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Human α-Defensins HNPs-1, -2, and -3 in Renal Cell Carcinoma

Influences on Tumor Cell Proliferation

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The α-defensins human neutrophil peptides (HNPs)-1, -2, and -3 have been described as cytotoxic peptides with restricted expression in neutrophils and in some lymphocytes. In this study we report that HNPs-1, -2, and -3 are also expressed in renal cell carcinomas (RCCs). Several RCC lines were found to express mRNA as well as the specific peptides of HNP-1, -2, and -3 demonstrated by reverse transcriptase-polymerase chain reaction, mass spectrometric, and flow cytometric analyses. At physiological concentrations HNPs-1, -2, and -3 stimulated cell proliferation of selected RCC lines *in vitro* but at high concentrations were cytotoxic for all RCC lines tested. As in RCC lines, α-defensins were also detected *in vivo* in malignant epithelial cells of 31 RCC tissues in addition to their expected presence in neutrophils. In most RCC cases randomly, patchy immunostaining of α-defensins on epithelial cells surrounding neutrophils was seen, but in six tumors of higher grade malignancy all tumor cells were diffusely stained. Cellular necrosis observed in RCC tissues in association with extensive patches of HNP-1, -2, and -3, seemed to be related to high concentrations of α-defensins. The *in vitro* and *in vivo* findings suggest that α-defensins are frequent peptide constituents of malignant epithelial cells in RCC with a possible direct influence on tumor proliferation. ***(Am J Pathol 2002, 160:1311–1324)***

Human defensins comprise a family of naturally occurring, closely related, cationic polypeptides of 29 to 42 amino acids in length. The peptides contain six conserved cysteines linked in characteristic disulfide bonds that stabilize the molecules as triple-stranded amphiphilic β-sheet structures in aqueous solutions.[1,2] *In vitro*, human defensins exhibit antimicrobial activity against different gram-positive and gram-negative bacteria, fungi, some enveloped viruses, and parasites.[3,4] At high concentrations they are cytotoxic to various types of eukaryotic cells[5,6] and also tumor cells *in vitro*.[7] Several studies showed that the antibiotic features of defensins are related to their structural characteristics. They preferentially bind to negatively charged lipid bilayers of bacterial membranes by electrostatic forces and make them permeable to molecules of masses up to several kilodaltons.[8] Because similar microbicidal and membranolytic peptides are also present in many other mammals,[9] human defensins have been suggested to belong to an ancestral system of innate host defense mechanisms against infection.[10]

To date, two classes of human defensins, termed "α-defensins" and "β-defensins," have been identified that differ with respect to their localization and linkage of cysteine residues, precursor peptide structure, and pattern of tissue expression. Whereas β-defensins are most abundant in epithelial cells of the lung,[11,12] skin,[13] and urogenital tract,[14] the α-defensins were first found in human polymorphonuclear leukocytes (PMNs) and intestinal Paneth cells.[15,16] Human neutrophil peptides (HNPs)-1, -2, and -3, and to a lesser extent HNP-4, are major constituents of the dense azurophilic granules of neutrophils, from which they are discharged into phagolysosomes on ingestion of microbes.[17] Within these compartments they are able to form pores that disrupt ion fluxes and eventuate in lysis of the engulfed microorganisms without major damage to host tissues. Two other α-defensins, HD-5 and HD-6, are sequestered

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in lysozyme-rich granules of the Paneth cells and are also thought to support local antimicrobial defense mechanisms in the gut.[18] Although normally sequestered in PMN and Paneth cells, α-defensins have also been recently described in the walls of coronary vessels.[19] Furthermore HNPs-1, -2, and -3 were found to be expressed in specific lymphocyte and monocyte subpopulations.[20]

In addition to their role in host defense against microbial infection, defensins are thought to contribute to tissue inflammation and repair. HNPs-1, -2, and -3 display various proinflammatory activities such as the induction of histamine release from mast cells[21] and chemotactic effects for human monocytes,[22] dendritic cells,[23] and PMNs as well as T cells.[24] α-Defensins have been also reported to exert mitogenic effects on murine fibroblasts and on epithelial cells with possible consequences on tissue repair.[25] They modulate binding and catabolism of low-density lipoproteins by vascular endothelial cells and therefore have been suggested to be involved in providing a link between inflammation and atherosclerosis.[26] Furthermore, α-defensins were shown in mice to activate T-helper cell responses *in vitro* and enhance systemic antigen-specific IgG production *in vivo*. Thus, they were suggested to provide signals for linking innate and acquired immunity.[27] In a recent study, α-defensins were found to represent dominant HLA-DR-binding peptides on normal and malignant hematopoietic progenitor cells suggesting major histocompatibility complex blockade for antigen presentation.[28]

Although defensins today seem to have diverse functional activities in innate antimicrobial immunity, a few reports also indicated the presence of defensins in tissues of epithelial tumors.[29–31] In this study, α-defensins are for the first time shown to be synthesized by renal cell carcinomas (RCCs) and to influence proliferation of a subset of renal malignant cells *in vitro*. In addition, endogenous binding of α-defensins to HLA-class II molecules in RCC was found suggesting a blockade of antigen-presenting function, as in myeloma and hematopoietic progenitor cells.[28] Thus, α-defensins could belong to tumor substrates that modulate malignant cell growth and enhance immune escape in RCC.

Materials and Methods

Cell Lines and Culture Conditions

In this study, eight RCC lines were used that included five lines (A-498, Caki-2, 786-0, 769-P, ACHN) purchased from the American Type Culture Collection (ATCC; Manassas, VA), one line (LE 9211-RCC) obtained from Dr. B. van den Eynde (Ludwig Cancer Institute, Brussels, Belgium) and two lines (TW33, N43) were established from renal tumor tissues in our laboratory according to the previously described method.[32] All cell lines represented tumor cells derived from typical clear cell, chromophilic/papillary, and chromophobe types of RCC. Immunohistochemical analyses showed a pure population of cytokeratin-positive cells in all cultures of these cell lines. In addition, an Epstein-Barr virus-transformed B-cell line Cox, as well as the promyelocytic cell line HL-60 also purchased from ATCC were used for comparison. Isolation, culture, and characterization of primary tubular epithelial cells, TK163, have been described previously.[33]

The RCC lines were routinely grown as monolayers and the cell lines Cox and HL-60 as suspension cultures in RPMI 1640 (Life Technology GmbH, Karlsruhe, Germany) supplemented with 10% fetal calf serum, penicillin, and streptomycin (standard culture medium) at 37°C in an atmosphere with 5% CO_2. All RCC lines were detached from culture flasks with trypsin/ethylenediaminetetraacetic acid (EDTA) (Life Technology GmbH, Karlsruhe, Germany) for fluorescence-activated cell sorting (FACS) and polymerase chain reaction (PCR) analysis.

Tissue Sampling

RCC tumor specimens were obtained from 31 patients undergoing nephrectomy. Five biopsies were derived from kidneys of healthy organ donors that were not transplanted because of vascular abnormalities. A small portion of each sample was submitted to conventional histopathological processing and light microscopic evaluation. The other parts of the RCC specimens and normal kidney tissues were put into cell culture medium RPMI 1640 (Life Technology GmbH) immediately after removal, snap-frozen, and stored in liquid nitrogen until further analysis.

Antibodies

For analysis of HNP-1, -2, and -3 expression, the murine monoclonal antibody (mAb) DEF-3 (Bachem Biochemica, Heidelberg, Germany), as well as another murine mAb prepared against highly purified HNP-1 (kindly provided by Dr. T. Ganz, Department of Pulmonary/Critical Care Medicine, School of Medicine, University of California, Los Angeles, CA)[34] were applied. Both antibodies also recognize HNPs-2 and -3, but not HNP-4. For fluorescence double staining a polyclonal anti-human defensin antibody was produced by immunizing rabbits with human α-defensins isolated from leukapheresis products and conjugated to keyhole limpet hemocyanin. IgG fractions of the immunized rabbit sera were separated on a protein A column. Specificity of the antibody was controlled by enzyme-linked immunosorbent assay with the HNP-1, -2, and -3 isolates as well as by comparative immunohistochemical staining with the mAbs against defensin.

In addition, mAb clone NP57 against human neutrophil elastase and clone 35H11 against cytokeratin 8 were obtained from DAKO (Hamburg, Germany). The mAb W6/32.HL directed against HLA-A, -B, and -C antigens and the mAb W6/32.HK,[35] an inactive variant of W6/32.HLK, as well as the mAb L243 with specificity for HLA-DR molecules were purchased from the ATCC.

