

Interferon- β is a potent inhibitor of cell growth and cortisol production *in vitro* and sensitizes human adrenocortical carcinoma cells to mitotane

Peter M van Koetsveld¹, Giovanni Vitale^{1,3,4}, Richard A Feelders¹, Marlijn Waaijers¹, Diana M Sprij-Mooij¹, Ronald R de Krijger², Ernst-Jan M Speel⁵, Johannes Hofland¹, Steven W J Lamberts¹, Wouter W de Herder¹ and Leo J Hofland¹

¹Division of Endocrinology, Department of Internal Medicine, Erasmus Medical Center, Erasmus MC, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

²Department of Pathology, Erasmus Medical Center, Rotterdam, The Netherlands

³Dipartimento di Scienze Mediche, Università degli Studi di Milano, Milan, Italy

⁴IRCCS Istituto Auxologico Italiano, Milan, Italy

⁵Department of Pathology, Grow-School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, The Netherlands

Correspondence
should be addressed
to L J Hofland
Email
l.hofland@erasmusmc.nl

Abstract

Adrenocortical carcinoma (ACC) is an aggressive tumor with very poor prognosis. Novel medical treatment opportunities are required. We investigated the effects of interferon- β (IFN- β), alone or in combination with mitotane, on cell growth and cortisol secretion in primary cultures of 13 human ACCs, three adrenal hyperplasias, three adrenal adenomas, and in two ACC cell lines. Moreover, the interrelationship between the effects of IGF2 and IFN- β was evaluated. Mitotane inhibited cell total DNA content/well (representing cell number) in 7/11 (IC₅₀: 38 \pm 9.2 μ M) and cortisol secretion in 5/5 ACC cultures (IC₅₀: 4.5 \pm 0.1 μ M). IFN- β reduced cell number in 10/11 (IC₅₀: 83 \pm 18 IU/ml) and cortisol secretion in 5/5 ACC cultures (IC₅₀: 7.3 \pm 1.5 IU/ml). The effect of IFN- β on cell number included the induction of apoptosis. IFN- β strongly inhibited mRNA expression of *STAR*, *CYP11A1*, *CYP17A1*, and *CYP11B1*. Mitotane and IFN- β induced an additive inhibitory effect on cell number and cortisol secretion. IGF2 (10 nM) inhibited apoptosis and increased cell number and cortisol secretion. These effects were counteracted by IFN- β treatment. Finally, IFN- β inhibited IGF2 secretion and mRNA expression. In conclusion, IFN- β is a potent inhibitor of ACC cell growth in human primary ACC cultures, partially mediated by an inhibition of the effects of IGF2, as well as its production. The increased sensitivity of ACC cells to mitotane induced by treatment with IFN- β may open the opportunity for combined treatment regimens with lower mitotane doses. The inhibition of the expression of steroidogenic enzymes by IFN- β is a novel mechanism that may explain its inhibitory effect on cortisol production.

Key Words

- ▶ adrenocortical carcinoma
- ▶ type 1 interferons
- ▶ interferon receptor
- ▶ mitotane
- ▶ insulin-like growth factor 2

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Introduction

Adrenocortical carcinoma (ACC) is a rare but highly malignant endocrine tumor. Surgery is the primary choice of treatment but recurrence rates after surgery are high (Schteingart *et al.* 2005, Fassnacht *et al.* 2011). In case of inoperable disease or tumor recurrence, therapy with the adrenolytic drug mitotane (1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-ethane; Lysodren) is usually applied (Hahner & Fassnacht 2005). Mitotane induces a cytotoxic effect on ACC cells and a response rate between 13 and 50% has been reported (Allolio & Fassnacht 2006, Veytsman *et al.* 2009). In addition, mitotane showed efficacy as adjuvant therapy in ACC (Terzolo *et al.* 2007, Veytsman *et al.* 2009). However, treatment with mitotane should be carefully considered and monitored due to the narrow therapeutic window and a common occurrence of severe adverse effects. Apart from mitotane monotherapy, combination therapy with other chemotherapeutic drugs has been used as well, although response rates are generally low (Johanssen *et al.* 2008). Therefore, the current treatment protocols are often disappointing and better therapies are clearly required (Allolio & Fassnacht 2006).

Type 1 interferons (IFNs), mainly IFN- α and IFN- β , have been shown to have potent *in vitro* and *in vivo* antitumor activities in several tumors (Vitale *et al.* 2007, Bekisz *et al.* 2010, Erdmann *et al.* 2011). In most studies, IFN- β is considerably more potent than IFN- α in its antitumor effect. Type 1 IFNs exert their tumor-suppressive activity through different mechanisms, including induction of cell cycle arrest and apoptosis (Bekisz *et al.* 2010). The activity of type 1 IFNs is mediated by a cell surface type 1 IFN receptor complex. The two major proteins of this complex are IFNAR1 and IFNAR2. IFNAR2 consists of a group of three splice variants of which the IFNAR2c isoform is the signal transducer (de Weerd *et al.* 2007).

We have recently shown that type 1 IFNs, in particular IFN- β , have a very potent antiproliferative effect on the human adrenal carcinoma cell lines H295 and SW13 (van Koetsveld *et al.* 2006). In both cell lines, type 1 IFNs block the S-phase of the cell cycle. In H295 cells, but not in SW13 cells, IFN- β strongly induced apoptosis, partially mediated via inhibition of IGF2 expression, an important growth factor in ACC (de Fraipont *et al.* 2005, van Koetsveld *et al.* 2006). There are no data with respect to the effects of both mitotane and type 1 IFNs in primary cultures of ACC. In this study, we evaluated the antitumor activity of type 1 IFNs, with or without mitotane, in primary ACC cultures. The effects of both drugs on cortisol production were studied and a comparison was made with the effects

observed in primary adrenal hyperplasia cells and adrenocortical adenoma (ACA) cultures. Finally, the interaction between the effects of IFN- β and IGF2 in primary ACC cultures was evaluated.

