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CATALYTICALLY-INACTIVE HUMAN CATHEPSIN D TRIGGERS FIBROBLAST INVASIVE GROWTH

Valérie Laurent-Matha[1#$], Sharon Maruani-Herrmann[1#&], Christine Prébois[1], Mélanie Beaujouin[1], Murielle Glondu[1§], Agnès Noël[2], Marie Luz Alvarez-Gonzalez[2], Sylvia Blacher[2], Peter Coopman[3], Stephen Baghdiguian[4], Christine Gilles[2], Jadranka Loncarek[5], Gilles Freiss[1], Françoise Vignon[1], and Emmanuelle Liaudet-Coopman[1*]

[1]INSERM U540 'Endocrinologie Moléculaire et Cellulaire des Cancers', Université de Montpellier 1, 60 rue de Navacelles, 34090 Montpellier, France. [2]Laboratory of Tumor and Developmental Biology, University of Liège, Tour de Pathologie (B23), Sart-Tilman, B-4000 Liège, Belgium. [3]CNRS UMR 5539, Université Montpellier 2, Place Eugène Bataillon, 34095 Montpellier, France. [4]CNRS UMR 5554, Université Montpellier 2, Place Eugène Bataillon, 34095 Montpellier France. [5]INSERM EMI 0229, Centre de Recherche en Cancérologie, CRLC Val d'Aurelle-Paul Lamarque, 34298 Montpellier, France.

Present addresses: [&]Dina Raveh's laboratory, Life Science Department, Ben Gourion University of the Negev, Box 653, Beer-Sheva 84105, Israel.[$]Cellular Biology Unit, IGH, CNRS UPR 1142, 141 rue de la Cardonille, 34396 Montpellier Cedex 05, France. [§]INSERM U145, IFR-50, Faculté de Médecine, 06107 Nice Cedex 2, France.

*Corresponding author: E Liaudet-Coopman, INSERM U540, 60 rue de Navacelles, 34090 Montpellier, France ; Tel (33) 4 67 04 30 80 ; FAX (33) 4 67 54 05 98, E-mail: liaudet@montp.inserm.fr

[#] Authors contributed equally to the work.

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ABSTRACT

The aspartyl-protease cathepsin D (cath-D) is over-expressed and hyper-secreted by epithelial breast cancer cells and stimulates their proliferation. As tumor epithelial-fibroblast cell interactions are important events in cancer progression, we investigated whether cath-D overexpression affects also fibroblast behavior. We demonstrate a requirement of cath-D for fibroblast invasive growth using a three-dimensional (3D) co-culture assay with cancer cells secreting or not pro-cath-D. Ectopic expression of cath-D in cath-D-deficient fibroblasts stimulates 3D outgrowth that is associated with a significant increase in fibroblast proliferation, survival, motility and invasive capacity, accompanied by activation of the *ras*-MAP kinase pathway. Interestingly, all these stimulatory effects on fibroblasts are independent of cath-D proteolytic activity. Finally, we show that pro-cath-D secreted by cancer cells is captured by fibroblasts and partially mimics effects of transfected cath-D. We conclude that cath-D is crucial for fibroblast invasive outgrowth and could act as a key paracrine communicator between cancer and stromal cells, independently of its catalytic activity.

INTRODUCTION

Cathepsin D (cath-D) (EC 3.4.23.5) is a ubiquitous lysosomal aspartyl endoproteinase extensively reported as being over-expressed and hyper-secreted by human breast cancer cells (Rochefort et al., 1987). Many independent clinical studies have associated cath-D over-expression with increased risk of clinical metastasis and shorter survival in breast cancer patients (Rochefort, 1992; Ferrandina et al., 1997; Foekens et al., 1999; Westley and May, 1999). During transport to lysosomes, 52 kDa human inactive pro-cath-D is proteolytically processed into a single-chain intermediate active enzyme of 48 kDa located in endosomes. Further proteolytic processing yields the mature active lysosomal protease which is composed of heavy (34 kDa) and light (14 kDa) chains (Richo and Conner, 1991). Human cath-D catalytic site includes two critical aspartyl residues (amino acid 33 and 231) located on the 14 kDa and 34 kDa chains, respectively (Metcalf and Fusek, 1993). Mannose-6-phosphate (Man-6-P) receptors are involved in cath-D lysosomal routing and in the cellular uptake of the secreted pro-cath-D (Von Figura and Hasilik, 1986) although cath-D may also be targeted to the lysosome and endocytosed independently of Man-6-P receptors (Capony et al., 1994, Laurent-Matha et al., 1998). Recently, cath-D was shown to be a rate limiting factor for outgrowth, tumorigenicity, and lung colonization of MDA-MB-231 breast cancer cells using an RNA antisense strategy (Glondu et al., 2002). Several reports have indicated that cath-D stimulates cancer cell proliferation (Vignon et al., 1986; Vetvicka et al., 1994; Liaudet et al., 1995) and increases metastatic potential *in vivo* (Garcia et al., 1990; Liaudet et al., 1994). Moreover we reported that D231N/cath-D mutated in its catalytic site, and hence devoid of proteolytic activity is still mitogenic for cancer cells both *in vitro* in three-

dimensional (3D) matrices and *in vivo* in athymic nude mice (Rochefort and Liaudet-Coopman, 1999; Glondu et al., 2001). Immunohistochemical studies indicated that cath-D, independently of its proteolytic activity, stimulates not only cancer cell proliferation by an autocrine and/or intracrine mechanism, but also tumor angiogenesis by a paracrine mechanism (Berchem et al., 2002). This revealed for the first time the potential paracrine action of cath-D in the context of a tumor.

Tumor progression requires a continually evolving network of interactions between neoplastic cells and their microenvironment consisting of an extracellular matrix, a stroma composed of fibroblasts, adipose, vasculature and resident immune cells, and a mixture of cytokines and growth factors (Pupa et al., 2002). Current observations increasingly point to the contribution of stromal components to oncogenic signals that mediate both phenotypic and genomic changes in epithelial cells (Tlsty and Hein, 2001). Carcinoma formation from its earliest stages seems to depend on the ability of transformed epithelial cells to first recruit and then subvert a variety of stroma cells originating from adjacent normal tissue to become infiltrating or invading (Elenbaas and Weinberg, 2001). In many common carcinomas including breast, colon, stomach, and pancreas, stroma comprises the majority of tumor mass, in some cases accounting for over 90%. In various experimental tumor models, the microenvironment affects efficiency of tumor formation, rate of tumor growth, extent of invasiveness, and ability of tumor cells to metastasize. In breast carcinomas, the influences of microenvironment are mediated, in large part, by paracrine signalling between epithelial tumor cells and neighboring stromal fibroblasts (Elenbaas and Weinberg, 2001). In addition to receiving signals from epithelial cells, the stromal fibroblasts stimulate tumorigenesis by releasing factors that act on epithelial tumor cells. Stromal and tumor cells exchange enzymes, growth factors and cytokines that

modify local extracellular matrix, stimulate migration and invasion, and promote proliferation and survival of tumor cells (Masson et al., 1998; Liotta and Kohn, 2001; Bisson et al., 2003). A stromal reaction immediately adjacent to transformed epithelial cells has been documented in several tumor systems (Basset et al., 1990; Olumi et al., 1999; Shekhar et al., 2001).

