The sensitivity of renal cell carcinoma cells to interferon alpha correlates with p53-induction and involves Bax

S. Wittnebel[1], A. Jalil[1], J. Thiery[1], S. DaRocha[1], E. Viey[1], B. Escudier[2], S. Chouaib[1], A. Caignard[1]

[1] INSERM Unité 487, Cytokines et immunologie des tumeurs humaines, Institut Gustave-Roussy, 39 rue Camille-Desmoulins, 94805 Villejuif Cedex
[2] Unité des thérapies innovantes, Institut Gustave-Roussy, 39 rue Camille-Desmoulins, 94805 Villejuif Cedex

Correspondence : A. Caignard
<caignard@igr.fr>

ABSTRACT. Interferon alpha (IFN-α) is an approved treatment in metastatic renal cell carcinoma (RCC). The underlying mechanisms are far from being clear, but are presumed to be a combination of stimulation of cell-mediated cytotoxicity, direct antiproliferative activity and antiangiogenic effects. Recently, the role of p53 in the cellular response to IFN-α has been proposed in other tumor models (hepatoblastoma). We therefore studied the expression of p53 during IFN-α treatment using two freshly established RCC cell lines RCC5 and RCC7. While IFN-α treatment significantly enhanced the expression of p53 in RCC7, no changes were observed in RCC5. Cell viability under IFN-α remained unchanged in both cell lines. Following γ-irradiation, a p53-activating stimulus, an enhanced cell death was observed in IFN-α-treated RCC7 but not in RCC5. We further demonstrate that there were no changes in Bcl-2- and Bax-expression, two target genes regulated by p53. However, intracellular staining revealed that cell death induced by IFN-α and γ-irradiation was preceded by a shift of Bax to the mitochondria in RCC7. Our results suggest a role of p53 and its downstream target Bax, in the control of RCC sensitivity to IFN-α.

Keywords: interferon α, renal cell carcinoma, p53, Bax

Renal cell carcinoma (RCC) is the most common renal tumor. It accounts for 2-3% of all adult malignancies and the incidence is rising. The most important prognostic factor for the clinical outcome is tumor stage, with a 5-year survival from 50-90% for localized disease, decreasing to 0-13% for metastatic disease [1].

Up to now, only surgical resection (mainly by radical nephrectomy) of localized disease (Stage I-II) offers a reasonable chance of curing the disease. Once the tumor has reached the metastatic stage, the treatment options remain limited. Chemotherapeutic drugs or γ-irradiation are not effective in RCC (response rate 2-6%) [2, 3].

At date, the standard treatment for metastatic renal cell carcinoma is based on the administration of interleukin-2 (IL-2) and IFN-α, alone or in combination, with response rates ranging between 10-15% [4, 5]. The mechanisms of action of IL-2 comprise, beside other effects, the stimulation of NK and T-cells, differentiation of lymphokine-activated killer (LAK) cells and maturation of antigen-presenting cells (APC), thus, a general stimulation of the immune system [6, 7]. Although IFN-α has been shown to be active in the treatment of RCC, its mode of action remains poorly understood. However, it has been reported to stimulate cell-mediated cytotoxicity, to exert a direct antiproliferative activity on tumours and to have antiangiogenic effects [8, 9].

Recently it has been shown that IFN-α increased the expression of p53 thereby boosting its responses to a variety of stress stimuli [10, 11]. It is well established that this tumor suppressor gene plays multiple roles in cell cycle control, differentiation, genomic stability, angiogenesis and apoptosis [12]. As a transcription factor, the protein becomes activated in response to DNA damage and to a variety of other stress signals including hypoxia, nucleotide depletion, hyperoxia, and activated oncogenes. It executes its function mainly by transactivating other genes, which are implicated in the control of the cell cycle and/or apoptosis (e.g. Bcl-2, Bax, Bcl-XL, p21WAF1, GADD45, etc.) [12, 13].

Evidence has been provided indicating that tumor cells bearing a mutant p53 are highly resistant to chemo- and radiotherapy and that the sensitivity to these agents could be increased upon restoration of p53-function. Furthermore, recent studies point to the implication of p53 to CTL-mediated cytotoxicitiy [12, 14].

