known partner molecules differs between cadherins.

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Part VI Discussion and perspectives

Invasion is crucial step in the development of cancer and happens when cells are genetically transformed and become sensitive or insensitive for host and environmental factors (Mareel and Leroy 2003, Hanagan and Weinberg 2000). An important invasion suppressor in epithelial cancer is E-cadherin, and the regulation of this cell-cell adhesion molecule happens at the different levels (part 5) (Van Aken *et al.* 2001). Loss of this molecule can be associated with transactivation of another cadherin, like P-cadherin (part 5) and N-cadherin (Derycke and Bracke 2004, part 2). Cells undergo changes and become fibroblastic, motile and invasive. A similar transition is observed during embryogenesis in the gastrulating stage. In the normal adult N-cadherin is expressed by neurons, (myo)fibroblast, endothelial cells, oocytes, spermatocytes and lens cells.

Furthermore, cancer cells secrete many growth factors, for their survival, and several proteases to degrade the extracellular matrix (Mott and Werb 2004). Proteases are able to generate different fragments of the cadherin proteins. N-cadherin is sensitive to several proteases like ADAM 10, MT1-MMP, MT5-MMP and plasmin (part 3). All these enzymes are releasing the 90 kD extracellular fragment of N-cadherin, coined soluble N-cadherin or sN-CAD. This shedded fragment is still functional, because it was shown to stimulate neurite outgrowth (Utton *et al.* 2001). Literature data show the presence of soluble cadherin fragments in different human biological fluids. The most convincing report was the correlation of sE-CAD with gastric cancer and the prediction of disease recurrence (Chan *et al.* 2005). However, until now almost no data where found about the presence of sN-CAD in any biological fluid. The lack of good antibodies recognizing the extracellular part of N-cadherin is probable one of the reasons why no data have been published so far. In the initial phase of this doctoral thesis we made some new monoclonal antibodies recognizing the HAV part in cadherin domain 1 and another one against cadherin domain 4 (in collaboration with Prof. J. Vandekerchove). However these were not suitable for ELISA, but the commercially available monoclonal antibody, GC-4, raised against the extracellular part of N-cadherin could be used for ELISA. Using this antibody an ELISA was established (part 3), which was applied to sera from persons with no evidence of disease (NED), cancer patients and patients suffering from other disease like heart disease, liver cirrhosis, arthritis, ... We could find ± 6 times higher concentration of sN-CAD in the cancer patient group (median value of 584 ng/ml) compared to NED group (median value 99 ng/ml). This difference between both

groups is higher than what was reported for sE-CAD, where the medians differenced by a factor 3, between the cancer group and the NED group. We also found a weak, but significant correlation with PSA. Now we are collecting well-documented serum samples from breast and prostate cancer patients, and we will test them to see if we can observe any correlation with the TNM staging or reaction to therapy. We assume that sN-CAD will be a better marker than sE-CAD because N-cadherin is not only expressed by the cancer cells but also reactionally by the stromal cells like (myo) fibroblast and endothelial cells, so different cells are a source of sN-CAD. Furthermore, sN-CAD can give idea if the tumour (cancer + stromal cells) is starting to be invasive and metastasize or give an idea how the tumour is reacting to the given therapy. We need to take into account that sN-CAD can also be elevated as a reaction to other diseases like inflammation.

sN-CAD is not only a possible marker, the protein fragment also has a biological function. It stimulates the migration of N-cadherin positive cells that can be fibroblast, endothelial cells or cancer cells. We could show that sN-CAD stimulates angiogenesis by using 2 *in vivo* assays as model for angiogenesis: the chorioallantoic membrane assay and rabbit corneal micropocket assay (part 4). Angiogenesis is correlated with cancer cell metastasis. Angiogenesis is the result of endothelial cells that degrade the basement membrane, migrate and proliferate in the nearby stroma, differentiate and form new contacts with the pericytes. N-cadherin knockout mice die at day 10 of gestation and embryos display major heart defects and malformed neural tubes and somites (Radice *et al.* 1997). Furthermore, selective knock down of N-cadherin in the endothelial cells caused a significant decrease in VE-cadherin expression, motility and proliferation of the endothelial cells (Luo and Radice 2005). So it seems that N-cadherin plays an important role during angiogenesis, not at least because it anchors endothelial cells with the pericytes and vascular smooth muscle cells. We observed that sN-CAD has a pro-angiogenic effect and this effect was mediated by the fibroblast growth factor receptor (FGF-receptor). It has been pointed out that N-cadherin interacts with the FGF-receptor via its HAV–binding domain present domain 4 (Williams *et al.* 2001), and by this direct interaction it induces a continuous cell activation (Suyama *et al.* 2002). The FGF-receptor mediates numerous signalling pathways important in angiogenesis (Gerwins *et al.* 2000). By activation of the FGF-receptor proteases are upregulated via the zinc finger transcription factor Ets-1 (Sato *et al.* 2000) and allows proteolytic activity at the front of migration. We assume that sN-CAD has an autocrine and paracrine effect on the endothelial cells. Because the source of sN-CAD is not only the endothelial cells, but can also be cancer cells and the surrounding stromal cells. By this cancer cells are provided of factors which

