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Antimicrobial protein hCAP18/LL-37 is highly expressed in breast cancer and is a putative growth factor for epithelial cells

Johan D. Heilborn[1], Margareta Frohm Nilsson[1], Clara I. Chamorro Jimenez[1], Bengt Sandstedt[2], Niels Borregaard[3], Emma Tham[4], Ole E. Sørensen[3], Günther Weber[1,4] and Mona Ståhle[1]*

[1]*Unit of Dermatology, Department of Medicine, Karolinska Institutet, Stockholm, Sweden*
[2]*Department of Clinical Pathology, Danderyds Hospital, Stockholm, Sweden*
[3]*Department of Hematology, Granulocyte Research Laboratory, Copenhagen, Denmark*
[4]*Department of Molecular Medicine, Karolinska Institutet, Stockholm, Sweden*

Human cathelicidin antimicrobial protein hCAP18/LL-37 is an effector molecule of the nonspecific innate immune system. hCAP18/LL-37 is present in leukocytes and is expressed in skin and other epithelia, where it is upregulated in association with inflammation and injury. In addition, antimicrobial proteins including cathelicidins have been proposed to play a role in the nonspecific defense against tumors. To assess its potential role in tumor host defense, we investigated the expression of hCAP18/LL-37 in a series of breast carcinomas. Unexpectedly, we found that hCAP18/LL-37 was strongly expressed in the tumor cells and not in the adjacent stroma. To test the hypothesis that hCAP18/LL-37 may provide a growth advantage for the tumor cells, we treated human epithelial cell lines with synthetic biologically active LL-37 peptide and found a significant increase in cell proliferation. In addition, transgenic expression of *hCAP18* in 2 different human epithelial cell lines resulted in increased proliferation of both cell types. These findings do not support the hypothesis that LL-37 has an antitumor effect, but rather suggest that hCAP18/LL-37 may promote tumor cell growth in breast cancer.

Key words: innate immunity; breast cancer; cathelin; growth factor; proliferation

Antimicrobial proteins are key effectors in the innate immune system. Human cathelicidin antimicrobial protein hCAP18, the only known cathelicidin in humans, consists of a conserved cathelin domain and a variable C-terminus, called LL-37.[1,2] Extracellular proteolytic processing of the holoprotein releases the LL-37 peptide, which has broad antimicrobial activity[1,3] as well as effects on host cells mediated by the G-protein-coupled receptor, formyl peptide receptor-like 1 (FPRL1).[4,5]

hCAP18 is present in leucocytes[6] and is expressed in skin and other epithelia, where it is upregulated in association with inflammation[7,8] and injury,[9,10] consistent with a role in innate barrier protection. Recently, antimicrobial proteins including cathelicidins have been proposed to play a role also in the nonspecific host defense against tumors.[11,12] In this context, we investigated the expression pattern of hCAP18/LL-37 in a series of breast carcinomas demonstrating a marked upregulation of hCAP18 mRNA and protein in the tumor cells but not in the adjacent stroma. Interestingly, the highest levels of hCAP18 protein were detected among tumors with the highest histologic grade, whereas hCAP18 levels in some low-grade tumors equaled those detected in the normal breast tissue. These findings clearly contrast with the hypothesized antitumor effect that has been proposed for antimicrobial peptides, but are consistent with recent findings that suggest a role for hCAP18/LL-37 in epithelial repair and angiogenesis.[5,10] Further supporting hCAP18/LL-37 as a growth-promoting factor, we here demonstrate that proliferation of epithelial cells was significantly enhanced both by treatment with synthetic biologically active LL-37 peptide and by transgenic expression of *hCAP18*.

Material and methods

Tissues

Frozen tumor tissue from 28 breast cancer patients was obtained from the Department of Pathology, Danderyd Hospital, Stockholm, Sweden (Table I). The tumors were scored according to Elston and Ellis I-III, following established guidelines.[13] Cyclin A was used as proliferation marker (Nova-Castra Laboratories, Newcastle Upon Tyne, U.K.). Estrogen receptor status was assessed on routinely processed paraffin sections. Uninvolved mammary tissue from 8 patients with breast cancer and from 2 healthy individuals undergoing reductive breast surgery served as controls. All samples were examined by the same pathologist (B.S.) and classified as normal (Table I). Written informed consent was given by all patients. The study was approved by the Regional Committee of Ethics.

In situ *hybridization for hCAP18*

A 435 bp hCAP18 full-length cDNA subcloned into pBluescript was used to transcribe [^{35}S]-labeled antisense and sense probes *in vitro* and *in situ* hybridization was performed essentially as described[8] on samples 0–17 (Table I).

Immunohistochemistry

Immunohistochemistry was performed on samples 0–17 (Table I). Cathelin affinity-purified rabbit antiserum against recombinant hCAP18[14] was used at 1:500 dilution as earlier described[10] according to the indirect peroxidase method using a Vectastain kit (Vector Laboratories, Burlingame, CA). To ascertain the specificity of the staining, immunoabsorption was performed as earlier reported.[10] For detection of the FPRL1 receptor, affinity-purified goat polyclonal antibody was used at 1:400 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) according to the indirect peroxidase method.