In the present study, we highlight the possibility that cath-D may participate in such paracrine signalling interactions between epithelial cancer cells and associated fibroblasts. The paracrine function of cath-D hyper-secreted by cancer cells was first examined both in a 3D co-culture assay and in an aortic ring assay. We then went on to study the effects of the ectopic human cath-D expression in cath-D–deficient fibroblasts on their behavior profile, e.g. outgrowth, proliferation, survival, migration, invasion and signalisation.

RESULTS

Cancer cells secrete paracrine factors crucial for fibroblast invasive growth.

The communication between epithelial cancer cells and fibroblasts and in particular the role of pro-cath-D secreted by cancer cells on stromal compartments was explored using a 3D assay of cath-D-deficient mouse CD55-/-SV40, human skin CCD45K, or human breast HMF fibroblasts co-cultured with cancer cells. Human breast cancer MCF-7 cells induced outgrowth of all fibroblast cell lines tested indicating that epithelial cancer cells were secreting paracrine factors capable of promoting fibroblast outgrowth in 3D matrices (Figure 1A).To assess whether secreted pro-cath-D might be one of these crucial paracrine factors, 3D co-culture assays were performed with cath-D-transfected rat 3Y1-Ad12 cancer cell lines secreting no cath-D (control), or hyper-secreting human wild-type cath-D (cath-D), or

D231N mutated cath-D devoid of proteolytic activity (D231N). Only cancer cells secreting wild-type or D231N pro-cath-D stimulated CD55-/-SV40 fibroblast outgrowth, whereas control cells had no effect (Figure 1B) suggesting that secreted pro-cath-D might promote 3D outgrowth of fibroblasts in a paracrine manner. Since the catalytic activity was clearly not involved, it might occur by binding to potential receptors present on fibroblast cell surfaces or to non-receptor proteins. We excluded the Man-6-P/IGF2 receptor as being the mediator of the cath-D induction of fibroblast invasive growth since addition of Man-6-P in excess sufficient to prevent the binding of cath-D to this receptor (Capony et al., 1987) had no effect in our 3D co-culture assays (data not shown).To strengthen the possibility of a direct paracrine action of secreted pro-cath-D, 3D co-culture assays were carried out with MCF-7 cells whose endogeneous pro-cath-D secretion was inhibited by 75% with small RNA-mediated gene silencing (cath-D siRNA) for 2 days before the co-culture assay (Figure 1C, panel b). MCF-7 cells transfected with cath-D siRNA were less effective in triggering CD55-/-SV40 fibroblast outgrowth when compared to MCF-7 cells transfected with control luciferase siRNA (luc siRNA) (Figure 1C, panel a). A 75% inhibition of pro-cath-D secretion by cath-D siRNA still occurred during our co-culture assay even after 4 days of co-culture (Figure 1C, panel b). We conclude therefore that inhibition of pro-cath-D secretion rapidly lead to a decrease in fibroblast invasive growth. Results similar to those depicted in Figure 1B-C were obtained with breast HMF fibroblasts indicating the possible relevance of the paracrine role of secreted pro-cath-D in the pathological context (data not shown). Moreover, secreted wild-type and D231N pro-cath-D also induced fibroblast outgrowth in an aortic ring assay (Sup Figure 1). Altogether, our results support the concept that pro-cath-D secreted by epithelial cancer cells might exhibit a crucial paracrine function on stromal cells.

Interestingly, all these stimulatory effects on fibroblasts were independent of the catalytical activity of cath-D.

Characterization of mouse fibroblasts engineered to express catalytically active or inactive human cath-D.

To dissect the effect of cath-D on fibroblast behavior, we stably transfected a cath-D–deficient fibroblast CD55-/- cell line with expression vectors for wild-type, D231N cath-D, or with an empty vector, generating CD55-/-cath-D, CD55-/-D231N and CD55-/-SV40 cell lines, respectively. A control cell line (CD53+/+) expressing endogenous mouse cath-D was stably transfected with an empty vector, generating the CD53+/+SV40 cell line. The cath-D concentration detected in cell extracts for CD55-/-cath-D and CD55-/-D231N cells were 7.9 ± 1 ng/µg DNA and 1.6 ± 0.1 ng/µg DNA corresponding to 15 and 3 pmoles/mg cytosolic protein. The amount of pro-cath-D secreted in 24 h by CD55-/-cath-D and CD55-/-D231N cells was respectively 2.6 ± 0.4 ng/µg DNA and 0.3 ± 0.1 ng/µg DNA. Expression of wild-type cath-D was significantly greater than that of D231N cath-D, suggesting that the D231N mutation might affect the protein stability. However, cellular processing of 52 kDa cath-D-D231N into 48kDa intermediate and 34 kDa mature forms was not prevented by the D231N mutation in fibroblasts (Sup Figure 2A) and the D231N mutant was devoid of catalytic function in our cellular model (Sup Figure 2B). The level of human wild-type cath-D expressed by transfected CD55-/- fibroblasts was comparable to that of endogeneous mouse cath-D produced by CD53+/+SV40 fibroblasts (Sup Figure 2B, top panel). However, mouse cath-D was not secreted (Sup Figure 2B, bottom panel).

Human cath-D triggers 3D outgrowth of fibroblasts independently of its proteolytic activity.

