

Alpha-Interferon and Its Effects on Signal Transduction Pathways

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Interferon- α (IFN α) is a recombinant protein widely used in the therapy of several neoplasms such as myeloma, renal cell carcinoma, epidermoid cervical and head and neck tumors, and melanoma. IFN α , the first cytokine to be produced by recombinant DNA technology, has emerged as an important regulator of cancer cell growth and differentiation, affecting cellular communication and signal transduction pathways. However, the way by which tumor cell growth is directly suppressed by IFN α is not well known. Wide evidence exists on the possibility that cancer cells undergo apoptosis after the exposure to the cytokine. Here we will review the consolidate signal transducer and activator of transcription (STAT)-dependent mechanism of action of IFN α . We will discuss data obtained by us and others on the triggering of the stress-dependent kinase pathway induced by IFN α and its correlations with the apoptotic process. The regulation of the expression of proteins involved in apoptosis occurrence will be also described. In this regard, IFN α is emerging as a post-translational controller of the intracellular levels of the apoptosis-related protein tissue transglutaminase (tTG). This new way of regulation of tTG occurs through the modulation of their proteasome-dependent degradation induced by the cytokine. Until today, inconsistent data have been obtained regarding the clinical effectiveness of IFN α in the therapy of solid tumors. In fact, the benefit of IFN α treatment is limited to some neoplasms while others are completely or partially resistant. The mechanisms of tumor resistance to IFN α have been studied *in vitro*. The alteration of JAK-STAT components of the IFN α -induced signaling, can be indeed a mechanism of resistance to IFN. However, we have recently described a reactive mechanism of protection of tumor cells from the apoptosis induced by IFN α dependent on the epidermal growth factor (EGF)-mediated Ras/extracellular signal regulated kinase (Erk) signaling. The involvement of the Ras \rightarrow Erk pathway in the protection of tumor cells from the apoptosis induced by IFN α is further demonstrated by both Ras inactivation by RASN17 transfection and mitogen extracellular signal regulated kinase 1 (Mek-1) inhibition by exposure to PD098059. These data strongly suggest that the specific disruption of the latter could be a useful approach to potentiate the antitumour activity of IFN α against human tumors based on the new mechanistic insights achieved in the last years. *J. Cell. Physiol.* 202: 323–335, 2005. © 2004 Wiley-Liss, Inc.

THE INTERFERON SUPERFAMILY

The interferons (IFNs) represent proteins with antiviral activity that are secreted from cells in response to a variety of stimuli (Pestka, 1981a,b, 1986, 1987). There are at least five classes of IFN alpha, beta, gamma, tau, and omega. The interferons are divided into two groups designated type I and type II interferons. IFN γ is the only type II interferon, whereas the type I interferons consist of four major classes: IFN α , IFN β , IFN ω , and IFN τ . There is only one Hu-IFN ω and one Hu-IFN β , but

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a family of multiple IFN α species exists. It is unlikely that any human IFN τ exists. In general, exposure of cells to viruses and doublestranded RNAs induce the production of IFN α and IFN β species. The classical function of these proteins is the protection against viral infections. However, soon the ability of IFN of inhibiting tumor cell proliferation emerged and, on the basis of the preclinical data, it entered the clinical practice and conquered a role in the therapy of a defined group of neoplasms. It appeared clearly, therefore, that IFN has a limited activity and several cancers are resistant to anti-proliferative action induced by this cytokine. On the other hand, the mechanisms at the basis of anticancer effects of IFN are not still completely clear even if the induction of programmed cell death has been recently involved. The potential role played by apoptosis and the new findings about the signal transduction elicited by this cytokine have given emphasis on the molecular pathways regulated by IFN in the view of potentiate its antitumour activity (Pestka, 2000).

