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The Putative Role of Human Peritoneal Adipocytes in the Fight against Bacteria: Synthesis of the Antimicrobial Active Peptide DEFA1–3

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Key Words
Defensin · Antimicrobial peptides · Host defense · Inflammation · Peritonitis · Dialysis · Omentum majus · Adipocyte

Abstract
Background: Spontaneous peritonitis is a rather rare event, even in peritoneal dialysis (PD). As defensins are natural antimicrobial peptides, we hypothesized that adipocytes as the major constituents of the omentum could play an important role in protecting against infection by generating defensin (DEFA1–3). ***Methods:*** We isolated adipocytes from the omentum majus and conducted qualitative and quantitative RT-PCR and immunohistochemical analyses. ***Results:*** For the first time described, we were able to detect DEFA1–3 mRNA in highly purified isolated omental adipocytes. The expression of DEFA1–3 in adipocytes was confirmed on the protein level by immunohistochemistry. ***Conclusion:*** Our report of DEFA1–3 expression by human omental adipocytes adds to the role of adipocytes in the primary defense against bacterial infection. This may include PD, where the presence of the catheter as a foreign body and the nonphysiological dialysis solution may require constant defense measures to prevent peritonitis, a hypothesis that will require further testing.

Introduction

Defensins are antimicrobial peptides secreted by various cells as a component of the innate host defense [1, 2]. Their bactericidal activity lies in their ability to form micropores in the phospholipid bilayer of bacterial membranes, causing a loss of structural integrity and collapse of the bacterial cell [3, 4]. Chemotactic activity has been attributed to different defensins. Human α-defensins 1–4 (DEFA1–4) act chemotactically upon monocytes, naive T cells and immature dendritic cells [5]. Human β-defensins 1 and 2 recruit memory T cells and immature dendritic cells via binding to the chemokine-receptor CCR6 [6]. Through these mechanisms, defensins may not only act as a local antimicrobial defense, but may also link the innate and acquired immune system in their protective response. In addition, α-defensins appear to be important antiinflammatory signal peptides regulating potentially tissue-destructive responses [7].

During renal replacement therapy by peritoneal dialysis (PD), bacterial peritonitis is a rare complication with an incidence of 1.1% per patient per year [8], despite the permanent peritoneal exposition to the dialysis solution and the catheter. Even in cases of a severe intestinal mucositis with loss of the normal intestinal epithelial barrier, a spontaneous peritonitis is a rare complication.

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Because of this relative resistance of the peritoneal cavity to infection, we hypothesized that adipocytes as the major constituents of the omentum could play an important role in protecting against peritonitis. We therefore examined the potential of omental adipocytes to generate defensin.

Material and Methods

Human Tissue

Under protocols approved by the local ethics committee, peritoneal tissue (omentum majus) was obtained from patients undergoing abdominal surgery. The anonymous samples were immediately snap frozen in liquid nitrogen and stored at –80°C. Peripheral blood leukocytes were obtained from healthy adult volunteers by venipuncture and served as controls for DEFA1–3.

Isolation of Adipocytes

The adipose tissue was placed in DME medium (Life Technologies), 1 ml medium for each gram tissue, and 1 mg of collagenase (Type 1, No. C1–22 Biochrom) and 15 mg of bovine serum albumin/ml medium. The procedure of adipocyte isolation was carried out under sterile conditions according to a modified method described by Rodbell [9]. The cell suspension containing adipocytes was filtrated through a 30-μm mesh in order to eliminate contaminating endothelial cells. Next, 3 centrifugation steps (400 *g* for 1 min), leading to the pelleting of all other cell types with the exception of adipocytes, and 3 intermittent washing steps were carried out. Furthermore, the overnight seeding of the cell suspension in our stimulation experiments led to a further 'purification' of adipocytes, as all other cell types, including granulocytes, adhered to the cell culture wells, while adipocytes remained floating. Thus, by microscopic inspection the isolated adipocytes used were essentially free of other contaminating cell types, including granulocytes.