Materials and methods

Patients

Adrenal tissue samples for *in vitro* functional studies were obtained from patients with adrenal hyperplasia ($n=3$), ACA ($n=3$), and ACC ($n=13$). Pathological diagnosis was performed by expert pathologists on the basis of the Van Slooten index (VSI; van't Sant *et al.* 2007). A VSI of >8 was considered for diagnosis of ACC. For measurement of type 1 IFN receptor mRNA expression, the series of tissues was enlarged with seven additional adrenal hyperplasias, six additional ACAs, and two additional ACCs. All ACA and ACC are primary tumors, except for case 9, which is a recurrence. Before surgery, none of the patients were pretreated with steroid inhibitors, mitotane, or chemotherapy. Circulating cortisol, testosterone, 17-OH-progesterone, estradiol, progesterone, and androstenedione were measured at baseline and after 1 mg dexamethasone overnight. Plasma aldosterone and plasma rennin were measured at baseline, as well as 2 \times 24-h urinary cortisol excretion. Ki-67 immunohistochemistry was performed according to standard techniques, using the MIB1 monoclonal antibody (Dako, Belgium). Scoring was done in areas with the highest labelling intensity, in which 1000 nuclei were scored, as described previously (Morimoto *et al.* 2008). Patient data are shown in Table 1. Informed consent was obtained from each patient. The medical ethics committee of the Erasmus MC has approved the protocol.

Primary cell culture of human adrenocortical tissues

Directly after surgery, the adrenal specimens were minced into small pieces and dissociated for 2 h at 37 °C, with collagenase type 1 (2 mg/ml; Sigma-Aldrich). Cell viability, after Ficoll density gradient separation, was determined by trypan blue exclusion and always exceeded 90%. The cells were cultured in DMEM/F12K medium (Invitrogen), supplemented with 5% FCS (Gibco), penicillin (1 \times 10⁵ U/l; Bristol-Meyers Squibb), and L-glutamine (2 mmol/l; Gibco). The cells were plated at a density of 100 000 cells/well in 24-well plates (Corning Costar). After 2 days of incubation at 37 °C in a humidified

Table 1 Patient and tissue characteristics.

Case no.	Histology	M/F	Age (years)	Weight (g)	VSI	Stage AJCC/ENS@T	Ki67	Functionality
1	H	M	46	ND	0	ND	ND	C
2	H	F	62	ND	0	ND	ND	C
3	H	F	57	ND	0	ND	–	C
4	ACA	F	64	40	5.7	ND	–	NF
5	ACA	F	56	43	0	ND	–	NF
6	ACA	F	43	16	0	ND	–	NF
7	ACC	F	70	1770	19.6	IV/IV	36	NF
8	ACC	F	56	205	24.3	IV/IV	18	NF
9	ACC	M	44	500	10.4	II/II	Not available	NF
10	ACC	M	45	305	19.6	IV/IV	9	C
11	ACC	M	42	95	21.7	IV/IV	16	NF
12	ACC	F	51	1900	18.4	IV/III	11	C
13	ACC	M	41	320	25.0	IV/IV	18	C,E
14	ACC	F	33	1167	28.4	IV/IV	17	C,A
15	ACC	F	39	542	18.0	IV/IV	14	NF
16	ACC	F	10	290	25.8	IV/IV	11	C,A
17	ACC	F	76	845	25.1	II/II	4	NF
18	ACC	F	46	531	21.0	II/II	11	NF
19	ACC	M	76	284	19.0	IV/IV	19	C,E

H, adrenal hyperplasia; ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma. M/F, male, female; VSI, Van Slooten index: >8 considered for diagnosis of ACC. Weight: weight of the tissue in grams (g). Stage according to AJCC; American Joint Committee on Cancer, ENS@T, European network for the study of adrenal tumors. ND, not determined. Functionality: *in vivo* production of C: cortisol, E: estrogens, A: androgens, NF: nonfunctional.

incubator containing 5% CO₂, the medium was refreshed without or with the indicated test substances in quadruplicate. Plates were further incubated until 7 days. Medium and test substances were refreshed once after 3 days. After 7 days, the medium was collected for cortisol and IGF2 determination. On day 7, the cells were washed twice with saline followed by lysis of the cells for DNA measurement and measurement of DNA fragmentation. Due to a limitation in the cell yield obtained per tissue, not all experiments could be performed in each individual case.

Cell lines and culture conditions

The human ACC cell lines NCI-H295R and SW13 were obtained from the American Type Culture Collection and ECACC respectively. Culture conditions of human NCI-H295R and SW13 ACC cells have been described previously (van Koetsveld et al. 2006). Briefly, the cells were cultured without (vehicle only) or with substances for 7 days. Medium and compounds were refreshed at day 4. Thereafter, the medium was collected for cortisol measurement. Cell growth and DNA fragmentation (apoptosis) assays have been described previously (van Koetsveld et al. 2006).

Quantitative RT-PCR

For the detection of type 1 IFN receptors (IFNAR1, IFNAR2-total, and IFNAR2c), IGF2, the cholesterol transporter

(STAR), and the steroidogenic enzymes (CYP11A1, HSD3B2, CYP17A1, CYP21A2, and CYP11B1), total RNA was isolated using a commercially available kit (High pure RNA isolation kit, Roche). cDNA synthesis and quantitative PCR using the TaqMan Gold nuclease assay were performed as described previously in detail (Hofland et al. 2005). The primer and probe sequences were purchased from Sigma-Aldrich. The sequence and the concentration of primers and probes used for quantitative PCR have been described previously (van Koetsveld et al. 2006, Vitale et al. 2007, Chai et al. 2010). mRNA expression levels were considered below the detection limit when Ct values were >35 . To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of cDNA template in parallel with cDNA samples. Samples were normalized against the expression of the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT). PCR efficiencies (E) were calculated for the primer–probe combinations used (Rasmussen 2001). The relative expression of genes was calculated using the comparative threshold method, $2^{-\Delta Ct}$ (Schmittgen & Livak 2008), after efficiency correction (Pfaffl 2001) of target and reference transcripts.

Cortisol determination

Cortisol concentrations were determined by a non-isotopic, automatic chemiluminescence immunoassay

system (Siemens Medical Solutions Diagnostics, Breda, The Netherlands). Intra- and interassay coefficients of variation (CV) were 6 and 8% respectively. *In vivo* hypercortisolism was established by a urinary free cortisol concentration of >850 nmol/24 h.

