Malignant germ cell tumours of the testis express interferon-γ, but are resistant to endogenous interferon-γ

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Interferon-γ (IFNγ) is a pleiotropic cytokine mainly secreted by activated T lymphocytes and natural killer (NK) cells (Mosman and Sad, 1996). The cellular response to IFNγ is mediated by a heterodimeric cell surface receptor (IFNγR), which consists of two subunits, the 90-kDa α chain (IFNγRα) and the 85-kDa β chain (IFNγRβ) (Valente et al., 1992; Pestka et al., 1997). Ligation of IFNγ to the α chain clusters the neighbouring β chain of the IFNγR followed by Janus kinase-mediated phosphorylation of the signal transducer and activator of transcription (STAT)-1, the homodimer of which migrates to the nucleus and stimulates gene transcription such as the interferon regulatory factor-1 (IRF-1) (Haque and Williams, 1998; Wagner et al., 2002).

After the first report of its ability to protect cells from viral infection, IFNγ has been demonstrated to play a crucial role in host defence, inflammation and autoimmunity. For instance, IFNγ has been shown to augment antigen presentation by upregulation of major histocompatibility complex class I and II molecules, to induce proinflammatory cytokines in effector cells and to orchestrate leukocyte-endothelium interaction by upregulation of adhesion molecules (Boehm et al., 1997). Regarding induction of apoptosis, however, IFNγ seems to play a paradoxical role in different normal and neoplastic cell types. Whereas in normal macrophages and neoplastic myeloid and NK cells, IFNγ prevents apoptosis (Lotem and Sachs, 1996; Mizuno et al., 1999; Xaus et al., 1999), it enhances apoptosis of tumour cells in malignancies such as pancreatic carcinoma, colon carcinoma and ovarian carcinoma (Adachi et al., 1999; Burke et al., 1999; Detjen et al., 2001).

Testicular germ cell tumours (TGCT) are the most common solid malignancy in young males from 20 to 40 years old and represent a heterogeneous group of different histological entities composed of seminomatous and nonseminomatous tumours (Ulbright, 1993) that are almost all infiltrated by various numbers of T lymphocytes (Torres et al., 1997). In an attempt to investigate mechanisms navigating T lymphocytes into the TGCT, we observed that neoplastic germ cells express IFNγ (Schweyer et al., 2002). Based on this evidence and considering the dual role of IFNγ in apoptosis of neoplastic cells, we asked whether TGCT-derived IFNγ possesses any effect on survival or death of TGCT.

**MATERIALS AND METHODS**

**Tissue samples**

Tumour specimens were obtained from 12 patients who underwent orchiectomy for testicular tumour. Patients had not received any chemotherapeutic or immunomodulatory treatment before operation. The mean age at the time of operation was 36.8 years, ranging...
from 28 to 55 years. Tumour tissues were classified according to the classification system of the World Health Organization (six cases of pure seminoma, six cases of combined tumour containing nonseminomatosum and seminomatous components) (Mostofi, 1980). Probes from normal testes were obtained from three patients who underwent bilateral orchietomy because of prostatic cancer. Two blocks of each testis were immediately frozen in liquid nitrogen and stored at −80°C until analysed by reverse transcription–polymerase chain reaction (RT–PCR). Samples of each tissue specimen were also fixed in neutral formalin and processed for histology, immunohistochemistry and in situ hybridisation (ISH).

Testicular germ cell tumour cell lines

The human TGCT cell lines used in this study were NTERA (American Type Culture Collection, ATCC, Manassas, VA, USA; CRL-1973) and NCCIT (ATCC, CRL-2073). The cell lines were grown as monolayers and maintained in HEPES-buffered RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (CC Pro, Neustadt, Germany), 100 IU ml⁻¹ penicillin (Sigma, Deisenhofen, Germany), 100 µg ml⁻¹ streptomycin (Sigma) and 2 mM L-glutamine (Life Technologies, Karlsruhe, Germany). Cultures were incubated at 37°C in a humid atmosphere with 5% carbon dioxide in air.