In RCC, in contrast to other malignancies, p53- mutations are rare, with a frequency of 10-30%, but the proapoptotic function of p53 seems to be repressed by a yet unknown mechanisms [15]. In this study, we demonstrate that IFN-α induces p53 expression in a RCC cell line, which could be correlated with an intensification of its death-inducing response to genotoxic stress. Furthermore, we show that cell death is preceded by a shift of the p53 target Bax to the mitochondria. Our results reveal a role of p53 and Bax in the sensitivity of RCC to IFN-α.

MATERIALS AND METHODS

Cells

Tumor cell lines derived from primary RCC were maintained in Dulbecco's modified Eagle's medium/Ham F12 1:1 with Glutamax (Invitrogen, Cergy, France) medium supplemented with 10% fetal bovine serum (FBS) and 1% Ultroser G (Gibco BRL, Scotland).

Cytokine treatment

IFN-α was purchased from Cell Signaling Technology. Cells were treated for the indicated time. Unless otherwise stated, the concentration of IFN-α was 750 U/mL.

Phenotypic analysis

RCC cells were treated with IFN-α (750 U/mL), controls were grown in medium without IFN-α. After 48 hours cells were harvested, washed twice with phosphate-buffered saline (PBS) and then incubated for 20 minutes at 4 °C with the first mAb directed against HLA class I (W6/32, IgG2a), washed twice with PBS followed by incubation with FITC-conjugated goat antimouse immunoglobulin, washed twice with PBS, and fixed before analysis on a FACS-Sort (Becton Dickinson, San Jose, CA, USA). Background levels were measured using isotypic controls. Low forward-scatter elements (dead cells or debris) were excluded from the analysis and 10 000 events were collected and analyzed using the Cellquest software (Becton Dickinson).

Western blot analysis

Total cellular extracts were prepared by lysing cells in ice cold buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1mM PMSF, 10 µg/mL aprotinin and 10 µg/mL leupeptin). Equivalent protein extracts (25-50 µg) were denatured by boiling in sodium dodecyl sulfate (SDS) and β-mercaptoethanol, separated by SDS-PAGE and transferred onto Hybond™ membranes (Amersham, Biosciences). The efficiency of the protein transfer was assessed by Ponceau Red staining of the membranes. Blots were blocked overnight with TBS containing 5% non-fat dried milk, 0.1% Tween and probed with the following Ab: p53 (AB-2, Oncogene, Boston, MA, USA), p21WAF1 (AB-1, Oncogene, Boston, MA, USA), BcL-2 and Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, blots were incubated with appropriate secondary, conjugated Ab-HRPO. The complexes were detected using an ECL™ detection kit (Amersham, Biosciences). Densitometric analysis including correction for background was performed by using the Bioprofil Bio1D Windows application V99-04 software.

Apoptosis assay – propidium iodide (PI) staining

Flow cytometry analysis of PI-stained cells was performed to analyze the effect of IFN-α treatment on cell viability. Renal tumor cells were pretreated with INF-α and/or submitted to γ-irradiation and cultured for an additional 72 h. Cells were then harvested, washed, and fixed in 70% ethanol. They were washed with PBS and stained with 1 mL of PI (20 µg/mL) containing 100 µg/mL RNase and 20 mM EDTA. DNA content was determined using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and the proportion of cells in a particular phase of the cell cycle was determined by CellQuest software (Becton Dickinson, San Jose, CA, USA). Induced apoptotic cell death was determined by measuring the proportion of subG_1 cells.

Confocal scanning immunofluorescence microcopy

Renal tumor cells were grown on sterile coverslips and subjected to treatments as described above. Coverslips were then washed once with PBS and fixed for 30 min in 4% paraformaldehyde (PFA) solution in PBS, and washed 3 times with PBS. After 5 min incubation with methanol, slides were washed 3 times with PBS, and cell membranes were permabilized for 10 min with 0,1% SDS in PBS and washed 3 times with PBS. Non-specific sites were blocked for 20 min with 10% FBS in PBS and washed once with PBS. The cells were incubated for 1 hour with anti-Bax polyclonal rabbit antiserum (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-cytochrome-c mouse monoclonal antibody (Becton Dickinson, San Jose, CA, USA). After three washes with PBS, coverslips were incubated with Alexa 546 conjugated antimouse antibody (Molecular Probes, Eugene, OR, USA) and Alexa 488 conjugated anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) for 1 hour. After washing with PBS, nuclei were stained using To-Pro®-3 iodide (Molecular Probes, Eugene, OR, USA). The coverslips were mounted with anti-fading Vectashield from (Vector, Burlingame, CA, USA). Confocal microscopy analysis was performed on a Zeiss LSM 510 microscope.