Interferon alpha and signal transduction

The IFN- α R1, IFN- α R2, CRFB4, IFN- γ R1, and IFN- γ R2 chains are members of the cytokine type 2 receptor family as described by Bazan (1990a,b) and by Thoreau et al. (1991) who proposed that the interferon receptors as well as other receptors for cytokines and some growth factors are composed of two folding domains that comprise the ligand binding site that resides in the crevice between the folds.

The primary cytokine-receptor interaction was suggested to involve one face of the ligand while another face of the bound cytokine can interact with accessory binding components. A summary of these receptors for the interferon-related receptor components is illustrated in a recent review (Kotenko and Pestka, 2000). These homologies relate the interferon receptor components to the fibronectin type III structure, which in turn relates all these structures to the immunoglobulin superfamily.

The biochemical effects elicited by the interaction between IFN α and its receptors

One example of how intracellular tyrosines are utilized in signaling is the type I interferon (IFN) pathway. Human type I IFNs (α , β , and ω) have been shown to induce the expression of a large number of genes involved in regulating a variety of important biological responses, including antiviral, antiproliferative, and immunomodulatory activities. The mechanisms by which type I IFNs initiate such a broad spectrum of biological activities is only beginning to emerge. Type I IFN-dependent signaling requires both type I IFN receptor chains, IFNAR1 (human type I interferon receptor chain 1) and IFNAR2c (human type I interferon α receptor chain 2) (Colamonici et al., 1994a,b; Yan et al., 1996a,b). Binding of type I IFNs induces the assembly of these receptor chains, which leads to the phosphorylation of tyrosine residues located in the intracellular domain of each receptor chain. These tyrosine phosphorylation events are thought to be carried out by the Janus kinases TYK2 and JAK1, which are themselves activated by tyrosine phosphorylation (Fu, 1992; Schindler et al., 1992). The subsequent substrates of the

TYK2 and JAK1 are the signal transducer and transactivator (STAT) proteins that are recruited at the phosphotyrosines located at the cytoplasmic tail of the receptor.

Signal transduction factors as substrate of type I IFN receptors

STAT family members. STAT proteins are a family of latent cytoplasmic transcription factors involved in cytokine, hormone, and growth factor signal transduction (Schindler et al., 1995; Ihle, 1996; Darnell, 1997; Imada and Leonard, 2000; Takeda and Akira, 2000; Williams, 2000; Bromberg, 2001). STAT proteins mediate broadly diverse biologic processes, including cell growth, differentiation, apoptosis, fetal development, transformation, inflammation, and immune response. Once activated, the tyrosine phosphorylated sites of the cytokine receptors become docking elements for SH2 and phosphotyrosyl-binding domain-containing proteins present in the membrane or the cytoplasmic compartment. Prominent among these are the STATs. Receptor-recruited STATs are phosphorylated on a single tyrosine residue in the carboxyl terminal portion. The modified STATs are released from the cytoplasmic region of the receptor subunits to form homodimers or heterodimers through reciprocal interaction between the phosphotyrosine of one STAT and the SH2 domain of another. Following dimerization, STATs rapidly translocate to the nucleus and interact with specific regulatory elements to induce target gene transcription. STAT proteins were originally discovered in interferon (IFN)-regulated gene transcription in the early 1990s (Sadowski et al., 1993; Shuai et al., 1993a,b; Darnell et al., 1994). Seven members of the STAT family of transcription factors have been identified in mammalian cells: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 and convincing evidence from genetic mapping studies indicates a common ancestral origin that gave rise to three chromosomal clusters of STAT genes through a series of duplication processes (Copeland et al., 1995).

STAT 1/2. IFN receptor activation classically leads to the phosphorylation and activation of STAT 1 and 2. STAT1 and STAT2 form a heterodimer that associates with a member (designated as 9) of the IFN regulatory factor (IRF) family, p48, resulting in the formation of the mature ISGF3 complex that translocates to the nucleus to initiate gene transcription by binding to interferon-stimulated response elements (ISRE) (Darnell et al., 1994; Darnell, 1997).

