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# Exploring Human Testicular Peritubular Cells: Identification of Secretory Products and Regulation by Tumor Necrosis Factor-α

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**Testicular peritubular cells are myofibroblastic cells, which represent the major cellular components of the wall of the seminiferous tubules. In men their phenotypic characteristics, including possible secretory activity and regulation, are not well known, in neither normal nor pathologically altered testes. Especially in testes of men with impaired spermatogenesis, the cytoarchitecture of the tubular wall is frequently remodeled and presents fibrotic thickening, increased innervation, and infiltration by macrophages and mast cells. The latter are two sources of TNF-α. The purpose of our study was to explore human testicular peritubular cells and mechanisms of their regulation. To this end we primarily studied cultured human testicular peritubular cells (HTPCs), isolated from adult human testes. Having established that HTPCs express TNF-α receptors 1 and 2 and respond to recombinant human TNF-α by a rapid phosphorylation of ERK1/2, we used complementary approaches, including gene array/RT-PCR studies, Western blotting/immunocytochemistry, and ELISA techniques to study phenotypic characteristics of HTPCs and actions of TNFα. We found that HTPCs express the nerve growth factor gene and TNF-α-stimulated mRNA levels and secretion of nerve growth factor in a dose- and time-dependent manner. Similarly, monocyte chemoattractant protein-1 was identified as a product of HTPCs, which was regulated by TNF-α in a concentration- and time-dependent manner. TNF-α furthermore strongly enhanced expression and/or synthesis of other inflammatory molecules, namely IL-6 and cyclooxygenase-2. Active cyclooxygenase-2 is indicated by increased prostaglandin D2 levels. In addition, intercellular adhesion molecule-1, which was not detected at protein level in the absence of TNF-α, was induced upon TNF-α stimulation. In conclusion, these results provide novel insights into the nature of human peritubular cells, which are able to secrete potent signaling molecules and are regulated by TNF-α. These results also hint to an as-yet-unknown role of peritubular cells in normal human testis and involvement in the pathomechanisms associated with impaired spermatogenesis in men. (*Endocrinology* 149: 1678–1686, 2008)**

THE WALL OF seminiferous tubules in man is built by several layers of very flat, elongated cells, which have been ascribed smooth muscle cell and/or fibroblast characteristics, according to morphological and immunohistochemical criteria (1). They can thus be viewed as structural cells bearing also the potential to contract (2, 3). A major functional role of these smooth-muscle like cells (myoid cells) in the human species is assumed to be involvement in sperm transport (4).

Rodent peritubular walls are not organized in multiple layers and normally consist of only one layer of myoid cells. Yet peritubular cells of rodents have been studied in much greater detail than human peritubular cells. Studies have indicated that rodent peritubular myoid cells can also contribute directly or more likely indirectly to the process of spermatogenesis or even testicular development via secreted factors. Although the precise nature of these factors remains unsolved, they affect Sertoli cell function [see so-called PmodS, peritubular cell factor that modulates Sertoli cell activity of rodent peritubular cells (5–7)]. Several pieces of evidence for secretory activity of peritubular cells in human and rat and involvement in testis development are provided by more recent studies. In contrast to fetal Sertoli cells, peritubular cells possess androgen receptors and via presumably secretory products of unknown nature they appear to mediate actions of androgens on Sertoli cell proliferation (8).

At least in rodents, a role for peritubular cells in pathologies has also emerged, especially in experimental autoimmune orchitis. Studies allow the conclusion that peritubular cells can release a number of cytokines, including TGFβ-2, monocyte chemoattractant protein (MCP)-1, and leukemia inhibitory factor (LIF) (9). In marked contrast, to the best of our knowledge, the possible nature of secretory products, cytokines, or other factors in human peritubular cells in health and disease is not known.

In man the extracellular matrix (ECM) composition of the peritubular wall and presumably the cellular phenotype of peritubular cells can undergo dramatic changes. These typical alterations are commonly found when spermatogenesis

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Abbreviations: COX, Cyclooxygenase; ECM, extracellular matrix; FCS, fetal calf serum; HTPC, human testicular peritubular cell; ICAM, intercellular adhesion molecule; LFA, leukocyte function-associated antigen; LIF, leukemia inhibitory factor; MC, mast cells; MCP, monocyte chemoattractant protein; MP, macrophage; NGF, nerve growth factor; TNFR, tumor necrosis factor receptor.

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is deranged leading to sub- or infertility (1, 10). The changes described include a loss of markers for contractility and accumulation of components of ECM proteins (1, 10, 11). The remodeling process can result in tubular fibrosis, a hallmark of male infertility due to spermatogenic defects in men (12).

Other testicular changes are likewise documented and typical in these cases. They include increased numbers of immune cells, mainly mast cells (MCs) and macrophages (MPs). These are found within the interstitial areas of human testes affected but also within the peritubular compartment, in which, furthermore, more nerve fibers are encountered (12–14).

How these documented changes in human testes are initiated and whether and how they are interlinked are unknown, as are their precise consequences. This lack of knowledge is certainly related to the fact that these processes cannot be monitored in men but is also due to the lack of an appropriate experimental approach, including studies on isolated human peritubular cells.

Previously only very few studies have dealt with human peritubular cell cultures (15, 16). Enzymatic dispersion techniques or infantile tissues as source of peritubular cells have been described. We recently established a method for isolation and cultivation of adult human testicular peritubular cells [HTPCs (17)]. The cells are derived from explants from small biopsies of the testes of men undergoing reconstructive surgery for obstructive azoospermia, and are characterized both *in vivo* and *in vitro* by expression of fibroblast markers (*e.g.* CD90) as well as smooth muscle cell markers (smooth muscle actin). HTPCs were found to produce ECM proteins, including collagens and fibronectin (17). Furthermore, we found that whereas they lack LH or FSH receptors, they possess functional receptors for histamine and tryptase, *i.e.* typical secretory products of MCs. Whether HTPCs can also secrete signaling molecules or growth factors has not been studied. In this context it is of importance that MCs, as well as MPs, although normally present in the human testis, are significantly increased in the testes of infertile/subfertile men, especially in the peritubular regions (12, 14, 18). We therefore hypothesized that MCs/MPs via their secreted mediators may influence and regulate nearby peritubular cells.