We next studied the effect of wild-type or D231N mutated cath-D expression on fibroblast outgrowth in 3D matrices (Figure 2). As shown in Figure 2A, human cath-D promoted outgrowth of mouse fibroblasts embedded into Matrigel. After 8 days of culture CD55-/-cath-D, CD55-/-D231N and CD53+/+SV40 cells had adopted a stellate morphology of growing and invasive colonies with protrusions sprouting into the surrounding matrix (Figure 2A). In contrast, CD55-/-SV40 fibroblasts presented a well-delineated spherical appearance of quiescent and/or dying cells and grew poorly, neither invading nor forming protrusions (Figure 2A). Similar results were obtained when fibroblasts were embedded into collagen I gels (data not shown). Moreover, when plated on Matrigel gels, CD55-/-cath-D and CD55-/-D231N cells grew as efficiently as CD53+/+SV40 cells (Figure 2B). This growth was shown by cell staining (Figure 2B, top panel) and by phase contrast microscopy (Figure 2B, bottom panel). In the latter case, higher magnification showed a network of interacting cells (Figure 2B, bottom panel). Cath-D-deficient CD55-/-SV40 fibroblasts grew poorly and displayed a stressed morphology of well-delineated rounded cells (Figure 2B, bottom panel). We finally compared migration of transfected fibroblasts in wound healing experiments performed on collagen I. As shown in Figure 3, CD55-/-cath-D and CD55-/-D231N fibroblasts migrated more efficiently into a wound area as compared to CD55-/-SV40 fibroblasts. Similar results were obtained with cells directly seeded on plastic (data not shown). Altogether, these results demonstrate that cath-D triggers fibroblast invasive outgrowth in matrices. Moreover, the mechanism is independent of its proteolytic activity since catalytically-inactive D231N cath-D is as effective in stimulating cell outgrowth.

Catalytically-inactive cath-D promotes proliferation and survival, as well as stimulates migration and invasion of fibroblasts.

We investigated whether stimulation of fibroblast outgrowth induced by cath-D was accompanied by increased proliferation, survival, migration, or invasiveness. As shown in Figure 4A, stable expression of wild-type or mutated D231N cath-D in cath-D-deficient CD55-/- cells lead to a significant stimulation of proliferation relative to that of CD55-/-SV40 cells. Similar proliferation was observed in CD53+/+SV40 fibroblasts expressing endogenous mouse cath-D (Figure 4A). Cell cycle analyses by flow cytometry revealed that the stimulation of cell proliferation induced by transfected wild-type and D231N cath-D was due to both a significant increase in the number of proliferating cells in S phase, 52.5 ± 2.5% , 61.7 ± 2.5% and 65.9 ± 2 for CD55-/-SV40, CD55-/-cath-D and CD55-/-D231N cells, respectively, and to a significant decrease in the number of apoptotic cells, 7.5 ± 2.9%, 0.79 ± 0.47% and 0.3 ± 0.25% for CD55-/-SV40, CD55-/-cath-D and CD55-/-D231N cells, respectively (Figure 4B, panel a). BrdUrd-incorporated S phase cells confirmed the increased S phase of CD55-/-cath-D (64%) and CD55-/-D231N cells (63%) relative to that of CD55-/-SV40 cells (50%) (Figure 4B, panel b). These results indicate that cath-D stimulates fibroblast proliferation in a manner independent of its catalytic activity. In addition to its positive effect on proliferation, cath-D also seems to rescue fibroblasts from apoptosis and promote their survival. The apoptotic status was further analyzed by flow cytometry with transfected fibroblasts grown for 3 days on Matrigel. A significantly higher proportion of CD55-/-SV40 fibroblasts appeared in a sub-G0/G1 (apoptotic) peak (9,8 ± 2.7%, n=5) as compared to CD55-/-cath-D (0.39 ± 0.55%, n=3), CD55-/-D231N (0.59%; 2.16%, n=2) and CD53+/+SV40 (0%, n=2) fibroblasts

(Figure 4C). Additionally, CD55-/-SV40 fibroblasts embedded in Matrigel and stained with cell permeable Hoechst 33342 presented an apoptotic phenotype characterized by the presence of apoptotic bodies, which could not be detected in CD55-/-cath-D fibroblasts (Figure 4D). These results were confirmed by transmission electron microscopy (Figure 4E). CD55-/-SV40 fibroblasts exhibited features of apoptotic cells characterised by the presence of a well-delineated cytoplasm and a complete chromatin condensation (Figure 4E, panels a, b). It is noteworthy that this primary apoptosis was followed by secondary necrosis under our culture conditions in Matrigel (Figure 4E, panel b). By contrast, CD55-/-cath-D cells never presented such an apoptotic phenotype (Figure 4E, panel c). These results demonstrate that cath-D-deficient CD55-/-SV40 fibroblasts were dying in matrices and that wild-type cath-D could promote their survival. We next evaluated whether cath-D could also play a role in fibroblast migration or invasiveness, important steps for tumor invasion and metastatic process. Indeed, results from Figure 2 and 3 already revealed that CD55-/-cath-D, CD55-/-D231N and CD53+/+SV40 cells migrated and invaded the surrounding matrices, suggesting a possible role of cath-D in migration and invasiveness. Chemotactic migration and invasiveness of fibroblasts were measured in parallel Boyden Chamber assays (Figure 4F-G). The basal level of migration of CD55-/-SV40 fibroblasts was relatively high as expected for mesenchymal cells, *e.g.* 30 $\pm$ 5% of cells crossed filters coated with collagen I (Figure 4F). Cath-D expressing cells (CD55-/-cath-D, CD55-/-D231N and CD53+/+SV40 cells) showed a small but significant increase of 1.4 fold in migrative capacity, when compared to cath-D-deficient CD55-/-SV40 cells (Figure 4F). In addition, invasive assays performed with a Matrigel barrier highlighted the fact that CD55-/-cath-D, CD55-/-D231N and CD53+/+SV40 cells were significantly more invasive than CD55-/-SV40 cells by a

factor of 2.7 fold (Figure 4G). A similar induction of invasiveness was also obtained in lower serum concentrations (1% FCS + 0.1% BSA) in the upper chamber (data not shown). Collectively, these results indicate that cath-D could contribute to fibroblast invasive growth by promoting not only proliferation and survival, but also by stimulating migration and invasiveness in a manner independent of its catalytic function.

Activation of MAP kinase in fibroblasts.