Human monocytes

Blood monocytes were obtained from healthy volunteers as described previously (Schwyer et al., 2002). A total of 2.5 × 10⁵ monocytes were cultured in basal medium containing RPMI 1640, 2 mM L-glutamine, 1% penicillin and streptomycin, and 15% human serum for 1 day. The cells were then stimulated for 12 h with 200 U ml⁻¹ of interleukin 1 (IL-1) or in the presence of control isotype-matched IgG (100, 500 or 1000 µg ml⁻¹) and the radioactivity was measured with a Microbeta Trilux (Hewlett-Packard, Meriden, CT, USA). Results were expressed as counts per minute (c.p.m.) ± s.e.m. Each experiment was performed in triplicate and was repeated three times.

To determine the apoptotic rate of NCCIT and NTERA cells, they were harvested at 24 or 48 h after application of neutralising antibody or control IgG. Then the cells underwent May–Giemsa–Grunwald (MGG) staining, or immunocytochemistry for the active form of caspase-3, or in situ end labelling (ISEL) for DNA fragmentation. For control, NCCIT cells were incubated with 50 µM cisplatin (Holzkirchen, Germany) for 24 h and proved to be apoptotic as described previously (Burger et al., 1999). Each experiment was performed in triplicate and was repeated three times.

May–Giemsa–Grunwald staining

Centrifuged cells (2 × 10⁵) were dried for 24 h, fixed in 100% aceton for 10 min, stained with MGG and embedded in 'Super-Mount Medium'. Apoptotic cells were identified by cellular shrinkage and nuclear condensation and fragmentation.

Immune staining

Antibodies The monoclonal antibody against placental-like alkaline phosphatase (PLAP, clone SA9) and the monoclonal antibody against cytokeratine (clone MNF116) were obtained from Dako (Hamburg, Germany); the monoclonal antibody against IFNγ/Rx (clone GIR-94), the polyclonal antibody against IFNγ/Rβ (C-20) and the polyclonal antibody against pStat-1 (Tyr701) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany); and the polyclonal antibody against IFNα (AF-285-NA) and the polyclonal antibody recognising the active form of caspase-3 were from R&D systems (Wiesbaden, Germany). The antibodies were applied at a working dilution of 1:25 (IFNγ) or 1:50 (PLAP, cytokeratine, IFNγ/Rx, IFNγ/Rβ, pSTAT-1 and active caspase-3).

Immunohistochemistry Immunohistochemical reactions for IFNγ/Rx and IFNγ/Rβ were performed on frozen sections. After incubation with the primary antibody, the sections were incubated with a horseradish peroxidase (HRP)-conjugated biotin–streptavidin amplified system (Dako) and the signals were visualised with 3,3′-diaminobenzidine (DAB; Dako) as described previously (Fayyazi et al., 2000). Immunohistochemical reactions for PLAP and cytokeratine were performed on paraffin-embedded serial sections. After incubation with the primary antibody, the sections were incubated with an AP-conjugated or an HRP-conjugated biotin–streptavidin amplified system (Dako) and the signals were visualised with fast red or AEC (Dako) as described elsewhere (Schwyer et al., 2000). All samples were counterstained with Mayer’s haematoxylin, mounted in Super-Mount Medium and analysed by light microscopy. Controls were stained as above omitting primary or secondary antibodies.

Immunofluorescence and immunocytochemistry Fresh tumour tissue was mechanically dissociated in a suitable volume of RPMI 1640 supplemented with 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 2 mM L-glutamine. Cells were spun down at 1500 r.p.m. for 5 min and the pellet was resuspended in 500 µl ice-cold phosphate-buffered saline (PBS). Then, 5 × 10⁵ cells were cytotoxic-fragmented on slides coated with 2% 3-aminopropyltriethoxy-silane, dried for 24 h and fixed with 100% acetone for 10 min at room temperature. For immunofluorescence staining, cells were incubated for 30 min with a primary antibody against IFNγ, IFNγ/Rx, IFNγ/Rβ, IFNγ/Rβ active form of caspase-3 or pSTAT-1, antibodies, cells were incubated with an FITC-labelled goat anti-rabbit IgG (working dilution 1:500) for 1 h (Dako), mounted with ‘Fluorescent Mounting Medium’ and observed with a Leica fluorescent microscope. To visualise bound primary anti-IFNγ-antibody, cells were incubated with an FITC-labelled rabbit anti-goat IgG (working dilution 1:500) for 1 h (Dako), mounted with ‘Fluorescent Mounting Medium’ and observed with a Leica fluorescent microscope. To visualise bound primary anti-IFNγ-antibody, cells were incubated with an FITC-labelled goat anti-rabbit IgG (working dilution 1:500) for 1 h (Dako), mounted with ‘Fluorescent Mounting Medium’ and observed with a Leica fluorescent microscope.
In situ end labelling (ISEL)