Immunohistochemistry

Five-μm-thick frozen sections cut from each tumor specimen were fixed in acetone for 10 minutes at −20°C and used for indirect immunoperoxidase staining. mAbs against defensin (diluted 1:500) and neutrophil elastase (diluted 1:250) were used as primary antibodies and goat anti-mouse EnVision horseradish-peroxidase conjugate (DAKO, Hamburg, Germany) as the second layer and detection system for antibody binding. All experiments included sections stained with undiluted supernatant of the mAb W6/32.HL as positive and of the mAb W6/32.HK as negative control to exclude nonspecific staining. All sections were counterstained with hemalaun (Merck, Darmstadt, Germany) and evaluated under a Zeiss light microscope (Zeiss, Jena, Germany).

Triple Immunofluorescence Labeling

After fixation the slides were incubated for 1 hour at room temperature with mAb against cytokeratin 8 (diluted 1:25), followed by a fluorescein isothiocyanate-conjugated goat anti-mouse antibody (diluted 1:200; Dianova, Hamburg, Germany). Then, the polyclonal antibody against α-defensins (diluted 1:100) was added followed by Cy3-conjugated goat anti-rabbit antibody (diluted 1:2000; Dianova, Hamburg, Germany). The cell nuclei were identified by counterstaining with 4,6-diamino-2-phenylindolyl-dihydrochloride (DAPI; 1 μg/ml). Negative controls were performed in all experiments by omitting the first antibodies. The slides were visualized by epifluorescence light microscopy (Olympus BX-60, Hamburg, Germany). Digital pictures from every fluorescence channel were taken and super imposed for the specific antibody stains as well as for each negative control labeling using the software DOKU from Soft Imaging Systems (Leinfelden-Echterdingen, Germany).

Flow Cytometry and Confocal Microscopy

Intracellular expression of HNP-1, -2, and -3 was assessed in RCC lines after thawing and culturing for 72 hours. For FACS analysis, the RCC lines were detached from the cell-culture flask by trypsin/EDTA, placed on ice, and washed twice with washing buffer [0.1% w/v bovine serum albumin in phosphate-buffered saline (PBS)]. For confocal microscopy, the RCC lines were directly cultured on round coverslips in 12-well plates (Greiner, Frickenhausen, Germany) for 12 hours. After washing with PBS, RCCs cultured on slides were fixed with 4% paraformaldehyde for 10 minutes at room temperature.

To block nonspecific labeling, cells were first incubated for 10 minutes with human standard immunoglobulin (Polyglobin N; Bayer AG, Leverkusen, Germany). After two further washes with washing buffer the RCC lines were fixed and permeabilized with the Fix and Perm kit (An der Grub, Kaumberg, Austria) according to the manufacturer's instructions. Thereafter, the cells were labeled with the mAbs against HNPs-1, -2, and -3 as primary antibody, and fluorescein isothiocyanate or Cy2-conjugated goat anti-mouse IgG (Dianova) as the secondary antibody layer. Fluorescence of the cells in suspension was evaluated on a FACScan (Becton Dickinson, Mountain View, CA) using the WinMDI flow-cytometry software version 2.5 (*http://facs.scripps.edu/*). Fluorescence of the cells cultured on the slides was evaluated with a LSM410 LaserScan microscope (Zeiss) using a 488-nm argon laser for excitation.

All experiments included labeling with the negative control mAb W6/32.HK and the positive control mAb W6/32.HL. The promyelocytic leukemia cell line HL-60 was used as a positive control cell line for intracellular expression of HNPs-1, -2, and -3.

Reverse-Transcriptase (RT)-PCR, Cycle-Sequencing of PCR Products

Total RNA was prepared from RCC lines using the RNeasy-Kit (Qiagen Ltd., Hilden, Germany). After DNA digestion with DNase I (Roche Molecular Biochemicals, Mannheim, Germany) for 20 minutes at 25°C, 1 μg of total RNA was reverse-transcribed with 0.5 μg of oligo $(dT)_{12\text{-}18}$ using the Advantage RT-for-PCR kit (Clontech, Heidelberg, Germany). For amplification of HNP-1, -2, and -3 cDNA, primer 1 (5′-CACTCCAGGCAAGAGCT-GATGAGGT-TG-3′, position 2688 to 2714) and primer 2 (5′-AATGCCCAGAGTCTTCCCTGGTAGATG-3′ position 3454 to 3482)[12,36,37] were synthesized on a LKB Gene Assembler Plus (Amersham Pharmacia Biotech, Freiburg, Germany) and purified with NAP 10 columns (Amersham Pharmacia Biotech). As an internal control in all tests, β-actin was amplified (product of 317 bp) using primer A (5′-TCAGAAGGATTCCTATGTGGGC-3′) and primer B (3′-CCATCA CGATGCCAG-TGGTA-5′). PCR amplifications were performed in a 50-μl reaction mixture of 250 ng cDNA, 5 μl GeneAmp10× PCR buffer (Applied Biosystems, Weiterstadt, Germany), 5% dimethyl sulfoxide, 200 μmol/L of each dNTP, 1 U AmpliTaq DNA Polymerase (Applied Biosystems, Weiterstradt, Germany), and 100 ng of each primer. Amplification was performed in a Primus 25 PCR System (MWG-Biotech, Ebersberg, Germany) starting with an initial denaturation step at 95°C for 7 minutes for 26 PCR cycles, each of which consisted of denaturation at 94°C for 15 seconds, annealing at 68°C for 45 seconds, and extension at 72°C for 30 seconds were followed by 20 cycles of denaturation at 95°C for 15 seconds, annealing at 62°C for 45 seconds, and extension at 72°C for 30 seconds. The last cycle was terminated by a final extension at 72°C for 10 minutes. Each amplification product was run on a 2% agarose gel and stained with ethidium bromide to monitor for specificity before direct cycle sequencing was performed.

For direct cycle-sequencing, 45 μl of the PCR products were purified by the QIAquick PCR Purification Kit (Qiagen Ltd., Hilden, Germany) to obtain clean, double-stranded DNA amplificates. Sequencing reactions were performed by the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and analyzed on an ABI Prism 377 DNA sequencer (Applied Biosystems).

Purification of α-Defensins

Synthetic HNP-1 peptide was purchased from Bachem Biochemica (Heidelberg, Germany). Natural HNPs 1, -2, and -3 were isolated from 1 to 2×10^{10} peripheral blood mononuclear cells (PBMCs) obtained by leukapheresis from healthy stem cell donors who were pretreated with 10 μg/kg of granulocyte colony-stimulating factor for 3 days. After MACS magnetic cell sorting (Miltenyi Biotec, Bergisch-Gladbach, Germany) of $CD34^+$ hematopoietic precursor cells, the residual leukocytes were used for isolation of HNPs-1, -2, and -3 using the protocol previously described with minor modifications.[38] Briefly, around 1×10^{10} white blood cells of the leukapheresis product were pelleted by centrifugation at 220 × *g* and 4°C for 10 minutes, washed once in PBS, and centrifuged. After resuspension in 100 ml of PBS, erythrocytes were lysed by adding 300 ml of EDTA-solution (4 mmol/L) and gently stirred for 1 minute. Lysis was stopped by adding 100 ml of salt-solution [3.5% (w/v) NaCl, 4 mmol/L EDTA]. After centrifugation for 20 minutes (220 × *g*), the resulting cell pellet containing the leukocytes and blood cell progenitors was resuspended in 20 ml of Ca^{2+}/EDTA-free Hank's balanced salt solution (with 2.5 mmol/L $MgSO_4$ and 1 g glucose/L) and homogenized in a Potter-Elvehjem homogenizer on ice (15 strokes, 600 rpm). After the addition of 100 ml of Ca^{2+}-free Hank's balanced salt solution containing 5 mmol/L EDTA, the cellular homogenate was centrifuged for 10 minutes at 1600 × *g*. Subsequently, the resulting supernatant containing the leukocyte granules was ultracentrifuged at 27,000 × *g* for 20 minutes. The pellet was resuspended in 5 to 10 ml of 5% (v/v) acetic acid and sonified eight times for 10 seconds on ice with a Sonifier B12 (Branson Ultrasonics Corp., Danbury, CT) and diluted with 5% (v/v) acetic acid to a final volume of 100 ml. This suspension was stirred for 12 hours on ice and ultracentrifuged at 27,000 × *g* for 20 minutes at 4°C. This extraction step was repeated twice with 50 ml of 5% (v/v) acetic acid. The defensin-containing supernatants were lyophilized and the resulting crude product was purified by reversed phase-high performance liquid chromatography (RP-HPLC) using a Nucleosil C18 column (150 × 4.6 mm, 3 μm; Grom, Herrenberg, Germany). The gradient was run from 10 to 80% (v/v) acetonitrile/0.05% (v/v) trifluoroacetic acid (TFA) with a flow-rate of 900 μl/min. The peak of the UV trace (214 nm) eluting at ~40% (v/v) acetonitrile was collected, lyophilized, and shown by electrospray-ionization-mass spectrometry on a TSQ 700 (Finnigan, Bremen, Germany) to consist of a mixture of HNPs-1, -2, -3 with a purity of more than 95%.