Protein extraction and Western blot analysis

Frozen tumor tissues (16–60 mg) were homogenized in lysine buffer using an electric homogenizer. Proteins from tumor tissues and cell lines were extracted in SDS-containing sample buffer according to standard protocols.[15] The protein concentration was determined by a spectrophotometric assay and adjusted with SDS-containing sample buffer to equal protein concentration.[16] For the detection of hCAP18/LL-37, the extracts were separated on 16.5% Tris-Tricine Ready gels (Bio-Rad Laboratories, Hercules, CA). Recombinant cathelin[17] and synthetic LL-37 peptide were used as size references. For the detection of ERK1/2 and FPRL1, protein was separated on 12% and 8% Tris-glycine gels, respectively. To confirm that approximately equal amounts of protein in each sample were blotted, the filters were reversibly stained with a 3% Ponceau S solution (Sigma-Aldrich, Poole, U.K.) in 3% TCA,

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***Correspondence to:** Unit of Dermatology, Department of Medicine, Karolinska Institutet, S-17176 Stockholm, Sweden. Fax: +46-8-51777851. E-mail: mona.stahle@medks.ki.se
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TABLE I – CLINICAL AND EXPERIMENTAL DATA

Sample[1] number	Age (year)	Type	Grading[2]	ER[3]	Cyclin A[4]	IH and ISH[5]	hCAP18[6] (ng/mg)	Real-time PCR[7]	Treatment[8]	Axillar LN[9]	Clinical status[10]
0	30	Healthy				Yes					
1	72	Healthy				Yes	0.7				
2	53	Lobular	I	+	L	Yes	2.3		M, TAM	−	0
3	65	Ductal	II	+	H	Yes	1.1		M,CT	−	CIS
3b		Normal				Yes					
4	37	Ductal	I	+	H	Yes	2.3		PM, Rx, TAM	−	0
5	69	Colloid	I	+	L	Yes	1.7		PM, Rx, TAM	−	0
6	84	Ductal	III	−	H	Yes	5.4		M	−	D
7	53	Ductal	III	−	H	Yes	35.8		M, Rx, CT	+	0
8	55	Ductal	III	−	H	Yes	5	8	PM, Rx, CT	+	Metastasis
9	73	Ductal	III	−	H	Yes	11.8		M, TAM	−	D
10	47	Ductal	III	−	H	Yes	5.3		M, CT, Rx	−	D
11	64	Ductal	II	+	H	Yes	1.6		M, Rx, TAM	−	0
12	52	Ductal	II	+	H	Yes	5		PM, Rx, CT	+	0
12b		Normal				Yes					
13	69	Ductal	I	+	L	Yes	0.9		PM, Rx, TAM	+	0
14	31	Ductal	II	+	L	Yes	4		M, Rx, CT	+	0
14b		Normal				Yes					
15	58	Ductal	I	+	L	Yes	3.9		PM, Rx, TAM	−	0
16	70 right	Ductal	I	+	L	Yes	4.12		PM, Rx, TAM	−	0
16	Left	Lobular	II	+	L	Yes	4.56		M, TAM	−	0
16b		Normal				Yes					
17	70	Tubular	I	+	L	Yes			M, Rx, TAM	−	0
18	76	Ductal	I	+	L	No	3.9		M, Rx, CT	+	0
19	64	Ductal	III	+	H	No	3.0		PM, Rx, CT	−	0
20	69	Ductal	I	+	L	No	4.7		PM, Rx, TAM	−	0
21	78	Lobular	III	+	H	No	38		M, CT	+	0
22	67	Ductal	III	+	H	No	4.0		M, CT	ND	0
23	82	Colloid	I	+	L	No	11.7		M, CT	ND	0
24	76	Ductal	II	−	L	No	3.7		M, Rx, CT	−	0
25	44	Ductal	III	+	H	No	7.0	11	M, Rx, CT	+	0
26	79	Medullary	III	+	H	No	4.8	18	M, Rx, CT	+	0
27	66	Ductal	I	+	L	No	8.7		PM, Rx, CT	−	D
28	58	Ductal	III	−	H	No	41	11	M, Rx, CT	+	D
29	65	Metastasis	−	+	H	No	29.5		CT	+	Metastasis
30	54	Lobular	III	+	H	No	5.8		M, Rx, CT	+	0
31	81	Normal				No	1.2	0.6			
32	60	Normal				No	2.9	1.1			
33	65	Normal				No	2.9	1.5			
34	55	Normal				No		1.1			

[1]Tissues from 28 patients with breast carcinoma, normal mammary tissue from 8 patients with breast carcinoma and from 2 healthy individuals undergoing reconstructive breast surgery (samples 0 and 1).–[2]Tumors graded according to Elston and Ellis.–[3]Assessment of estrogen receptor (ER) status performed with immunohistochemistry.–[4]Percentage of cells expressing proliferation marker cyclin A. Low (L) $< 5\%$; high (H) $\geq 5\%$.–[5]Tissues investigated with immunohistochemistry (IH) and *in situ* hybridization (ISH) for hCAP18.–[6]Protein extraction from tissues; hCAP18 levels measured with ELISA and presented as ng hCAP18 per mg total protein.–[7]RNA extraction from tissues; hCAP18 mRNA measured with real-time PCR (TaqMan). The mean expression of the normal samples was arbitrarily set to 1.–[8]M, mastectomy; PM, partial mastectomy; Rx, radiation; CT, chemotherapy; TAM, tamoxifene.–[9]Axillary lymph nodes status at surgery. ND, not done.–[10]Clinical status was assessed 1.5–2 years after diagnosis. D, deceased; 0, no clinical relapse; CIS, carcinoma *in situ*.

before incubating with the primary antibody. Affinity-purified anticathelin antiserum,[17] affinity-purified anti-LL-37 antiserum,[10] anti-FPRL1 antiserum (sc18191; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal anti-ERK1/2 antibody (Cell Signaling Technology, Beverly, MA) were all used at 1:1,000 dilution. After electroblotting onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) and sequential incubation with primary antibodies and horseradish-peroxidase-conjugated IgG (Santa Cruz Biotechnology), signals from enhanced chemiluminiscence (ECL; Amersham Biosciences, Piscataway, NJ) were captured with a CCD camera (LAS 1000; Fujifilm, Tokyo, Japan).