mediate growth and dissemination. We could prove that the HAV-peptide exerts the same effect as the extracellular fragment on stimulation of angiogenesis. Several studies showed already that dimeric N-cadherin-peptides stimulated neurite outgrowth (Williams *et al.* 2002), while the antagonistic cyclic peptide induces apoptosis in endothelial cells (Erez *et al.* 2004).

Finally, we can speculate about future therapeutic possibilities. One possibility would be the interference with the molecular cross talk between cancer cells and stromal cells, and between cancer cells and endothelial cells via blocking the N-cadherin signalling with N-cadherin antibodies or antagonistic peptides (Kelland 2005). The company Adherex Technologies already synthesized an N-cadherin antagonist (ADH-1, Exherin^{TM}) and is now testing them in phase II trials. Another possibility might be the use of protease inhibitors, to halt the formation of cadherin fragments and their effects on invasion and angiogenesis. However, clinical trials using protease inhibitors were disappointing so far (Lah *et al.* 2006).

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Part VII Summary – Samenvatting -Résumé

Summary

In the present thesis N-cadherin and its cleaved form soluble N-cadherin (sN-CAD) are studied in relation to cancer. Cadherins are calcium-dependent intercellular adhesion molecules that are often deregulated in cancer. E-cadherin, which is expressed by normal epithelial cells, is downregulated when cells become cancer cells and this sometimes coincides with transactivation of another cadherin, for example N-cadherin. As a result, cells change their phenotype and become fibroblastic, motile and invasive. The role of N-cadherin during cell adhesion, differentiation, embryogenesis and cancer is reviewed, and several examples are given of N-cadherin expression in tumour cell lines and biopsies.

Extracellular proteases, also known as the cancer degradome, play an important role during tumour progression; they are involved in processes as invasion and metastasis but also cell proliferation, apoptosis and angiogenesis. The proteases, such as ADAM10, MT1-MMP and plasmin are able to shed a 90 kD extracellular fragment from N-cadherin. We could establish an ELISA for the detection of this sN-CAD. We tested body fluids from persons with no evidence of disease and cancer patients for the presence of sN-CAD. Significantly elevated levels of sN-CAD were measured in the cancer patient group and a weak but significant correlation was found with prostate specific antigen, which is the most frequently used circulating tumour marker for prostate cancer.

Furthermore, sN-CAD also has a biological function: it was able to stimulate the motility of N-cadherin positive cells and more specifically of the endothelial cells. By using the chorioallantoic membrane assay and the rabbit corneal micropocket assay we could identify sN-CAD as a proangiogenic molecule. Unravelling the signalling pathway, we proved that the fibroblast growth receptor is an important player in the sN-CAD mediated effects.

In conclusion, sN-CAD is a potentially interesting molecule in oncology, both as a circulating tumour marker and as a target for angiostatic treatment.

Samenvatting

N-cadherine en zijn gekliefde vorm soluble N-cadherine (sN-CAD) in relatie met kanker zijn het onderwerp van deze thesis. Cadherines zijn calciumafhankelijke intercellulaire adhesie moleculen, en zijn meestal gedereguleerd in kanker. E-cadherine komt tot expressie op normaal epitheel cellen, maar gaat verloren wanneer de cellen muteren naar een kanker cel en dit fenomeen gaat dikwijls gepaard met de transactivatie van een ander cadherine, bijvoorbeeld N-cadherine. Door deze verandering verkrijgen de kanker cellen een fibroblastisch fenotype, worden motieler en invasief. Er is een overzicht gemaakt over de rol van N-cadherine in cel adhesie, differentiatie, embryogenese en kanker, en verschillende voorbeelden van N-cadherine expressie in kanker cellijnen en biopsies zijn weergegeven.