Stimulation Experiments

Isolated adipocytes were disseminated in 6- or 12-well plates in DME medium (+10% FCS, heat-inactivated) and were then either left unstimulated or were stimulated with tumor necrosis factor (TNF)-α (20 ng/ml; R&D Systems, Minneapolis, Minn., USA), interferon (IFN)-γ (40 ng/ml; Sigma Chemical, St. Louis, Mo., USA), lipopolysaccharide (LPS; R595 from *Salmonella minnesota*, Sigma, in RPMI-1640; end concentration 1 μg/ml) or heat-inactivated bacteria (100°C for 30 min) [*Staphylococcus aureus* American Type Culture Collection (ATCC) 29213, 10^{10} CFU/ml; *S. aureus* ATCC 25923, 10^{10} CFU/ml; *Pseudomonas aeruginosa* ATCC 27583, 10^9 CFU/ml; *Escherichia coli* ATCC 25922, 10^9 CFU/ml]. Unless stated otherwise, the exposure time was 20 h.

RNA Isolation

This was performed according to the method by Chomczynski and Sacchi [10]. The quality of a RNA preparation was evaluated by separation on a 1% agarose gel in 1× morpholinopropanesulfonic acid buffer.

RT-PCR for Human DEFA1–3

Total RNA (1–2 μg) was reverse transcribed with SuperScript reverse transcriptase (200 U/μl; Gibco) using oligo dTs. Specific primers (Invitrogen, Karlsruhe, Germany) for DEFA1–3 were: 5′-TCCCCAGAAGTGGTTGTTTCC-3′ and 5′-GCAGAATGCCC-AGAGTCTTC-3′, generating a 161-bp product. The PCR conditions for DEFA1–3 were denaturation for 75 s at 95°C, annealing for 60 s at 57°C, and extension for 30 s at 72°C for a total of 40 cycles. The PCR was performed in the 'Robo Cycler Gradient 96 Stratagene'. For each of the probes, a β-actin control was run in parallel (361 bp).

Sequencing of the DNA

The PCR product was isolated using the QiaQuick Gel Extraction Kit (Qiagen) before sequencing. The nucleotide sequence was blasted with the standard NCBI nucleotide-nucleotide blast program (www.ncbi.nlm.nih.gov/blast). The detection was carried out by MediGenomix (Martinsried, Germany; sequenzing@medigenomix.de).

TaqMan PCR

The TaqMan PCR was carried out in the TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany). Each 20 μl reaction consisted of 2 μl cDNA, 10 μl 2× PCR master mix (Applied Biosystems No. 4318157), 1 μl of the forward and reverse primer, and the assay by design TaqMan probe (DEFA1–3), as well as 7 μl ddH_2O. The TaqMan conditions for DEFA1–3 were 2 min at 50°C followed by 10 min at 95°C, then 40 cycles at 95°C for 15 s and 60°C for 60 s. The sequences of the primers and the probe used were as following: forward: CCTTG-CATGGGACGAAAGCT; reverse: GCACGCTGGTATTCTG-CAAT; fluorescence probe: FAM-CTTGAGCCTGGATGCTTT. Glyceraldehyde-3-phosphate dehydrogenase and ribosomal RNA served as house keeping genes (Applied Biosystems). Genomic contamination was excluded as all samples had been DNAse treated and RT controls were negative.

Immunohistochemistry

Immunohistochemical analyses were carried out on 5-μm frozen tissue sections of the omentum majus using the alkaline phosphatase/antialkaline phosphatase method. Frozen sections were incubated for 2 h at room temperature with a monoclonal mouse anti-human DEFA1–3 antibody (1:2,000; BMA, Basel, Switzerland). The incubation with the secondary antibody took place at room temperature for 30 min. The secondary antibody was a rabbit anti-mouse antibody (Z259, dilution 1:40; DAKO). Then, a further incubation with an alkaline phosphatase-specific monoclonal mouse antibody (D651, dilution 1:40; DAKO) was carried through for 1 h at room temperature. All dilutions were carried out in phosphate-buffered saline (pH 7.6). For staining, sections were exposed for 15 min to a solution of sodium nitrite (28 mM), new fuchsin (basic fuchsin 21 mM), naphthol-AS-B1-phosphate (0.5 M), dimethylformamide (64 mM), and levamisole (5 mM) in 50 mM Tris-HCl buffer (pH 8.4) containing 164 mM NaCl. The specificity of the DEFA1–3 staining was confirmed by preabsorption with the synthetic DEFA1–3 peptide (0.5 μg/μl; Bachem, Germany).