We focused our interest on TNF-α, a cytokine with pleiotrophic actions (9, 19–22), which is assumed to be released by testicular MPs (12) and presumably MCs of the human testis as well. The present study was thus performed primarily to investigate the secretory potential of human peritubular cells and to study the possible influence of TNF-α.

## Materials and Methods

### *Isolation of HTPCs, cell culture, and human testicular samples*

Isolation of HTPCs was performed as previously described (17). Briefly, testicular tissue was obtained by open biopsy from vasectomized men with obstructive azoospermia as a standardized procedure before religation of the spermatic duct at the Academic Teaching Hospital Freising, Germany. All patients displayed normal spermatogenesis based on histological analyses (for detailed information see Ref. 17). Cells from a total number of seven different patients, without obvious changes in spermatogenesis, were cultured in DMEM + 10% fetal calf serum (FCS; both from PAA GmbH, Cölbe, Germany). Treatment with human recombinant TNF-α (Sigma Aldrich, Hamburg, Germany) was performed as detailed below, using routinely concentrations of 5 and 100 ng/ml and in some cases also 50 ng/ml. For all experiments, freshly isolated or cryopreserved cells from passages 2–9 were used. All participants granted written informed consent. The study was approved by the local ethics committee. For immunohistochemistry studies, human testicular samples of fertile and infertile men obtained from the archives of the Department of Dermatology and Allergy were used (fixed and embedded in paraffin; see Refs. 12–14).

### *Immunohisto- and cytochemistry*

Immunohistochemistry and immunofluorescence methods were performed as described elsewhere (17, 18) using cells of at least three different patients for each immunostaining. For some experiments, cells were cultured in the presence of TNF-α in DMEM + 10% FCS. Monoclonal antibodies against TNF-α (Chemicon, Hampshire, UK; 1:50) and tryptase (Dako, Hamburg, Germany; 1:50) were used for immunohistochemistry on consecutive sections of paraffin-embedded biopsies. Immunocytochemical staining was performed with the following antibodies: TNF receptor (TNFR)-1 and TNFR-2 monoclonal antibodies (Biozol, Eching, Germany; 1:1.000 and 1:50), cyclooxygenase (COX)-2 monoclonal antibody (BD Biosciences, Heidelberg, Germany; 1:50), MCP-1 monoclonal antibody (R&D Systems, Minneapolis, MN; 1:50), IL-6 polyclonal antibody (R&D Systems; 1:1000), intercellular adhesion molecule (ICAM)-1 monoclonal antibody (Acris, Hiddenhausen, Germany; 1:50). Controls consisted of incubation with nonimmune normal serum (rabbit 1:5.000 and mouse 1:10.000) instead of specific antibodies or omission of the primary antibody.

### *Isolation of RNA, RT-PCR, and semiquantitative PCR*

Cells were grown to subconfluence and TNF-α (5 and 100 ng/ml) was added to DMEM medium containing 10% FCS for 3 h. Cells from at least three patients per experiment were washed twice with PBS and suspended in RLTbuffer (QIAGEN GmbH, Hilden, Germany) containing 1% β-mercaptoethanol (according to the manufacturer's protocol). Isolation of RNA was done with QIAGEN RNeasy minikit, followed by reverse transcription using random hexamer primers (17). Two hundred nanograms of total RNA were used for further semiquantitative RT-PCR experiments [(13); for primers, annealing temperatures and cycle numbers see Table 1]. PCR products were visualized by ethidium bromide staining in agarose gels. Commercially available human testis and spleen cDNAs (CLONTECH, Palo Alto, CA) were used as positive controls in all PCR experiments. Negative controls were performed by omitting the respective input cDNA. The identities of all PCR products were verified by sequencing (12).

### *Gene arrays*

Gene expression profiles were evaluated using human extracellular matrix gene array kits (SuperArray; Biomol GmbH, Hamburg, Germany). Cells from three different patients were treated with/without recombinant human TNF-α (100 ng/ml) for 3 h in DMEM containing 10% FCS. RNA was pooled and used for further analysis (for details see Ref. 17).

### *Western blotting*

HTPCs were seeded on 60-mm dishes (Nunc GmbH & Co. KG, Wiesbaden, Germany) in DMEM containing 10% FCS and incubated with/without TNF-α for various periods of time (10, 20, and 60 min and 24 h). Immunoblotting was performed as described (13) using a monoclonal mouse antihuman phospho-ERK1/2 antibody (Cell Signaling Technology, New England Biolabs GmbH, Frankfurt am Main, Germany; 1:1.000), a polyclonal rabbit antihuman ERK1/2 antiserum (Cell Signaling Technology; 1:1.000), a polyclonal rabbit antihuman COX-2 antiserum (Oxford Biomedical Research, Oxford, MI; 1:1.000), a polyclonal goat antihuman IL-6 antiserum (R&D Systems; 1:100), and a monoclonal mouse antihuman β-cytoplasmic actin antibody (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany; 1:5000). Western blot bands were quantified by densitometry using ImageJ (National Institutes of Health, Bethesda, MD; version 1.37) and normalized to β-actin