IGF2 protein assay

IGF2 protein was measured in the culture supernatant by a non-extraction enzymatically amplified 'two-step' sandwich-type immunoassay (DSL Germany GmbH–Benelux, Assendelft, The Netherlands), according to the manufacturer's recommendations. Intra- and interassay CV values were 5.2 and 6.9% respectively. IGF2 protein is expressed in nmol/l.

Drugs and reagents

Human recombinant IFN- β 1a (Rebif) and human recombinant IFN- α 2b (intron-A) were obtained from Serono Benelux BV (Den Haag, The Netherlands) and Schering Plough Corporation (Utrecht, The Netherlands) respectively. Both IFNs were stored and diluted in aquadest. Mitotane was purchased from Sigma–Aldrich. Mitotane was dissolved in absolute ethanol at a concentration of 10 mM and stored at 4 °C. For each experiment, fresh dilutions of mitotane in ethanol absolute were prepared. IGF2 was obtained from Invitrogen. A stock solution of 50 nM was prepared in 0.01 M acetic acid and stored at -20 °C. Further dilutions were made in culture medium.

Statistical analysis

For statistical analysis, GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA) and SPSS15 were used. Dose–response curves for mitotane and IFN- β in primary cultures were only calculated if there was a significant effect by 50 μ M mitotane and 500 IU/ml IFN- β . A comparative statistical evaluation among treatment groups was performed by ANOVA. When significant, overall differences were found, and a Newman–Keuls multiple comparison test between groups was made. Values of $P < 0.05$ were considered statistically significant. Data are indicated as mean \pm S.E.M.

Results

Patient and tumor characteristics

In the series of patients with ACC (age: 46 ± 5 years, age range: 10–76 years), 8 out of 13 (62%) were females

(Table 1). The mean weight of the ACC was 722 ± 190 g. Six out of 13 patients (46%) had hypercortisolism. In two patients, there was co-secretion of androgens and in two patients of estrogen. In ACC, there was a significant association between the *in vivo* and *in vitro* presence or absence of cortisol production ($P < 0.01$, Fisher's exact test). Nine out of 13 (69%) patients had metastasis in one or more organs. The mean VSI of the ACC was 21.5 ± 1.5 . In tumor cultures from 11 patients (nos 7–17), the effects of mitotane and IFN were studied, whereas in two additional cases (nos 18 and 19), the effects of IFN on IGF2 mRNA expression were evaluated.

Expression of type 1 IFN receptor mRNA

All adrenal samples had a detectable mRNA expression of *IFNAR1*, *IFNAR2*-total, and *IFNAR2c* receptors (Fig. 1A, B and C). In order of magnitude, expression levels were *IFNAR1* \gg *IFNAR2* \gg *IFNAR2c*. There were no statistically significant differences in the level of *IFNAR1*, *IFNAR2*-total, and *IFNAR2c* mRNA expression between adrenal hyperplasia, ACA, or ACC tissues. In ACC, there was no significant correlation between the mRNA expression of the IFN receptors and the effect of IFN on cell number, cortisol production, or DNA fragmentation (data not shown).

Effect of mitotane on primary cultures of human ACC and human ACC cell lines

Mitotane treatment for 6 days induced a statistically significant, dose-dependent inhibition of cell number, as measured by the DNA content/well, in H295 and SW13 human ACC cell lines, as well in 7 of 11 (64%) of the primary human ACC cultures (Fig. 2A). The IC_{50} values of inhibition were 2.6 ± 1.7 , 45 ± 2.8 , and 38 ± 9.2 μ M for H295, SW13, and primary ACC cultures (range 1.3–62 μ M) respectively. Mitotane had no significant effect on DNA fragmentation in the primary ACC cultures and cell lines (Fig. 2B). Mitotane inhibited cortisol secretion in H295 cells (SW13 cells are non-secreting) with an IC_{50} of 1.3 ± 0.2 μ M (Fig. 2C).

Five out of 11 primary human ACC cultures produced cortisol *in vitro*. Cortisol secretion was inhibited by mitotane in a dose-dependent manner with an IC_{50} value of 4.5 ± 0.1 μ M (Fig. 2C). The percentage of inhibition of cell number (DNA content) and cortisol production by mitotane was time dependent (data not shown). There was a statistically significant difference

between IC₅₀ value of inhibition of cortisol secretion and the IC₅₀ of inhibition cell number in cortisol-producing ACC cultures ($n=5$; $P<0.01$). This suggests that significant lower concentrations of mitotane are required for

inhibition of cortisol secretion compared with inhibition of cell number.

Effect of IFN- β on cell number, DNA fragmentation, and cortisol production in primary ACC cultures

Figure 2D, E, and F shows the effect of IFN- β on cell number ($n=11$), DNA fragmentation ($n=7$), and cortisol secretion ($n=5$) in primary ACC cultures. IFN- β inhibited cell number in a dose-dependent fashion (Fig. 2D; IC₅₀ value 83 ± 18 IU/ml, range 0.4–170 IU/ml). Moreover, IFN- β stimulated DNA fragmentation in a dose-dependent manner (Fig. 2E; EC₅₀ value 13 ± 1.9 IU/ml, range 5–23 IU/ml). Finally, IFN- β also inhibited cortisol secretion in primary ACC cultures with an IC₅₀ value of inhibition of 7.3 ± 1.5 IU/ml (range 5–41 IU/ml, Fig. 2F). IFN- $\alpha 2b$, at concentrations up to 500 IU/ml, did not significantly change DNA content nor did DNA fragmentation. Cortisol secretion was slightly inhibited by IFN- $\alpha 2b$ (only at 500 IU/ml) by $26 \pm 4.0\%$ compared with untreated control cells ($P<0.01$; data not shown).

Effect of combination treatment with mitotane and IFN- β on human primary ACC cultures and ACC cell lines

In order to evaluate potential additive or synergistic effects of mitotane and IFN- β on ACC cells, we evaluated the effect of the combination of both compounds on primary ACC cultures. In the primary ACC cultures, an additive inhibition of cell number and cortisol secretion was observed for the combination of IFN- β (100 IU/ml) and mitotane (1 μ M) (Fig. 3A and C). A similar additive effect of both compounds was found in H295 cells (data not shown). In primary ACC, IFN- β , but not mitotane, stimulated DNA fragmentation. The concomitant incubation of mitotane with IFN- β did not significantly enhance the pro-apoptotic activity of IFN- β (Fig. 3B).