Fixed centrifuged cells (2 × 10^5) were incubated with TBS (50 mm Tris-HCl; 150 mm NaCl; pH 7.5) containing 10% FCS and 0.3% H_2O_2 for 15 min. The cells were then incubated for 60 min at 37°C with 50 μl of the labelling mix (250 μl -1 terminal transferase, 20 μl ml -1 Digoxigen-DNA labelling mix at 10 × concentration and 1 mmol l -1 CoCl_2 in reaction buffer for terminal transferase (Roche, Mannheim, Germany)). After rinsing in TBS, the cells were blocked with 10% FCS and incubated for further 60 min with a rabbit HRP-conjugated F(ab)_2 fragment against digoxigenin (working dilution: 1:500, Dako). 3,3'-Diaminobenzidine was next applied as chromogen. Cells with fragmented DNA revealed nuclear brown signals. DNA-fragmented cells with intact plasma membrane were considered to be apoptotic. Negative controls were stained as above but without terminal transferase.

Identification and quantification of apoptosis and statistical analysis

Apoptotic cells were identified by nuclear condensation and fragmentation in MGG staining, by positive cytoplasmic signals for the active form of caspase-3 in immunocytochemistry or by DNA fragmentation in ISEL. All experiments were performed in triplicate and were repeated three times with similar results.

Percentage of apoptotic cells was calculated as the ratio of apoptotic cells to 500 cells counted. Results are expressed as the mean ± s.e.m. The differences were analysed with a t-test and were considered significant if P < 0.05.

RNA extraction and RT – PCR

Total RNAs were extracted from TGCT cell lines as well as from IFNγ-stimulated human monocytes using the Qiagen RNA isolation kit (Qiagen, Hilden, Germany), digested with DNaseI and transcribed to cDNA using oligo-d(T) primers and SuperScript II reverse transcriptase (RT) (Life Technologies). In brief, 1 – 5 μg of total cellular RNA was incubated for 50 min at 37°C with 50 μl of RT mixture and 20 U of RNase Inhibitor in a 20-μl volume containing 2.5 mmol l -1 oligo-d(T) primers, 5 mmol l -1 MgCl_2, 50 mmol l -1 KCl, 10 mmol l -1 Tris-HCl and 1 mmol l -1 of each of the deoxynucleoside-triphosphate, heated to 70°C and subsequently cooled to 5°C.

The quantity and quality of the extracted RNA were determined by spectrophotometry and agarose gel electrophoresis. Polymerase chain reaction amplification of β-actin cDNA was performed on each sample as a control for efficient cDNA synthesis. Negative controls, which were performed without cDNA adjunction in the reagent mixture, were included for every PCR analysis. Specific primers were custom synthesised (MWG, Ebersberg, Germany) and specific fragments were amplified using primer pairs as follows: IFNγ, 5'-GATGACACAGAGATCCAAAGAG-3', 5'-GCAATCTGACTCTTATTGCGTTC-3'; IFNγR2, 5'-CCGAGAAGAACATCGAGGAA3-3', 5'-GGAGGTTGCGGGCTTTATTA-3'; IFNγRβ, 5'-GGAGGAATCCAAAGGTTCA-3', 5'-GCAATCTGACTCTTATTGCGTTC-3'. The PCR reaction mixture consisted of 1.5 mmol l -1 MgCl_2, 50 mmol l -1 KCl, 10 mmol l -1 Tris-HCl, 0.2 mmol l -1 each of the two primers and 2.5 μl of Taq DNA polymerase (Pharmacia, Freiburg, Germany) in a 0.1 ml volume containing 20 μl of the above-mentioned RT reaction mixture. Reaction conditions were: 2 min at 96°C followed by 35 cycles of 30 s at 95°C, 45 s at specific annealing temperature (IFNγ: 56°C, IFNγR2: 58°C, IFNγRβ: 58°C) and 45 s at 72°C, and a final extension at 72°C for 10 min. Amplified products were electrophoresed in ethidium bromide-stained 1.5% agarose gel.
RESULTS