**TABLE 1.** Information about the oligonucleotides used in PCR experiments, PCR conditions, and amplified sequences

| Name | Sequence (5′–3′) | GenBank accession no. | Amplicon (bp) | Annealing temperature (C)/cycles |
|---|---|---|---|---|
| COX-2 | | NM_000963.1 | 368 | 55/27 |
| Forward | GCA AAT CCT TGC TGT TCC | | | |
| Reverse | GGA GGA AGG GCT CTA GTA | | | |
| Cyclophilin | | NM_006347 | 325 | 56/27–35 |
| Forward | CTC CTT TGA GCT GTT TGC AG | | | |
| Reverse | CAC CAC ATG CTT GCC ATC C | | | |
| ICAM-1 | | NM_000201 | 190 | 58/27 |
| Forward | TTG AAC CCC ACA GTC ACC TAT | | | |
| Reverse | CCT CTG GCT TCG TCA GAA TCA | | | |
| IL-6 | | NM_000600 | 159 | 59.5/27 |
| Forward | AAC CTG AAC CTT CCA AAG ATG G | | | |
| Reverse | TCT GGC TTG TTC CTC ACT ACT | | | |
| MCP-1 | | NM_002982 | 190 | 59/27 |
| Forward | CAG CCA GAT GCA ATC AAT GCC | | | |
| Reverse | TGG AAT CCT GAA CCC ACT TCT | | | |
| NGFb | | BC032517 | 185 | 59/27 |
| Forward | AGG GAG CAG CTT TCT ATC CTG | | | |
| Reverse | GGC AGT GTC AAG GGA ATG C | | | |
| TNFR-1 | | NM_001065 | 198 | 57/35 |
| Forward | ACC GGC ATT ATT GGA GTG AAA A | | | |
| Reverse | GGG GTA GGC ACA ACT TCG TG | | | |
| TNFR-2 | | NM_001066 | 105 | 58.5/35 |
| Forward | CGC TCT TCC AGT TGG ACT GAT | | | |
| Reverse | CAC AAG GGC TTC TTT TTC ACC T | | | |

(23). All Western blots were repeated with cells derived from three different patients.

### *Nerve growth factor (NGF) ELISA*

Cells were stimulated with TNF-α (5, 50, and 100 ng/ml) in DMEM without serum and supernatants were collected after 6 and 24 h. All samples for this assay were prepared under serum-free conditions to prevent interference of serum contents with NGF detection, as recommended by the manufacturer. The 24-h samples were used to study in detail responses to different concentrations of TNF-α. All samples were stored at −80 C until use. Measurement of secreted NGF levels was performed using NGF Emax immunoassays (Promega, Madison, WI) according to the manufacturer's protocol (assay sensitivity: minimum detection of 15.6 pg/ml NGF; assay specificity: less than 3% cross-reactivity with other related neurotrophic factors). Briefly, Nunc maxisorp 96-well plates (Nunc) were coated with polyclonal NGF antibodies overnight. The following day samples were added and captured by a monoclonal antibody against NGF followed by signal detection. Absorbance was measured at 450 nm wavelength using a Fluostar photometer (Fluostar Optima; BMG Labtech, Offenburg, Germany). All measurements were performed at least in duplicate with cells from three to four different patients and were normalized to cellular protein (13).

### *MCP-1 ELISA*

HTPCs were stimulated as described in the NGF ELISA section, except for 2.5% FCS supplement to the media (according to the manufacturer's protocol). For the measurement of secreted MCP-1 in culture supernatants a commercial MCP-1 ELISA (Quantikine CCL-2/MCP-1 ELISA; R&D Systems) was used (assay specifications: detection limit 5.0 pg/ml; no significant cross reactivity to other chemoattractant proteins). After indicated stimulation periods (6 and 24 h with 5 ng/ml TNF-α and 24 h for the dose response experiments) supernatants were collected, stored at −80 C or assayed immediately. For MCP-1 measurements material from four different patients were used and were normalized to cellular protein (13).

### *Prostaglandin D2 ELISA*

Treatment with TNF-α (5 ng/ml) and sample collection after 3 h were performed as described in the NGF-ELISA section in serum-free medium. For the measurement a commercial prostaglandin D2 ELISA was used as described (Cayman Chemicals, Ann Arbor, MI; for reference see Ref. 24).

### *Statistical analyses*

Data analysis and statistics were performed using PRISM 4.0 (GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed using Kruskal-Wallis tests or repeated-measures ANOVA test. Differences between the groups were evaluated with the appropriate posttest (Dunn or Newman-Keuls). Data represent the mean ± SEM.

## Results

### *TNF-α immunopositive MCs are located in the human testis*

Sections of testicular biopsies displaying normal and impaired spermatogenesis contain TNF-α-positive cells in the interstitial and the peritubular compartments, as seen by immunohistochemistry. Because TNF-α expression by human testicular MPs was shown previously (12), we examined possible expression by testicular MCs. By using antibodies directed against TNF-α and the typical MC marker tryptase on consecutive sections, immunohistochemistry revealed that some of the TNF-α immunopositive cells are indeed tryptase-reactive MCs. These cells were present in samples with normal (Fig. 1) and impaired spermatogenesis (Fig. 2).

### *HTPCs bear functional TNFRs (TNFR-1 and TNFR-2)*

RT-PCR followed by sequencing and/or immunocytochemistry indicated TNFR-1 and TNFR-2 to be expressed in human testes and HTPCs from men with normal spermatogenesis (Fig. 3, A and B). Phosphorylation of ERK1/2 indicative of consecutive activation of MAPK cascade is reported to be involved in TNF-α signaling (25). We detected strong phosphorylation of ERK1/2 in HTPCs after 10, 20, but not 60 min of stimulation with 5 ng/ml TNF-α in cultures derived from three individuals (Fig. 3C).