When comparing the dose-dependent effect of mitotane on H295, SW13 cells and primary ACC cultures, without or with a sub-maximal concentration of IFN- β (10 IU/ml for H295, 50 IU/ml for SW13, and 100 IU/ml for ACC), we observed a three- to sixfold lower IC₅₀ of the growth inhibitory effect of mitotane (H295: IC₅₀ 3.7 ± 0.4 μ M for mitotane alone vs 0.6 ± 0.2 μ M for mitotane plus IFN- β ; SW13: IC₅₀ 60.0 ± 7.2 μ M for mitotane alone vs 16.4 ± 9.6 μ M for mitotane plus IFN- β ; ACC: IC₅₀ 5.8 ± 0.6 μ M for mitotane alone vs 1.7 ± 0.7 μ M for mitotane plus IFN- β), indicating an increased sensitivity to mitotane by combined treatment with IFN- β .

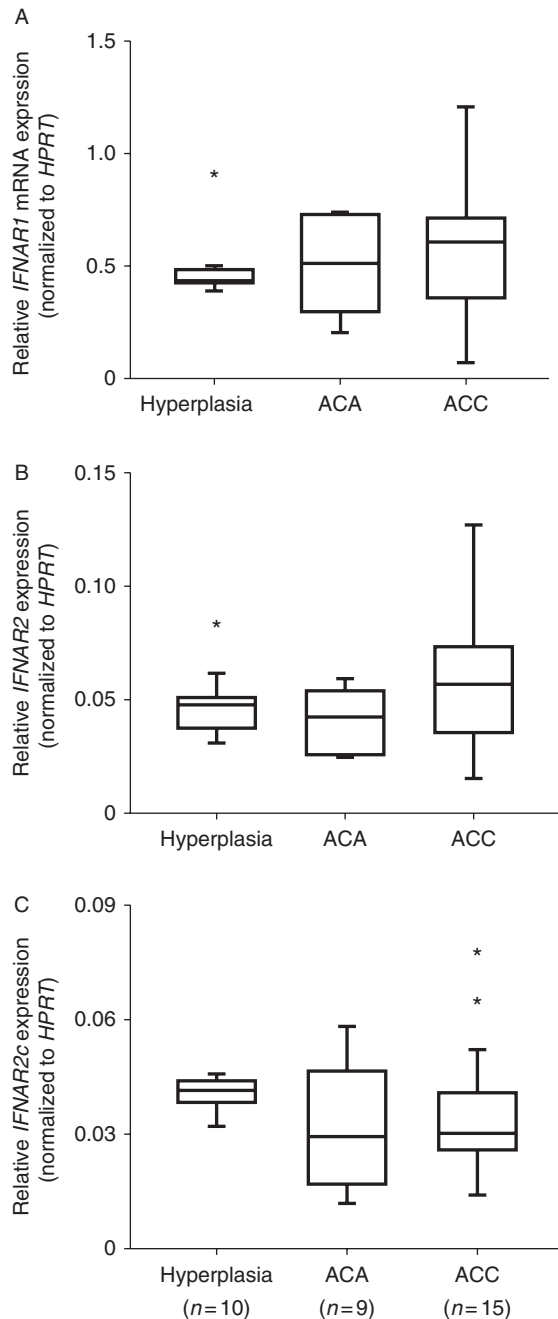
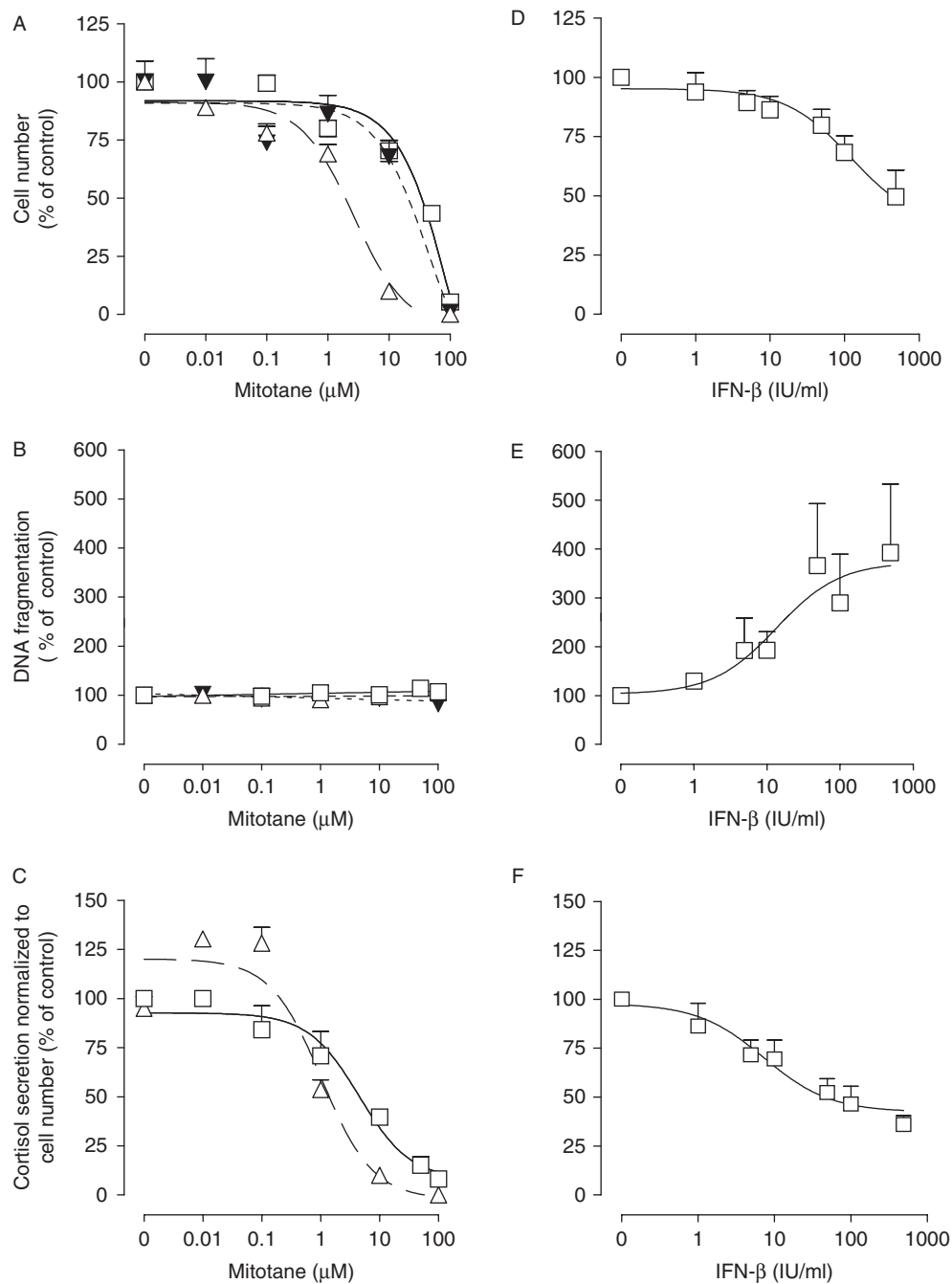