Stat 1:1 homodimers, Stat 3:3 homodimers, Stat 1:3 heterodimers, Stat 5:5 homodimers, and CrkL:Stat5 heterodimers are also formed during engagement of the type I IFN receptor since, as described below, also these STAT molecules can be activated by IFN α . These complexes move to the nucleus where they bind to GAS regulatory elements in the promoters of IFN-activated genes (Meinke et al., 1996; Darnell, 1997). Thus, signaling specificity via the IFN α -activated Jak/Stat pathway is established by the formation of multiple different complexes that activate distinct regulatory elements in the promoters of IFN-regulated genes.

Cross talks with other STATs and signal transducers. Additional transcription factors have been found to be activated by type I IFNs in the last decade. In fact, it

has been demonstrated the IFN α -dependent activation of STAT 3 in human peripheral blood-derived T cells and the leukemic T cell line Kit225. In this experimental model, the observation that IL-2 and IFN α activate JAK1 to a comparable degree, but only IFN α activates STAT1, indicates that JAK1 activation is not the only determining factor for STAT1 activation (Beadling et al., 1994). Moreover, the data show that JAK1 stimulation is also not sufficient for STAT3 activation. It has been moreover shown that STAT3 binds to a conserved sequence in the cytoplasmic tail of the IFNAR1 chain of the receptor and undergoes interferon-dependent tyrosine phosphorylation (Constantinescu et al., 1994; Mullersman and Pfeffer, 1994).

The p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), which activates a series of serine kinases, binds to phosphorylated STAT3, and subsequently undergoes tyrosine phosphorylation (Pfeffer et al., 1997). Thus, STAT3 acts as an adapter to couple another signaling pathway to the interferon receptor: the PI3K (Yang et al., 1998). Consequently, PI3K is activated and can transduce its signals through Akt activation which is involved in cell survival. Akt was discovered as the product of the oncogene *v-akt* that is able to transform lymphoid cells (Franke et al., 1995). Based on homology to the PKA and PKC family of protein kinases, Akt was also named protein kinase B and RAC-PK (Burgering and Coffey, 1995). The PI-3K/Akt pathway provides cell survival signals in response to nerve growth factor, insulin-like growth factor 1, platelet-derived growth factor, interleukin 3, and the extracellular matrix (Franke et al., 1997). Akt apparently promotes cell survival by phosphorylating multiple targets, including the Bcl-2 family member BAD (Datta et al., 1997), the apoptosis-inducing enzyme caspase-9 (Cardone et al., 1998), and the Forkhead transcription factor (FKHRL)1 that regulates Fas ligand gene expression (Brunet et al., 1999). The recent results by Yang et al. have shown in lymphoma cell models that IFN activates Akt enzymatic activity and that kinase-dead Akt blocks IFN-promoted NF- κ B activation, indicating that Akt is important for IFN-promoted NF- κ B activation. Moreover, a constitutively active Akt construct promotes NF- κ B activation. These data suggest that the main target of the IFN α -induced Akt activation is NF- κ B that in this experimental system mediates anti-apoptotic signals. It will be important to establish which possible substrates for Akt undergo IFN-dependent phosphorylation and determine their physiological significance in IFN-promoted cell survival (Yang et al., 2001). Recent data suggests a role of Akt activation induced by IFN α in the regulation of monocyte adhesion (Navarro et al., 2003).

Also STAT5 has been demonstrated to be activated by IFN α in lymphoma and tumour cells (Fish et al., 1999). A recent report has implicated STAT5 in the engagement of CrkL in IFN signaling, as shown by the requirement of STAT5 as a docking site for the SH2 domain of CrkL. CrkL, in cooperation with STAT5, binds DNA, and this complex functions as a transcription factor in IFN α / β -induced signaling (Barahmand-pour et al., 1995). Recent reports have suggested that STAT5 is involved in IFN α signaling also in myeloid cell lines and HeLa cells (Meinke et al., 1996), and its activation has been observed in response to differentiation and growth

arrest signals (Eilers et al., 1994; Barahmand-pour et al., 1995; Grumbach et al., 2001).