Extracellulaire proteasen, ook het kanker “degradome” genoemd, spelen een belangrijke rol tijdens tumor progressie. Zij zijn van belang in invasie en metastase maar ook tijdens cel proliferatie, geprogrammeerde celdood en angiogenese. De proteasen, ADAM10, MT1-MMP en plasmine kunnen het extracellulaire fragment van N-cadherine afklieven. We konden een ELISA opstellen die ons de mogelijkheid gaf om sN-CAD te detecteren in verschillende biologische vochten. We testten sera van gezonde mensen en kanker patiënten voor de aanwezigheid van sN-CAD. Significant verhoogde waarden van sN-CAD werden waargenomen in sera van patiënten met kanker en een kleine maar significante correlatie met prostaat specifiek antigen (PSA) werd vastgesteld. PSA wordt frequent gebruikt als circulerende tumor merker in prostaat kanker.

sN-CAD heeft ook een biologische functie, het stimuleert de migratie van N-cadherine positieve cellen, meer in het bijzonder van endotheel cellen. Door gebruik te maken van de kippen chorioallantoisch membraan en de konijn cornea micropocket test, konden wij sN-CAD identificeren als een proangiogene molecule. Ontwarren van de signalisatie weg bevestigde ons dat de FGF-receptor een belangrijke rol speelt in de sN-CAD gemedieerde effecten.

Tot besluit, sN-CAD is een mogelijk interessante molecule in de oncologie, als een circulerende tumor merker en als doel voor angiostatisch behandeling.

Résumé

Dans la présente thèse, la N-cadhérine et sa forme clivée, la N-cadhérine soluble (sN-CAD), sont étudiées en relation avec le cancer. Les cadhérines sont des molécules d'adhésion intercellulaires calcium-dépendantes qui sont souvent dérégulées par le cancer. La E-cadhérine, exprimée par des cellules épithéliales normales, est régulée vers le bas lorsque des cellules deviennent cancéreuses. Ce phénomène coïncide parfois avec la transactivation d'une autre cadhérine, par exemple la N-cadhérine. Le résultat est que les cellules modifient leur phénotype pour devenir fibroblastiques, motiles et invasives. Le rôle de la N-cadhérine dans l'adhésion et la différentiation cellulaires, l'embryogenèse et le cancer est étudié et nous donnons plusieurs exemples d'expression de N-cadhérine dans des lignées de cellules tumorales et de biopsies.

Les protéases extracellulaires, connues également comme le "dégradome" du cancer, jouent un rôle important lors de la progression tumorale. Elles sont impliquées dans des processus comme l'invasion et la métastase, mais aussi dans la prolifération cellulaire, l'apoptose et l'angiogenèse. Les protéases comme ADAM10, MT1-MMP et la plasmine sont capables de séparer un fragment extracellulaire de 90kD de la N-cadhérine. Nous avons pu établir un ELISA pour la détection de cette sN-CAD. Nous avons examiné des fluides corporels de personnes sans signe de maladie et de patients cancéreux, à la recherche de la présence de sN-CAD. Des taux significativement élevés de sN-CAD ont été mesurés dans le groupe de patients cancéreux et une corrélation faible mais significative a été trouvée avec l'antigène prostatique spécifique (PSA) qui est le marqueur tumoral circulant le plus utilisé dans le cancer de la prostate.

En outre, sN-CAD exerce, elle aussi, une fonction biologique: elle stimule la migration de cellules positives pour la N-cadhérine et plus spécifiquement des cellules endothéliales. Par l'application du test de la membrane chorioallantoïque d'embryon de poulet et de la micropoche dans la cornée de lapin, nous avons pu établir que la sN-CAD était une molécule pro-angiogène. L'éclaircissement des voies de signalisation nous a permis de démontrer que le récepteur de croissance fibroblastique joue un rôle important dans les effets médiés par la sN-CAD.

En conclusion, la sN-CAD est une molécule potentiellement intéressante en cancérologie, en tant que marqueur tumoral circulant et comme cible d'un traitement angiostatique.

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- **Derycke L**., De Wever O., Van Aken E. and Bracke M., The extracellular fragment of N-cadherin promotes migration and angiogenesis, Wetenschapsdag on the University Hospital Ghent, Belgium, Marc 30, 2006

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- Van Aken E., Morbidelli L., **Derycke L**., Mareel M., and De Laey JJ. Plasmin produces a proangiogenic fragment. European Association for Vision and Eye Research, Alicante, Spain, October 4, 2002
- **Derycke L**., Van Aken E. and Bracke M. The extracellular fragment of N-cadherin promotes migration and angiogenesis". M. BACR meeting, Brussels, Belgium, January 28, 2006

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