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Interferon-γ and interleukin-6 inhibit proliferation in human melanoma cells by different signalling pathways

V. Fontaine, M. Mahieu and J. Content*
Laboratoire de Virologie, Institut Pasteur, 642 rue Engeland, 1180 Bruxelles, Belgium. Fax: (+32) 2 3733174; Email: jcontent@ben.vub.ac.be

Interferon regulatory factor-1 (IRF-1) is a cell growth inhibitor, induced by cytokines, which transactivates downstream effector genes. The role of IRF-1 in the antiproliferative effect of interleukin-6 (IL-6) was investigated using the A375 human melanoma cell line. IL-6 is a stronger inhibitor of A375 proliferation compared with interferon-γ (IFNγ). However, in contrast to IFNγ, IL-6 triggered lower IRF-1 DNA binding activity and induced barely detectable IRF-1-dependent transactivation activity. Furthermore, although IFNγ induces only activation of signal transducer and activator of transcription (STAT) 1, IL-6 activates mainly STAT3. These data suggest that IRF-1 plays a minor role in the antiproliferative effect of IL-6, which uses alternative signalling events to induce growth inhibition in A375 melanoma cells. © 1998 Rapid Science Ltd

Key words: interferon-γ, interferon regulatory factor-1, interleukin-6, melanoma cells

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine that exerts a wide range of biological activities on a large variety of cells. Depending on the cell type, IL-6 can trigger proliferation, growth arrest or differentiation.[1] Melanocytes and early-stage melanoma cells are frequently growth inhibited by IL-6, whereas cell lines established from advanced melanomas are often resistant to this inhibition or are even growth stimulated under the same conditions.[2] In addition, during tumour progression melanoma cells tend to acquire resistance to other antiproliferative factors, including oncostatin M (OSM), IL-1, transforming growth factor-β (TGFβ) and tumour necrosis factor (TNF).[2] Constitutive IL-6 production, downregulation of the IL-6 receptor and inhibition of unknown post-receptor signal transduction pathways have been associated with IL-6 resistance in melanoma cells.[2–5] In order to gain insight into the biochemical changes leading to such resistance, it is essential to clarify the molecular events associated with the antiproliferative effect of IL-6 on melanoma cells.

Interferon regulatory factor 1 (IRF-1) is a transcriptional activator which has been identified as part of the signal transduction of interferon; IRF-2, which recognizes the same DNA sequences, acts as its antagonist. The expression of IRF-1 in fibroblasts suggests that IRF-1 mediates cell growth inhibition by transactivation of downstream genes.[6] The IRF-2:IRF-1 ratio seems to be critical in the regulation of cell growth. In growing cells, IRF-2 is more abundant than IRF-1. However, after stimulation by virus, interferon (IFN) or other cytokines (such as IL-1, IL-2, IL-6 or TNF), the amount of IRF-1 increases relative to IRF-2 and may consequently lead to an antiproliferative response.[7] Increased IRF-1 synthesis and DNA-binding activity have already been observed during the growth arrest induced by IL-6 in M1 myeloleukaemic cells (preceding terminal differentiation) and in T47D breast carcinoma cells.[8–10] However, to date, it is unclear whether IRF-1 plays a general role in the antiproliferative effect of IL-6.

Here we report that IL-6 induces the formation of IRF-1 DNA complexes in A375 human melanoma cells that are growth inhibited by IL-6. However, in view of the weak IRF-1 transcriptional activity induced by IL-6 in A375 cells, IRF-1 is unlikely to be essential in the IL-6-induced growth inhibition of these cells.

Materials and methods

Cell culture

A375 cells, kindly provided by Carl Figdor (Dutch Cancer Institute), were cultured in Iscove's Modified Dulbecco's medium supplemented with 5% fetal calf serum (FCS).

*To whom correspondence should be addressed

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Cytokines

Purified recombinant human IL-6 (5×10^8 IU/mg) obtained from *Escherichia coli*[11] or from CHO cells (InterPharm Laboratories, IPL, Nes-Ziona, Israel) were used. Recombinant IFNγ (10^8 IU/mg) was obtained from InterPharm Laboratories or from Boehringer Mannheim.