The *Ras*-MAP kinase signaling cascade has been reported as being involved in proliferation, survival, motility and invasion (Bar-Sagi and Hall, 2000; Schlessinger et al., 2000; Iwabu et al., 2004). Therefore, we examined whether cath-D could activate the MAP kinase cascade in fibroblasts. Specific anti-pERK monoclonal antibodies enabled us to follow levels of ERK1/2 kinase phosphorylation, reflecting activation of the MAP kinase pathway. Results from Figure 5A (panel b) revealed that ERK1 and ERK2 phosphorylation levels were significantly induced by 2.4, 3.5 and 5.4 fold in CD55-/-cath-D, CD55-/-D231N and CD53+/+SV40 fibroblasts, respectively, relative to CD55-/-SV40 fibroblasts. This effect was observed by culturing cells in 2% FCS, which corresponded to the experimental conditions used in our proliferation assays (Figure 4A). We then investigated whether fibroblast invasive growth induced in 3D matrices by transfected cath-D could be linked to a stimulation of the MAP kinase pathway. CD55-/-cath-D cells were either pre-treated or not with a MEK1/2 inhibitor, U0126, and were then embedded in Matrigel. U0126 efficiently inhibited ERK1/2 kinase phosphorylation (Figure 5B, panel a) and thereby prevented invasive growth of CD55-/-cath-D fibroblasts (Figure 5B, panel b). Taken together, these findings suggest that cath-D may be responsible for positive regulation of proliferation,

survival, motility and/or invasion of fibroblasts by triggering activation of *ras*/MAPK/ERKs.

Paracrine function of cath-D on fibroblasts.

To provide further support for a direct paracrine action of pro-cath-D secreted by breast cancer cells on fibroblasts, we next studied the binding and endocytosis of secreted [^{35}S]methionine-labeled pro-cath-D by recipient CD55-/- fibroblasts (Figure 6A). The labeled 52 kDa pro-enzyme was totally transformed into 48 kDa intermediate and 34 kDa mature enzymes following its binding and endocytosis after 24 h (Figure 6A, panel b, 0). The addition of Man-6-P in excess, described as preventing cath-D binding to Man-6-P receptors (Capony et al., 1987), partly inhibited pro-cath-D internalization by 65 % (Figure 6A, panel b, + Man-6-P). These results support the hypothesis of a paracrine action of pro-cath-D secreted by breast cancer cells being bound and captured by fibroblasts *via* Man-6-P receptors and another putative cell surface receptor. To further characterize the cath-D paracrine action, we next studied the effect of conditioned medium from mock-transfected (control), wild-type (cath-D) or D231N cath-D (D231N) transfected 3Y1-Ad12 cancer cell lines on CD55-/-SV40 fibroblast outgrowth, proliferation and apoptosis, invasion, and activation of the MAP kinase signaling pathway. Figure 6B shows that conditioned media containing wild-type or D231N secreted pro-cath-D stimulated fibroblast outgrowth, whereas conditioned medium from control cells was inefficient in this respect. The paracrine effect of secreted pro-cath-D on CD55-/-SV40 fibroblast outgrowth was associated with a significant 10% increase of proliferating cells in S phase (Figure 6C, panels a-b), a significant 1.4 fold increase in their invasive capacity (Figure 6D) and a 3 fold induction of ERK1 and ERK2 phosphorylation

(Figure 6E). However, no significant effect on apoptosis was observed following addition of pro-cath-D (Figure 6C, panel a). Overall, these results show that secreted pro-cath-D partially mimics the effects of transfected cath-D and strongly suggest that pro-cath-D is acting extracellularly *via* a paracrine loop in a manner independent of its catalytic activity.

DISCUSSION

Interactions between stromal and epithelial cells are important in many steps of tumor progression (Elenbaas and Weinberg, 2001). Stromal and tumor cells interchange numerous growth factors and proteases to activate the adjacent extracellular matrix and, in turn, induce the selection and expansion of neoplastic cells (Liotta and Kohn, 2001). Fibroblasts are a major cell type of the stromal compartment, and are intimately involved in orchestring the stromal part of the dialogue in tissue homeostasis (Grinnell, 1994). Whereas the role of matrix metalloproteinases and urokinase plasminogen activator in the stromal compartment has been documented in various studies (Sieuwerts et al., 1999; Liotta and Kohn, 2001; Singer et al., 2002; Kataoka et al., 2003; Tang et al., 2004), the potential role of cath-D in fibroblasts had not yet been fully determined. It has been proposed that cath-D localized at the surface of breast fibroblasts might be mitogenic (Koblinski et al., 2002) or that intracellular cath-D in fibroblasts might assist cancer cells in the digestion of extracellular matrix during tissue invasion (Heylen et al., 2002). Here, we show for the first time that the lysosomal aspartic protease cath-D induces fibroblast invasive growth in a manner independent of its catalytic activity.

The paracrine effect of cath-D secreted by epithelial cancer cells on promoting invasive 3D outgrowth of fibroblasts may have important implications with regard to

carcinoma progression. Fibroblasts in stromal tissue surrounding the primary tumor site may become invasive and proliferative in response to secretion of pro-cath-D by tumor cells. Many epithelial tumors need to undergo a distinct epithelial to mesenchymal transition to become fully aggressive and invasive (Vincent-Salomon and Thiery, 2003). A part of this transition may involve activation of stromal fibroblasts (Basset et al., 1990; Olumi et al., 1999; Shekhar et al., 2001). In this respect, in addition to being a marker of poor prognosis in breast cancers (Rochefort, 1992; Ferrandina et al., 1997; Foekens et al., 1999; Westley and May, 1999), cath-D may be a potential target for therapeutic inhibitors of tumor progression.

Invasive growth is generally associated with epithelial cells but had also been attributed to other cell types (Comoglio et al., 1999) and involves proliferation, survival, motility, invasion of extracellular matrices and induction of polarity (Tamagnone and Comoglio, 1997). We demonstrate here that both catalytically active and inactive cath-D stimulate proliferation, survival, motility and invasion of fibroblasts. The mitogenic activity of cath-D had been widely described for cancer cells (Vignon et al., 1986; Vetvicka et al., 1994; Liaudet et al., 1995; Glondu et al., 2001; Glondu et al., 2002) and has been suggested for endothelial cells (Berchem et al., 2002). Our data also clearly highlight the facts that cath-D-deficient fibroblasts undergo apoptosis in matrices and that the expression of catalytically active or inactive cath-D in fibroblasts induced their survival. The anti-apoptotic function of cath-D had been previously suggested from the apoptotic and necrotic phenotypes observed after invalidation of its gene (Saftig et al., 1995; Nakanishi et al., 2001; Koike et al., 2003) and in tumor xenografts (Berchem et al., 2002). We also observed a positive effect of cath-D in the migration and invasive potential of fibroblasts. The involvement of cath-D proteolytic activity in degradation of the extracellular matrix, an

important step for invasion, is unlikely because cath-D requires an acidic pH to be proteolytically active (Briozzo et al., 1988). Since D231N cath-D was as potent as wild-type cath-D in stimulating invasion, the mechanism responsible seems more likely to implicate either an interaction of cath-D with other factors involved in this process, or an indirect regulation of gene expression. We did not detect any significant change in the mRNA expression levels of a variety of proteases and their inhibitors in transfected fibroblasts (data not shown), but our preliminary data suggest that cath-D could bind to an inhibitor of one specific class of proteases and therefore increase protease activity. Invasive growth also results from the concomitant activation of *ras*-MAP kinase, PI3 kinase, and signal transducer and activator of transcription (Comoglio et al., 1999). Our data suggest a possible link between the biological effects of cath-D in fibroblasts and the MAP kinase pathway, which is well known for its role in promoting cell proliferation (Bar-Sagi and Hall, 2000). In addition, activation of ras/MEK is frequently associated with up regulation of protein kinase C, particularly PKCdelta which is known to be associated with changes in cellular motility (Iwabu et al., 2004), another effect seen following cath-D transfection.