Detection of HNPs-1, -2, and -3 in a RCC Line

Cells (10^9) of the cultured RCC line TW33 were harvested and washed twice in PBS. The pelleted cells were resuspended in 10 ml of 10% (v/v) acetic acid, freeze-thawed on ice three times under vigorous shaking, and subsequently centrifuged at 1600 × *g* for 10 minutes at 4°C. The resulting supernatant was recentrifuged at 26,000 × *g* for 20 minutes at 4°C, lyophilized, and resolved in 0.05% TFA/water (v/v) and applied to RP-HPLC using a Grom-Sil 120 DDS4HE column (250 × 10 mm, 5 μm; Grom, Herrenberg, Germany). The gradient was run from 10 to 80% (v/v) acetonitrile/0.05% (v/v) TFA for 50 minutes with a flow rate of 2.2 ml/min, and the eluant was collected as 2-minute fractions. Four hundred μl of each fraction were tested for recognition by the HNP-1-, -2-, and -3-specific antibody DEF-3 in a slot-blot analysis. Briefly, the 400-μl fractions were drawn through a Nytran N transfer membrane (pore size 0.2 μm; Schleicher & Schuell, Dassel, Germany) in a Milli-Blot chamber (Millipore, Bedford, MA). The membrane was blocked for 1 hour with 2% powdered milk (m/v) in washing buffer [9% (m/v) NaCl, 10 mmol/L Tris-HCl, 0.05% (v/v) Tween 20, pH 7.8]. After washing the membrane was incubated with the DEF-3 mAb for 1 hour, washed again, and incubated for 1 hour with goat anti-mouse $F(ab)_2$ fragment horseradish peroxidase (Dianova). The development was performed with the ECLplus Western blotting kit (Amersham Pharmacia Biotech). Positive fractions were concentrated in a SpeedVac and analyzed by MALDI mass spectrometry on a Voyager DE STR (Applied Biosystems, Langen, Germany).

Identification of HLA-DR-Associated Peptides

Preparation of HLA-associated peptide fractions was performed as described previously.[39] Briefly, 10^{10} interferon-γ-treated RCC A-498 cells were pelleted at 1000 × *g* and lysed with 2% (v/v) Triton X-100. The lysate was cleared by ultracentrifugation and submitted to affinity chromatography of HLA-DR molecules using the monoclonal antibody L243. For prevention of nonspecific adsorbance, a mock precolumn made of Sepharose material was used. Elution of the HLA molecules and the bound peptides was performed using TFA/water, pH 2.0. The peptide-released HLA-class II isolate was checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Released peptides were separated from the HLA molecules with an ultrafiltration unit (Amicon, Danvers, MA) using a 20-kd cut-off membrane (Sartorius, Göttingen, Germany). The peptide-containing fraction was lyophilized and subsequently resolubilized in acetonitrile/water (1:1) containing 0.1% (v/v) TFA.

Peptides were separated by microbore-reversed phase HPLC using a 125 × 2.1 mm C_{18} column (Vydac, Hesperia, CA) connected with an HPLC intelligent pump model L 6200 (Merck-Hitachi, Darmstadt, Germany) and a diode array detector model 1000S (Applied Biosystems). An elution gradient consisting of 0 to 80% acetonitrile/0.05% TFA for 70 minutes with a flow rate of 150 μl per minute was applied. From the absorbance trace at 220 nm, each fraction containing absorbance peaks of the eluate was collected and stored at −80°C until sequence analysis. All collected peaks have been examined for their peptide masses and for homogeneity by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). Pure fractions were submitted directly to an Edman sequencer. Sequencing results were

compared with the Swissprot database at the European Bioinformatics Institute (Hinxton Hall, UK) (*http://www.ebi.ac.uk/swissprot/*).

Tumor Cell Proliferation Assays

RCCs (10^4/well) were incubated in triplicate in 96-well round-bottom microtiter plates (Greiner, Frickenhausen, Germany) with and without different concentrations of the synthesized HNP-1 or the isolated HNP-1, -2, and -3 peptide fraction (0 to 100 μg/ml) in RPMI 1640 (Life Technology, GmbH, Karlsruhe, Germany) supplemented with 2 mmol/L of L-glutamine, 25 mmol/L Hepes, 50 μg/ml gentamicin, without serum at 37°C and 5% CO_2. After 24 hours cellular proliferation was quantified by measuring nuclear incorporation of tritiated thymidine ([^{3}H] TdR) (specific activity 185 GBq/mmol used at 37 kBq/well; Amersham-Buchler, Braunschweig, Germany) after a 16-hour pulse label. Radioactivity was measured by liquid scintillation counting and data reduction using in-house programs. The results are expressed as mean corrected counts per minute (ccpm) and stimulation indices (SI; mean ccpm of the sample/mean ccpm of the control). Blocking studies of cell proliferation were performed by preincubation of defensin with the polyclonal or the monoclonal antibody DEF3.

Tumor cell proliferation was also assessed by evaluation of viable cells before and after incubation with HNPs-1, -2, and -3 using a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (Roche Molecular Biochemicals, Mannheim, Germany) by mitochondrial dehydrogenases. Tumor cells (10^4) cultured in 96-well flat-bottom microtiter plates under serum-free culture conditions with and without HNPs-1, -2, and -3 for 40 hours as described above were incubated with 20 μl of the ready-to-use WST-1 reagent in a final cell culture volume of 220 μl/well at 37°C and 5% CO_2 for 10 minutes. After thoroughly shaking for 1 minute, formazan dye produced by metabolically active cells was determined by measuring the absorbance of the cell culture medium (RPMI 1640 without phenol red) against a background control (absorbance of culture medium plus WST-1 in the absence of cells) with an enzyme-linked immunosorbent assay reader at 440-nm wavelength. A calibration curve of formazan dye absorption was established with 3×10^4 to 8×10^2 titrated unmanipulated tumor cells. Cellular equivalents of the tumor cell test cultures were determined by comparison of their absorption values with the calibration curve.

Proliferation was also assessed by direct cell counting of tumor cells cultured on 8-well Lab-Tek Permanox chamber slides (Nalge Nunc Int. Corp., Naperville, IL) with and without 12.5 μg/ml of defensin in duplicates in serum-free medium as described above. After 40 hours the cell nuclei were stained on the slides with DAPI (1 μg/ml) and evaluated by epifluorescence light microscopy (Olympus BX-60). The cell nuclei of the tumor cells adherent to the bottom of each culture well (~0.25 cm^2 per well) were quantified on digital pictures taken from the blue fluorescence channel using the Scananalytics Software IPLab Evaluation (*http://www.scananalytics.com*).