Figure 1 Relative mRNA expression levels of type 1 interferon receptors *IFNAR1* (A), *IFNAR2*-total (B), and *IFNAR2c* (C) in adrenal hyperplasia, adrenocortical adenoma (ACA), and adrenocortical carcinoma (ACC) evaluated by quantitative RT-PCR. The amount of *IFNAR* mRNA was calculated relative to the expression of *HPRT*. *Outliers, defined as cases with values that were between 1.5 and 3 box lengths from the either end of the box.

**Figure 2**

(A, B, and C) Dose-dependent effect of mitotane on H295 cells (open triangle), SW13 cells (closed down triangle), and primary cultures of human ACC cells (open square; $n=7$) on cell number expressed as DNA content after 6 days of incubation (A), on apoptosis, expressed as DNA fragmentation (B), and on cortisol secretion normalized to cell number in H295 and human ACC cells ($n=5$) (C); (D, E, and F) Dose-dependent effect of

IFN- β in primary cultures of human ACC cells on cell number ($n=11$), expressed as DNA content (D), on apoptosis ($n=7$), expressed as DNA fragmentation (E), and on cortisol secretion normalized to cell number ($n=5$) (F) after 6 days of incubation. Data are expressed as percentage of control and represent the mean \pm s.e.m. Control is set as 100%.

The effect of IFN- β and mitotane on steroidogenic enzyme expression in cortisol-producing primary ACC cultures.

In two cortisol-producing ACC and H295 cells, we measured the effects of a 7-day incubation with IFN- β (100 IU/ml) or mitotane (1 μ M) on mRNA expression of the steroidogenic enzymes. The cholesterol transporter (*STAR*) and steroidogenic enzymes *CYP11A1*, *CYP17A1*, and *CYP11B1* were expressed at detectable mRNA levels,

except for *CYP11B1*, which was not detectable in H295 cells. *CYP11B2*, *CYP21A2*, and *HSD3B2* were expressed at levels below the assay detection limit in unstimulated primary ACC and in H295. IFN- β (100 IU/ml), but not mitotane, significantly inhibited mRNA expression of *STAR*, *CYP11A1*, *CYP17A1*, and *CYP11B1* in the primary ACC cultures (Fig. 3D), as well as in H295 cells (except for *CYP11B1*, which was below detection limit in unstimulated H295 cells, data not shown).