The question remains as to how cath-D induces such a major change in fibroblast phenotype. Since fibroblasts can become activated by catalytically active or inactive cath-D or by the pro-cath-D precursor, its proteolytic activity is clearly not directly involved in the stimulatory effect. At present, the only receptor known to interact with secreted pro-cath-D is Man-6-P/IGF2**,** which has a well defined function in the transport of various ligands *via* the endosomal pathway (Clague et al., 1998). However, the ability of this receptor to stimulate cellular responses *via* signaling pathways remains controversial. Although a recent study indicates that it may transduce IGF2 mitogenic activity *via* the MAP kinase pathway (McKinnon et al.,

2001), our results have ruled out the Man-6-P/IGF2 receptor as being the mediator of cath-D induction of fibroblast invasive growth in 3D co-culture assays (data not shown).

Another possibility is that secreted pro-cath-D binds to a yet unidentified cell surface receptor coupled to the MAP kinase transduction pathway. Indeed it has been proposed that such a receptor exists at the cell surface of cancer and endothelial cells to mediate cath-D mitogenic activity (Vetvicka et al., 1994; Laurent-Matha et al., 1998; Glondu et al., 2001; Berchem et al., 2002).

In conclusion, this study demonstrates that cath-D plays a crucial role for fibroblast outgrowth in 3D matrices. We therefore propose that cath-D may favor tumor progression not only by affecting the epithelial compartment but also by promoting fibroblast outgrowth *via* a paracrine loop. Under these circumstances, cath-D over-expressed and hyper-secreted by cancer cells is captured *in vivo* by stromal cells and may not only promote proliferation and survival, but also stimulate motility and invasion of fibroblasts, and consequently enhance tumor-host homeostasis. If we view the cancer state as a product of its micro-environment, the identification of factors such as cath-D that participate in the tumor-host communication interface activating the host micro-environment is crucial for the development of new stromal therapy.

MATERIALS AND METHODS

Cell lines, Transfections, siRNAs and growth assays.

Cath-D-deficient CD55-/- and CD53+/+ immortalized mouse fibroblasts were kindly provided by Dr Christoph Peters (University of Freiburg, Germany). Human normal skin CCD45K fibroblasts were purchased from ATCC. Human mammary HMF

fibroblasts, kindly provided by Dr Jadranka Loncarek and Dr Jacques Piette (CRLC Val d'Aurelle-Paul Lamarque, Montpellier, France), were obtained from reduction mammoplasty tissues from a patient without cancer. Cells were cultured in DEM medium with 10% fetal calf serum (FCS, GibcoBRL). Cells were co-transfected with a 10-fold excess of wild-type, D231N cath-D, or empty pSG5-neo vector and a pTK-Hyg hygromycin-resistance expression vector (Clontech) using Lipofectamine (Gibco-BRL). Colonies growing in the presence of hygromycin B (100 µg/ml, Invitrogen) were pooled and human cath-D was quantified using an immunoradiometric assay (Glondu et al., 2001). Duplexes of 21-nucleotide human cath-D siRNA (target sequence AAGCUGGUGGACCAGAACAUC, residues 666-684) (Bidere et al., 2003) or firefly luciferase siRNA (target sequence AACGUACGCGGAAUACUUCGA residues 515-534) were synthesized by MWG Biotech S.A. (France).15,000 MCF-7 cells were plated in 24-well plates and after 3 days cells were transiently transfected with 1 µg siRNA using 6µl Oligofectamine (Invitrogen). The expression (33 µg protein) and secretion (30 µl medium) of cath-D were monitored by Western blot using 1 µg/ml anti-human monoclonal cath-D antibody (#610801, BD Biosciences). For proliferation assays, 3,000 cells were plated in 24-well plates and then covered with culture medium with 2% FCS. Cells were fixed with methanol and DNA content determined by a diaminobenzoic acid fluorescence assay (Vignon et al., 1986). For outgrowth assay, 100,000 cells were re-suspended at 4°C in Matrigel (0.2 ml, 10 mg/ml) (Becton and Dickinson), and quickly added to a pre-set layer of Matrigel in 24-well plates as described previously (Glondu et al., 2001). The top Matrigel layer was gelled at 37°C for 30 min and then covered with culture medium containing 10% FCS (0.5 ml). In some experiments, cells were pre-treated for 6 h with the MEK1/2 inhibitor, U0126 (10µM, Cell Signaling Technology, Inc.) and were then embedded

into Matrigel. For treatment of cells embedded into Matrigel, conditioned medium (0.5 ml) supplemented with 5% FCS was directly added after the top Matrigel layer had gelled. To prepare the conditioned media, mock-transfected, cath-D and D231N transfected 3Y1-Ad12 cancer cells were plated at a 70 % density and after 24 h, cells were washed twice with medium without FCS and then cultured with 0% FCS. After 48 h, cells reached 90-100% confluence and conditioned medium was removed and centrifuged at 800 g for 10 min and was finally stored at -80°C. For growth on Matrigel, 100,000 cells were directly plated into 24-well plates on a pre-set Matrigel layer and then covered with culture medium containing 10% FCS. In 3D Matrigel co-culture outgrowth assays, 50,000 MCF-7 cells were re-suspended at 4°C in Matrigel (0.2 ml, 10 mg/ml) and then added to a pre-set layer of Matrigel in 24-well plates. CD55-/-SV40, CCD45K, or HMF fibroblasts were re-suspended in 0.2 ml Matrigel, added to the Matrigel layer containing embedded MCF-7 cells and were then covered with culture medium with 10% FCS (0.5 ml), as previously described for collagen I 3D co-culture assays (Nakashiro et al., 2000). In other co-culture outgrowth assays, 15,000 MCF-7 cells previously transfected with cath-D or luc siRNAs, or 200,000 cath-D transfected 3Y1-Ad12 cell lines were plated in 24-well plates. After 2 days, cells were covered with a layer of Matrigel (0.2 ml), then with a layer of Matrigel (0.2 ml) containing 50,000 CD55-/-SV40 fibroblasts, and finally were covered with culture medium with 10% FCS (0.5 ml). Cells were photographed with an Olympus inversed phase-contrast microscope using an Olympus camera with a 10x objective and negatives were scanned and processed using Adobe Photoshop software.