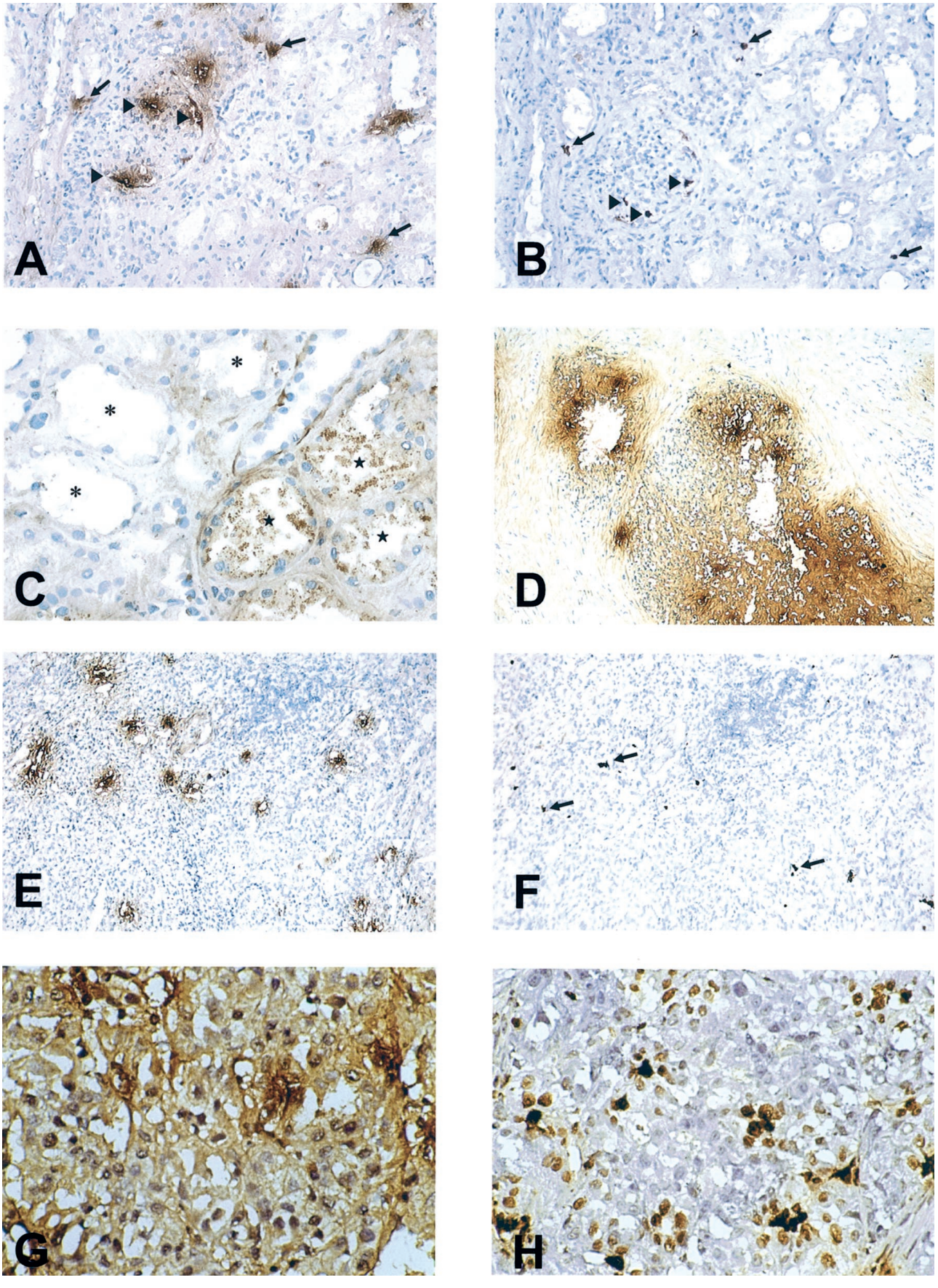
Lymphocyte Proliferation Assay

An HLA-DRB1*0301-restricted T cell line isolated from a normal donor after priming of PBMCs with the Epstein-Barr virus-transformed HLA-DRB1*0301 homozygous B-cell line Cox was restimulated with untreated Cox and Cox pulsed with defensin. Cox cells (1×10^6) were preincubated with 35 μg/ml of HNPs-1, -2, and -3 peptide isolates in RPMI 1640 for 2 hours at 37°C. Pulsed Cox cells were washed twice with RPMI 1640 and irradiated with 80 Gy. Then 5×10^4 irradiated Cox cells with and without preincubation with defensin were co-cultured with 5×10^4 cells/well of the T cell line in round-bottomed microtiter plates for 1 or 3 days, respectively, at 37°C. After 24 hours, 48 hours, and 72 hours 37 kBq of [^{3}H]-TdR (Amersham-Buchler, Braunschweig, Germany) was added and cells harvested after 16 hours on glass-fiber filtermats using a semiautomated cell harvester. T cell proliferation was determined by ^{3}H-TdR incorporation by liquid scintillation counting. Identical plates were cultured and harvested at 24 hours, 48 hours, and 72 hours; all assays were performed in triplicate. The results are expressed as mean ccpm and SI (mean ccpm of the sample/mean ccpm of the control).

Results

Detection of HNPs-1, -2, and -3 in Biopsies of Normal Kidneys

Five biopsies of kidneys from healthy donors were investigated by indirect immunoperoxidase staining with two different murine mAbs as well as with the polyclonal antibody specific for the α-defensins HNPs-1, -2, and -3. All tested antibodies against HNPs-1, -2, and -3 revealed two different staining patterns: patchy staining within glomeruli as well as in the interstitium (Figure 1A) and fine granular intracytoplasmic labeling of proximal tubular epithelial cells (Figure 1C). The patchy staining correlated mostly with the presence of granulocytes as demonstrated by labeling of consecutive sections with antibodies against neutrophil elastase (Figure 1B) and suggested that HNPs-1, -2, and -3 were released into the surrounding tissues from the neutrophils. Proximal epithelial cells stained for intracytoplasmic granular deposits of α-defensins often showed signs of cellular necrosis. In control experiments, preincubation of the applied mAbs with HNP-1, -2, and -3 isolates from leukapheresis products completely blocked staining of normal renal tissue (data not shown). Triple immunofluorescence labeling of normal kidney sections confirmed the presence of HNPs-1, -2, and -3 in and at the surroundings of elastase-positive neutrophils (Figure 2E), but also in proximal tubular epithelial cells (Figure 2F). Occasionally, HNPs-1, -2, and -3 appeared to be also present in endothelial cells of some interstitial capillaries and of small as well as larger arterial vessels.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Detection of HNPs-1, -2, and -3 in Tissues of RCC

Thirty-one samples of different RCC tissues were investigated by indirect immunoperoxidase labeling with both mAbs as well as the polyclonal antibody specific for HNPs-1, -2, and -3. Two different patterns of HNP-1, -2, and -3 presence in the analyzed tumors were seen. In most of the analyzed RCC biopsies, random distribution of patchy stains of HNP-1, -2, and -3 was noticed (Figure 1E). In those 25 tumors, the patchy stains for HNPs-1, -2, and -3 were mostly found to be associated with the presence of PMNs located between tumor cells as shown by staining for neutrophil elastase in consecutive sections (Figure 1F). In six RCC samples, however, moderate to strong diffuse labeling of all tumor cells was seen with the monoclonal and polyclonal antibodies against HNPs-1, -2, and -3 (Figure 1G). Again, staining for neutrophil elastase often showed neutrophils to be dispersed between the malignant cells (Figure 1H), but adjacent to neutrophils, in addition, epithelial tumor cells appeared to be positive for HNPs-1, -2, and -3. In some parts of these tumors this strong labeling for HNP-1, -2, and -3 deposits seemed to correlate with large areas of cellular necrosis (Figure 1D). Because these tumors were diagnosed grade II to III, diffuse expression of HNPs-1, -2, and -3 seemed to correlate with a higher grade of tumor malignancy. Triple-immunofluorescence labeling with antibodies against HNP-1, -2, and -3, DAPI, and neutrophil elastase or cytokeratin 8 confirmed the presence of defensins in association with neutrophils in most tumor tissues (Figure 2G), but also showed that HNPs-1, -2, and -3 were clearly present in single epithelial cells of 25 tumor tissues (Figure 2A). Corresponding to the immunoperoxidase staining a small number of RCC tissues showed the presence of HNPs-1, -2, and -3 in almost all malignant cells also expressing cytokeratin 8 (Figure 2; B, C, and D).

FACS Analysis of HNPs-1, -2, and -3 in RCC Lines

In FACS analyses, all eight RCC lines tested showed moderate to strong intracellular expression of the α-defensins after labeling with the mAbs specific for HNPs-1, -2, and -3. Representative histograms of intracellular labeling of HNPs-1, -2, and -3 are shown for five RCC lines in comparison to the promyelocytic-positive control cell line HL-60 (Figure 3).

Confocal scanning microscopy of the RCC line A-498 revealed that in contrast to the cell line HL-60 and normal PMNs, HNPs-1, -2, and -3 were not localized to specific granules, but appeared to be diffusely distributed within the cytoplasm without being confined to a specific cellular compartment (Figure 4).