Growth inhibition assay

Cells were seeded at a density of 2×10^3 cells/microwell in a final volume of 0.2 ml. Cultures were incubated at 37°C in 5% CO_2 in the presence of serial dilutions of cytokines. After 5–6 days, the viable cells were stained with 20% crystal violet. The dye was eluted with 0.1 M sodium citrate and 50% ethanol, and absorbance was measured at 540 nm.

DNA electrophoretic mobility shift assays (EMSAs)

The C13, palindromic IFN response element (pIRE)/α_2-macroglobulin and pIRE/IRF-1 probes and the nuclear extracts were prepared and used as previously described.[8,9,12] Binding reactions were performed for 20 min at 25°C, and the DNA–protein complexes were separated by electrophoresis in 5% acrylamide/bisacrylamide gels in 25 mM Tris-borate pH 8.2, 0.5 mM EDTA for 2–3 h at 175 V. The gels were then dried and subjected to autoradiography. When used, antibodies were added as 1 µl of immune sera to the extracted proteins in 5 µl with 3-fold concentrated salt and buffer solution for 30 min at 0°C before adding the labelled probe, together with 1 µg salmon sperm DNA and 2.5 µg poly(dI-dC) in a final volume of 20 µl.[9] The monoclonal antibodies anti-STAT1α/β was purchased from Transduction Laboratories (Lexington). The STAT3 rabbit polyclonal antibodies (K15) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Transfection and luciferase assay

A375 cells were transfected with 10 µg of p[(AAGTGA)$_4$]$_5$tkΔ(-39) Lucter plasmid,[13] kindly provided by Dr S. Goodburn (St George's Hospital Medical School, London), using calcium phosphate coprecipitation. At 8–12 h after transfection cells were trypsinized, pooled and seeded in triplicate in six-well plates. Cells were treated with cytokines (in triplicate) and assayed after 48 h with Promega's extraction (1% Triton X-100; 0.25 ml/well) and luciferase assay kit. The relative luciferase activity was obtained after correcting the luciferase values to cell viability to normalize for cell number.

Results

The growth of A375 cells is inhibited by various cytokines including IL-1, TNF, IL-6 and OSM.[14,15] The effect of human IL-6 and IFNγ on the proliferation of the A375 melanoma cell line was examined (Figure 1). As reported previously, IFNγ inhibited the growth of A375 cells.[16] However, compared with the effect of IL-6, the antiproliferative effect of IFNγ was moderate (with a maximum inhibition of 60% at a concentration of 420 ng/ml). In contrast, IL-6 markedly inhibited the growth of A375 cells, with a maximal inhibition of 80% at a concentration of 1 ng/ml.

IRF-1 and IRF-2 DNA binding activity induced by IL-6 and IFNγ in A375 cells was determined with an EMSA using a ^{32}P-labelled multimeric $(AAGTGA)_3$ oligonucleotide (C13) that comprises a strong binding site for proteins of the IRF family.[17–19] In nuclear extracts of A375 cells treated for 75 min with IFNγ