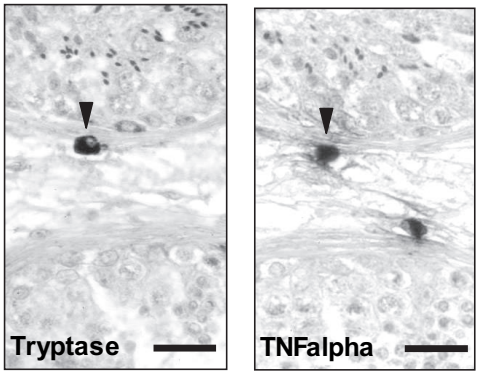


FIG. 1. Localization of TNF-α immunopositive MCs in a human testis with normal spermatogenesis. Using immunohistochemistry, some tryptase-positive MCs were identified in close proximity to the peritubular region (*left*, *black arrowhead*). As seen on consecutive sections (~5–7 μm thick) these MCs were also immunopositive for TNF-α, (*right*, *black arrowhead*). *Scale bars*, 20 μm. Sections were slightly counterstained with hemalaun-eosin.

### *Identification of secreted factors of HTPCs: NGF and MCP-1*

We used different approaches, namely RT-PCR, gene arrays, and immunocytochemistry as well as Western blot and ELISA measurements to evaluate phenotypic characteristics and secretory factors of HTPCs.

RT-PCR experiments and sequencing showed that HTPCs produce NGF mRNA, which was also found in human testes and spleen (Fig. 4A). To investigate the release of NGF, we used NGF ELISAs (Fig. 4B). We detected that with time NGF accumulates in the cell culture medium under basal conditions (n = 4 individual patient samples).

Likewise, MCP-1 expression was detected in HTPCs by RT-PCR/sequencing and in human testes and spleen. Immunocytochemistry revealed MCP-1 protein expression in the cytosol of some but not all HTPCs, and MCP-1 protein was secreted into the cell culture medium under basal conditions in a time-dependent manner (Fig. 5, B and C; n = 4 individual patient samples).

### *TNF-α increases NGF and MCP-1 and induces inflammatory markers in HTPCs*

Results obtained from four individual patients showed that NGF mRNA levels within 3 h of TNF-α stimulation (5 and 100 ng/ml) were significantly higher when compared with untreated cells (Fig. 4A). The release of NGF by HTPCs,

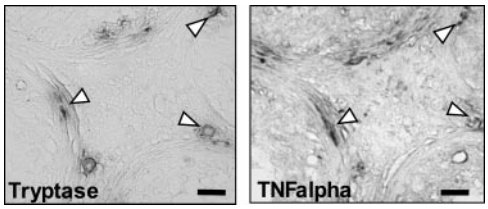


FIG. 2. Localization of TNF-α immunopositive MCs in a human testis with impaired spermatogenesis. Using immunohistochemistry, several tryptase-positive MCs were identified and localized in close proximity to or within the peritubular cell layer (*left*, *arrowheads*). The labeled MCs were also immunopositive for TNF-α, as seen on consecutive sections (*right*, *arrowheads*). *Scale bars*, 15 μm.

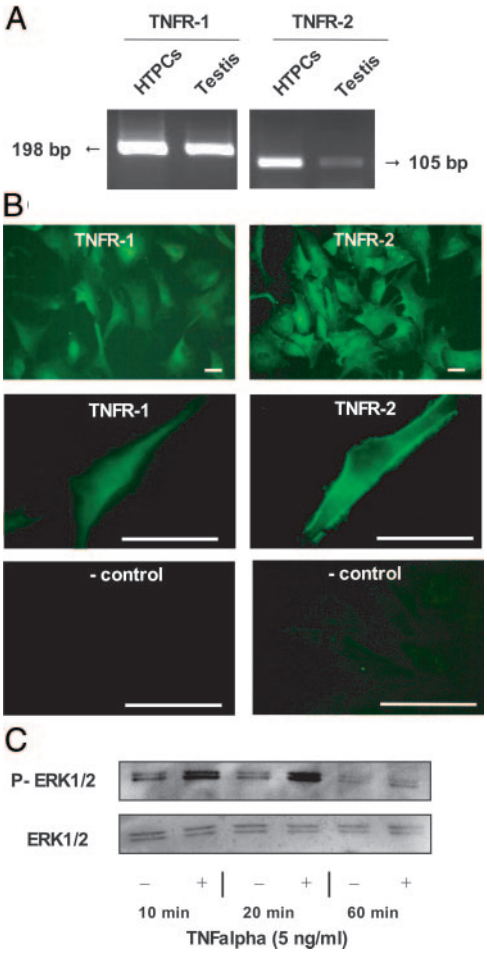


FIG. 3. HTPCs bear functional TNFRs (TNFR-1 and TNFR-2). A, In RT-PCR experiments, TNFR-1 and TNFR-2 mRNAs were detected to be expressed in human testis and by HTPCs. B, Immunocytochemistry studies confirmed the results at protein level and detected TNFR-1 and TNFR-2 proteins in the cytosol and/or membrane of HTPCs. *Bars*, 15 μm. Negative controls were performed by omitting the primary antibody (− control). C, Western blot studies performed at different time points revealed phosphorylation of ERK1/2 (P-ERK1/2) after 10 and 20 min, but not after 60 min, of TNF-α stimulation. Levels of total ERK1/2 are shown below.

as detected after 24 h, was raised nearly 6-fold upon TNF-α stimulation, reaching levels of 150 pg/mg protein (n = 4 individuals; Fig. 4B). Increasing concentrations of TNF-α (up to 50 ng/ml) enhanced NGF secretion, whereas the highest concentration used (100 ng/ml) was less effective (n = 3 individuals), as shown in Fig. 4C.