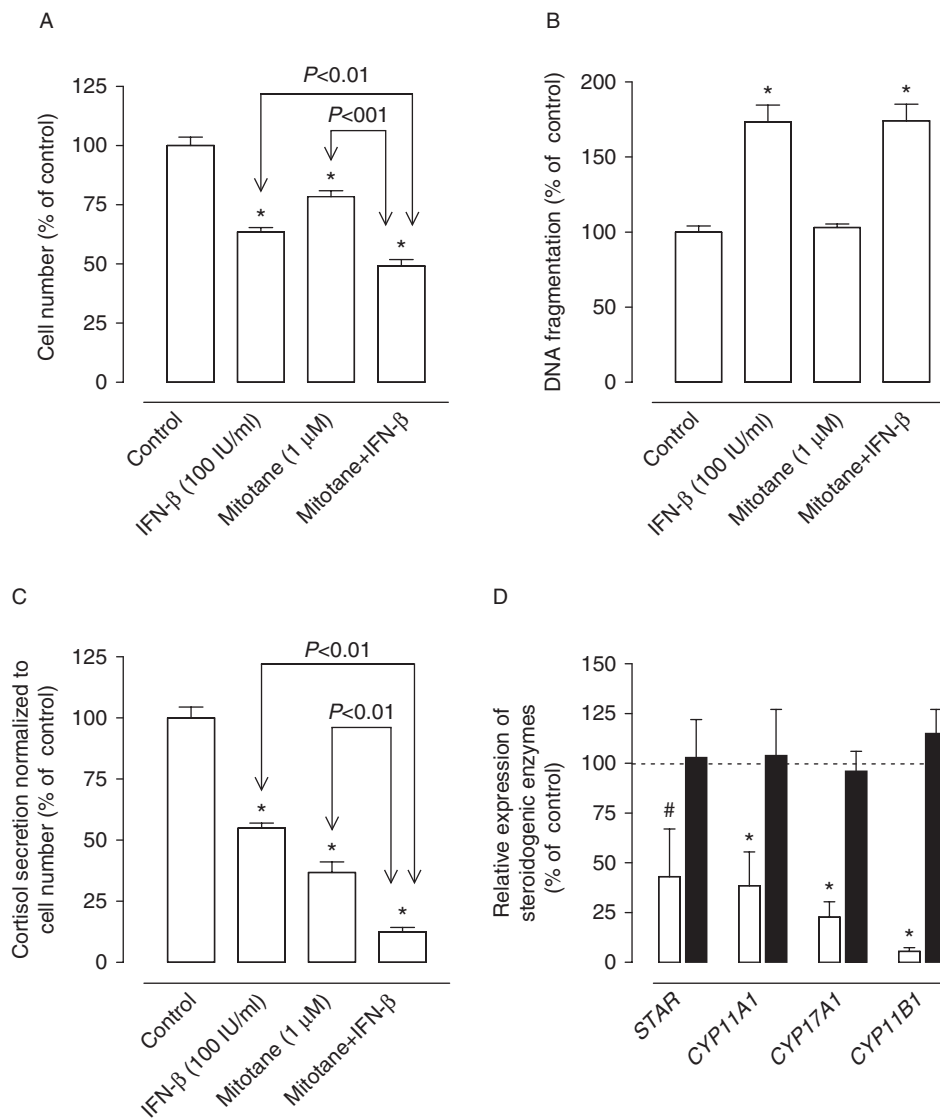


Figure 3

(A, B, C, and D) Effect of IFN- β (100 IU/ml), mitotane (1 μ M), and the combination of both drugs in primary ACC. (A) Effect on cell number ($n=8$), (B) effect on apoptosis, expressed as DNA fragmentation, (C) effect on cortisol production, normalized to cell number, after 6 days of incubation, (D) effect of IFN- β (100 IU/ml; open bars) or mitotane (1 μ M; filled bars) on mRNA expression of *STAR*, *CYP11A1*, *CYP17A1*, and *CYP11B1* in cortisol-

producing ACC ($n=2$). Data are expressed as the percentage of control and represent the mean \pm s.e.m. Control is set as 100%. # $P<0.05$ and * $P<0.01$ vs untreated control. Unstimulated relative expression levels, normalized to *HPR1*, were 0.83 ± 0.22 for *STAR*, 2.29 ± 0.47 for *CYP11A1*, 6.26 ± 0.56 for *CYP17A1*, and 1.15 ± 0.16 for *CYP11B1*.

Effect of combination treatment with mitotane and IFN- β in primary cultures of adrenal hyperplasia and ACA

We did not observe a statistically significant effect on cell number of cells from adrenal hyperplasia and ACA after 6 days of treatment with IFN- β (100 IU/ml) and/or mitotane (1 μ M) (data not shown). DNA fragmentation was only slightly increased in ACA cells when treated with IFN- β (+22%, $P < 0.05$ vs untreated control) or with the combination of mitotane and IFN- β (+23%, $P < 0.05$ vs untreated control). There was no modulation of DNA fragmentation on cells from adrenal hyperplasia (data not shown).

In cells from adrenal hyperplasia and ACA, the cortisol secretion decreased significantly after treatment with IFN- β (–51%, $P < 0.01$, vs untreated control and –61%, $P < 0.05$, vs untreated control respectively) or mitotane (–47%, $P < 0.05$, vs untreated control and –69%, $P < 0.05$, vs untreated control respectively). Treatment with the combination of mitotane and IFN- β induced a strong additive inhibitory effect on cortisol secretion (–80%, $P < 0.01$, vs untreated control for adrenal hyperplasia; –87%, $P < 0.01$, vs untreated control for ACA).

Effects of IGF2, alone and in combination with IFN- β , on primary ACC cultures

Figure 4 shows the effect of incubation with exogenous IGF2 (10 nM) in primary ACC ($n = 5$). IGF2 inhibited DNA fragmentation (–33%, $P < 0.01$ vs untreated control, Fig. 4B), which resulted in an increase in cell number (+30%, $P < 0.01$ vs untreated control, Fig. 4A). There was no statistically significant effect of IGF2 on cortisol secretion, normalized to cell number (Fig. 4C). The effects of IGF2 on cell number and DNA fragmentation were significantly counteracted by 100 IU/ml IFN- β (Fig. 4A and B).

Effect of IFN- β on IGF2 production by primary ACC cultures

We have previously shown that IFN- β induced a strong inhibitory effect on IGF2 mRNA expression in H295 cells. Here, we detected secreted IGF2 protein in medium, collected from days 3–6, from nine primary cultures of human ACC cells, as well as from H295 cells. We found IGF2 concentrations in the media ranging from 0.8–77.6 nmol/l per 100 000 plated cells. IFN- β (100 IU/ml) induced a significant decrease in IGF2 secretion in H295 cells (–39 \pm 7%). The secretion of IGF2 protein, corrected for the effect of IFN- β on cell

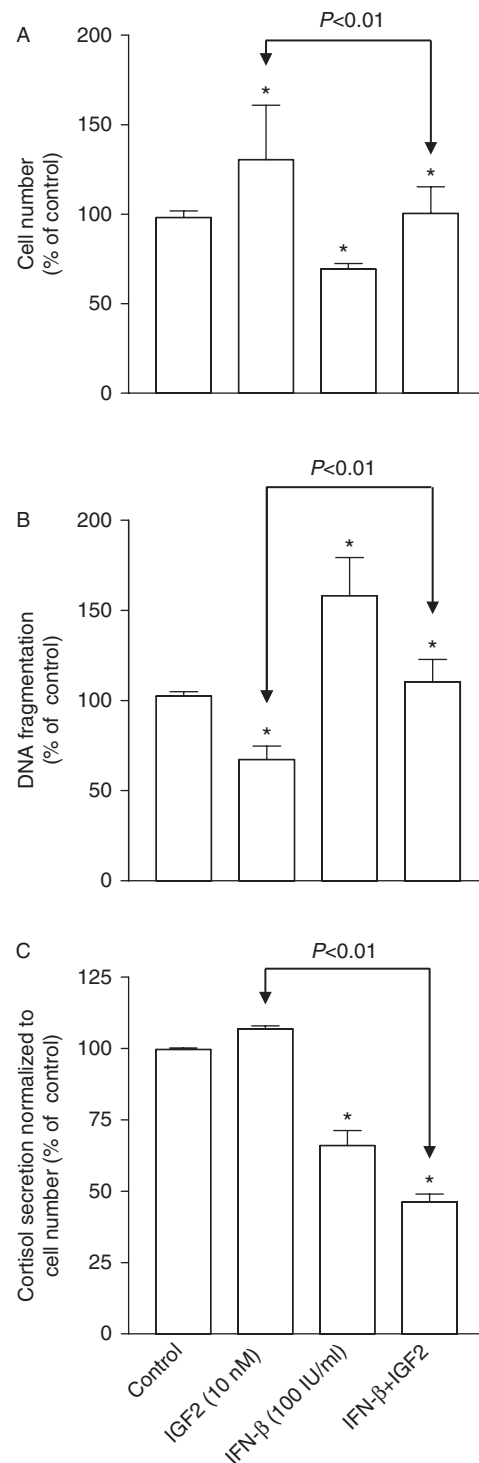


Figure 4

Effect of IGF2 (10 nM), IFN- β (100 IU/ml), and the combination of both compounds on primary cultures of human ACC cells. Effect on cell number ($n = 5$), expressed as DNA content (A), apoptosis ($n = 5$), expressed as DNA fragmentation (B), and on cortisol secretion ($n = 3$; C) after 6 days of incubation. Data are expressed as the percentage of control and represent the mean \pm S.E.M. Control is set as 100%. * $P < 0.01$ vs control.