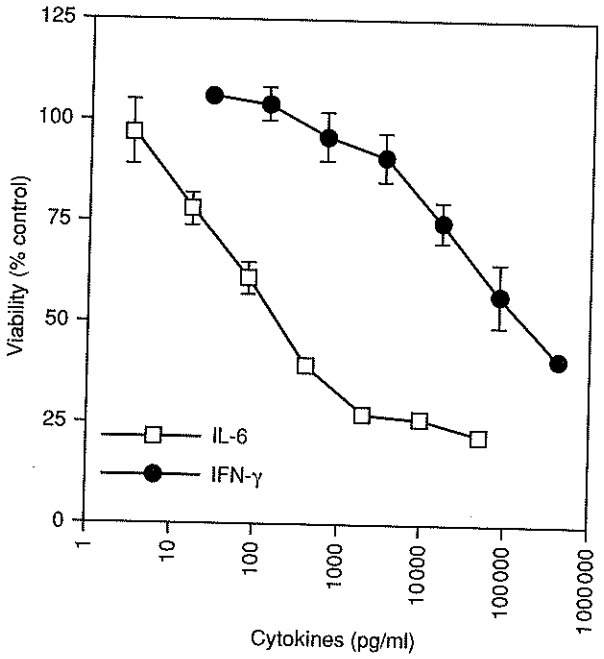

Figure 1. Effects of IL-6 and IFNγ on the proliferation of A375 cells. The growth inhibition assays were performed as described in Materials and methods. Data are expressed as the mean (± SE) of triplicate determinations from one representative experiment. Untreated cells were used as controls (100%).

or IL-6 there was an increased formation of DNA–protein complexes (Figure 2). To distinguish between IRF-1- and IRF-2-containing complexes, the nuclear extracts were reacted prior to the EMSA with antisera raised against the IRF-2 or IRF-1 proteins, respectively. Positive controls demonstrated the specific reactivity of the anti-IRF antibodies. IRF-1 DNA binding activity was only detected in the presence of the cytokines. IFNγ was clearly a stronger activator of IRF-1 DNA binding activity than IL-6. In contrast, IRF-2 DNA binding activity was constitutive and was not significantly affected by either cytokine.

To evaluate the effect of increased IRF-1 DNA binding activity on transcription, we transfected the A375 cell line with a reporter plasmid, p[(AAGTGA)$_4$]$_5$tkΔ(-39) Lucter, which contains a luciferase gene under the control of a synthetic promoter