Levels of MCP-1 mRNA were likewise affected after 3 h of treatment with TNF-α (5 and 100 ng/ml; Fig 5A). MCP-1 accumulation in culture medium was time and dose-dependently enhanced by TNF-α, as found in HTPCs derived from four individuals (Fig. 5, C and D). The dose-response curve was similar as found in the case of NGF: concentrations of TNF-α up to 50 ng/ml increased MCP-1 secretion, whereas the highest concentration used was less effective (n = 4 individuals).

We screened for other secretory factors influenced by TNF-α. Results obtained by RT-PCR showed, for example, increased IL-6 mRNA levels and elevated levels of the in-

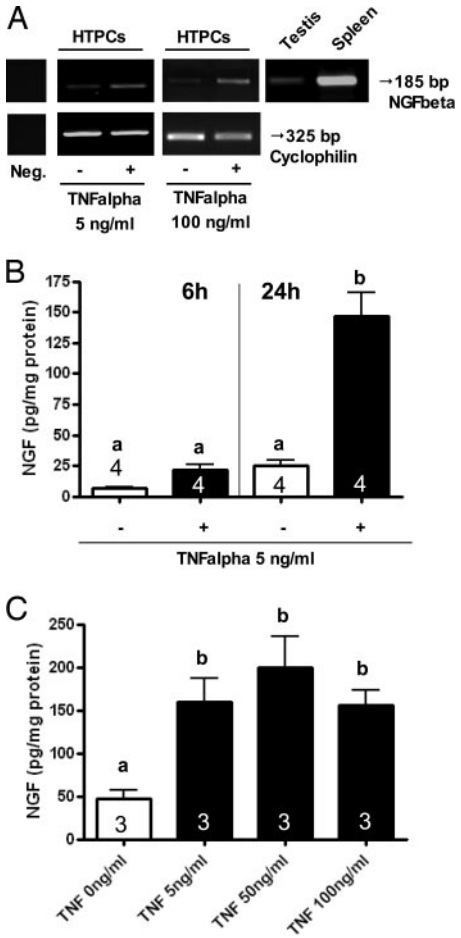


FIG. 4. TNF-α increases the expression and release of NGF by HTPCs. A, RT-PCR experiments showed that HTPCs express NGF mRNA at low levels. Stimulation with TNF-α (5 and 100 ng/ml, respectively) led to an increase in NGF gene expression. Cyclophilin amplifications were used to evaluate differences in applied cDNA amounts. Testes and spleen cDNAs were used as positive controls. Negative controls (Neg.) were performed by omitting the respective cDNA in the PCR. B, NGF ELISAs were used to quantify secreted amounts of NGF protein released from HTPCs into the media from four different patients. TNF-α strongly increased NGF levels after 6 and 24 h, respectively, with a statistically significant, approximately 6-fold increase in NGF release after 24 h. *Different letters above the columns* indicate statistically significant differences ($P < 0.001$). C, The effect of TNF-α was dose dependent. Measurements were performed using HTPCs from three different patients. *Different letters above the columns* indicate statistically significant differences ($P < 0.05$).

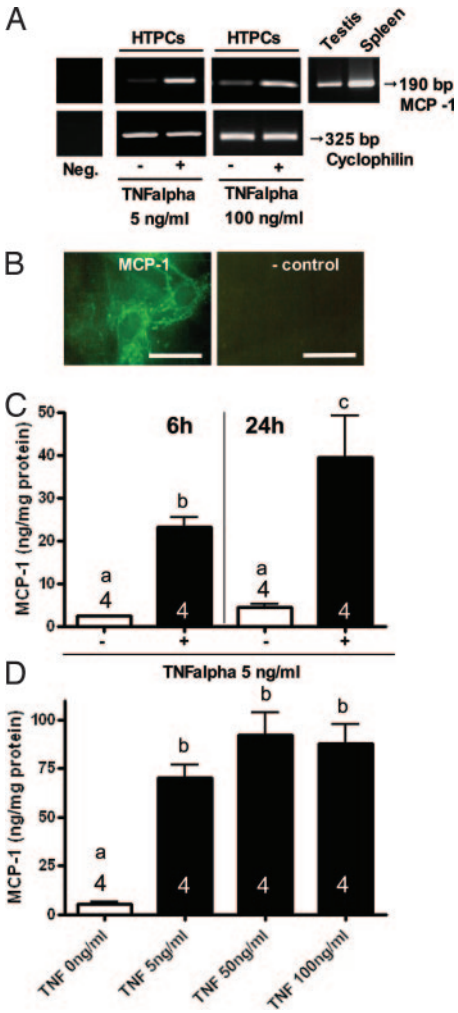


FIG. 5. TNF-α increases the expression and release of MCP-1 by HTPCs. A, RT-PCR experiments showed that unstimulated HTPCs express MCP-1 mRNA. Stimulation with TNF-α (5 and 100 ng/ml, respectively) led to an increase in MCP-1 mRNA levels. Cyclophilin amplifications were used to evaluate differences in applied cDNA amounts. Testes and spleen cDNAs were used as positive controls. Negative controls (Neg.) were performed by omitting the respective cDNA in the PCR. B, Immunocytochemistry confirmed the PCR results, showing MCP-1 protein expression in the cytosol of HTPCs, suggesting localization of MCP-1 mainly in the endoplasmic reticulum. *Scale bars*, 15 μm. Negative controls were performed by omitting the primary antibody (− control). C, MCP-1 ELISAs were used to quantify secreted amounts of MCP-1 protein in HTPC culture media from four different patients. Statistically significant stimulatory effects of TNF-α on MCP-1 secretion became evident after 6 and 24 h. *Different letters above the columns* indicate statistically significant differences ($P < 0.05$). D, The effect of TNF-α on MCP-1 release was dose dependent. Measurements were performed using HTPCs from four patients. *Different letters above the columns* indicate statistically significant differences ($P < 0.05$).

ducible form of COX-2, the rate-limiting enzyme in prostaglandin synthesis (n = 4 individuals for each, Fig. 6A). Using a commercial antibody, IL-6 protein could, however, not be detected by Western blotting or by immunocytochemistry in HTPCs (data not shown).