HNP-1, -2, and -3 mRNA Expression

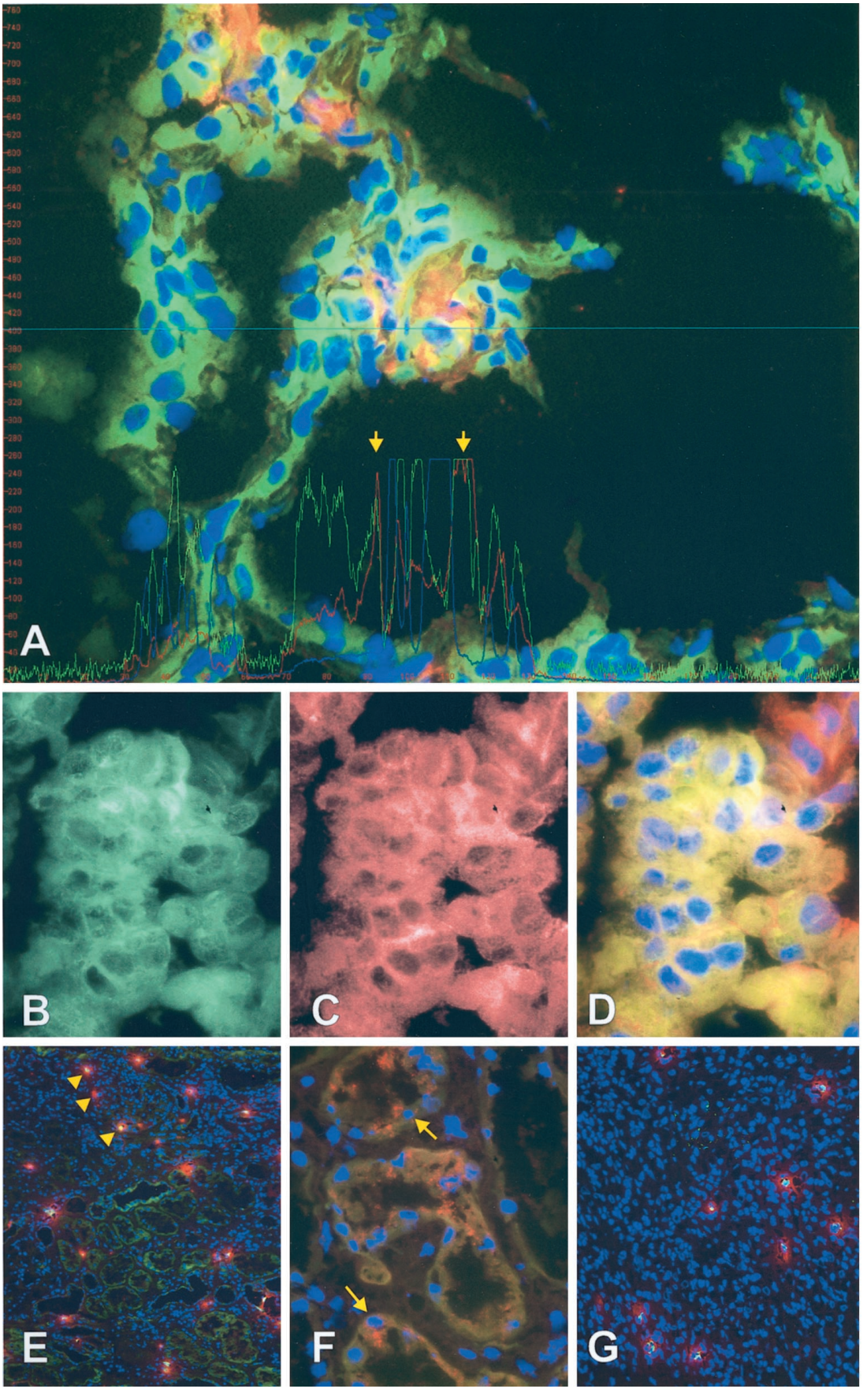
In RT-PCR analysis, the intron 2-spanning primers used allowed amplification of specific HNPs-1, -2, and -3 mRNA products of 251 bp from cDNA. The amplified fragments contained sequences coding for part of the propiece (positions 112 to 242) as well as of the mature peptides (positions 243 to 326) of HNPs-1, -2, and -3.[37] HNP-1, -2, and -3 transcripts were constantly found in PBMCs of leukapheresis products as well as in five of the eight tested RCC lines (Figure 5). The transcripts were confirmed by direct cycle sequencing to exactly match the expected coding sequences of specific mRNA[37] for HNP-1 or -3 products. However, the level of HNP-1, -2, and -3 transcripts varied between the individual HNP-1, -2, and -3 mRNA-positive RCC lines as well as between cultures of the same cell line from different time periods. Freshly thawed cells usually showed stronger transcription of the α-defensin genes than long-standing cultures of these RCC lines. The other three of the eight tested RCC lines A-498, 769-P, and LE 9211 as well as cultures of normal primary tubular cells TK163 revealed weak expression of HNP-1, -2, and -3 mRNA in several experiments if freshly thawed, but prolonged, cultures of these cells were negative. Presence of HNP-1, -2, and -3 mRNA was also observed by RT-PCR analysis in five RCC tissues as well as in two normal renal kidneys, but could be expected to represent HNP-1, -2, and -3 mRNA from neutrophils also (data not shown).

Detection of HNP-1, -2, and -3 Peptides in RCC Lines

Presence of HNP-1, -2, and -3 peptides in RCCs was demonstrated by mass-spectrometric analysis of lysates of the RCC line TW33. Mass-spectral profiling of acid-eluted proteins/peptides of the lysate of this cell line by MALDI showed three prominent average mass peaks for the mature peptides of HNP-1 (ACYCRIPACIAGERRYGTCIYQGRLWAFCC) at m/z 3442.98 [theoretical average mass $(M + H)^+$3443.09u], HNP-2 differing from HNP-1 only in the lack of alanine in the first position (CYCRIPACIAGERRYGTCIYQGRLWAFCC) at m/z 3371.84 [theoretical average mass $(M + H)^+$3372.01u] and HNP-3 with an aspartic acid instead of an alanine compared to HNP-1 (DCYCRIPACIAGERRYGTCIYQGRLWAFCC) at m/z 3486.93 [theoretical average mass $(M + H)^+$3487.10u] (Figure 6).

Within the RCC line A-498, HNP-1 and -2 peptides were not only found as cytoplasmic substrates, but also

Figure 1. Immunohistochemical localization of HNP-1, -2, and -3, and neutrophil elastase in normal kidney and in RCC tissues. Normal kidney (**A–C**): patchy staining of HNP-1, -2, and -3 detected with the antibody DEF3 in glomeruli (**arrowheads**) and interstitium (**arrows**) in **A,** corresponding presence of granulocytes stained for neutrophil elastase in glomeruli (**arrowheads**) and interstitium (**arrows**) of a consecutive section (**B**). Granular cytoplasmatic staining of proximal tubular epithelial cells (**C**). RCC tissues (**D–H**): HNP-1, -2, and -3 expression was found to be either patchily (**E**) or diffusely distributed (**G**) with signs of central cellular necrosis in larger labeled areas (**D**). Presence of tumor-infiltrating granulocytes stained for neutrophil elastase (**F** and **H**) in consecutive sections of RCC tissues analyzed in **E** and **G**. DAKO Envision labeling; original magnifications: ×200 (**A**, **B**); ×400 (**C**, **G**, **H**); ×100 (**D, E, F**).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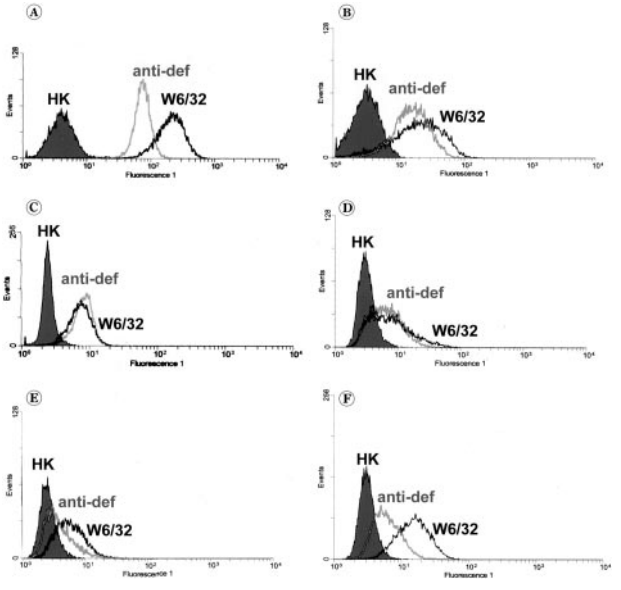

Figure 3. FACS analysis with the mAb against HNP-1, -2, and -3 kindly provided by T. Ganz (Department of Pulmonary/Critical Care Medicine, School of Medicine, University of California, Los Angeles, CA) shows intracellular expression of α-defensins in the promyelocytic cell line HL-60 (**A**) as expected, but also in normal tubular epithelial cells (**B**) and RCC lines (**C–F**) at variable intensities. A-498 (**C**), ACHN (**D**), 769-P (**E**), 786-0 (**F**) [**gray line**, labeling with the anti-defensin antibody; **black line**, labeling with the antibody W6/32. HL (positive control); **black shadowed area**, labeling with the antibody W6/32. HK (negative control)].