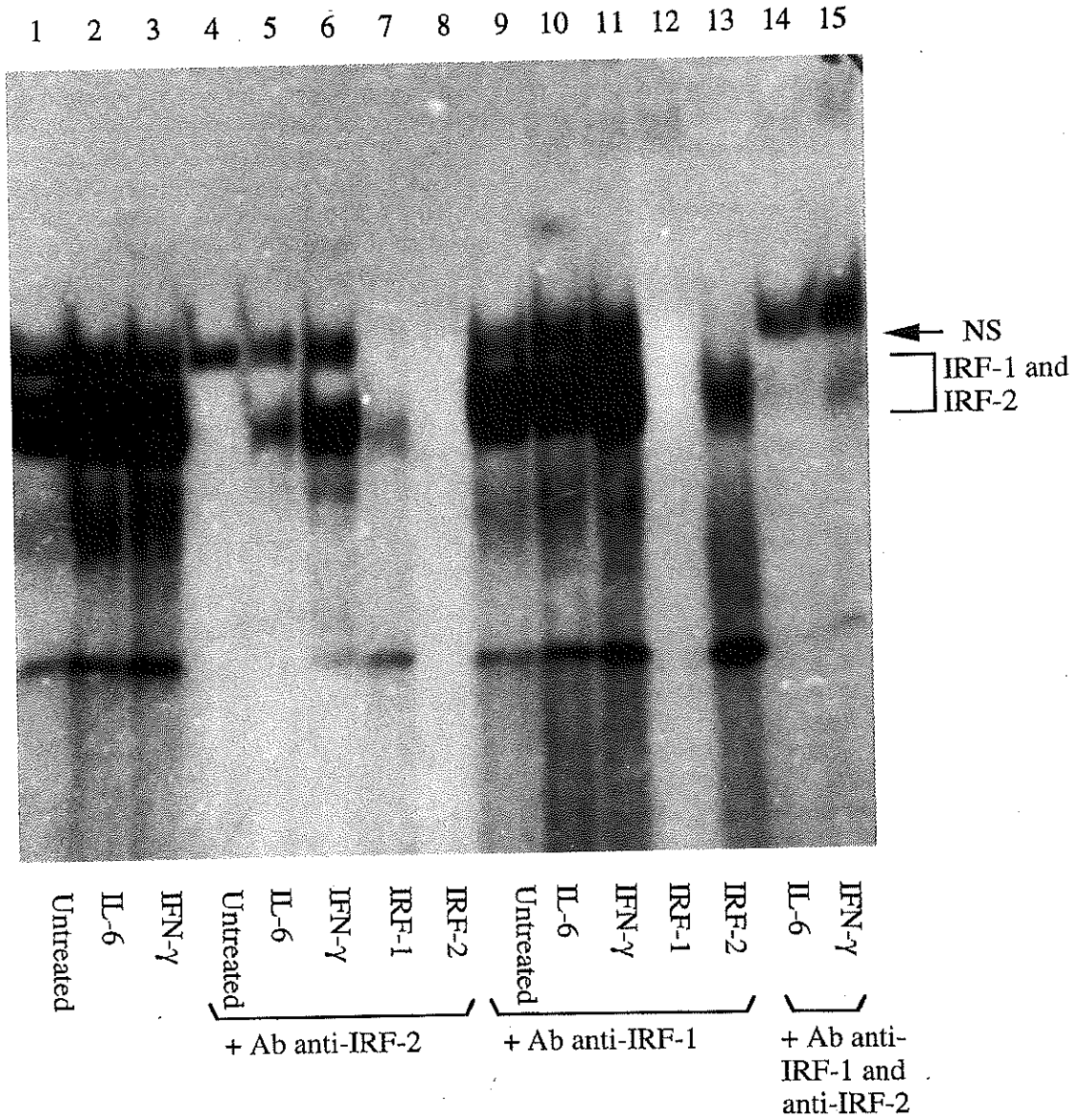

Figure 2. DNA binding activities of IRF-1 and IRF-2 in A375 cells. Nuclear extracts from untreated A375 cells (lanes 1, 4 and 9) and those treated for 75 min with 20 ng/ml IL-6 (lanes 2, 5, 10 and 14) or 3000 U/ml IFNγ (lanes 3, 6, 11 and 15) were assayed in an EMSA with the C13 (IRF family binding site) probe. In order to visualize IRF-1, IRF-2 or other proteins binding to the C13 probe separately, antibodies anti-IRF-2 (lanes 4–8), anti-IRF-1 (lanes 9–13) or both (lanes 14 and 15) were incubated with the extracts. IRF-1 (lanes 6 and 12) and IRF-2 (lanes 7 and 13) cDNA translation products in reticulocyte lysates (1 μl per lane) were assayed in an EMSA with the C13 probe as controls. NS = non-specific complexes.

consisting of the weakly acting herpes simplex virus thymidine kinase TATA box and multimers of the AAGTGA sequence that comprises a strong binding site for IRF-1 and IRF-2.[13] In the absence of cytokines a very low luciferase activity was detected in the transfected cells. Consistent with the IRF-1 DNA–protein complexes formation induced by IFNγ and IL-6 in the A375 cells, we measured a 45-fold induction of luciferase activity by IFNγ and a very weak induction (1.8-fold) when transfected cells were treated with IL-6 (Figure 3).

The interaction between cytokines and their cognate receptors activates tyrosine kinases of the JAK family which in turn trigger tyrosine phosphorylation of signal transducer and activator of transcription (STAT) proteins. Subsequent to tyrosine phosphorylation, STAT proteins dimerize and translocate to the nucleus where they bind specific DNA sequences.[20] Therefore, we addressed the question of whether the same STAT would be activated by both IL-6 and IFNγ in A375 cells. Nuclear extracts from untreated and IL-6- and IFNγ-stimulated A375 melanoma cells were analysed in EMSAs, using two ^{32}P-labelled oligonucleotides, pIRE/α_2-macroglobulin and pIRE/IRF-1, which contain the distinct palindromic interferon-regulated elements of either the α_2-macroglobulin or IRF-1 genes, respectively. Although pIRE/IRF-1 comprises a binding site for STAT1 but not for STAT3, the pIRE/α_2-macroglobulin is more widely recognized by STAT1, STAT3 and STAT5.[12,21] As shown in Figure 4, stimulation of A375 cells with IFNγ resulted in the formation of a single DNA–protein complex, binding equally well to both radiolabelled probes. Supershift assays suggested that this complex contained STAT1 homodimers. In contrast, three complexes were visible after IL-6 stimulation. Supershift assays suggested that the lower retarded band consisted of STAT1 homodimers and the upper band consisted of STAT3 homodimers. However, the STAT3 homodimeric complexes were the most abundant.