Controversial data are, on the other hand, available, on the interaction between IFN α and the extracellular signal-regulated kinase-dependent signaling. It was, in fact, found that the serine-threonine kinase mitogen-activated protein kinase (MAPK) [specifically, the 42-kDa MAPK or 2 (erk2)], directly involved in cell growth induction, interacts with the alpha subunit of IFN- α / β receptor in vitro and in vivo. Treatment of cells with IFN β induces tyrosine phosphorylation and activation of MAPK and caused MAPK and STAT1 alpha to coimmunoprecipitate (David et al., 1995). Furthermore, expression of dominant negative MAPK inhibits IFN β -induced transcription. Other groups have shown that short-term treatment with IFN α can activate the mitogen extracellular signal regulated kinase (MEK)/ERK pathway (Arora et al., 1999; Lund et al., 1999) in haematological experimental models. Romerio et al. have recently demonstrated that long term exposure of leukemic and lymphoma cells to IFN α induces a decrease of the activity of MEK and ERK through a ras \rightarrow raf-1-independent pathway. Moreover, the addition of a MEK inhibitor (and thus of MAPK activity) increases the growth inhibition induced by IFN α (Romerio et al., 2000; Romerio and Zella, 2002).

Another molecular target of type I IFN receptor is protein kinase C (PKC) δ , a member of the PKC family of proteins, that is activated during engagement of the Type I IFN receptor and, consequently, associates with STAT1. Such an activation of PKC δ appears to be critical for phosphorylation of STAT1 on serine 727, as inhibition of PKC δ activation diminishes the IFN α - or IFN β -dependent serine phosphorylation of STAT1. In addition, treatment of cells with the PKC δ inhibitor rottlerin or the expression of a dominant-negative PKC δ mutant results in inhibition of IFN α - and IFN β -dependent gene transcription via ISRE elements. Interestingly, PKC δ inhibition also blocks activation of the p38 MAP kinase, the function of which is required for IFN α -dependent transcriptional regulation, suggesting a dual mechanism by which this kinase participates in the generation of IFN α responses (Uddin et al., 2002). The complex signal transduction network activated by IFN α is summarized in Figure 1.

MECHANISMS OF CELL GROWTH INHIBITION BY IFN α : APOPTOSIS

The caspase and mitochondrial involvement

Apoptosis plays an important role in the control of many normal physiological processes, such as embryonic development, immune regulation, and maintenance of tissue homeostasis (Krammer, 2000). Decreased sensitivity to apoptotic stimuli is also a trait commonly shared by cancer cells. This feature provides the tumour cells with a survival advantage, facilitating the outgrowth of malignant clones and may also explain a variable susceptibility to various anti-cancer drugs (Los et al., 1997; Raza, 2000). Induction of apoptosis is thus a highly attractive mechanism for IFN α 's antitumoural activity, and it could also play a role in the clearing of virus-infected cells.

IFN α can indeed induce apoptosis in some transformed cell lines as well as in primary tumour cells

caspase-3-positive cells. Bak activation, moreover, occurred early in the apoptotic response, prior to the cyt c release and loss of DC, whereas Bax activation followed these events (Panaretakis et al., 2003). The same authors show a transient initial increase of Bcl-xL and Mcl-1 that could explain the late onset of the apoptosis induced by IFN α (Puthier et al., 2001). In this regard, it was reported that IFN α , similar to interleukin-6 (IL-6), extends the survival of human myeloma cells through an upregulation of the Mcl-1 anti-apoptotic molecule although it was previously reported that IFN α induces growth inhibition of other multiple myeloma cells (Matsui et al., 2003). The latter data confirm the dual effect of this cytokine on the expression and, presumably, activity of the mitochondrial bcl-related molecules. Taken together these results suggest a mitochondrial involvement in the apoptosis triggered by IFN α .