ELISA

A sandwich ELISA previously described[17] was used to quantify hCAP18 in protein extracts from normal mammary gland and tumor tissues.

Expression analysis of hCAP18 *by real-time PCR*

RNA from 4 normal samples and 4 tumors was extracted with the Qiagen RNeasy kit (Operon Biotechnologies, Cologne, Germany) and reverse-transcribed with a first-strand synthesis kit (Amersham Biosciences). RNA was quantified by real-time PCR on an ABI Prism 7700 (Applied Biosystems, Foster City, CA) using 10 ng of cDNA according to standard protocols. The samples were evaluated in triplicates. Sequences were 5′-GTCACCAGAGGATTGTGACTTCAA-3′ and 5′-TTGAGGGTCACTGTCCCCATA-3′ for the primers and 6-FAM-5′-CCGCTTCACCAGCCCGTCCTT-3′-BHQ1 for the fluorigenic probe. The samples were normalized by quantification of 18S-RNA (Assay on Demand; Applied Biosystems). The mean expression of the normal samples was arbitrarily set to 1.

Synthetic LL-37 peptide

LL-37 peptide was synthesized and purified by HPLC to a purity of 98% (PolyPeptide Laboratories Hillerød, Denmark). Biologic activity of the peptide was confirmed in an antibacterial assay.[18]

LL-37 peptide treatment of epithelial cells

A spontaneously immortalized human keratinocyte cell line (HaCaT)[19] was cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco-BRL Life Technologies, Paisley, U.K.) supplemented with 10% FCS (fetal calf serum; Hy-Clone, Boule Nordic

AB Huddinge, Sweden) and antibiotics (PEST = penicillin 50 U/l and streptomycin 50 mg/ml; Gibco-BRL). Cells were harvested at 70% confluence and seeded in 96-well plates, 2000 cells in 100 μl medium (DMEM + 10% FCS and PEST). After 12 hr, medium was changed to serum-free medium (DMEM + PEST) and cells were synchronized in G0/G1 by serum starvation for 72 hr and then treated with 100 μl of medium (DMEM + 5% FCS + PEST) containing synthetic biologically active LL-37 peptide at 10 μg/ml. Cells treated with only DMEM + 5% FCS and PEST served as control. The experiments were performed in quadruplicates. Cell proliferation was evaluated by [^{3}H]-thymidine incorporation. Cells were treated with 1 μCi/well of [^{3}H]-thymidine (20.00 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA) for 12 hr and harvested (Harvester 96; Tomtec, Orage, CT) onto a glass fiber filter (Wallac, Turku, Finland). The incorporation of [^{3}H]-thymidine was determined using a liquid scintillation counter (Microbeta Pluss; Wallac). The experiment was repeated twice in 30 replicates.

Transgenic expression of hCAP18 *in HEK293 and HaCaT cells*

A *Bfa1* fragment from Image clone 3057931[20] containing the entire coding sequence, including the 16 bp of the 5′ untranslated region, was subcloned into the *Sma1* site of the bycistronic vector pIRES2-EGFP (BD Biosciences, Bedford, MA). HEK293 and HaCaT cells were transfected using Fugene (Roche Diagnostics, Indianapolis, IN) under standard conditions and selected for 2 weeks with 400 ng/ml G418 (Invitrogen, Paisley, U.K.). Cells were sorted for EGFP expression with a MoFlo high-speed cell-sorting flow cytometer (DakoCytomation, Fort Collins, CO) using Summit software for data analysis, and their expression of CAP18 was quantified by immunoblotting. Control cell lines were similarly established by transfection with the vector expressing EGFP only. The cell lines maintained a stable expression of CAP18 during several months of continued cultivation without any selection.

Proliferation assays for HEK293 and HaCaT cells stably transfected with hCAP18

HEK293 cells transfected with *hCAP18* were harvested at 70% confluence and seeded in 24-well plates. After 24 hr, medium was changed and cells were cultured in 2 ml of medium (Optimem; Gibco-BRL Life Technologies) supplemented with 5% FCS and PEST. Cells were harvested at day 6 and counted by flow cytometry (Becton Dickinson, Bedford, MA). Cell viability was measured with Trypan blue; under all conditions, less than 5% of the cells were Trypan blue-positive. All conditions were performed in triplicates. HEK293 cells transfected with the vector expressing only EGFP served as control.