Wound healing, invasion and motility assays.

For wounding experiments, 500,000 cells were seeded on type I collagen-coated 6-well plates (5µg / cm2) and cell layers were wounded by tip-scraping. Cells were washed with fresh medium to remove floating cells and re-fed with fresh medium supplemented with 10% FCS and wound healing was monitored with an Olympus inversed phase-contrast microscope. The Boyden chamber chemo-invasion and migration assays were performed essentially as described previously (Glondu et al., 2002). For invasion assays, polycarbonate filters (12-µm pore) were coated with 35 µg of Matrigel. Cells were harvested in 4 % FCS containing medium and added to the top chamber (600,000 cells / chamber) and 10% FCS containing DEM medium was used in the lower compartment. In some experiments, conditioned medium from transfected 3Y1-Ad12 cells was used in the lower compartment either lacking pro-cath-D, or containing (30 nM) wild-type pro-cath-D supplemented with 10% FCS. Chambers were incubated for 24 h at 37°C, after which living cells that had traversed the Matrigel and spread on the lower surface of the filter were quantified by the mitochondrial deshydrogenase enzymatic assay using 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenol tetrazolium bromide (MTT) and determination of OD_{540nm}.Migration assays were performed as described for the chemo-invasion studies, with filters coated with 5 µg of rat tail collagen I and the cells were incubated in the upper chamber in 0% FCS + 0.1 % BSA.

Flow cytometry, incorporation of BrdUrd, electron microscopy and *in situ* apoptosis.

500,000 cells plated onto Matrigel (0.5 ml, 10 mg/ml) in 12-well plates were recovered after 3 days with 2.5 ml Matrisperse (Becton Dickinson), resuspended in 1 ml of a solution containing 0.1% tri-sodium citrate dehydrate, 0.1% Triton X-100, 25 µg/ml propidium iodide and 100 µg/ml RNAse, prior to FACScan analysis (Becton

Dickinson, CA) with an argon ion laser turned at 488nm, 20 mW. Propidium iodide fluorescence was measured at 585 nm. Data were collected and analyzed with Cellquest (Becton Dickinson, CA) and ModFit (Verity Software, ME) softwares, respectively. In other experiments, 60,000 cells plated in 6-well plates in the absence or presence of conditioned media supplemented with 2% FCS containing either no pro-cath-D, 30 nM wild-type or 10 nM D231N pro-cath-D were analyzed by flow cytometry after 3 days. For staining for incorporated BrdUrd in nuclei, cells cultured on coverslips were labelled with BrdUrd (10 µM) followed by incubation with anti-BrdUrd monoclonal antibody (Beckton Dickinson), as previously described (Nakayasu, and Berezney, 1989). Secondary antibody was an FITC conjugated anti-mouse IgG (Jackson Laboratories). Cells were then incubated with PBS containing DAPI (0.5 µg/ml) and coverslips were mounted on microscopy slides and counted under a fluorescent microscope (Axioplan MC100, Zeiss). For electron microscopy, ultra thin sections of cells embedded in Matrigel were classically observed using a Jeol 1200X transmission electron microscope. For *in situ* apoptosis, cells embedded in Matrigel were incubated for 15 min at 37°C with 10 µM cell permeant Hoechst 33342 (Molecular Probes) in complete DMEM medium. After three washes with complete DMEM medium, live cells in complete medium were observed with a Leica DMRA2 microscope equipped with a water immersion 63x/0.90 apochromatic objective and a Coolsnap FX cooled CCD camera (Princeton Instruments), both controlled by the MetaMorph imaging software (Universal Imaging Corps).

Immunoprecipitation and endocytosis of cath-D.

For cath-D immunoprecipitation, fibroblasts were incubated in 70 % methionine-free DEM supplemented with 150 µCi/ml [^{35}S]methionine (>1000 mCi/mmol, Amersham)

and 10% FCS for 16 h. Labeled proteins (100 x10^6 cpm for cell extract and 15 x10^6 cpm for medium of total TCA-precipitable proteins) were immunoprecipitated with M1G8 anti-cath-D antibody and analysed by 10% SDS-PAGE and fluorography (Capony et al., 1987). For cath-D endocytosis, MCF-7 cells were labeled with 200 µCi/ml [^{35}S]methionine for 24 h in DEM without methionine and FCS, and labeled conditioned culture medium was used directly for internalization studies. CD55-/- fibroblasts plated in 6-well plates were incubated for 18 h in 1 ml serum-free DEM medium supplemented with the conditioned medium prepared from MCF-7 cells corresponding to 3 x 10^6 cpm of total TCA-precipitable proteins with an excess of non-radioactive methionine (10 mM). After incubation, the medium was discarded and cath-D endocytosed into cells was analyzed by immunoprecipitation (Laurent-Matha et al., 1998).

Western blot analysis of ERK1/2 activation.

For ERK1/2 activation, cells were grown to 80% confluency in medium supplemented with 2% FCS for 3 days and ERK1/2 activation was analysed with either an anti-phospho-p44/42 MAP kinase antibody (Cell Signaling Technology) or an anti-ERK2 antibody (sc-1647, Santa Cruz Biotechnology, Inc.).

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LEGEND TO FIGURES

Figure 1. 3D co-culture of fibroblasts and cancer cells.

(A) Outgrowth of CD55-/-SV40 fibroblasts (a), **breast HMF fibroblasts (b), and CCD45K skin fibroblasts (c) co-cultured with MCF-7 breast cancer cells.** Fibroblasts were embedded in Matrigel alone (left panel) or in the presence of MCF-7 cells embedded in a bottom layer of Matrigel (right panel). Phase contrast optical photomicrographs after 3 days of co-culture for CD55-/-SV40 fibroblasts and after 6 days of co-culture for HMF and CCD45K fibroblasts are shown. One representative experiment out of 3 is shown.

(B) Outgrowth of CD55-/-SV40 fibroblasts co-cultured with 3Y1/Ad12 cancer cells. CD55-/-SV40 fibroblasts were embedded in the presence of a bottom layer of 3Y1-Ad12 cancer cell lines secreting no cath-D (control), or human wild-type (cath-D), or D231N cath-D (D231N). Phase contrast optical photomicrographs after 3 days of co-culture are shown (panel a). Pro-cath-D secretion was analyzed after 3 days of co-culture by western blot (panel b). One representative experiment out of 3 is shown.