RESULTS

HLA class I induction in renal cell carcinoma cell lines in response to IFN-α

We selected two of our RCC-cell lines (RCC5 and RCC7) on the basis of their capacity to express functional p53 as assessed by their capacity to induce p21 after γ-irradiation (data not shown). In order to examine the global responsiveness of these cell lines to IFN-α, we measured the HLA-class I-induction following treatment with this cytokine. The cell lines were treated for 48 hours with IFN-α (750 U/mL) and then submitted to FACS-analysis for HLA-I expression. As shown in *figure 1*, a significant increase in HLA-I expression after exposure to IFN-α was detectable in both cell lines in a comparable manner.

Differential p53 expression in RCC during IFN-α treatment

On the basis of their global responsiveness to the IFN-α treatment, we then explored the influence of IFN-α on p53-expression. The cells were treated with IFN-α (750 U/mL) and the expression of p53 was probed by westernblot at different time points. As demonstrated in *figure 2*, a notable increase in p53 was detected in RCC7, reaching a maximum after 8 hours, whereas no changes were detected in RCC5.

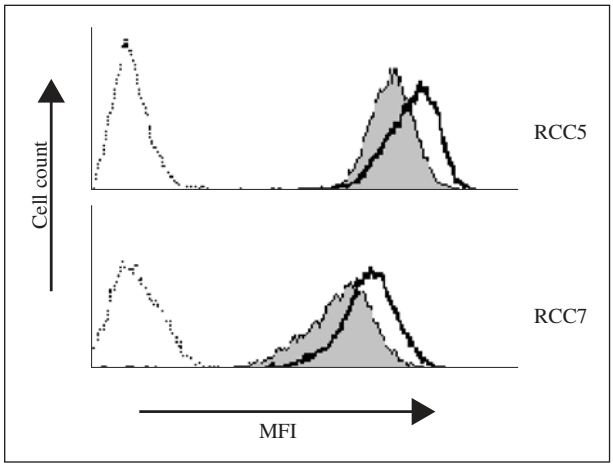

Figure 1
HLA class-I induction following IFN-α treatment in RCC.
The renal cell carcinoma cell lines RCC5 and RCC7 were treated with IFN-α (750 U/mL). Control cells were grown in medium without IFN-α. After 48 hours, cells were harvested and labeled with anti-HLA-I (W6/32). Basal HLA-I expression on control cells is shown as solid profiles, HLA-I expression after IFN-α treatment is shown as an open profile. Isotypic controls are depicted by a thin open profile. MFI indicates mean fluorescence intensity in a logarithmic scale.

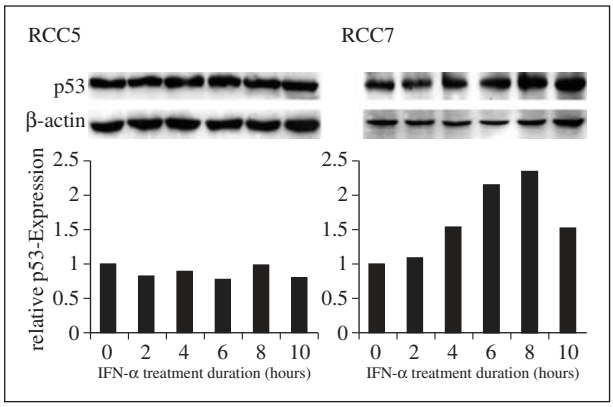

Figure 2
INF-α increases p53-expression in RCC7.
p53 expression following the time course of IFN-α-treatment of RCC cell lines. RCC5 and RCC7 were treated with INF-α (750 U/mL) and p53-expression was probed by Western blot at the indicated time points. Top: Western blot. Bottom: quantitative display of the induction levels of p53 protein. Specific band intensities were measured using a densitometer and p53 induction was calculated as p53/β-actin ratio.