HaCaT cells transfected with *hCAP18* were harvested at 70% confluence and seeded at 2,000 cells per well in 96-well plates in DMEM with 10% FCS + PEST. Medium was changed 12 hr later to DMEM supplemented with 5% FCS + PEST. After 24 hr of culture, the cells were treated 12 hr with 1 μCi/well of [^{3}H]-thymidine, harvested and analyzed as described above. HaCaT cells transfected with the vector only expressing EGFP served as control.

Expression analysis of FPRL1

RNA from HaCaT cells was extracted with the RNeasy kit (Qiagen, Chatsworth, CA) and reverse-transcribed with a first-strand synthesis kit (Amersham Pharmacia, Cambridge, U.K.). FPRL1 RNA was quantified by real-time PCR and normalized against 18S RNA as described above. Sequences were 5′-TCTGCTGGCTACACTGTTCTGC-3′ and 5′-GACCCCGAGGACAAAGGTG-3′ for the primers and 6-FAM-5′-CCCAAGCACCACCAATGGGAGGA-3′-BHQ1 for the fluorigenic probe.

Pertussis toxin assay

To assess the involvement of FPRL1 in mediating the stimulation of epithelial cell proliferation induced by hCAP18/LL-37, HaCaT cells were treated with the G-protein-coupled receptor inhibitor pertussis toxin. Cells were preincubated with pertussis toxin (Sigma-Aldrich) 24 hr before the LL-37 treatment in a final toxin concentration of 20 ng/ml. Medium was changed 48 hr after cell seeding and the HaCaT cells were treated with 100 μl of medium (DMEM + 5% FCS and PEST) containing synthetic biologically active LL-37 peptide at 5 or 10 μg/ml, respectively. Cells treated with only DMEM + 5% FCS and PEST served as control.

Assay of phosphorylated ERK1/2 in LL-37-treated HaCaT cells

HaCaT cells were seeded at 10% confluence and kept in DMEM with 0.2% FCS for 36 hr. For the next 48 hr, cells were cultured in DMEM with 1% or 5% FCS, respectively, and in the presence or absence of LL-37 at 10 μg/ml, with daily changes of medium. EGF at 10 ng/ml served as positive control. The expression of phosphorylated ERK 1/2 was evaluated by Western blot analysis with a mouse monoclonal antibody (Cell Signaling Technology).

Statistical analysis

Values are presented as mean number of cells or counts per minute (CPM) ± SD. Comparisons between groups were analyzed by 2-sided *t*-tests. Results were considered statistically significant for p-values < 0.05. For the analysis of the expression in tumors, a one-tailed *t*-test was performed on hCAP18 protein levels at a significance level of < 0.05.

Results

hCAP18/LL-37 is expressed in breast cancer

Patient details are presented in Table I. By *in situ* hybridization, there was low signal for *hCAP18* mRNA (not shown) and weak immunoreactivity for hCAP18 protein in breast tissue from a healthy control (Fig. 1*g*) and in uninvolved breast cancer (not shown). All breast cancer tissues showed immunoreactivity for hCAP18 in the tumor cells and not in the stroma (Fig. 1*a*, *c* and *d*). The tumor cell population was not homogeneous with regard to hCAP18 immunoreactivity, strongly positive cells being found adjacent to cells devoid of detectable hCAP18 (Fig. 1*c*). Immunoabsorption with cathelin recombinant protein abolished the hCAP18 immunoreactivity (Fig. 1*e* and *f*). By *in situ* hybridization, positive signal for *hCAP18* mRNA was detected in the same areas closely matching the expression pattern obtained with immunohistochemistry (Fig. 1*b*). Signal intensity varied and was most prominent among high-grade tumors. Control sections hybridized with the sense hCAP18 cRNA probe lacked specific signal for *hCAP18* mRNA (not shown).

Quantification of hCAP18 protein by ELISA in breast cancer tissue extracts revealed no difference between Elston I and II grade tumors, but clearly higher hCAP18 levels in tumors of the highest malignancy grade (Table I). The difference between Elston III grade and the remaining tumors was statistically significant ($p < 0.01$). Ten of the 13 grade III tumors reached or exceeded an hCAP18 concentration of 5 ng/mg total protein. Only 2 of the remaining 18 tumor samples reached this level. We also assayed 4 specimen of healthy breast tissue, which revealed similar levels as Elston I or II tumors. To verify the expression pattern obtained by ELISA, we performed real-time PCR on 4 normal samples and on 4 of the tumors. The results of transcript quantification were in line with the data on protein expression (Table I).

By immunoblotting, all tumors and normal breast tissues investigated showed immunoreactive bands corresponding to the intact nonprocessed 18 kDa holoprotein (Fig. 2). In 4 of the 5 investigated grade III tumors (Table I, samples 6–10), we also detected bands corresponding to LL-37, the processed hCAP18 protein (Fig. 2). The antiserum used is raised against the hCAP18 holoprotein and detects LL-37 at high concentrations even though it is affinity-purified against the cathelin peptide.[10]