Testicular germ cell tumours express IFNγ

To determine the expression and cellular localisation of IFNγ in TGCT, in situ analyses were performed. Whereas no IFNγ expression was noted within the normal testes (data not shown), nonradioactive ISH revealed that numerous tumour-infiltrating mononuclear cells and almost all neoplastic germ cells independent of their histological type expressed IFNγ mRNA (Figure 1). Consequently, we asked whether IFNγ mRNA is translated into IFNγ protein. To answer this question, tumour cells were isolated from TGCT and subjected to immunofluorescence. Results demonstrated that primary TGCT not only produce IFNγ mRNA but also IFNγ protein (Figure 1).

For an autocrine effect, IFNγ must be secreted by tumour cells. To prove this, we analysed two well-established human TGCT cell lines NCCIT and NTERA, for the expression and secretion of IFNγ. Reverse transcription–polymerase chain reaction RT–PCR showed that both cell lines expressed IFNγ mRNA (data not shown). Applying ELISA, significant amounts of secreted IFNγ (343–112 pg ml−1) were found in culture supernatants of NCCIT and NTERA, as described previously (Schweyer et al, 2002) (data not shown). Based on this background, we next asked whether TGCT-derived IFNγ influences the proliferation and/or apoptosis of the testicular tumour cells in an autocrine manner.

Testicular germ cell tumour-derived IFNγ does not affect proliferation or apoptosis of TGCT cell lines

To study the effect of the secretory IFNγ on multiplication and/or death of TGCT cells, endogenous IFNγ was blocked by adding different concentrations of a neutralising antibody to the cultured NCCIT and NTERA cells for 24 or 48 h. Results from the [3H]thymidine assay demonstrated that IFNγ has no effect on proliferation of the TGCT cell lines when compared to controls. In accordance, immunocytochemistry for the active form of caspase-3, ISEL for DNA fragmentation and MGG staining for the detection of nuclear condensation and fragmentation showed that although the apoptosis rate of tumour cells was increased following IFNγ neutralisation when compared to controls, the differences among the groups remain, however, not significant (P > 0.05). Figure 2 demonstrates results from proliferation and apoptosis assays on TGCT cell lines 24 h after IFNγ neutralisation. Similar results were also seen 48 h after beginning IFNγ neutralisation (data not shown).

Testicular germ cell tumours express both IFNγR subunits α and β

Since it is known that IFNγ mediates its effects by binding to a specific high-affinity receptor (Boehm et al, 1997), we asked whether the unresponsiveness of TGCT to the endogenous IFNγ is due to the lack of dysfunction of IFNγR on neoplastic germ cells. Nonradioactive ISH revealed that in addition to numerous tumour-infiltrating mononuclear cells, almost all tumour cells independent of their histological type expressed IFNγRα and IFNγRβ mRNAs (Figure 3). Consequently, the expression of IFNγRα and IFNγRβ proteins in primary tumours was examined by immunohistochemistry and immunocytochemistry. Results illustrated both IFNγR subunits on neoplastic germ cells in primary TGCT as well as on the surface membrane of tumour-infiltrating mononuclear cells (Figure 3). Within the normal testes, however, no expression of IFNγRα or IFNγRβ was noted in germ cells (Figure 3). In situ hybridisation and immunohistochemistry, however, indicated that IFNγRβ, but not IFNγRα, was expressed in Leydig cells (Figure 3). In addition to primary tumours, both human TGCT cell lines were also examined for the IFNγR expression. RT–PCR, Western blot and FACS analyses demonstrated that NCCIT and NTERA cell lines express IFNγRα and IFNγRβ (Figure 4).