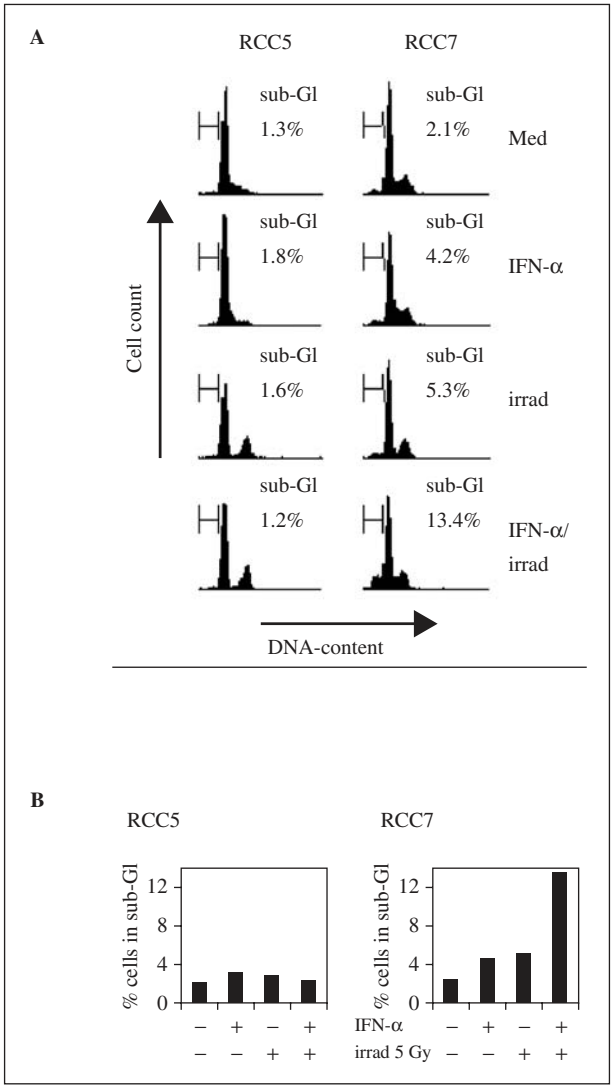

Figure 3
INF-α enhances γ-irradiation-induced cell death in RCC7.
Effect of IFN-α treatment on the sensitivity to γ-irradiation of RCC5 and RCC7. Cells were pretreated with INF-α (750 U/mL) and γ-irradiated (5Gy). Following additional incubation for 72h, floating and adherent cells were harvested and stained for cell-cycle analysis using PI staining. The percentage of dead cells was determined by the percentages of cells in sub-G1. **A**) cell cycle analysis. **B**) display of cells in sub-G1.

IFN-α-induced p53 expression correlates with increased sensitivity to genotoxic stress

One major role of p53 besides regulation of the cell cycle is the induction of cell death after genotoxic stress stimuli such as γ-irradiation. To investigate, whether the increase in p53-protein level in RCC7 resulted in a greater susceptibility to γ-irradiation, cells were treated with IFN-α (750 U/mL) before γ-irradiation. After an additional 72h, the cells were probed for cell death by FACS, using PI-staining. As outlined in *figure 3*, IFN-α increases the sensitivity of RCC7 to γ-irradiation (5Gy), a dose, which by itself does not induce cell death in RCC7. In contrast, no increase in cell death is observed in RCC5, in which IFN-α-induced p53 expression is not observed.

Expression of Bax and Bcl-2 is not affected by IFN-α-induced p53 expression

It is thought that p53 influences the balance between the pro- and anti-apoptotic members of the Bcl-2 family by means of transcriptional control. Furthermore, evidence has been provided indicating that the ratio of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 plays a crucial role in the control of the intrinsic pathway of apoptosis. We therefore reasoned that the enforced cell death in RCC7 treated with IFN-α and γ-irradiation might be due to changes in this ratio. The expression of Bcl-2 and Bax was probed by Western blot in cells treated with IFN-α and/or γ-irradiation. As shown in *figure 4*, no change was observed in the Bax/Bcl-2 ratio in either of the cell lines.