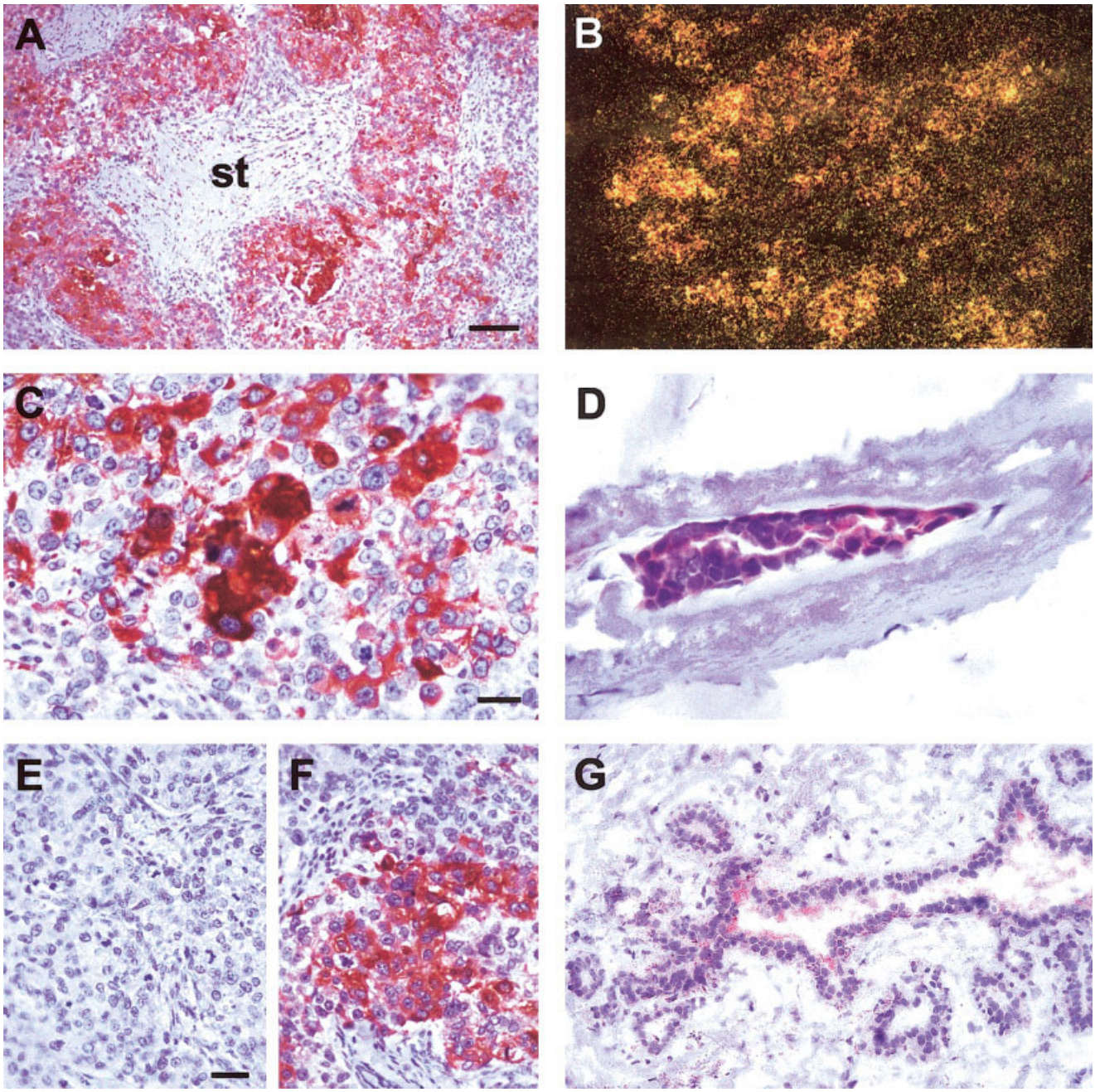

FIGURE 1 – hCAP18/LL-37 is highly expressed in breast cancer. (*a*) Section of ductal breast carcinoma grade III (patient 7, Table I) demonstrating strong immunoreactivity for hCAP18 protein in tumor cells (red precipitate) surrounding a stromal island (st). (*b*) *In situ* hybridization shows a matching signal for hCAP18 mRNA in a section from the same tissue. Intense autoradiographic signals appear as white grains under dark-field illumination. (*c*) High-power view of carcinoma cells demonstrates strongly immunoreactive cells adjacent to tumor cells devoid of immunoreactivity. (*d*) hCAP18 immunoreactive breast carcinoma cells within a blood vessel. (*e*) Immunoabsorption with cathelin recombinant peptide completely abolished the hCAP18 immunoreactivity (same tissue as *a*). (*f*) Regular immunostaining for hCAP18 as positive control during immunoabsorption (same tissue as *a*). (*g*) Normal mammary gland epithelium shows weak immunoreactivity for hCAP18. Photomicrographs (*a*, *c*–*g*) show results obtained with the hCAP18 antibody at 1:500 dilution. Scale bars = 100 μm (*a* and *b*); 25 μm (*c* and *d*); 10 μm (*e*–*g*).

hCAP18/LL-37 increases proliferation of epithelial cells

HEK293 and HaCaT cells transfected with an *hCAP18* (hCAP18/E) expression vector demonstrated significantly higher proliferation rate than control cells transfected with the vector expressing EGFP only (E; Fig. 3). By immunoblotting of protein extracts from the transfected HEK293 and HaCaT cells, we confirmed that these *hCAP18* vector-containing cells produced the holoprotein (Fig. 3) and a 4 kD immunoreactive band corresponding to LL-37 was detected in the cell medium (data not shown). In addition, HaCaT cells cultured at 5% fetal calf serum and treated with synthetic biologically active LL-37 peptide at 10 μg/ml demonstrated a significant increase in cell proliferation (Fig. 4).