(C) Outgrowth of CD55-/-SV40 fibroblasts co-cultured with MCF-7 cells whose pro-cath-D secretion was inhibited by siRNA silencing. CD55-/-SV40 fibroblasts were embedded in the presence of a bottom layer of MCF-7 cells transfected with cath-D or luc siRNAs. Phase contrast optical photomicrographs after 4 days of co-culture are presented (panel a). One representative experiment out of 2 is given. Expression and secretion of pro-cath-D were monitored in MCF-7 cell lysates (C) and media (S) before the beginning of the co-culture and then in the media at day 1 to 4 of co-culture by western blot (panel b). *, non specific contaminant protein. Arrows indicate fibroblasts. *Bars* (---, 50 µm; ⁻, 500 µm). K= molecular mass in kDa.

Figure 2. Outgrowth of CD55-/- and CD53+/+ transfected fibroblasts.

(A) Matrigel outgrowth. Cells were embedded in Matrigel gels. Phase contrast optical photomicrographs after 8 days of culture are shown. One representative experiment out of 3 is shown. *Bars*, 50 µm.

(B) Growth on Matrigel gels. Cells were plated on Matrigel gels. **Upper rows**: p-nitrotetrazolium violet cell staining after 7 days of culture in a 24-well plate. **Bottom rows**: phase contrast optical photomicrographs after 4 days of culture. One representative experiment out of 3 is shown. *Bars* (── , 2 mm; ----- , 50 µm).

Figure 3. Migration of CD55-/- transfected fibroblasts induced by wound healing.

Sub-confluent cell layers were wounded by tip-scraping and wound healing was monitored under a phase-contrast microscope. The same area of each dish was monitored microscopically at 0 and 24 h. Experiments were done in triplicate. *Bars,* 500 µm.

Figure 4. Proliferation, apoptosis, migration, and invasion of CD55-/- and CD53+/+ transfected fibroblasts.

(A) Proliferation assay. Cells were cultured in DEM medium supplemented with 2% FCS and DNA was quantified at the indicated days. Cell growth was expressed as µg of DNA (mean ± s.e.m. of 7 independent experiments performed in triplicate). *, $p<0.001$ *versus* CD55-/-SV40 cells (day 8) (Student's *t*-test).

(B) Cell cycle and S phase analysis. Cells were cultured in DEM medium supplemented with 2% FCS for 3 days and cell cycle was monitored by flow

cytometry (panel a). *, p<0.005 versus CD55-/-SV40 cells. S = S phase; A = apoptotic or sub-G0/G1 peak. Experiments were done in triplicate. BrdUrd-incorporated S phase cells were detected with a FITC anti-BrdUrd antibody and counted (panel b).

(C) Apoptosis evaluation. Cells grown for 3 days on Matrigel gels were analyzed by flow cytometry. Cell cycle phases are indicated; A=apoptotic peak. Experiments were done in duplicate.

(D) In situ apoptosis. Cells were embedded in Matrigel and after 24 h were incubated with 10 µM cell permeable Hoechst 33342 (Molecular Probes) and live cells were observed with a microscope equipped with a water immersion objective. Arrows indicate apoptotic bodies. *Bars*, 10 µm.

(E) Electron microscopic appearance of CD55-/-SV40 and CD55-/-cath-D fibroblasts. Cells were embedded in Matrigel (M) and after 24 h were processed for TEM. **(a)** Ultra thin section in the cytoplasm of an apoptotic CD55-/-SV40 fibroblast. *Bars*, 0.9 µm **(b)** Primary apoptosis as evidenced by complete chromatin condensation followed by secondary necrosis of CD55-/-SV40 fibroblast. *Bars*, 2.5 µm. **(c)** Typical morphology of the nuclei of CD55-/-cath-D fibroblast. *Bars*, 1.4 µm. N, nucleus; nu, nucleolus; v, vacuole.

(F) Migration assay. Cells were tested for their ability to migrate through filters coated with collagen I. Data represent the percentage of cells that cross through filters relative to total seeded cells and are the mean ± s.d. of 3 independent experiments performed in triplicate. *, $p < 0.05$ *versus* CD55-/-SV40 cells (Student's *t*-test).

(G) Invasion assay. Cells were tested for their ability to migrate through filters coated with Matrigel. Data given as in panel F are the mean ± s.d. of 3 independent

experiments performed in triplicate. **, $p < 0.005$ *versus* CD55-/-SV40 cells (Student's *t*-test).

Figure 5. Activation of ERKs in CD55-/- and CD53+/+ transfected fibroblasts.

(A) Activation of the MAP kinase pathway. Cells were cultured in DEM medium supplemented with 2% FCS and cell lysates were analyzed by immunoblotting with an antibody specific for phospho-ERK1/2. Equivalent amounts of ERK2 were confirmed by reprobing the blots with anti-ERK2 antibody. A representative Western blot from 4 independent experiments is shown in panel a, and the quantitation is shown in panel b. Signals were quantified by scanning densitometry, and phosphorylation level was normalized to ERK2. *, $p < 0.005$ *versus* CD55-/-SV40 cells (Student's *t*-test).

(B) Effect of U0126 on CD55-/-cath-D invasive growth. CD55-/-cath-D cells in DEM with 10% FCS were treated or not with 10 µM U0126 for 6 h. Cells were then embedded in Matrigel. Panel a shows ERK1/2 phosphorylation. Panel b illustrates fibroblast invasive growth after 2 days of culture. Similar results were obtained in 2 independent experiments. *Bars*, 50 µm.

Figure 6. Paracrine action of pro-cath-D on fibroblasts.

(A) Endocytosis of pro-cath-D by fibroblasts. Conditioned media containing secreted labeled pro-cath-D was produced by incubating MCF-7 breast cancer cells with [S^{35}]methionine for 24 h. Immunoprecipitated 52 kDa precursor pro-cath-D from labeled medium is shown in panel a. CD55-/- fibroblasts were incubated for 18 h with ^{35}S-labeled conditioned medium containing the secreted labeled pro-cath-D in the absence or presence of 10 mM Man-6-P (panel b). After washing, cell lysates

containing endocytosed [S^{35}]methionine-labeled cath-D were analyzed by SDS-PAGE after immunoprecipitation with M1G8 antibody. Immunoprecipitations were performed in triplicate.

(B) Effects of pro-cath-D on fibroblast outgrowth.