For COX-2, RT-PCR studies indicated and Western blot experiments confirmed an approximately 50% increase of COX-2 levels in HTPCs, compared with unstimulated controls (3 h of TNF-α stimulation; Fig. 6, A and B). An increase was also evident when immunocytochemistry was performed. Low levels of COX-2 were seen under basal conditions, whereas TNF-α stimulation increased immunoreactive COX-2 protein in the cytosol of HTPCs (n = 3 individuals; Fig. 6C). COX-2 protein was, however, not detected in all HTPCs in culture (data not shown). Further experiments showed that induction of COX-2 starts as early as 10 min after TNF-α addition to the cultures (data not shown) and that COX-2 is active. The latter was indicated in experiments with HTPCs from two patients, in which prostaglandin D2 levels in the cell culture medium doubled within 3 h of stimulation of 5 ng/ml TNF-α (1.9- and 2.1-fold, respectively).

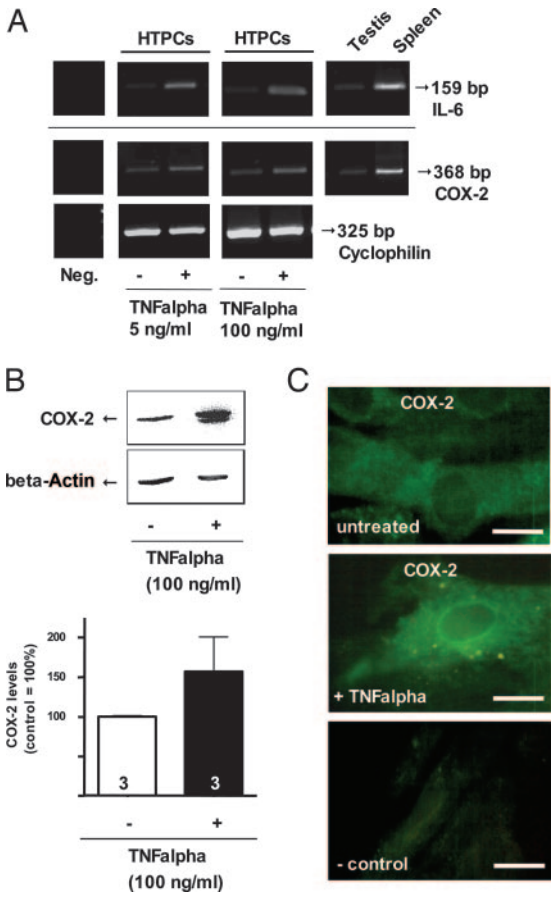


FIG. 6. TNF-α increases the expression of IL-6 and COX-2 in HTPCs. A, RT-PCR experiments showed that HTPCs express minute amounts of IL-6 and COX-2 and that the levels of both mRNAs are increased by TNF-α (5 and 100 ng/ml, respectively). Cyclophilin amplifications were used to evaluate differences in applied cDNA amounts. Testes and spleen cDNAs were used as positive controls. Negative controls (Neg.) were performed by omitting the respective cDNA in the PCR. B, Protein expression of COX-2 was evaluated by Western blot. Results were densitometrically evaluated and showed that COX-2 protein production is increased by about 50% in TNF-α-treated HTPCs (n = 3 patients, 3 h of stimulation). C, Immunocytochemistry confirmed the Western blot results, showing COX-2 protein expression mainly in the cytosol of HTPCs stimulated for 24 h with 100 ng/ml TNF-α. *Scale bars*, 5 μm. Negative controls were performed by omitting the primary antibody (− control).

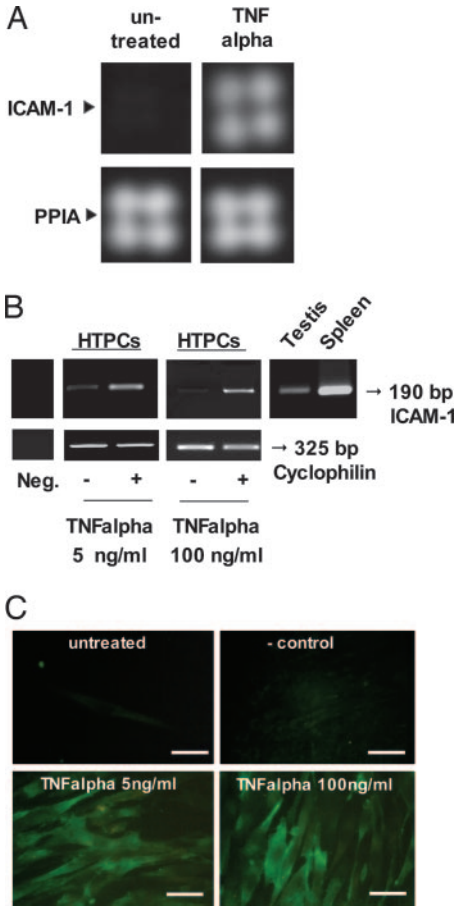


FIG. 7. ICAM-1 is induced by TNF-α. A, Gene array studies suggested strong induction of ICAM-1 expression by TNF-α (3 h, 100 ng/ml). PPIA, Peptidylprolyl isomerase A (housekeeping gene). B, RT-PCR experiments showed that unstimulated HTPCs possess low levels of ICAM-1 mRNA. Stimulation with TNF-α (5 and 100 ng/ml, respectively) led to an increase in ICAM-1 gene expression. Cyclophilin RT-PCRs were used to evaluate differences in applied cDNA amounts. Testes and spleen cDNAs were used as positive controls. Negative controls (Neg.) were performed by omitting the respective cDNA in the PCR. C, Regulatory effects of TNF-α (5 and 100 ng/ml TNF-α) on ICAM-1 protein were also found in immunocytochemistry studies: ICAM-1 was undetectable in untreated control cells, whereas TNF-α stimulated HTPCs express vast amounts of ICAM-1 protein. *Scale bars*, 20 μm. Negative controls were performed by omitting the primary antibody (− control).