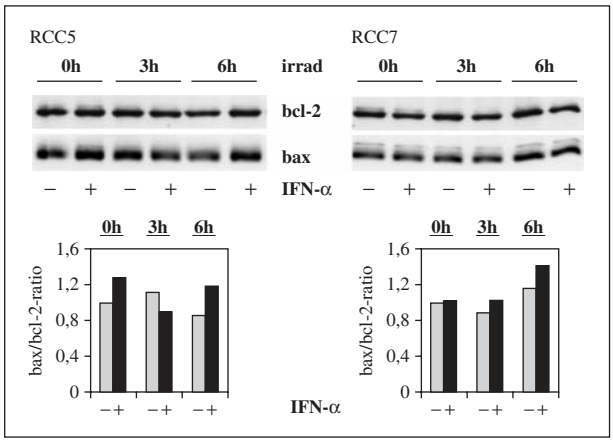

Figure 4

Bax/Bcl-2 ratio in RCC5 and RCC7.
Expression of Bcl-2 and Bax in RCC5 and RCC7 after γ-irradiation (5Gy) alone or in combination with IFN-α (750 U/mL). Top: Western blot. Bottom: display of the Bax/Bcl-2 ratio. The quantities of both proteins were determined at the indicated time points using a densitometer.

Enforced Bax-shift to the mitochondria following IFN-α treatment and γ-irradiation

Recently it has been shown that p53, besides its action as transcription factor, can activate Bax through a mechanism that is transcription-independent. To evaluate, whether treatment with IFN-α could enhance the translocation of Bax to the mitochondria, we performed confocal microscopy with intracellular staining of Bax and cytochrome c. As shown in *figure 5*, such pretreatment enhances Bax translocation to the mitochondria after γ-irradiation in RCC7, but not in RCC5. Interestingly, in RCC5, Bax co-localized with the mitochondria even in non-treated cells, without an obvious release of cytochrome c.

DISCUSSION

IFN-α is used in the treatment of metastatic renal cell carcinoma [16]. However, the way in which tumor cell growth is suppressed by IFN-α is not well understood [17]. Recently it has been reported that IFN-α may execute its anti-neoplastic action at least in part through induction of the tumor suppressor protein p53, thereby boosting the cellular response to a variety of stress stimuli activating p53, including γ-irradiation and chemotherapeutic drugs [10, 11].

The present study was performed to explore the impact of the tumor suppressor protein p53 on the action of IFN-α in renal cell carcinoma using two, well characterized RCC cell lines (RCC5 and RCC7). Although both cell lines express a functional p53, we have shown that IFN-α induced p53 expression only in RCC7, whereas the p53-level in RCC5 remained unaffected. The mechanisms underlying the deficit in the p53 induction in RCC5 are not clear. It has been reported that resistance of renal cell carcinoma to IFN-α treatment may be due to defects in the signal transduction, mainly through defective induction of Stat1 [18]. However, the interferon signaling, as assessed by the induction of HLA-class I upon treatment with IFN-α is not disturbed in our cell lines. Furthermore, the overexpression of the multidrug resistance (MDR) gene and the MDR gene product, P-glycoprotein (Pgp) frequently observed in RCC, has been associated with resistance of these tumors to IFN-α [19]. Whether this or other mechanisms account for the deficit in p53 induction of RCC5 needs further investigation.

The treatment of our RCC cell lines with IFN-α did not affect cell viability by itself but it potentiated cell death following γ-irradiation, a stimulus that typically induces p53-regulated responses such as cell cycle arrest and/or apoptosis [12], in RCC7 but not in RCC5. Up to now, the mode of action of IFN-α in RCC has been poorly understood, but is presumed to be a combination of the stimula-