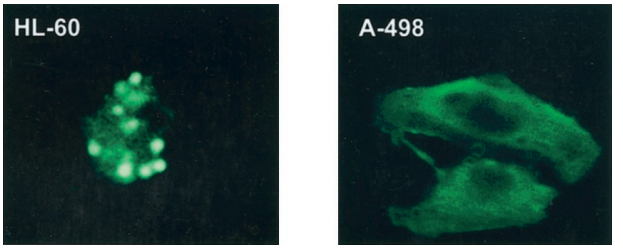

Figure 4. Confocal microscopy of the promyelocytic cell HL-60 and of the RCC line A-498 after indirect intracellular immunofluorescence labeling with the mAb DEF3. The promyelocytic cell line HL-60 shows HNP-1, -2, and -3 peptides concentrated in intracellular granules, whereas in the RCC line A-498 HNP-1, -2, and -3 peptides appeared to be diffusely distributed within the cytoplasm.

bound to human HLA class II molecules. A-498, which is constitutively HLA-class II-negative, could be stimulated to express HLA-DR molecules by incubation with interferon-γ. The induced HLA-DR-peptide complexes were isolated from the RCC line A-498 by immunoaffinity chromatography with the monoclonal antibody L243. RP-HPLC analysis of the acid-eluted HLA-DR-bound peptides revealed one dominant peptide fraction at the retention time 38.35 minutes that was shown by MALDI-MS analysis and consecutive Edman sequencing to consist of the mature HNP-1 and -2 peptides (Figure 7).

Modulation of RCC Proliferation by HNPs-1, -2, and -3

To investigate a possible functional significance of α-defensin expression in RCC, different RCC lines were incubated in serum-free RPMI 1640 medium with varying concentrations of the isolated natural HNP-1, -2, and -3 peptide fraction. In addition, a synthetic peptide HNP-1 was used. As shown in Figure 8A, incubation with the HNP-1, -2, and -3 peptide fraction at 6 to 25 μg/ml concentrations strongly stimulated DNA synthesis as measured by ^{3}H-thymidine incorporation after 24 hours in two (A-498 and 786-0) of five tested RCC lines in comparison to untreated control cultures of the same cell lines. At higher concentrations (>25 μg/ml), the mixture of α-defensins revealed suppression of DNA synthesis in all cell lines tested. Mean SIs of cell proliferation in several repeated experiments were 4.9 and 1.9 for A-498 and 786-0, respectively. Induction of cell proliferation (SI 3.2) was also reproduced in the RCC line A-498 by incubation with 12.5 μg/ml of the synthetic peptide HNP-1. On the contrary, proliferation of the RCC line A-498 induced by the α-defensins could be inhibited by preincubation of the HNP-1, -2, and -3 peptide fraction with the mAb DEF3 (12.5 μg/ml) (Figure 8B).

Further information to support that HNP-1, -2, and -3 could stimulate cell proliferation in certain RCC lines was obtained in the WST-1 proliferation assay. A similar dose-response curve for formazan dye production as sign of an increase of viable cells in the cultures of the RCC lines A498 and CRL 1932 treated with various concentrations of HNP-1, -2, and -3 was observed, but not in the three other RCC lines investigated (Figure 8A, inset). On incubation with higher concentrations of defensins (>25 μg/ml), all RCC lines showed a strong decline of viable cells even below the levels of untreated cultures. Direct counting of fluorescence-labeled cell nuclei of A498 tumor cells cultured on chamber slides also confirmed a significant increase of total cell numbers after 40 hours of stimulation with 12.5 μg/ml of defensin in comparison to untreated tumor cell cultures (Figure 8C).

Figure 2. Cryostat sections of three different RCC tissues (**A–D**, **G**) and two normal kidneys (**E**, **F**) were triple-stained with fluorescence-labeled antibodies against cytokeratin 8 or neutrophil elastase (green), defensin (red), and DAPI (blue). Overlapping binding of the antibodies against cytokeratin/neutrophil elastase and defensin is visualized as yellow areas. **A:** RCC tissue. Overlay of stains for cytokeratin, defensin, and DAPI. Intensities of the single antibody stains were evaluated by the software DOKU at the level of the **horizontal turquoise line** and are displayed as inserted curves. Simultaneous presence of cytokeratin 8 and HNP-1, -2, and -3 (yellow spots) in RCCs are documented as complete overlap of the green and red curves (**arrows**). **B–D:** RCC tissue. Superimposed stains for cytokeratin (**B**, green) and defensin (**C**, red) in the overlay (**D**, yellow) show almost complete overlap on tumor cells; DAPI stain (blue) for nuclei. **E** and **F:** Normal kidney. Superimposed stains for neutrophil elastase (green), defensin (red), and DAPI (blue) with yellow spots indicating neutrophils simultaneously labeled for defensin and neutrophil elastase, intraglomerular neutrophils marked by **arrowheads** in **E**; intracytoplasmatic staining of defensin in proximal tubular epithelial cells (**arrows** in **F**). **G:** RCC tissue. Overlaid stains for defensin, neutrophil elastase, and DAPI with overlapping labeled neutrophils (yellow spots). Original magnifications: ×400 (**A**, **F**); ×600 (**B–D**); ×200 (**E**, **G**).

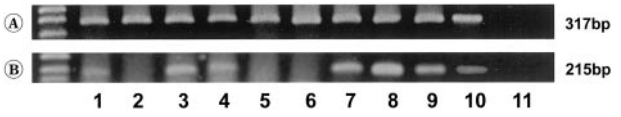

Figure 5. RT-PCR analysis of HNP-1, -2, and -3 mRNA expression: 250 ng of cDNA from different cultured RCC lines, from the normal kidney cell line TK163, and PBMC of leukapheresis products were amplified for β-actin (**A**, control product, 317 bp) and HNP-1, -2, and -3 sequences (**B**, product 215 bp). **Lane 1**, Caki-2; **lane 2**, A-498; **lane 3**, ACHN; **lane 4**, 786-0; **lane 5**, 769-P; **lane 6**, LE 9211; **lane 7**, N43; **lane 8**, TW33; **lane 9**, PBMC; **lane 10**, TK163; **lane 11**, no cDNA.

Modulation of Antigen-Presenting Function by HNPs-1, -2, and -3

Functional effects of α-defensin binding to HLA-DR molecules on immune recognition of renal carcinoma cells could not be directly evaluated because the RCC lines were constitutively negative for HLA-class II antigens. After stimulation with interferon-γ all RCC lines except CRL 1932 expressed HLA-DR molecules, but were unable to stimulate alloreactive proliferative T cell responses (data not shown). Dominant endogenous binding of α-defensins to HLA-DR molecules as shown in the RCC line A-498, however, could not be blocked and discriminated from other possible mechanisms inhibiting T cell allorecognition of the tumor cells. Therefore, the Epstein-Barr virus transformed B-cell line Cox was preincubated with 35 μg/ml of defensins for 2 hours, washed, and used to stimulate an HLA-DR restricted alloreactive T cell line in comparison to the unmanipulated B cell line. After 24 hours, 48 hours, or 72 hours of co-culture of the T cells and of the irradiated B cell line, ^{3}H-TdR was added and incorporated radioactivity was measured 16 hours later. As shown in Figure 9, the T cells were highly reactive against the unmanipulated B cell line, but revealed a 13% reduced proliferative activity after 48 hours and 75% after 72 hours stimulation with Cox pulsed with defensins by comparison.

Discussion

In this study we demonstrate for the first time that the α-defensins HNP-1, -2, and -3 can represent peptide

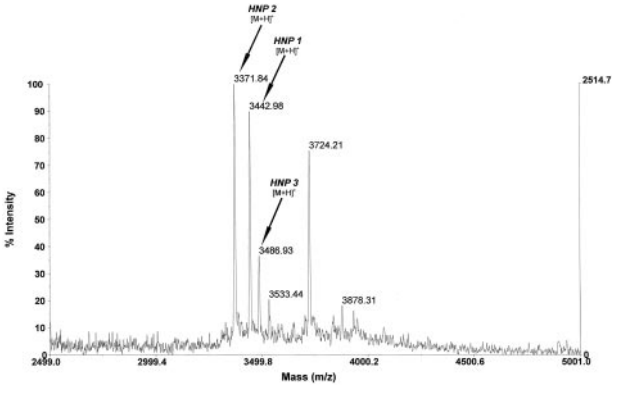

Figure 6. Direct identification of HNP-1, -2, and -3 peptides in lysates of the RCC line TW33 by mass spectrometry. MALDI-MS analysis of an acidic cell extract from the RCC line TW-33 revealed three prominent mass peaks at m/z 3371.84, 3442.98, and 3486.93 that correlated to the expected average masses of the mature HNP-1, -2, and -3 peptides.