### *ICAM-1 is induced by TNF-α*

Data of gene array studies (Fig. 7A) indicated a dramatic induction of ICAM-1 by TNF-α. These results were verified by RT-PCR and immunocytochemistry. TNF-α induced ICAM-1 mRNA expression as early as 3 h after stimulation (5 and 100 ng/ml TNF-α, n = 4 individuals; Fig. 7B). ICAM-1 protein was not detected in HTPCs unless TNF-α (5 and 100 ng/ml) was added for 24 h (HTPCs from n = 3 individuals; Fig. 7C).

## Discussion

This study exploits a recently established culture technique of HTPCs to elucidate the unknown nature of human peritubular cells and their secretory products. Our results identify for the first time NGF, MCP-1, and other potent mediators, including prostaglandins and IL-6, as secretory products. Importantly, TNF-α strongly affects HTPCs by stimulating expression of secretory products and by inducing ICAM-1. Because peritubular cells are in close anatomical proximity to immune cells releasing TNF-α, we suggest that human peritubular cells are an integral component of the paracrine network controlling human testicular functions in health and disease.

Human peritubular cells composing the wall of the human seminiferous tubule are at least from a functional point of view largely unexplored cells. The lack of knowledge about this human cell type is in part due to their extremely thin and elongated phenotype *in vivo*. It is evident from the literature that only few reports have been able to perform reliable immunohistochemical analyses of these cells. We also did not

succeed in our attempts to show by immunohistochemistry the testicular sites, which presumably include the peritubular compartment, of MCP-1, IL-6, or NGF (our unpublished data) in human testicular biopsies available to us. Successful visualization in testes sections was reported mainly in the case of major structural proteins (*e.g.* smooth muscle actin, desmin; *e.g.* see Ref. 17), ECM proteins (*e.g.* collagens, laminin, fibronectin), nuclear receptors (26), or adrenomedullin (27). Localization of cytokines (*e.g.* LIF) by immunohistochemistry failed, although rodent peritubular cells clearly produce LIF (28). Thus, we reasoned that to be able to further explore human peritubular cells, cellular models are required. We recently introduced such a model (17), which was further characterized in the present study.

Human peritubular cells *in vivo* and HTPCs *in vitro* do not possess receptors for FSH or LH (17) and therefore are not directly regulated by gonadotropins. Yet regulation of phenotype and functions of peritubular cells is to be assumed because the cytoarchitecture of the peritubular wall undergoes dramatic changes, especially in testes of men with impaired spermatogenesis. A loss of desmin expression and accumulation of ECM products resulting in fibrotic remodeling are among the most pronounced changes known to the very day (1). Increased innervation density and numbers of MCs and MPs are other well-documented alterations associated with male sub-infertility and tubular wall remodeling (12, 14, 20). In particular, the accumulation of immune cells within the peritubular compartment has led us to suspect that local factors, derived from immune cells, could be involved in the regulation of peritubular cells. Our study shows that the cytokine TNF-α, a product of both testicular MCs and MPs as well as other testicular sources (29), is a paracrine factor involved in the regulation of human peritubular cells. We provide evidence for increased secretory activity and altered phenotypic characteristics of HTPCs after treatment with human recombinant TNF-α.

It is clear by now that, in general, immune cells and structural cells, including fibroblasts or smooth muscle cells, can produce and release neurotrophins, namely NGF, neurotrophin 4, and brain-derived neurotrophic factor (30). It has been thought that NGF is synthesized primarily by sympathetic target organs because NGF is a prototype survival factor for sympathetic neurons. HTPCs secrete NGF, as we found, and the peritubular wall compartment is innervated by sympathetic nerve fibers. Importantly, innervation is increased in testes of men with impaired spermatogenesis, in which peritubular remodeling and increase in numbers of MCs/MPs occur (18). Thus, NGF (derived from peritubular cells) presumably induced by TNF-α (derived from MCs/MPs) in testes of men with impaired spermatogenesis could be involved in the development of hyperinnervation in fibrotically remodeled tubular walls (18).

Are there other roles of NGF in the testis? The answer(s) to this question may depend on species, developmental state and health or disease. In human testes, NGF could, for example, be involved in activation of MCs, which was described by electron microscopy in a previous study to occur in peritubular walls of infertile men (14). Based on published observations in other organs, NGF could attract and stimulate MCs to produce and release higher amounts of profibrotic tryptase and histamine (31), thus possibly augmenting tubular wall remodeling (32–34). This may create a situation, in which MCs, sympathetic innervation of the peritubular wall and peritubular cells interact and act in concert, *e.g.* MCs with neural structures via inflammatory signaling molecules as well as via NGF, which is also a product of these cells (33). Another possible direct action and involvement of NGF in tubular wall remodeling, similar to actions described in the cornea, also remains to be studied in human testis (35).

Reports imply that NGF could also be produced by Leydig cells and, importantly, germ cells (34–40) and that Sertoli cells, as well as germ cells, possess NGF receptors (41, 42). The latter in conjunction with a recent report makes it likely that NGF is involved in spermatogenesis (34). Other reports imply action in differentiation of myoid cells (40) and Leydig cells in addition to the above mentioned multiple interactions with MCs and other immune cells (43).