Testicular germ cell tumours lack STAT-1 activation

After demonstration of the IFNγR expression on TGCT, the functional activity of the IFNγR was analysed. For this, we examined whether the transcription factor STAT-1 is phosphorylated in IFNγ-expressing neoplastic germ cells because it is known that a sufficient stimulation of IFNγR results in a STAT-1 activation through phosphorylation. Immunocytochemistry on tumour cells isolated from primary TGCT and Western blot analysis of NCCIT and NTERA cell lines revealed that STAT-1 is not constitutively phosphorylated in neoplastic germ cells (Figure 5). To examine whether the IFNγR/STAT-1 signaling pathway is intact in TGCT cells, we stimulated NCCIT and NTERA cell lines with different doses of rhIFNγ. Western blot analysis demonstrated that upon stimulation STAT-1 was phosphorylated and IRS-1 was induced in both cell lines (Figure 5), suggesting that both IFNγR and STAT-1 are biologically intact.
DISCUSSION

In the present study, we investigated the effect of IFNγ on proliferation and apoptosis of TGCT. Analyses showed IFNγ in almost all neoplastic germ cells of primary TGCT. The data presented in this report extended our previous findings (Schweyer et al, 2002) because they demonstrated that primary TGCT not only expressed IFNγ mRNA but also IFNγ protein. These findings were surprising, because IFNγ is normally expressed and secreted by inflammatory leucocytes (Elgert et al, 1998) but not by tumour cells. Moreover, IFNγ is primarily known as a cytokine with several antitumour properties. For instance, it has been shown that IFNγ possesses direct cytotoxic effects on ovarian carcinoma cell lines (Kim et al, 2002), augments apoptosis-inducing capacity of TNFz in cervical carcinoma cells (Suk et al, 2001), reduces the proliferation activity of colon carcinoma cells and melanoma cells (Raitano and Korc, 1993; Krasagakis et al, 1995), and is able to upregulate MHC molecules on renal cell carcinomas, thus leading to a better recognition of neoplastic cells by cytotoxic T cells (Totpal and Aggarwal, 1991; Hillman et al, 1997). In addition to these antitumour activities, however, IFNγ seems to have also some powerful protumour effects. For instance, it is well known that IFNγ is a potent inhibitor of apoptosis in some haematological malignancies (Lotem and Sachs, 1996; Mizuno et al, 1999).

Based on this background, we hypothesised that endogenous IFNγ affects the proliferation and/or apoptosis of neoplastic germ cells. To prove this hypothesis, we first analysed two human TGCT cell lines for the expression and secretion of IFNγ. Results demonstrated that both NCCIT and NTERA cell lines produce and release significant amounts of IFNγ. Next, we neutralised the IFNγ in culture supernatants of the TGCT cell lines by applying a specific antibody to study the role of secretory IFNγ on the neoplastic germ cells. Using independent proliferation and apoptosis assays, we did not however note any evidence showing that the endogenous IFNγ influences the multiplication and/or the

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Figure 2: Proliferation and apoptosis of two TGCT cell lines (A and B). NCCIT and NTERA cells were incubated with different concentrations of a neutralising antibody (Ab) against human IFNγ (100, 500 or 1000 μg ml⁻¹) or with control isotype-matched IgG (100, 500 or 1000 μg ml⁻¹). After 24 h, the proliferation activity and the apoptosis rate of the cells were assessed by [³H]thymidine assay and morphological methods, respectively. The [³H]thymidine assay does not reveal any significant difference in the proliferation activity of NCCIT or NTERA cells following IFNγ neutralisation (white columns) when compared with controls (black columns). Results are expressed as counts per minute (c.p.m.) ± s.e.m. (A). To prove the effect of IFNγ neutralisation on apoptosis of NCCIT and NTERA cells, they were stained with MGG for the detection of nuclear condensation and fragmentation, with immunocytochemistry for the active form of caspase-3 and with ISEL for DNA fragmentation. Representative photomicrographs of active caspase-3 (black cytoplasmic signals) and of DNA fragmentation (black nuclear signals) illustrate no significant difference in apoptosis rate of cells incubated with the anti-IFNγ Ab (1000 μg ml⁻¹) or incubated with control IgG (1000 μg ml⁻¹) (B). The diagrams show quantification of tumour cell apoptosis with MGG staining following application of anti-IFNγ Ab (white columns) or control IgG (black columns) (B). Each experiment was performed in triplicate and was repeated three times. The values given (⁎P > 0.05, Student’s t-test) are for the statistical significance of the difference between the two groups.