The stress kinase cascade involvement

In eukaryotic cells, enzymatic isoforms of MAPK, such as Jun kinase-1 (JNK1) and p38 kinase, which can mediate anti-proliferative stimuli and apoptosis, have been identified. They have large sequence homology, but are functionally different from proliferative pathway-associated erk1/2. In fact, JNK1 and p38 kinase are part of enzymatic cascades activated by anti-proliferative agents such as ionizing and ultraviolet rays and cytokines. Recently, it has been demonstrated a role of JNK1 and p38 kinase in the onset of apoptosis in several cell models. In this regard, in addition to the STAT pathway, type I IFNs activate members of the MAPK family, including erk (David et al., 1995) and the p38 MAPK (that belongs to the stress-activated kinases) (Goh et al., 1999; Uddin et al., 1999, 2000). It was recently shown that activation of p38 is required for transcriptional activation of IFN-sensitive genes (Goh et al., 1999; Uddin et al., 1999, 2000). In addition, it was demonstrated that such transcriptional regulation of IFN-sensitive genes is unrelated to effects on DNA binding of STAT complexes or serine phosphorylation of STATs (Uddin et al., 2000), apparently involving a STAT-independent nuclear mechanism. Thus, coordination of the functions of the IFN-activated STAT and p38 pathways is necessary for full transcriptional activation in response to interferons (Goh et al., 1999; Uddin et al., 1999, 2000). It was, moreover, found that p38 MAPK pathway is engaged in type I IFN signaling in primary human hematopoietic progenitors and its function is required for the generation of the suppressive effects of interferons on normal hematopoiesis. In details, p38 and its downstream effector, mitogen-activated protein kinase activated protein kinase 2 (MapKapK-2), are rapidly activated by IFN α treatment of enriched primary human progenitor cells and pharmacological inhibition of p38 MAPK activation reverses the type I IFN-dependent inhibition of hematopoietic progenitor colony formation (Verma et al., 2002). Moreover, p38 MAPK, is involved in the generation of the antileukemic effects of IFN α in break cluster region (BCR)-ABL-expressing cells of acute myeloid leukemia (Mayer et al., 2001). We have reported that IFN α increases the expression of the epidermal growth factor receptor (EGF-R) at the surface of human

epidermoid carcinoma cells (Budillon et al., 1991). We have also found that IFN α enhances the activity of EGF on these cells. In fact, IFN α -treated KB cells (human epidermoid carcinoma) are sensitized to the growth promoting effects of EGF. Moreover, the EGF-induced tyrosine phosphorylation of total cellular proteins and of the EGF-R is increased in the IFN α -treated cells (Caraglia et al., 1995). On the bases of these findings, we have hypothesized that the increased expression and function of the EGF-R could represent a protective response of tumour cells (STRESS RESPONSE) to the antiproliferative effect of IFN α (Tagliaferri et al., 1994). In order to verify this hypothesis we have studied, in KB cells, the expression of heat shock proteins (HSP) which are molecules involved in the protective response of eukaryotic cells to stress. IFN α increases the expression of HSP27, HSP90, and HSP70 inducible forms while it does not change the levels of the constitutive form of HSP70. After EGF addition to IFN α -treated KB cells, the levels of the HSPs are resumed to the levels of untreated control cells (Caraglia et al., 1999). We have moreover found that IFN α induces apoptosis on human epidermoid cancer KB cells and that also this effect is antagonized by EGF. We have evaluated the effects of IFN α and EGF on the stress-induced pathway of MAPK isoenzymatic activity JNK1 and MAPKp38 in KB cells. We have found that IFN α induces an about fivefold increase of activity of these proteins while the addition of EGF to IFN α -treated cells causes a progressive reduction of the activity of the two enzymes which reaches almost basal levels after 6 h of exposure to EGF. However, EGF alone does not induce any change in the activity of JNK1 and MAPKp38 in untreated KB cells. We have evaluated the involvement of JNK1 in the triggering of IFN α -induced apoptosis by transfecting KB cells with a plasmid encoding for a wild type form of JNK1 (JNK1^{wt}). Either the treatment of parental cells with IFN α or the overexpression of JNK1^{wt} in transfected cells induce apoptosis and the exposure of JNK1^{wt}-transfected cells to IFN α causes a potentiation of apoptosis. The addition of EGF to JNK1^{wt}-transfected cells exposed to IFN α is again able to revert this effect. Therefore, the effects of EGF and IFN α on apoptosis are paralleled by changes of the activity of the stress-inducible JNK1 that appears responsible, at least in part, for the apoptotic effects of IFN α (Caraglia et al., 1999).