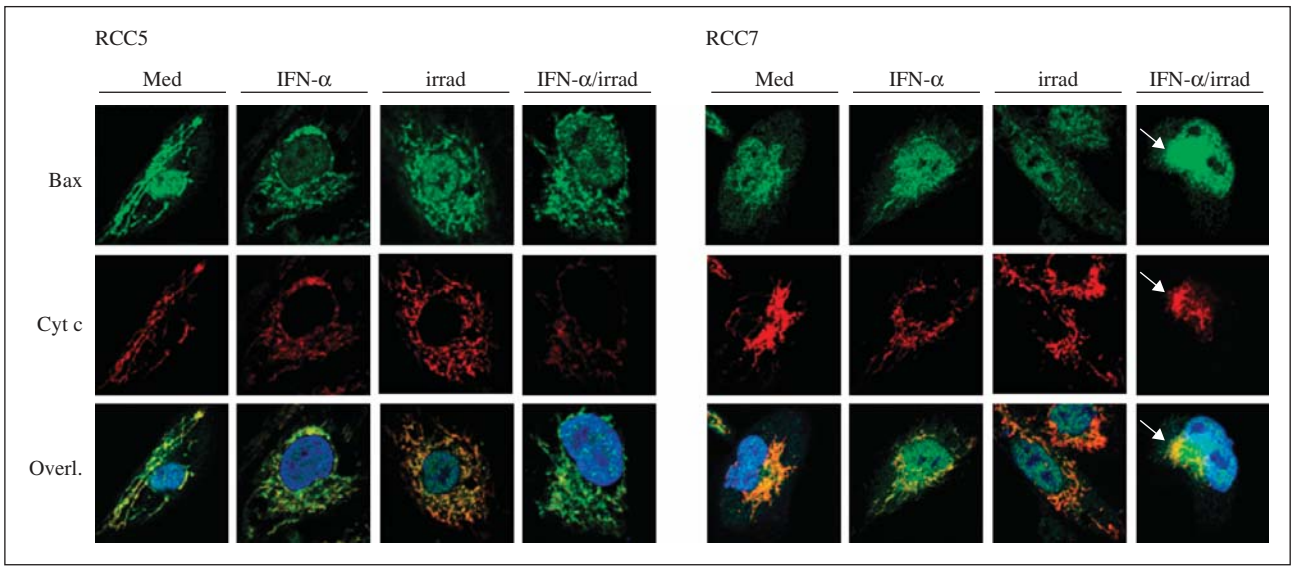

Figure 5

Determination of sub-cellular Bax-location.
Intracellular staining for Bax and cytochrome c in RCC5 and RCC7 after γ-irradiation (5Gy) alone or in combination with IFN-α (750 U/mL). Immunostaining was performed with anti-Bax mAb (green), anti-cytochrome c mAb (red) and TO-PRO-3 for the nucleus (blue). Note the enforced shift of Bax to the mitochondria, and an initial release of cytochrome c in RCC7 following pretreatment with IFN-α and γ-irradiation.

tion of cell-mediated cytotoxicity, a direct antiproliferative activity, and antiangiogenic effects [9]. Our findings indicate that p53 may be implicated in the effects of IFN-α in the treatment of RCC taking into account the crucial role of p53 in controlling cell growth, apoptosis and angiogenesis [12]. Furthermore it has been demonstrated in other tumor models that the p53 function is important with respect to tissue target susceptibility to specific effector killer cells [14]. Although it has been hypothesized that the p53-pathway is repressed in RCC by a factor that remains to be defined [15], there is evidence that the activation of this pathway can sensitize RCC to additional treatments such as anti-Fas or anti-TRAIL Ab [20, 21].

It is well established that p53 engages the so-called "intrinsic", mitochondrial apoptotic pathway, mainly by regulating the transcription of the pro- and anti-apoptotic proteins of the Bcl-2 family such as Bcl-2 and Bax [13, 22]. This results in the permeabilizing of the mitochondrial membrane and subsequent release of apoptogenic factors including SMAC/DIABOLO, HtrA2/Omi, AIF and cytochrome c, which leads to effector caspase activation [13, 22].

In our experimental system, despite the increased cell death, we were not able to demonstrate changes in the expression of Bcl-2 or Bax after combination of IFN-α and γ-irradiation in RCC7. This may be explained by the rather short period of observation, as up-regulation of Bax 24 hours after p53 induction in RCC has been reported [20]. On the other hand, it has been shown, that p53 can induce cell death through the mitochondrial pathway in the absence of transcription [23, 24]. In fact, the intracellular staining revealed a pronounced shift of Bax to the mitochondria after combination of IFN-α with γ-irradiation in RCC7. Interestingly, in RCC5, Bax co-localized with the mitochondria regardless of the treatment, without obvious signs of ongoing apoptosis. We therefore hypothesise, that the dynamics in the localization of the pro-apoptotic protein Bax may be important in the response to genotoxic stress stimuli and that the enforced expression of p53 may be implicated in this process even though the exact mechanistic connection requires further investigation.

Taken together, our results indicate a role for p53 in the control of renal cell carcinoma sensitivity to IFN-α by mechanisms implicating Bax translocation and mitochondrial apoptosis pathway activation. Whether and how this can contribute to new treatment strategies needs further experimental studies.