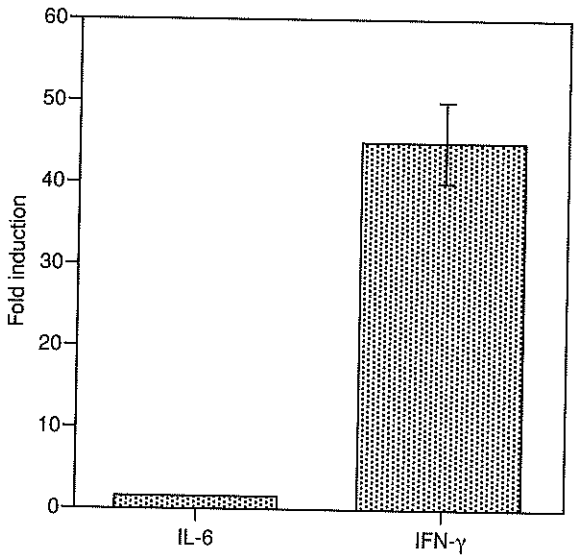

Figure 3. Induction of the IRF-1-dependent transactivation AAGTGA element in A375 cells. A375 cells, pooled and seeded in triplicate 12 h after transfection (see Materials and methods) with the IRF-1-dependent p[(AAGTGA)$_4$]$_5$tkΔ(-39) Lucter plasmid, were treated in triplicate with 50 ng/ml IL-6 or 3000 U/ml IFNγ. Cell lysates were assayed 48 h later using a luciferase assay kit (Promega). Untreated cells were used as controls (no induction).

Discussion

IRF-1 is a negative regulator of cell growth, a tumour suppressor and a mediator of apoptosis[22–24] whose expression and transcriptional activity are tightly regulated by cytokines. These properties make it an attractive candidate as mediator of IL-6 and IFN antiproliferative activities. We examined the IRF-1 activity induced by cytokines in A375 cells. We observed that, in contrast to IFNγ, IL-6 induces low IRF-1 DNA binding activity. Consequently, the IRF-1 transcriptional activity detected in the presence of IL-6 is weak and may therefore not be an important IL-6 signalling event. However, IRF-1 may interact with other transcription factors (such as NF-κB, high mobility group I(Y) or STAT1), which might facilitate its DNA binding and transcriptional activity to various promoters.[25,26] These complexes could be missed by an artificial promoter.

We observed that IFNγ is a strong activator of STAT1 in A375 cells. Recent works have indicated that STAT1 transcriptional activity is required for the antiproliferative effects of both IFNα and IFNγ.[27,28] With regard to the absence of IRF-1 induction by IFN in STAT1$^{-/-}$ mice, IRF-1 could be a mediator of STAT1-induced growth inhibition.[29] On the other hand, we observed that IL-6 is a weak inducer of STAT1 and IRF-1 transcriptional activities but a strong activator of STAT3. Therefore it seems that IL-6, which is a stronger inhibitor of A375 proliferation than IFNγ, used preferentially distinct signalling events to achieve such an impressive biological activity. In addition, we also observed a discrepancy between IRF-1 induction by IL-6 and the growth inhibition activity of this cytokine in other melanoma cells. Indeed, although a human melanoma cell line, SCHN 280 (New York University Medical Center), was totally and specifically resistant to IL-6 inhibition, IL-6 was still able to induce IRF-1 (data