LL-37 receptor FPRL1 is expressed in breast cancer

The G-protein-coupled receptor FPRL1 has been shown to mediate LL-37-induced effects in eukaryote cells;[4,5] to assess its potential role in the present setting, we investigated the expression of FPRL1 protein in mammary tissue and found strong immunoreactivity for FPRL1 both in breast cancer cells and in normal glandular epithelium (Fig. 5*a* and *b*). Immunoblotting confirmed that FPRL1 was expressed in both tissues (Fig. 5*c*). In addition, transgenic expression of *hCAP18* significantly increased the expression of FPRL1 mRNA (Fig. 5*d*) in HaCaT cells, which may further support the involvement of FPRL1 in hCAP18/LL-37 signaling. However, pretreatment of HaCat cells with pertussis toxin did not abolish but suppressed the proliferation of these cells by approximately 50% (not shown), indicating that FPRL1 may not be uniquely involved in mediating hCAP18/LL-37 growth-stimulatory effects in these cells. To test the possible involvement of ERK1/2 in activation of epithelial cell proliferation, we treated HaCaT cells with synthetic biologically active LL-37 but there was no significant activation of ERK1/2, which indicates that EGFR is not involved in mediating the LL-37-stimulatory effect on HaCaT cell proliferation.

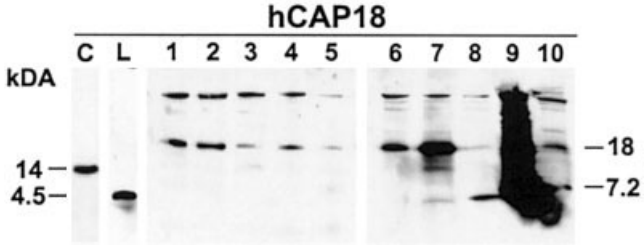

FIGURE 2 – hCAP-18/LL-37 is detected by immunoblotting in breast cancer. Clinical data of patients are presented in Table I (samples 1–10). Recombinant cathelin (C) and LL-37 peptide (L) were used as size references. Normal breast tissue is presented in lane 1. Elston grade I tumors are presented in lanes 2, 4 and 5. A grade II tumor is presented in lane 3 and grade III tumors are presented in lanes 6–10. In all tissues, there were immunoreactive bands corresponding to the intact nonprocessed 18 kDa holoprotein. The processed LL-37 peptide (4 kD) was visible in 4 of the 5 grade III tumors (numbers 7–10).

Discussion

In the present study, we demonstrate that hCAP18/LL-37 is constitutively produced in normal mammary gland epithelium. This is consistent with a role for LL-37 in antimicrobial barrier protection in human and agrees with earlier reports where low constitutive expression of LL-37 was found in normal quiescent epithelium, in contrast to the pronounced expression seen in association with injury and inflammation.[7–10] Constitutive expression of antimicrobial peptides has previously been detected in various exocrine glands such as the human cathelicidin LL-37 in sweat glands, the cathelicidin CRAMP in murine salivary glands and β-defensins in human salivary glands.[21–23] Expression of human β-defensin-2 (hBD-2) mRNA in mammary glands was reported by Bals *et al*.[24] in 1998 and recently other groups have found constitutive hBD-1 expression in mammary glandular tissue of nonlactating women as well as in breast tissue during lactation and in breast milk.[25,26]

Our finding that hCAP18/LL-37 is expressed in breast cancer cells is novel. Interestingly, the production of hCAP18 was most