Thus, NGF is an emerging local factor involved in the complex paracrine regulation of the testes as well as the ovary (44, 45). In the ovary, gonadotropins appear to be involved in regulation of NGF expression (46), whereas our study shows that TNF-α stimulates NGF production in HTPCs. This is in agreement with observations that many factors, including cytokines released by immune cells, have been shown to be involved in the regulation of NGF production (47).

In our study we also found that TNF-α induced the expression and the secretion of MCP-1 and IL-6, which are considered prototype inflammatory markers and are involved in the attraction of immune cells to sites of inflammation (48, 49). Production of cytokines, in general, and MCP-1 and IL-6 in particular, is in line with reports of rodent peritubular cells, which are known to secrete a number of cytokines (9). Various cytokines have been shown to induce steroidogenesis in rat Leydig cells (50) and Sertoli cell proliferation (51), whereas other ILs are involved in Sertoli cell-germ cell interactions (51, 52). In human peritubular cells production of such factors was, however, unknown. With regard to the human testis, such actions of cytokines remain to be studied, but they may explain significantly increased numbers of immune cells within the peritubular compartments of many men suffering from sub-infertility (12). Interactions between peritubular cells and immune cells in the human testis may be the basis of a vicious circle associated with male infertility.

COX-2, the inducible form of cyclooxygenase, plays a pivotal role in the synthesis of prostaglandins, which are important mediators of inflammation, angiogenesis, and progression of cancer diseases. In the testes of infertile patients, increased expression levels of COX-2 mRNA and protein were readily observed in interstitial cells, which consist mainly of Leydig cells (13). Testicular peritubular cells have, in the study mentioned, not been reported to possess COX-2 immunoreactivity. Most likely, as mentioned, this is due to the extremely thin and elongated phenotype of these cells *in vivo*. We revealed, however, that in cultured HTPCs, COX-2 is expressed at least by some cells and furthermore that TNF-α is able to markedly increase COX-2 protein levels. COX-2 is active, as evidenced by increased levels of prostaglandin D2 in the culture media of HTPCs. We did, however,

not attempt to measure which other prostaglandins are being formed by HTPCs as a consequence of COX-2 action because the testicular effects of prostaglandins are only about to be deciphered. Nevertheless, recent studies of our group demonstrated a local prostaglandin D2 and prostaglandin F2$\alpha$ system in the human testis, with possible important implications (24, 53). We speculate that prostaglandins can act in a paracrine manner by restricting or enhancing endocrine functions of interstitial cells and/or, as can be concluded from different fibroblast models, could be initiators of fibrogenic responses (13).

Besides secretion of cytokines, cell-cell interactions and migration of immune cells are important steps in inflammatory processes, in which the adhesion molecule ICAM-1 plays an essential role. ICAM-1 is a member of the immunoglobulin supergene family, a natural ligand for $\beta$2-integrins [leukocyte function-associated antigen (LFA-1), MAC-1] present on immune cells, including leukocytes and MCs. ICAM-1 is regulated by several factors including lipopolysaccharide or TNF-$\alpha$ in multiple cell types (54). Interestingly, ICAM-1 was found in one of the very few studies addressing human peritubular cells (16). The authors of this study used enzymatic digestion to prepare their cultures and found strong expression of ICAM-1, whereas in our cell system of explant cultures, it was not found. Nevertheless, we detected a rapid and massive induction of ICAM-1 protein after TNF-$\alpha$ stimulation.

The consequences of ICAM-1 expression by peritubular cells remain to be shown (55), but interestingly, in testes of infertile men, the number of MCs within the peritubular compartment is significantly increased (14). Activated MCs are able to interact with other cells via binding of LFA-1 to ICAM-1 (56). This interaction can then cause proliferation by activation of mitogenic signals via ERK1/2 kinases or can evoke cytokine release (54). The morphological proximity between TNF-$\alpha$-positive immune cells bearing LFA-1 and the peritubular compartment (19) therefore implies intensive cell-cell interactions between MCs and HTPCs, with ICAM-1 being one factor possibly involved in these events.

In summary, our study provides for the first time insights into the secretory potential of human peritubular cells and shows that TNF-$\alpha$ acts as a regulator of these cells. We used different approaches, namely RT-PCR and gene arrays and immunocytochemistry as well as Western blot and ELISA measurements to evaluate phenotypic characteristics and secretory factors of HTPCs. Although some of the antibodies used in the study (*e.g.* MCP-1, IL-6, NGF) were not valuable for immunocytochemical/immunohistochemical and/or Western blot experiments, other lines of evidence (RT-PCR, gene arrays, ELISAs) support the hypothesis that HTPCs are not solely structural cells but also have a major secretory capacity.

Despite the fact that these results are mainly obtained by studying human cells in culture, circumstantial evidence for the importance of our results is provided by the known sum of changes observed in testes of infertile/subfertile men with impaired spermatogenesis. Briefly, in these cases the peritubular wall is remodeled and TNF-$\alpha$ sources, namely MPs and MCs, are increased. Proinflammatory agents such as IL-6 and MCP-1 may be responsible in part for accumulation of immune cells and ICAM-1 expression could allow a close physical interaction with MCs. NGF could interact with MCs and nerve fibers. Consequences of increased NGF production may include increased innervation, spermatogenic damage, and activation of MCs. Peritubular cells via COX-2 could furthermore contribute to the production of testicular prostaglandins with yet unexplored consequences.

We conclude that the recently established human peritubular cellular model allows novel insights into paracrine (*e.g.* MC and MP mediated) processes in the human testis. The results strongly suggest that peritubular cells via secretors' products, besides structural functions, are likely to be crucially involved in controlling human testicular functions in health and disease.

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