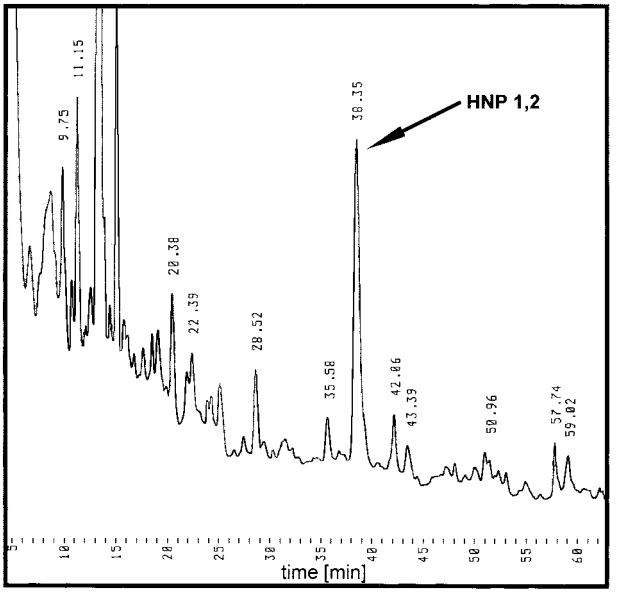

Figure 7. Identification of HNP-1 and -2 as HLA-DR-associated peptides on the RCC line A-498. HLA-DR-associated peptides were isolated from 10^{10} cells of the RCC line A-498 by immunoaffinity chromatography of the respective HLA complexes with the mAb L243 and subsequent acid release; RP-HPLC separation of the HLA-DR-associated peptide pool showed a dominant peptide peak at 38.35 minutes retention time, which was identified by MALDI-MS analysis and consecutive Edman sequencing to consist of HNP-1 and HNP-2 peptide masses and sequences.

constituents of renal carcinoma cells. By RT-PCR, immunohistological, and mass-spectrometrical analysis HNPs-1, -2, and -3 were found to be expressed in different RCC lines in culture. Of the six human α-defensins, HNPs-1, -2, and -3 have first been described to be exclusively expressed in neutrophils and were only recently shown to be also present in specific lymphocytes.[15,20] In the kidney, only β-defensins have previously been detected in tubuli.[14,40] Through immunohistological analysis, HNPs-1, -2, and -3 were shown to be also present *in situ* in normal tubular epithelial cells of the kidney as well as in tumor cells of a large group of RCC tissues adjacent to their expected expression in neutrophils. With regard to their functional role in RCC, α-defensins were observed to possess mitogenic activity on a subset of RCC lines. Identification of HNPs-1, -2, and -3 as peptides endogenously bound to HLA-DR molecules on a RCC line suggested that α-defensins may also influence immune recognition of RCC.

By FACS analysis, all investigated RCC lines exhibited intracellular labeling for α-defensins at similar or lower levels as the myeloid cell line HL-60. Presence of HNP-1, -2, and -3 in RCC lines was further established by mass-spectrometric identification of the respective mass-peaks for mature HNP-1, -2, and -3 peptides in cell lysates of the RCC line TW33. Contrary to FACS analyses, only five RCC lines repeatedly showed mRNA transcripts for HNP-1, -2, and -3 peptides similar to HL-60 or PBMCs from leukapheresis products. However, the other three RCC lines tested varied in their positivity for HNP-1, -2, and -3 mRNA in different subcultures. Although the α-defensin HNPs-1, -2, and -3 have been claimed to represent microbicidal peptides with exclusive cell lineage speci-

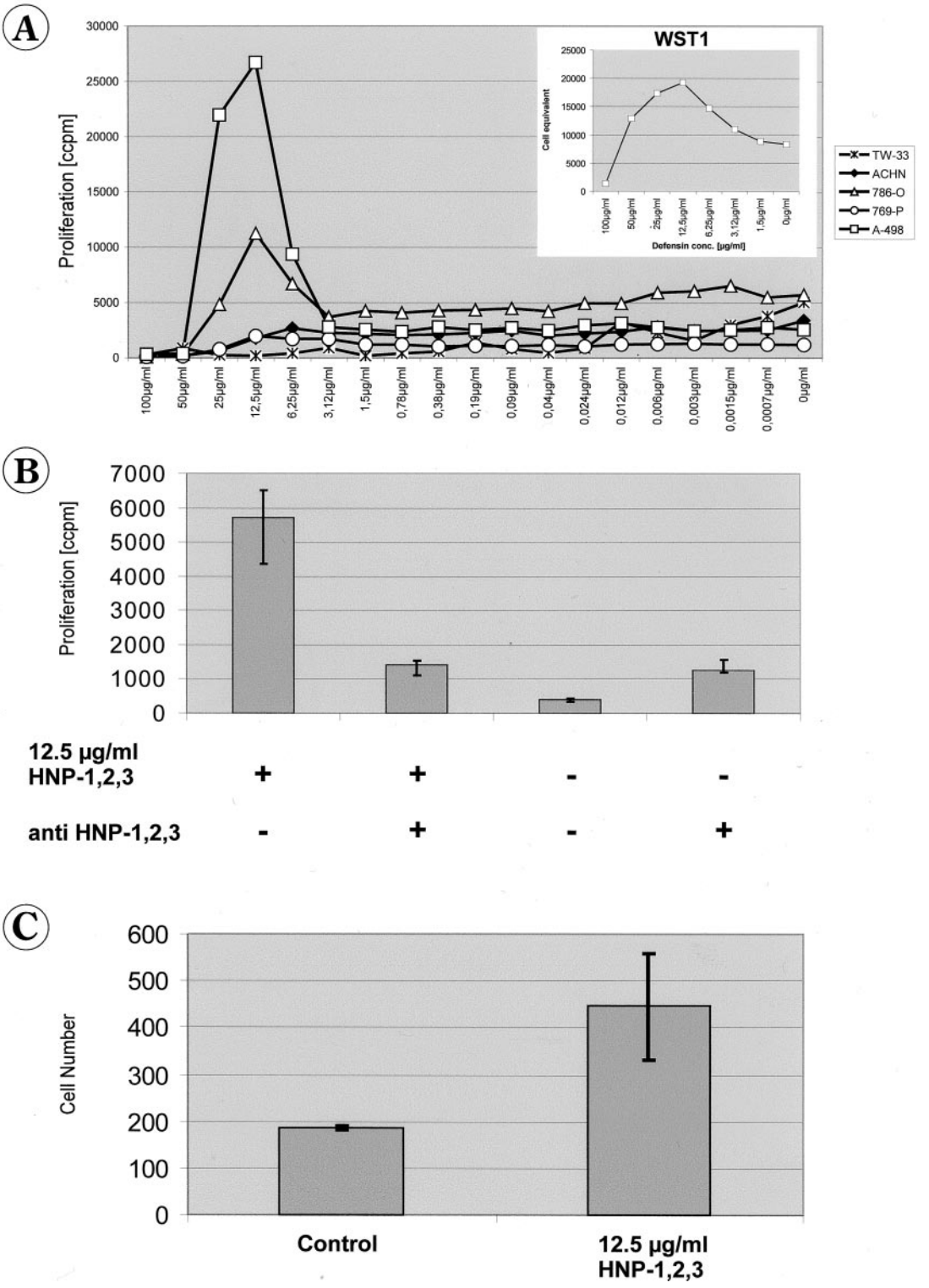

Figure 8. Influences of HNP-1, -2, and -3 on cell proliferation of different RCC lines. **A:** RCC lines were incubated with and without different concentrations of the isolated HNP-1, -2, and -3 peptide fraction. Dose-dependent proliferative responses were measured by [^{3}H]-TdR incorporation given as mean counts per minute (cpm) or as formazan-dye production in viable cells (**A**, **inset**); the RCC lines A-498 and ACHN showed a dose-dependent, significant increase of proliferative activity after stimulation with HNP-1, -2, and -3 in comparison to background proliferation in cultures without HNP-1, -2, and -3. Similar results were obtained in six independent stimulation experiments as well as with the synthetic HNP-1 peptide. **B:** Inhibition of cell proliferation of the RCC line A-498 stimulated with 12.5 μg/ml of isolated HNP-1, -2, and -3 is shown by preincubation of HNP-1, -2, and -3 with 12.5 μg/ml of the mAb DEF3 in comparison to cultures of the cell line incubated with defensins only (control of autocrine proliferation: incubation with RPMI 1640 medium only) **C:** Evaluation of total cell number by fluorescence microscopy after culture of the RCC line A-498 for 40 hours both with 12.5 μg/ml of isolated HNP-1, -2, and -3 and without. A significant increase of cell nuclei indicating cell proliferation was observed after stimulation with defensins.