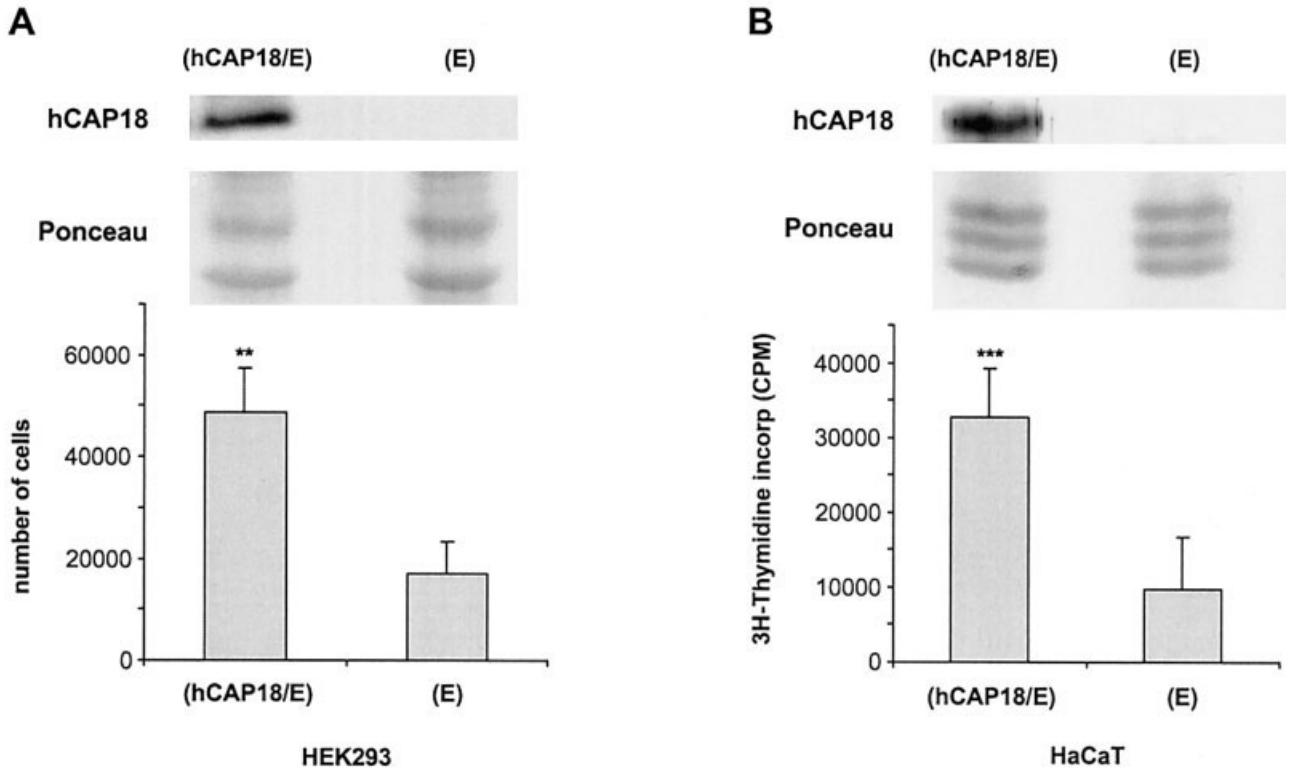

FIGURE 3 – Transgenic expression of hCAP18 in epithelial cells increases cell proliferation. (*a*) Top left: immunoblotting on HEK293 extracts with anti-LL37 antiserum. Cells transfected with a bicistronic vector hCAP18 + EGFP (hCAP18/E) show hCAP18 protein expression. Top right: HEK293 cells transfected with only EGFP (E). Bottom: HEK293 cells (hCAP18/E) demonstrate significantly higher proliferation rate (evaluated with flow cytometry) compared with control cells (E). Ponceau staining is shown as loading control. (*b*) Top left: HaCaT cells transfected as described in (*a*). Bottom: hCAP18-transfected HaCaT cells demonstrate significantly higher proliferation rate (evaluated with ^{3}H-thymidine incorporation) compared with control cells.

notably increased in the breast epithelium of high-grade tumors compared with normal mammary epithelium or low-grade tumors. However, the hCAP18 expression was neither universal nor uniform, *i.e.*, not all cancer cells were positive for hCAP18, but distinctly positive cells were found adjacent to cells devoid of detectable hCAP18 mRNA and protein (Fig. 1*c*), and the degree of expression varied considerably among cells in all tumor types. This may reflect a complex yet strictly controlled regulation of hCAP18, as has been suggested for human α-defensins in renal cell carcinoma.[27]

In our study, the highest hCAP18/LL-37 levels were detected among tumors with the highest histologic grade. Although the difference in hCAP18 expression between high-grade tumors on the one hand and low-grade and normal breast tissues is statistically significant, there is no strict correlation. Within all groups, there were tumors expressing hCAP18 at the level of the healthy samples and 2 of the grade I tumors showed a relatively high expression otherwise only observed among the grade III tumors. However, given the limitations by the sample numbers, our observations suggest a potential correlation between degree of malignancy and expression of hCAP18/LL-37. One may argue that the overexpression of hCAP18 in breast cancer may result from failures in intracellular pathways affecting the regulation of *hCAP18*, and that hCAP18 expression reflects these alterations rather than providing a growth advantage for the tumor. However, coupled with the *in vitro* studies presented here, we believe that the data underline the potential role for LL-37 in promoting tumor growth.

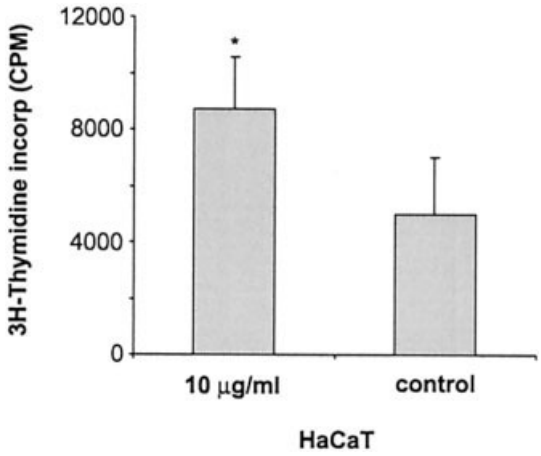

FIGURE 4 – Treatment with synthetic LL-37 peptide increases cell proliferation of epithelial cells. HaCaT cells synchronized by serum starvation for 72 hr and then treated for 36 hr with 10 μg/ml of synthetic biologically active LL-37 peptide (in DMEM + 5% FCS + PEST) show significantly increased cell proliferation compared with nontreated (control) HaCaT cells. Proliferation rate evaluated with [^{3}H]-thymidine incorporation.