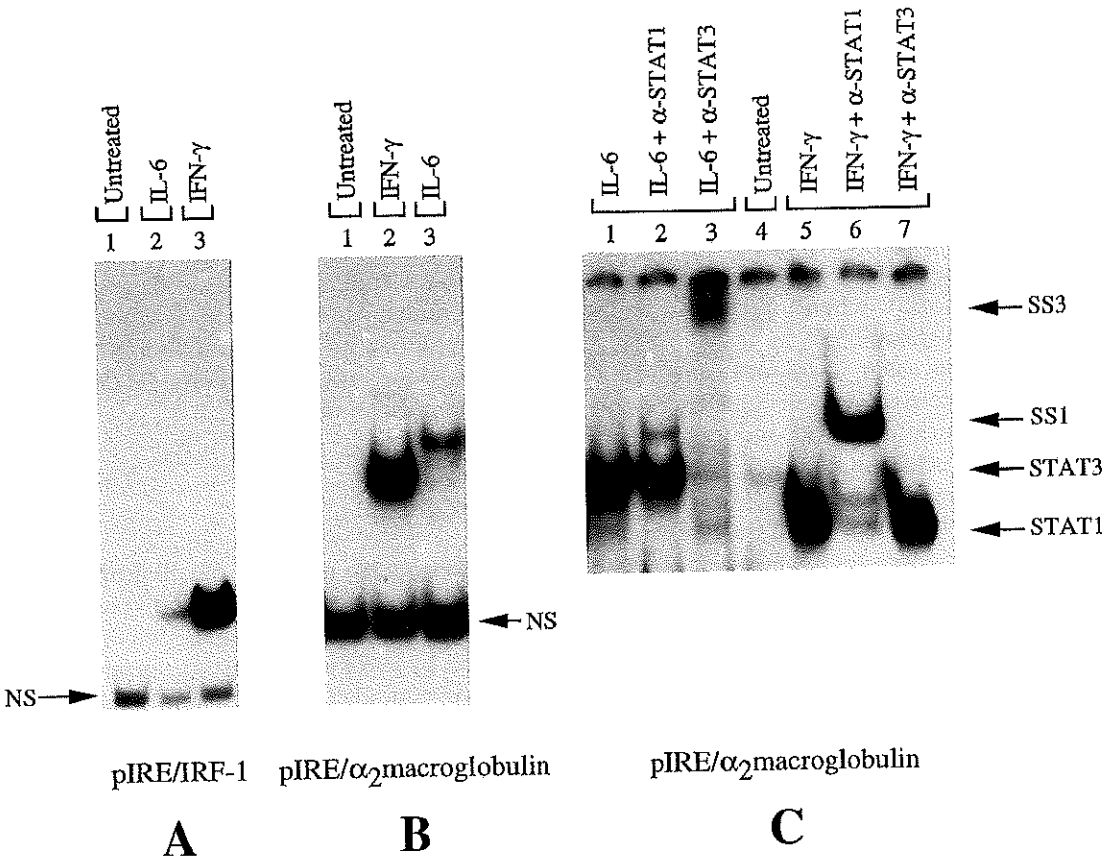

Figure 4. DNA binding activities induced by IL-6 and IFNγ in A375 cells on related pIRE sequences. Nuclear extracts from the A375 cells were subjected to an EMSA (see Materials and methods) using pIRE sequences from IRF-1 (**A**) or α_2-macroglobulin (**B** and **C**) genes. Nuclear extracts were prepared from cells left untreated for 15 min (lanes 1A, 1B and 4C) or treated for 15 min with 20 ng/ml IL-6 (lanes 2A, 3B and 1–3C) or 3000 U/ml IFNγ (lanes 3A, 2B and 5–7C). **C** represents a supershift analysis. The extracts were incubated with either anti-STAT1 (lanes 2 and 6) or anti-STAT3 (lanes 3 and 7) antibodies for 30 min before the addition of the ^{32}P-labelled probe. Gels were subjected to autoradiography for 16 h (**A** and **B**) or 3 days (C). NS = non-specific complexes; SS = supershifted complexes.

not shown). Recently, dominant negative forms of STAT3 were demonstrated to inhibit the growth arrest, differentiation and apoptosis induced by IL-6 in mouse myeloid leukaemia M1 cells.[30] Further studies are currently being performed to investigate the role of STAT3 in the IL-6 antiproliferative effect observed in A375 cells.

In summary, although IRF-1 could play a prominent role in the antiproliferative effect of IFNγ, it seems unlikely to be a major element in the IL-6 growth inhibition response in A375 melanoma cells.

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