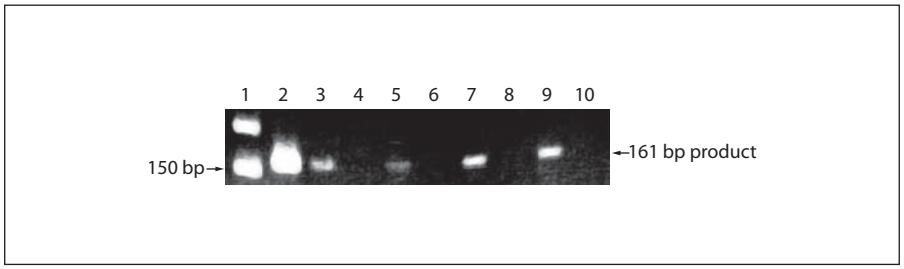

Fig. 1. RT-PCR for DEFA1–3. 1 = Marker; 2 = positive control (granulocytes) RT+; 3 = adipocytes unstimulated RT+; 5 = adipocytes stimulated with cytokines RT+; 3, 7, 9 = unstimulated adipocytes from different preparations RT+; 4, 6, 8, 10 = adipocytes RT–.

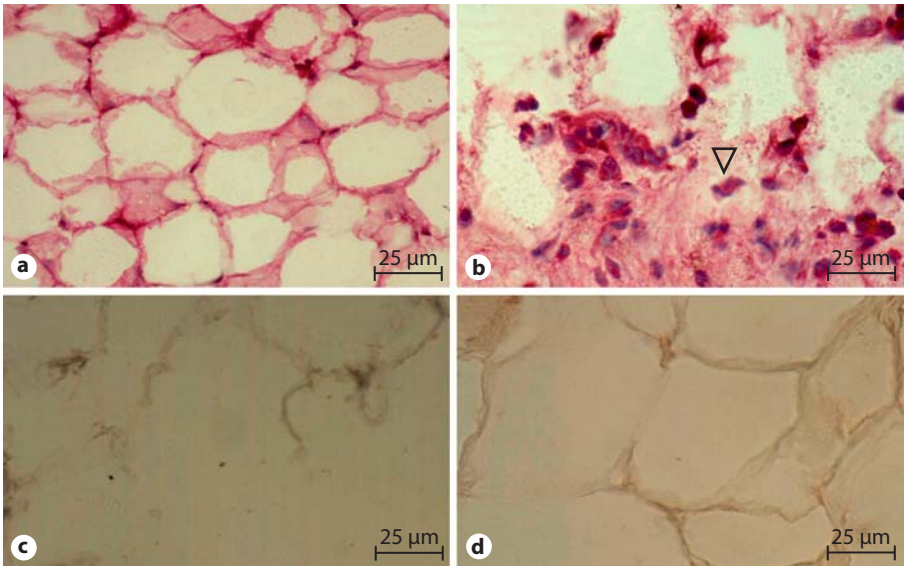

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Fig. 2. Immunohistochemistry for DEFA1–3. **a**, **b** Omental tissue stained with DEFA1–3 monoclonal antibody showed a strong positive signal for the adipocyte cytoplasm and the infiltrating leukocytes (▽). **c** Negative control with omission of the first antibody. **d** Disappearance of the positive signal for DEFA1–3 in the omentum and the leukocytes after absorption of the antibody with the synthetic DEFA1–3 antigen.

Results

RT-PCR for DEFA1–3

The adipocytes isolated from whole omentum and used in subsequent experiments were highly purified. The second filtration step through the 30-µm mesh during the isolation procedure (see Material and Methods) eliminates contaminating endothelial cells. The high degree of purity was further achieved by including 3 centrifugation steps followed by 3 washing steps in the isolation procedure. These centrifugation steps led to pelleting of all other cell types, with the exception of adipocytes, which float to the surface. Stromal-vascular cells (capillary, endothelial, mast, macrophage and epithelial cells) were absent from the adipocyte preparation upon histological examination.

By RT-PCR, a band of the appropriate size for DEFA1–3 (161 bp) was observed in granulocytes (positive control) and adipocytes. These results were consistently reproducible (n = 10) with adipocyte preparations from different donors (n = 5). In the next step, we stimulated adipocytes with 20 ng TNF-α/ml + 40 ng IFN-γ/ml of DME culture medium containing 1% FCS and examined the inducibility of defensin expression. Both unstimulated and stimulated adipocytes revealed specific expression (fig. 1). The PCR product was sequenced and found to be homologous with the published DEFA1–3 gene sequence.