number, was significantly inhibited by IFN- β (100 IU/ml) in five of nine ACC cultures ($-72 \pm 12\%$; range -64 to -91% ; Fig. 5A). In two additional cases of ACC (nos 18 and 19), we found that IFN- β treatment induced a 50–80% reduction in *IGF2* mRNA expression. Finally, IFN- α and mitotane did not suppress IGF2 protein secretion (data not shown). Moreover, there was no detectable IGF2 protein in media from primary cultures of ACA ($n=3$) and adrenal hyperplasia ($n=3$).

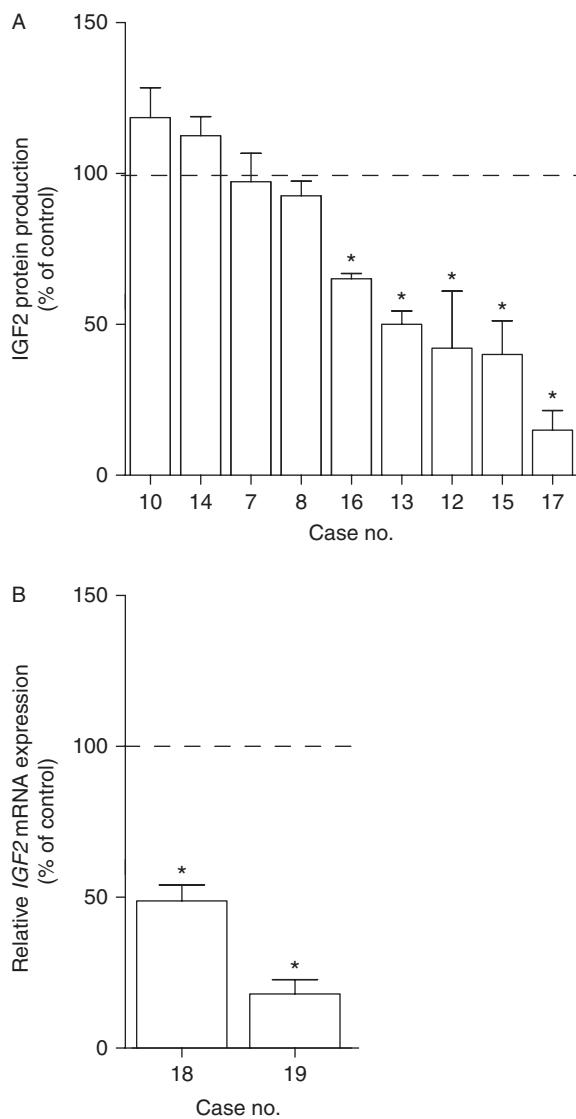


Figure 5
The effect of IFN- β (100 IU/ml) on IGF2 protein secretion (A) and *IGF2* mRNA expression (B) in primary ACC cultures. Values are expressed as the percentage of untreated controls. * $P < 0.01$ vs control.

Discussion

ACC is a rare endocrine malignancy with a very poor prognosis. There is a clear need for more effective diagnostic and therapeutic approaches to this deadly cancer (Allolio & Fassnacht 2006, Fassnacht et al. 2011). *In vitro* and *in vivo* studies have demonstrated the efficacy of type 1 IFNs in the treatment of several types of cancer (Vitale et al. 2007, Bekisz et al. 2010). In most studies, IFN- β has greater antitumor effects than IFN- α . IFN- β is an essential mediator for the host defense system against oncogenesis, interacting with the same type 1 IFN receptors as IFN- α , but with a tenfold higher affinity (Johns et al. 1992).

In this study, we demonstrated the potent antitumor activity of IFN- β in a series of primary cultures of human ACC. In primary ACC cultures, expressing both type 1 IFN receptors, IFN- β inhibited cell number, through the induction of apoptosis, as well as cortisol secretion, in a dose-dependent manner. The inhibition of cortisol secretion was achieved at a significantly lower dose of IFN- β compared with the dose required for inhibition of cell number and appeared the result of an inhibition of mRNA expression of the steroidogenic enzymes *STAR*, *CYP11A1*, *CYP17A1*, and *CYP11B1*. To the best of our knowledge, the inhibition of the expression of steroidogenic enzymes by IFN- β has not been described before. In contrast, mitotane at a dose of 1 μ M (approximately IC_{50} of cortisol inhibition) had no effect on the expression of steroidogenic enzymes. Mitotane, however, is known to inhibit the functional activity of these enzymes (Veysman et al. 2009). In addition, previous studies in H295 cells showed inhibitory effects of mitotane at higher concentrations ($\geq 10 \mu$ M) on the expression of several steroidogenic enzymes (Asp et al. 2010, Lin et al. 2012). These higher mitotane concentrations are generally required for cytotoxic effects as well. IFN- α , at concentrations up to 500 IU/ml, did not significantly modulate cell number and DNA fragmentation in the primary human ACC cells. In primary cultures of adrenal hyperplasia and ACA, we found similar levels of type 1 IFN receptor mRNAs compared with ACC. However, in adrenal hyperplasia and ACA cells, we observed only an inhibition of cortisol secretion similar to that found in ACC cultures, whereas there was no effect on cell number during IFN- β incubation. This suggests that additional pathways are induced in ACC, which mediate the cell growth inhibitory effect of IFN- β . Indeed, genomic studies clearly show a differential expression level of many genes and proteins between ACC and normal adrenal tissues

(Slater *et al.* 2006, Volante *et al.* 2008). *In vivo* studies in humans show that the acute administration of type 1 IFNs induces an activation of the hypothalamic–pituitary–adrenal axis, resulting in marked increases in plasma ACTH and cortisol levels, while this response is attenuated or abolished during long-term treatment (Vitale *et al.* 2009). If our current observations on the inhibitory effects of IFN- β on cortisol production by ACC, as well by cells of adrenal hyperplasia, also apply to normal physiology, it can be hypothesized that the acute stimulatory effects of type IFNs on this axis are overruled after more long-term treatment by the inhibitory effect of IFN- β on cortisol production at the level of the adrenal.