A tumour suppressor gene specifically activated after a genotoxic stress is p53. Takaoka et al. have recently shown that transcription of the p53 gene is induced by IFN α/β , accompanied by an increase in p53 protein level. IFN α/β signaling itself does not activate p53; rather, it contributes to boosting p53 responses to stress signals. In these experimental conditions p53 gene induction by IFN α/β contributes to tumour suppression, is activated in virally infected cells to evoke an apoptotic response and is critical for antiviral defence of the host (Takaoka et al., 2003). The role of NF- κ B in the apoptosis induced by IFN α is controversial. In fact, it has been demonstrated that IFN α can activate NF- κ B through STAT3 and via phosphatidylinositol 3 kinase (PI3K) and Akt activation in lymphoma cells and promotes survival of human primary B-lymphocytes via PI3K (Yang et al., 2001). Other studies demonstrate that IFN α sensitizes

human hepatoma cells to TRAIL-induced apoptosis through DR5 upregulation and NF- κ B inactivation or suppresses the antiapoptotic effect of NF- κ B and sensitizes renal cell carcinoma cells in vitro to chemotherapeutic drugs (Steiner et al., 2001; Shigeno et al., 2003).

THE PROTEIN SYNTHESIS AS A TARGET OF IFN α ACTION

In the past years, the attention of scientists has focused mainly on the study of the genetic information and alterations that regulate eukaryotic cell proliferation and that lead to neoplastic transformation. All therapeutic strategies against cancer are, to date, directed at DNA either with cytotoxic drugs or gene therapy. Little or no interest has been aroused by protein synthesis mechanisms. However, an increasing body of data is emerging about the involvement of translational processes and factors in control of cell proliferation, indicating that protein synthesis can be an additional target for anticancer strategies (for a review see Caraglia et al., 2000). One of the more studied molecular targets of IFN α is the protein kinase dependent from dsRNA, PKR. PKR activation induced by the cytokine regulates translational and transcriptional pathways (eIF-2a and NF- κ B-dependent) resulting in the specific expression of selected proteins (Fas, p53, Bax, and others) that triggered cell death by engaging with the caspase pathway. Through an unknown mechanism, upon PKR activation, FADD recruits procaspase 8, activating it to its active form, caspase 8 that, in turn, activates down-stream caspases such as caspase 3, 6, 7, which cleave multiple targets triggering cell death. The role of the caspase 9 pathway in these events is unknown (Gil and Esteban, 2000).