Quantitative RT-PCR for DEFA1–3

Using quantitative TaqMan PCR, we examined adipocyte cDNA after separate stimulation with TNF-α, IFN-γ, LPS, or heat-inactivated bacteria (including *E. coli*, *P. aeruginosa*, and two *S. aureus* strains). None of these conditions resulted in a significant alteration of the mRNA for DEFA1–3 (data not shown).

Immunohistochemistry for DEFA1–3

We next performed immunohistochemistry on tissue sections from omentum majus (n = 5). DEFA1–3 staining could be attributed to adipocytes, as shown in figure 2a,

excluding endothelial cells of capillaries in the omentum majus as potential sources. It should be noted that omental leukocytes were also strongly positive for DEFA1–3, as shown in figure 2b, but that the staining of omental adipocytes was independent of any surrounding leukocytes. The negative control (omission of the primary antibody) is shown in figure 2c. The specificity of the DEFA1–3 staining was also confirmed by preabsorption with the synthetic DEFA1–3 peptide, which served as the antigen in the DEFA1–3 antibody generation. This resulted in the complete disappearance of the adipocyte and leukocyte staining, thus confirming the specificity of the signal as shown in figure 2d.

Discussion

While multiple tissues, including the peritoneum of patients on PD [11, 12], have been examined for their defensin expression, we are not aware of defensin detection in adipose tissue and isolated adipocytes. Based on the isolation procedure also used in other publications, we consider our isolated adipocytes to be highly purified. We therefore believe that our data support the presence of DEFA1–3 in adipocytes. We were able to show the mRNA expression for DEFA1–3 in highly purified isolated omental adipocytes from different donors. The expression of DEFA1–3 was not inducible by TNF-α or IFN-γ, LPS or exposure to bacteria. The expression of DEFA1–3 in peritoneal adipocytes was also confirmed on the protein level by immunohistochemistry, adding fat cells and specifically omentum to the tissues capable of generating defensin.

At this point, we can only speculate about the concentrations of defensins produced by omental adipocytes. Hypothetically, adipocytes could play a reinforcing role in the context of well-studied and established immune response pathways through the production of defensins. Interestingly, we found DEFA1–3 production not only in omental adipocytes, but also in subcutaneous adipose tissue (data not shown). Thus, a constitutive production of DEFA1–3 by adipocytes in the subcutaneous and omental fat may represent an additional defense against the external environment in the skin as well as in the peritoneal cavity. It is the ubiquitous occurrence of adipocytes that turns them into a pool of antimicrobial peptide production.

We could not stimulate expression of DEFA1–3 in omental adipocytes. Potentially during infection generated DEFA1–3 constitutively would be released, similar to the constitutive expression of the human β-defensin 1 (hBD-1) in epithelia, that is then released upon inflammatory signals [13, 14].

Unfortunately, we could not get a usable protein extract from the adipocytes for Western blot analysis due to interference from the very high fat content. Various methods to get around this still resulted in a protein smear on the gel. Finally, we therefore took recourse to the immunohistochemistry method, which has the additional advantage of localizing DEFA1–3 to specific cell types.

Our observations add to the reports that postulate a novel function of adipose tissue in immunity. For over 40 years, it is known that the omentum plays a role in host defense, as it is a place of antibody synthesis [15]. Over several decades, omental transposition has been successfully applied to reduce infection and accelerate the wound-healing process [e.g. 16, 17]. Adipose tissue can produce adipokines that include potent proinflammatory mediators [18]. The fundamental importance of the interplay between immunological tissue (lymphomyeloid cells) and adipose tissue has recently emerged in concepts about the inflammatory basis of the metabolic syndrome [19, 20]. Our report of DEFA1–3 expression by human omental adipocytes adds to the role of adipocytes in primary defense against bacterial infection. The finding of the adipocyte as a site of defensin production is of interest, as adipocytes are ubiquitous in the human body. The release of DEFA1–3 constitutively synthesized by and stored in omental adipocytes could contribute to local microbicidal effects and chemotactic properties as protective mechanisms in the peritoneum. This may include PD, where the presence of the catheter as a foreign body and the nonphysiological dialysis solution may require constant defense measures to prevent peritonitis, a hypothesis that will require further testing. While our results provide initial evidence for DEFA1–3 generation by omental adipocytes, further studies will be needed to identify factors controlling synthesis and release of defensins by peritoneal adipocytes and their contribution to the local antibacterial defense mechanism.

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