The biologic role of antimicrobial peptides in carcinomas is unclear. High hBD-2 protein concentration and marked immunoreactivity for both human α- and β-defensins have been found in various oral carcinomas and it has been suggested that the increased levels of these antimicrobial peptides may be the result of infection and/or stimulation by cytokines.[28–30] Other studies have proposed that antimicrobial peptides isolated from insects, *e.g.*, melittin- and cecropin-related peptides, exert antitumor effects on mammalian tumor cells.[31–34] Moreover, vector-mediated delivery and expression of the coding sequences for cecropin and mellitin in a human bladder carcinoma cell line suppressed tumorigenicity in nude mice.[11] Likewise, transgenic expression of the porcine cathelicidin PR-39 reduced the invasive capacity of human hepatocellular carcinoma.[12]

Although further studies are required to elucidate the functions of antimicrobial peptides in cancer, a multifunctional role for these peptides is becoming increasingly manifest. In addition to pathogen inactivation through a direct membrane effect, LL-37 exerts chemotactic effects *in vitro*, inducing migration of human neutrophils, monocytes, subsets of T-cells and mast cells.[4,35,36] This chemotactic activity is dependent on binding of LL-37 to FPRL1, a pertussis toxin-sensitive membrane-bound G-protein-coupled receptor.[4] Additional suggested functions for hCAP18/LL-37 include a role in epithelial repair and angiogenesis by promoting reepithelialization of skin wounds and neovascularization.[5,10]

Thus, the marked hCAP18/LL-37 expression in breast cancer cells presented herein may reflect a growth advantage for these tumor cells. To test this hypothesis, we transfected the human epithelial cell lines HEK293 and HaCaT with an *hCAP18* expression vector and found a significant increase in proliferation of transfected cells. In addition, synthetic biologically active LL-37 peptide significantly increased proliferation of HaCaT cells. These findings clearly contrast with the suggested antitumor effect proposed for antimicrobial peptides, but are consistent with recent findings by Müller *et al.*[27] that human α-defensins may modulate progression of renal cell carcinoma (RCC). These defensins were found in tumor cells of RCC as well as

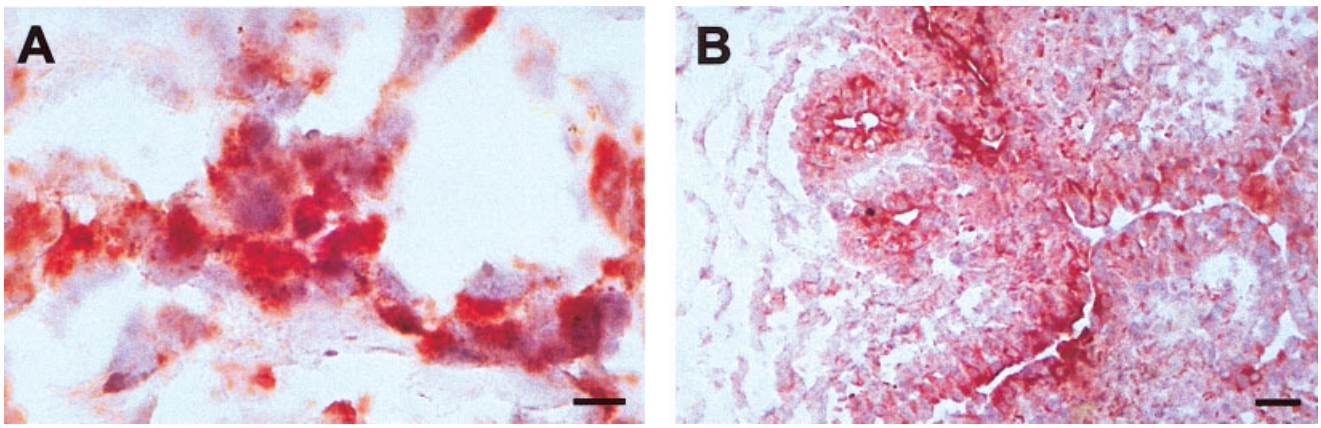

FIGURE 5 – The LL-37 receptor FPRL1 is expressed in breast cancer and in normal mammary gland epithelium. (*a*) Section of ductal breast carcinoma Elston grade II (patient 12, Table I) with prominent immunoreactivity for FPRL1 receptor in tumor cells (red precipitate). (*b*) Section of normal mammary gland epithelium demonstrating immunoreactivity for FPRL1 in the ductal region (red precipitate). Photomicrographs show results obtained with the FPRL1 antiserum at 1:400 dilution. Scale bars = 50 μm (*a*); 10 μm (*b*). (*c*) Immunoblotting revealed that the LL-37 receptor FPRL1 was expressed in both normal (N) and breast cancer (T) tissue. (*d*) HaCaT transfected with a bicistronic vector hCAP18 + EGFP (hCAP18/E) shows significantly increased expression of FPRL1 receptor mRNA by real-time PCR. HaCaT cells transfected with only EGFP (E) served as control.

in normal tubular epithelial of the kidney and at physiologic concentration-stimulated tumor cell proliferation.

Our *in vitro* studies suggest that LL-37 stimulates proliferation of epithelial cells partially through FPRL1 since blocking the receptor with pertussis toxin decreased the exogenous LL-37 proliferation effect by approximately 50%, possibly indicating the involvement also of other receptors. In a recent study, it was suggested that LL-37 activates airway epithelial cells by activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK kinase = MEK) via transactivation of the epidermal growth factor receptor (EGFR).[37] However, in our experiments, we did not detect any significant activation of ERK1/2.

In conclusion, the results presented here do not support that hCAP18/LL-37 acts as an antitumor agent; rather, in line with previous data indicating that LL-37 promotes neovascularization, we suggest a role for hCAP18/LL-37 in tumor growth.

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