Presently, mitotane is the most frequently used drug for treatment of ACC. However, the toxicity of mitotane may limit the clinical application of this drug. We observed that H295 and SW13 cell lines, as well as seven out of 11 (64%) human primary ACC cultures, responded to treatment with mitotane, as shown by the dose-dependent inhibition of cell number and cortisol secretion. The inhibition of cortisol secretion seems independent of the effect on cell number, as inhibition occurs with an IC₅₀ of 4.5 μ M, whereas cell number inhibition occurs at an IC₅₀ of 38 μ M. The range for an objective tumor response to mitotane in patients is 14–20 mg/l (43–63 μ M) (Hahner & Fassnacht 2005, Hermesen *et al.* 2011). This difference in sensitivity to mitotane between cortisol inhibition on the one hand and cell number inhibition on the other hand may explain the observed decrease in cortisol level without effect on tumor mass in certain patients with ACC (Abiven *et al.* 2006). In H295, SW13, and primary ACC cultures, we did not observe any induction of apoptosis or inhibition of IGF2 protein during incubation with mitotane. Pushkarev *et al.* (2007) showed DNA fragmentation by gel electrophoresis in tissue slices of adrenal tissues, including tumors. As tissue slices were used, containing the tumoral–stromal compartment (fibroblasts, endothelial, and/or immune cells) as well, it is difficult to speculate in which cells DNA fragmentation was induced. Therefore, the precise mechanism of mitotane-induced cell death remains to be established.

Few authors have attempted to use low doses of mitotane in combination with chemotherapeutic agents in order to obtain objective responses and attenuating the severe toxicity of mitotane observed at high doses (Khan *et al.* 2000, Berruti *et al.* 2005). In the presented *in vitro* studies, IFN- β (10–100 IU/ml), in combination with a low concentration of mitotane (1 μ M), showed additive antitumor effects. In fact, in H295 and in primary ACC

cultures, the combination of both drugs induced a significant additive decrease in cell number, as well as cortisol secretion. The additive inhibitory effect of mitotane and IFN- β on cortisol secretion may be explained by a combined effect on both the expression (IFN- β) and the functional activity (mitotane) of steroidogenic enzymes. Moreover, in ACC cells, mitotane even enhances the pro-apoptotic activity of IFN- β , whereas IFN- β lowered the IC₅₀ value of growth inhibition by mitotane by three- to sixfold. Studies *in vivo* and *in vitro* have already demonstrated a synergistic effect of IFN- β and chemotherapeutic drugs in hepatocellular carcinoma and human liver cancer cells (Ogasawara *et al.* 2007, Uyama *et al.* 2007). Whereas treatment with IFN- β and mitotane also induced a strong additive inhibitory effect on cortisol production in primary cultures of adrenal hyperplasia and adrenal adenomas, no effect on cell number was observed, thereby excluding toxicity of the combination treatment. It is worthwhile to observe that the concentration used for mitotane (1 μ M) in the combination treatment with IFN- β in our *in vitro* studies is much lower than the mitotane serum levels associated with frequent side effects (>62 μ M) in patients with ACC. This may provide a rationale for the development of a novel therapeutic regimen in ACC using the current drug of choice mitotane at lower dose, in combination with IFN- β . The clinical use of IFN- β , however, has not yield impressive results so far. This may be related to the relatively low serum concentrations of IFN- β (maximum concentration 22.5 \pm 4.1 IU/ml, after administration of 1.0 million IU/kg) that can be achieved *in vivo*, which is likely due to the very short half-life of IFN- β (Buchwalder *et al.* 2000). Pegylated forms of IFN- β may improve the pharmacokinetic and pharmacodynamic properties of IFN- β (longer half-life and achievable serum concentration of 100 IU/ml IFN- β or higher for 36 h) (Pepinsky *et al.* 2001). When human pegylated IFN- β becomes available, studies in animal models of ACC should prove its efficacy *in vivo*.

Finally, in order to further study the mechanism of action of IFN- β , we also evaluated the effect of IFN- β on IGF2 expression and secretion. IGF2 is considered to be an important autocrine/paracrine growth factor involved in the pathogenesis of ACC (Almeida *et al.* 2008, Volante *et al.* 2008). Indeed, we detected a high concentration of IGF2 in conditioned medium of primary ACC cultures and H295 cells. Incubation of human primary ACC cultures with exogenous IGF2 stimulated cell proliferation and cortisol secretion and inhibited apoptosis (DNA fragmentation). A strong inhibition of IGF2 secretion by IFN- β was observed in five out of nine primary cultures of ACC, due

to a potent inhibition of IGF2 mRNA expression, as was demonstrated in two cultures. Moreover, the cell growth and cortisol stimulatory effects of IGF2 could be counteracted by the incubation of ACC cells with IFN- β . The suppression of IGF2 production could, therefore, represent one of the mechanisms involved in the antitumor activity of IFN- β in ACC.

In conclusion, this study demonstrates for the first time the potential antitumor and cortisol production inhibitory activity of IFN- β in primary ACC cultures. The inhibition of cortisol production is likely mediated via a reduction of the expression of steroidogenic enzymes, pointing to a novel mechanism of action of IFN- β . The combined treatment with IFN- β and a low dose of mitotane induces an additive antitumor activity. One of the mechanisms involved in the antitumor activity of IFN- β is the inhibition of IGF2 production by ACC cells, as well as inhibition of the effects of IGF2. *In vivo* studies are required to further explore the potential clinical application of IFN- β , alone or in combination with mitotane, in ACC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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