The eukaryotic initiation factor-5A of protein synthesis (eIF-5A)

The eukaryotic initiation factor 5A (eIF-5A) is peculiar because its activity is modulated by a series of post-translational modifications that culminates in the formation of the unusual amino acid hypusine. Hypusine [N^c-(4-amino-2-hydroxybutyl)lysine] is formed by the transfer of the butylamine portion from spermidine to the ϵ -amino group of a specific lysine residue of eIF-5A precursor (Wolff et al., 1990) and by the subsequent hydroxylation at carbon 2 of the incoming 4-aminobutyl moiety (Abbruzzese et al., 1986; Park et al., 1993). eIF-5A probably acts in the final stage of the initiation phase of protein synthesis by promoting the formation of the first peptide bond (Hershey, 1991). Hypusine plays a key role in the regulation of eIF-5A function because its precursors, which do not contain hypusine do not have activity (Park et al., 1991). These biochemical correlates make eIF-5A peculiar. In fact, only the hypusine-containing eIF-5A form is active and, consequently, the dosage of intracellular hypusine content measures also the activity of eIF-5A since hypusine is contained only in this factor. The correlation between hypusine, and thus eIF-5A activity, and cell proliferation (Abbruzzese, 1988) suggests that activated eIF-5A might play a role in cell growth and differentiation (Shnier et al., 1991). More recently a correlation has been found between the polyamine-dependent modification of eIF-5A and the triggering of apoptosis in tumour cells (Abbruzzese et al.,

1989). In fact, excess putrescine accumulation in hepatoma tissue culture DH23A/b cells induces apoptosis and suppresses the formation of hypusine-containing eIF-5A (Abbruzzese et al., 1989). Furthermore, we have evidenced an in vitro post-translational modification of eIF-5A catalyzed by tissue transglutaminase (tTG) (Beninati et al., 1998) that is involved in apoptosis regulation and if stably transfected in Balb-C 3T3 cells strongly reduces hypusine levels. These effects occurred together with a significant reduction of cell proliferation and apoptosis (Beninati et al., 1998). We have reported that IFN α induces growth inhibition and reduction of the activity of eIF-5A in human epidermoid cancer KB cells (Caraglia et al., 1997). The activity of eIF-5A was evaluated through the determination of hypusine levels since this amino acid is essential for the function of this translational factor that is involved in the regulation of cell proliferation and transformation (Caraglia et al., 1997). The cell proliferation regulatory properties of eIF-5A could be correlated by its reported mRNA chaperon functions since eIF-5A is involved in the transport of mRNAs from the nucleus to the cytoplasm (Lipowsky et al., 2000). It has been also proposed that these mRNAs could encode for proteins involved in the regulation of cell proliferation (Caraglia et al., 2000). We have, moreover, found that IFN α induces cell growth inhibition and apoptosis in human epidermoid cancer cells and these effects are antagonized by EGF. We also found that IFN α is able to induce a strong inhibition of eIF-5A activity since a reduction of hypusine synthesis is recorded with a parallel increase of eIF-5A protein expression. This finding suggests a further reduction of the active fraction of eIF-5A (hypusine-containing eIF-5A:total eIF-5A ratio). On the other hand, when EGF antagonized the apoptosis induced by IFN α a restoration of hypusine synthesis caused by the cytokine and an increase of erk activity are recorded in cancer cells. In the same experimental conditions, we have also found that PD098059, a specific inhibitor of MEK-1 and thus of erk, reduces hypusine synthesis and enhanced the decrease of intracellular hypusine content caused by IFN α (Caraglia et al., 2003a,b). Moreover, PD098059 is also able to antagonize the recovery of hypusine synthesis induced by EGF (Caraglia et al., 2003a,b). The reduction of hypusine synthesis could be even higher if tumour cells treated with IFN α did not show an anti-apoptotic response based on the hyperactivation of the MEK \rightarrow ERK pathway. Therefore, the addition of PD098059 to IFN α -pretreated cells overcome this survival pathway inducing a potentiation of both hypusine level reduction and apoptosis. On the other hand, the addition of EGF to IFN α -treated cells overstimulated this survival pathway inducing a recovery of both hypusine levels and apoptosis (Caraglia et al., 2003a,b). On the basis of these results, we have investigated if eIF-5A could be really critical for the biological effects induced by IFN α . We have used the specific deoxyhypusine synthase inhibitor N1-guanyl-1,7-diaminoheptane (GC7) that avoids hypusine formation and thus blocks eIF-5A activity (Lee and Park, 2000). We have found that this agent synergized with IFN α in inducing cell growth inhibition and apoptosis suggesting a critical role for eIF-5A in the modulation of cell proliferation induced by IFN α in human epidermoid cancer cells (Caraglia

et al., 2003a,b). All these data support the hypothesis of an involvement of eIF-5A, another protein synthesis regulator, in the apoptosis induced by IFN α in human epithelial cells.