Acknowledgements. *This work was supported by a grant from the ARC to AC (grant 4613) and to SC (grant 4744). SW was supported by a fellowship from the Dr Mildred Scheel Stiftung.*

REFERENCES

1. Martel CL, Lara PN. Renal cell carcinoma: current status and future directions. *Crit Rev Oncol Hematol* 2003; 45: 177.
2. Amato RJ. Chemotherapy for renal cell carcinoma. *Semin Oncol* 2000; 27: 177.
3. George CM, Stadler WM. The role of systemic chemotherapy in the treatment of kidney cancer. *Cancer Treat Res* 2003; 116: 173.
4. Bleumer I, Oosterwijk E, De Mulder P, Mulders PF. Immunotherapy for renal cell carcinoma. *Eur Urol* 2003; 44: 65.
5. Bukowski RM. Cytokine therapy for metastatic renal cell carcinoma. *Semin Urol Oncol* 2001; 19: 148.
6. Fishman M, Seigne J. Immunotherapy of metastatic renal cell cancer. *Cancer Control* 2002; 9: 293.
7. Motzer RJ, Russo P. Systemic therapy for renal cell carcinoma. *J Urol* 2000; 163: 408.
8. Sen GC, Lengyel P. The interferon system. A bird's eye view of its biochemistry. *J Biol Chem* 1992; 267: 5017.
9. Nathan PD, Eisen TG. The biological treatment of renal-cell carcinoma and melanoma. *Lancet Oncol* 2002; 3: 89.
10. Takaoka A, Hayakawa S, Yanai H, *et al.* Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature* 2003; 424: 516.
11. Vilcek J. Boosting p53 with interferon and viruses. *Nat Immunol* 2003; 4: 825.
12. May P, May E. Twenty years of p53 research: structural and functional aspects of the p53 protein. *Oncogene* 1999; 18: 7621.
13. Shen Y, White E. p53-dependent apoptosis pathways. *Adv Cancer Res* 2001; 82: 55.
14. Thiery J, Dorothee G, Haddada H, *et al.* Potentiation of a tumor cell susceptibility to autologous CTL killing by restoration of wild-type p53 function. *J Immunol* 2003; 170: 5919.
15. Gurova KV, Hill JE, Razorenova OV, Chumakov PM, Gudkov AV. p53 pathway in renal cell carcinoma is repressed by a dominant mechanism. *Cancer Res* 2004; 64: 1951.
16. Negrier S, Escudier B, Gomez F, *et al.* Prognostic factors of survival and rapid progression in 782 patients with metastatic renal carcinomas treated by cytokines: a report from the Groupe Francais d'Immunotherapie. *Ann Oncol* 2002; 13: 1460.
17. Caraglia M, Marra M, Pelaia G, *et al.* Alpha-interferon and its effects on signal transduction pathways. *J Cell Physiol* 2004: 9.
18. Brinckmann A, Axer S, Jakschies D, *et al.* Interferon-alpha resistance in renal carcinoma cells is associated with defective induction of signal transducer and activator of transcription 1, which can be restored by a supernatant of phorbol 12-myristate 13-acetate stimulated peripheral blood mononuclear cells. *Br J Cancer* 2002; 86: 449.
19. Frank MH, Pomer S. Interferon alpha2b differentially affects proliferation of two human renal cell carcinoma cell lines differing in the P-glycoprotein-associated multidrug-resistant phenotype. *J Cancer Res Clin Oncol* 1999; 125: 117.
20. Mizutani Y, Nakanishi H, Yoshida O, Fukushima M, Bonavida B, Miki T. Potentiation of the sensitivity of renal cell carcinoma cells to TRAIL-mediated apoptosis by subtoxic concentrations of 5-fluorouracil. *Eur J Cancer* 2002; 38: 167.
21. Wu XX, Mizutani Y, Kakehi Y, Yoshida O, Ogawa O. Enhancement of Fas-mediated apoptosis in renal cell carcinoma cells by adriamycin. *Cancer Res* 2000; 60: 2912.
22. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004; 305: 626.
23. Chipuk JE, Kuwana T, Bouchier-Hayes L, *et al.* Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 2004; 303: 1010.
24. Erster S, Mihara M, Kim RH, Petrenko O, Moll UM. *In vivo* mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol Cell Biol* 2004; 24: 6728.