Figure 3: Expression of IFNγR in primary TGCT and normal testis (A–H). Nonradioactive ISH demonstrates IFNγRα mRNA (A and C, brown signals) and IFNγRβ mRNA (B and D, brown signals) in a representative TGCT with seminomatous differentiation (A and B) and in a representative normal testis (C and D). Immunostaining shows the expression of IFNγRα (E and G, brown signals) and IFNγRβ proteins (F and H, brown signals) in the same tumour (E and F) and in the same normal testis (G and H). Note that neoplastic but not normal germ cells in tubuli seminiferi (asterisks) express both IFNγRα and IFNγRβ. Also note that, in the normal testis, Leydig cells (arrow) express IFNγRβ, but not IFNγRα.
Experimental Therapeutics

Through a high-affinity receptor consisting of a ligand-binding domain, IFN-γ mediates its effects. In TGCT cells, the receptor chain α (IFN-γRα) and β (IFN-γRβ) are expressed, as shown in our previous report (Schweyer et al., 2002), and considering the fact that IFN-γ mediates its effects through a high-affinity receptor consisting of a ligand-binding polypeptide chain α and a signal-transducing chain β (Pestka et al., 1997), and many tumours (e.g. hepatocellular carcinoma, prostatic carcinoma, basal cell carcinoma) do not express both receptor chains, thus providing tumour resistance to IFN-γ. Applying nonradioactive ISH and immunohistochemistry, however, IFN-γR mRNA and protein for both α and β chains were detected in primary TGCT. Immunoblot and flow cytometry revealed that not only primary tumours but also TGCT cell lines express both receptor chains on their cell surface. Based on the fact that TGCT express IFN-γ, we considered three possibilities. Firstly, the concentration of the endogenous IFN-γ is too low to stimulate the IFN-γR; secondly, IFN-γR is functionally inactive, as demonstrated in renal cell carcinomas (Dovhey and Ghosh, 2000); and, finally, STAT-1 lacks TGCT, as demonstrated in renal cell carcinomas. For this phenomenon, we considered three possibilities. Firstly, the concentration of the endogenous IFN-γ is too low to stimulate the IFN-γR; secondly, IFN-γR is functionally inactive, as demonstrated in renal cell carcinomas (Dovhey and Ghosh, 2000); and, finally, STAT-1 lacks TGCT, as demonstrated in renal cell carcinomas (Dovhey and Ghosh, 2000); and, finally, STAT-1 lacks TGCT, as demonstrated in renal cell carcinomas.
interferon-regulated proteins such as IRF-1. Results demonstrated that upon application of rhIFNγ in excess (on an average five times the concentration of endogenous IFNγ measured in supernatants of the TGCT cell lines), STAT-1 was phosphorylated and IRF-1 was induced. Thus, we suggest that IFNγ/R and STAT-1 are biologically intact in TGCT, but the level of the endogenous IFNγ is not able to activate the IFNγR/STAT signalling pathway in an autocrine and/or paracrine manner. Despite the lack of direct influence on neoplastic germ cells, an outstanding question may be whether endogenous IFNγ alters the stromal microenvironment in TGCT, including enhancement of angiogenesis, modification of extracellular matrix composition, recruitment of inflammatory cells and dysbalance of protease activity and thereby the tumour development and progression.

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