CD55-/-SV40 fibroblasts embedded in Matrigel were treated in the absence (0) or presence of media conditioned (CM) by 3Y1-Ad12 cancer cell lines secreting no human cath-D (control), 30 nM wild-type (cath-D), or 10 nM D231N cath-D (D231N). After 4 days of culture, cell growth was analyzed by phase contrast microscopy. Experiments were performed in triplicate*. Bars,* 50 µm.

(C) Effects of pro-cath-D on fibroblast proliferation and apoptosis. CD55-/-SV40 cells were cultured for 3 days in the presence of CM containing no human cath-D (control), 24 nM human cath-D or 8 nM D231N cath-D supplemented with 2% FCS and cell cycle was monitored by flow cytometry (panel a). S = S phase; A = apoptotic peak. Similar results were obtained in 3 independent experiments. *, $p < 0.005$ *versus* control CM (Student's *t*-test). NS, non specific. BrdUrd-incorporated S phase cells were detected with a FITC anti-BrdUrd antibody and counted (panel b).

(D) Effects of pro-cath-D on fibroblast invasion. Cells were tested for their ability to invade in the presence of CM containing no human cath-D (control) or 30 nM pro-cath-D. Data are the mean ± s.d (n = 5 independent experiments). *, $p < 0.025$ *versus* control CM (Student's *t*-test).

(E) Effects of pro-cath-D on MAP kinase pathway. Cells were cultured for 3 days in the presence of CM supplemented with 2% FCS and containing either no human cath-D (control), 30 nM cath-D, or 10 nM D231N cath-D and ERK1/2 activation was analyzed as described in Figure 5.

ONLINE SUPPLEMENTAL MATERIAL

Materials and Methods

Aortic ring assays.

Rat aorta rings prepared as described (Blacher et al., 2001) were maintained for 9 days at 37°C in MCDB131 (Life Technologies, Paisley, Scotland) and conditioned media from mock-transfected, cath-D and D231N transfected 3Y1-Ad12 cancer cells were prepared as described above (1:1). The cultures were examined by phase contrast microscopy with an Olympus microscope at the appropriate magnification. Image analysis was performed on a Sun SPARC30 WorkStation, using the 'Visilog5.0' Noesis software. Images were digitized in 760x540 pixels with 256 grey levels. Grey level image transformation and binary image processing have been carrying out using traditional tools and mathematical morphology (Blacher et al., 2001). To identify endothelial cells, gels containing aorta were fixed in 70% EtOH. Dried gels were incubated with 1.5% milk in PBS, with biotinylated isolectin IB4 that have a strong affinity for endothelial cells (5 µg / ml in 1 mM $CaCl_2$, pH 7.2) (Molecular Probes) for 2 h, with streptavidin-FITC (dilution 1:40 in PBS) (Amersham) for 30 min and were counter-stained with bisbenzimide (10µg/ml in PBS).

Proteolytic activity of cath-D

Conditioned media (1 ml) were collected after 24 h incubation of transfected cells in 10% FCS medium in 6-well plates and cell extracts were prepared as described (Glondu et al., 2001). Total proteins from conditioned media (100 µl) or from cell extracts (75 µg) were incubated with 30,000 cpm [^{14}C]methemoglobin (New England Nuclear, Boston, MA), 2 µg of unlabeled methemoglobin (Sigma), in a citrate reaction

buffer (pH 3.5) and in the absence or presence of 2 mM pepstatin (Sigma) (Capony et al., 1987). The proteolytic activity was calculated from the TCA-soluble radioactivity per microgram of DNA extracted from the corresponding cells.

Legends to supplemental figures

Sup Figure 1. Paracrine action of pro-cath-D on fibroblasts in an aortic ring.

(A) Photomicrographs of aortic rings cultured for 9 days in the absence (MCDB) or presence of media conditioned by 3Y1-Ad12 cancer cell lines secreting no human cath-D (control), 30 nM human wild-type (cath-D) or 10 nM D231N cath-D (D231N). Points correspond to isolated cells spreading around aorta rings. Arrow indicates a micro-vessel. *Bars*, 1 mm.

(B) Representative illustration of cells spreading around the aortic ring in presence of D231N CM. Endothelial cells stained in green were identified by their capacity to bind to isolectin IB4 as described in Materials and methods. Nuclei were stained in blue with bisbenzimide. Arrows indicate endothelial cells (E) and fibroblast-like cells (Fl). *Bars*, 100 µm.

(C) Quantification of isolated cells escaping aorta rings. Cell number was quantified by computer-assisted image analysis and plotted as a function of the distance to the ring.

(D) Quantification of maximal distance of isolated cell migration (Dmax) from ring. Dmax was quantified by computer-assisted image analysis. *, $p < 0.01$ *versus* control (ANOVA analysis; p values < 0.05 were considered significant). Each experiment was performed with 3 rings in each condition and in triplicates. Similar results were obtained in another experiment performed with different conditioned media.

Sup Figure 2. Characterization of human wild-type and D231N mutated cath-D in CD55-/- transfected fibroblasts.

(A) Maturation and secretion of human wild-type and D231N cath-D. Cells were labeled for 16 h with [S^{35}]methionine. Cell lysates (C) and media (S) samples were immunoprecipitated with M1G8 antibody, analysed on a 10% SDS-PAGE and fluorographed. Arrows indicate migration of the 3 forms of cath-D. K= molecular mass in kDa.

(B) Proteolytic activity of cath-D .

Upper panel: Cell extracts from CD55-/-cath-D and CD53+/+SV40 transfectants were assayed at the indicated times for proteolytic activity at pH 3.5 with [^{14}C]methemoglobin in the absence or presence of pepstatin. Cell extracts from CD55-/-SV40 and CD55-/-D231N cells were assayed for 6 h for proteolytic activity at pH 3.5 with [^{14}C]methemoglobin in the absence or presence of pepstatin. Cellular human cath-D proteolytic activity was calculated by subtracting the TCA-soluble radioactivity obtained with pepstatin and was expressed as cpm per microgram of DNA.

Bottom panel: Conditioned media from transfected fibroblasts containing equivalent amount of proteins secreted by 24 h were assayed for 16 h for proteolytic activity. Secreted cath-D proteolytic activity was obtained by subtracting the TCA soluble radioactivity obtained with pepstatin.

Reference

Blacher, S., L. Devy, M.F. Burbridge, G. Roland, G. Tucker, A. Noël, and J.M. Foidart. 2001. Improved quantification of angiogenesis in the rat aortic ring assay. *Angiogenesis* 4:133–142.