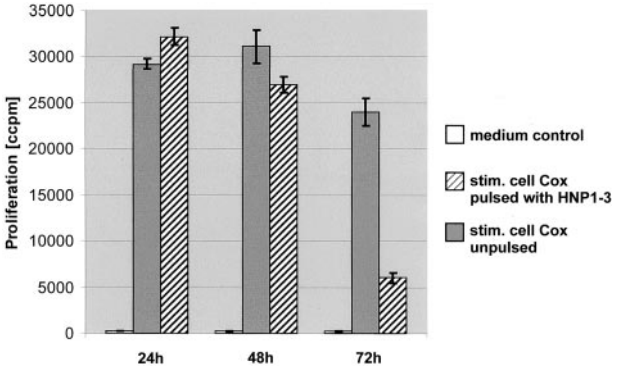

Figure 9. Use of a HNP-1, -2, and -3 prepulsed antigen-presenting cell influences proliferative T cell responses. The Epstein-Barr virus-transformed HLA-DRB1*0301-positive B-cell line Cox prepulsed for 2 hours with 17 μg/ml of the isolated HNP-1, -2, and -3 peptide fraction or RPMI 1640 medium as control, was washed and used to restimulate an HLA-DRB1*0301 restricted alloreactive T cell line. Proliferative activity of the T cell line as measured by [^{3}H]-TdR incorporation after 48 hours and 72 hours of incubation and given as mean corrected counts per minute (ccpm) was reduced in response to the prepulsed Cox (**striped bars**) in comparison to Cox incubated with medium only (**black bars**). **White bars,** background proliferation of the T cell line incubated with medium only.

ficity for hematopoetic cells such as neutrophils and some lymphocytes, specific genomic elements or transcription factors controlling myeloid lineage-restricted gene activation of α-defensins have not yet been identified.[41,42] Our observation that the amount of α-defensin mRNA varied greatly between different RCC lines and was not stable in the same RCC line if tested after different culture periods may reflect a complex, strictly controlled regulation of HNP-1, -2, and -3 mRNA, as it has been also claimed for myeloid cells.[42] Although α-defensin transcription may be modulated by certain cytokines such as IL-8 (Mueller CA, Flad T, Klatt T, Steiert I, Beir H, Mueller GA, manuscript in preparation), regulatory mechanisms responsible for the observed temporal or individual variations of α-defensin mRNA expression in RCC lines are still unknown. In this respect, it may also be of interest that a loss of heterozygosity at chromosome 8p23, which may include the defensin gene cluster,[43] in RCC tumors could play a role in the regulation of α-defensin expression and tumor progression.[44]

By triple immunohistological stainings of biopsies from normal kidneys and RCC tissues, HNPs-1, -2, and -3 were found to be also present *in situ* in normal as well as malignant renal epithelial cells co-expressing cytokeratin. In the normal kidney, presence of HNP-1, -2, and -3 peptides appeared to be concentrated to proximal tubular cells in contrast to reports on β-defensins primarily localized to epithelial layers of the loops of Henle, the distal tubuli, and the collecting duct.[14] Although weak expression of HNP-1-, -2-, and -3-specific mRNA had been observed in subcultures of primary tubular epithelial cells, we could not entirely exclude that the labeling of proximal tubular cells in the normal kidney also reflected reabsorption of α-defensins from the primary glomerular ultrafiltrate. As with β-defensins, HNPs-1, -2, and -3 might contribute to the local antimicrobial defense of the nephron and thus participate in protection from ascending urinary infections in the normal kidney. HNPs-1, -2, and -3, however, were not only found in tubular epithelial cells of the normal kidney, but also in neutrophils double-immunostained for neutrophil elastase as expected. PMNs were rarely present within the tubulointerstitial space and also within the glomerular capillary loops. Both mAbs specific for HNPs-1, -2, and -3 labeled intracellular granules but, surprisingly, also halos surrounding such neutrophils in the normal renal tissues. Such patchy stains suggested release of HNPs-1, -2, and -3 from neutrophils and diffusion into the encircling parenchyma, as it has been reported for brain tissue.[45]

In 25 of 31 RCC tissues tested a similar patchy pattern of HNP-1, -2, and -3 immunostaining was observed. The α-defensin patches mostly correlated with the presence of single elastase-positive polymorphonuclear cells in their center and several tumor epithelial cells clearly double-stained for cytokeratin in their surroundings. Unfortunately in all cases, we could not discriminate *in situ*, whether HNPs 1, -2, and -3 were sequestered from adjacent located neutrophils or also produced by the tumor cells. However, because of the *in vitro* finding of defensin synthesis in the RCC lines, and in particular in the RCC line TW33 that was established from an analyzed tumor biopsy, malignant epithelial cells contributing to HNP-1, -2, and -3 synthesis is most likely. α-Defensin-synthesizing tumor cells appeared to be primarily present in six RCC biopsies of different histological subtypes in which all epithelial tumor cells were stained diffusely and strongly for HNPs-1, -2, and -3.

Interestingly, cytolytic effects in tissues were occasionally correlated with presence of the α-defensins. In particular, groups of tumor cells in the center of primarily extended defensin patches tended to exhibit morphological signs of necrosis. Although the *in vivo* implications of defensin expression in RCC are still unknown, *in vitro* experiments on RCC lines suggested that, depending on the amount of HNP-1, -2, and -3 present in the tumor tissue, HNPs-1, -2, and -3 may exert potent mitogenic activity on tumor cells or elicit cytotoxic effects. At higher concentrations than 25 μg/ml, HNPs-1, -2, and -3 exerted cytotoxic effects on all tested RCC lines in an *in vitro* serum-free culture system, but interestingly at lower concentrations they stimulated cell growth of a subset of tumor cell lines. Similar mitogenic effects of defensins have been previously described on mouse fibroblasts and epithelial cells.[25] According to our *in vitro* and *in vivo* observations, RCCs may be heterogeneous in their content of tumor cells that are sensitive or resistant to stimulation with α-defensins. Thus, it is indeed plausible that α-defensins could contribute to tumor cell selection by promoting proliferation of a subset of malignant cells. At present it is unknown whether such selection mechanisms could favor RCC subpopulations that produce α-defensins and are associated with a higher grade of malignancy.

In neutrophils α-defensins are initially synthesized as inactive prodefensins with an NH_2-terminal neutralizing propiece that is cleaved by as yet unknown proteases. From our immunocytological studies with antibodies recognizing the mature as well as the immature peptides we could not determine whether renal carcinoma cells in

tissues contain prodefensins or the mature HNP-1, -2, and -3 peptides. At least from mRNA analysis prodefensins were most likely to be synthesized in RCC lines and mass-spectrometric analysis had shown that also low amounts of the mature HNP-1, -2, and -3 peptides were also present. From an analysis of RCC lines by confocal microscopy, we recognized that α-defensins were not confined to a specific subcellular compartment in RCC lines as in contrast to neutrophils. This finding suggested that RCC lines might accumulate α-defensins mainly as precursor peptides to avoid self-destruction. It is, however, still unknown how RCC lines synthesizing α-defensins are able to process these peptides. As with the defensins HD5 and HD6 in intestinal epithelial cells,[46] processing of HNP-1, -2, and -3 precursors could rely on the concomitant expression of specific proteases such as the metalloprotease matrilysin. Further analyses are required to elucidate such regulatory mechanisms controlling the level of functional defensin production in RCC.

As another consequence of HNP-1, -2, and -3 production, endogenous binding of these peptides to HLA-DR molecules in RCC lines could be found after stimulation of the malignant cells with interferon-γ. As it has been previously shown, α-defensins were observed to down-regulate HLA-class II-specific alloreactive T cell responses after preincubation of the stimulator cells with the HNP-1, -2, and -3 isolate in a dose-dependent manner.[28] Also in this study, proliferative responses of an alloreactive T cell line were reduced after pulsing the stimulating cells with defensins. This indicated that defensins bound to HLA-class II molecules in RCC as in other tumor cell lines could inhibit major histocompatibility complex class II-restricted RCC recognition by T cells. This could also account for the known difficulties in using RCC lines as stimulatory cells of tumor-reactive T cells *in vitro*.

Although it is claimed that defensins have their predominant functional role as neutrophil-specific endogenous antibiotics in humans,[3] several other activities, in particular chemotactic properties for immune cells, have been attributed to these peptides.[22,23] This study provides evidence for the first time that α-defensins may also be expressed in specific epithelial cells of the kidney and possibly involved in the pathogenesis of renal tumors. By influencing tumor cell proliferation and immune recognition, α-defensins could potentially modulate tumor progression of RCC.

Acknowledgments

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