THE MODULATION OF PROTEIN DEGRADATION

The proteasome is a multisubunit enzyme complex that plays a central role in the regulation of proteins that control cell-cycle progression and apoptosis, and has therefore become an important target for anticancer therapy. In fact, the expression of proteins essential for the regulation of cell growth and survival can be also controlled at post-transcriptional and post-translational levels, the latter through the regulation of protein degradation. Before a protein is degraded, it is first flagged for destruction by the ubiquitin conjugation system, which ultimately results in the attachment of a polyubiquitin chain on the target protein. The proteasome's 19S regulatory cap binds the polyubiquitin chain, denatures the protein, and feeds the protein into the proteasome's proteolytic core. The proteolytic core is composed of two inner beta rings and two outer alpha rings. The two beta rings each contain three proteolytic sites named for their trypsin-like, post-glutamyl peptide hydrolase-like (i.e., caspase-like), or chymotrypsin-like activity. Inhibition of the proteasome generally results in cell-cycle arrest and apoptosis (for a review see Adams, 2003). Since 1996 it has been demonstrated that type I IFNs (IFN τ) can regulate the expression of a 16-kDa protein that is produced by the bovine endometrium during early pregnancy and that shares epitopes with hUCRP and ubiquitin (Austin et al., 1996). These results were confirmed by a study performed with metabolic labeling and two-dimensional gel electrophoresis followed by MS and database searches to identify potentially new IFN α -induced proteins in human T cells. By this analysis, it was shown that IFN α induces the expression of ubiquitin cross-reactive protein (ISG15) and two ubiquitin-conjugating enzymes, UbcH5 and UbcH8. Northern-blot analysis showed that IFN α rapidly enhances mRNA expression of UbcH5, UbcH6, and UbcH8 in T cells. In addition, these genes were induced in macrophages in response to IFN α . Similarly, IFNs enhanced UbcH8 mRNA expression in A549 lung epithelial cells, HepG2 hepatoma cells, and NK-92 cells. Cycloheximide, a protein synthesis inhibitor, did not block IFN-induced upregulation of UbcH8 mRNA expression, suggesting that UbcH8 is the primary target gene for IFN α (Nyman et al., 2000). More recently, it was demonstrated that administration of interleukin 1 β (IL-1 β) in vivo attenuates IFN α -induced STAT1 tyrosine phosphorylation in the liver but not in the spleen. The inhibitory action of IL-1 β in vivo is not affected by depleting hepatic Kupffer cells, suggesting that IL-1 β may directly target IFN α signaling in hepatocytes. Indeed, pretreatment of human hepatocellular carcinoma HepG2 cells with IL-1 β suppresses IFN α -induced antiviral activity and protein MxA mRNA expression. Furthermore, IL-1 β attenuated IFN α -induced STAT1 binding and tyrosine phosphorylation without affecting the level of STAT1 protein. This inhibitory effect can be reversed by pretreatment with either proteasome inhibitors or transfection of dominant negative NF- κ B

inducing kinase mutants. Taken together, these findings suggest that IL-1 β attenuates IFN α -induced STAT1 activation by a proteasome-dependent mechanism (Tian et al., 2000). Moreover, we have recently found that IFN α induces apoptosis through, at least in part, the increase of the expression and activity of tTGase in human epidermoid lung cancer cells (Esposito et al., 2003). The increase of the expression of tTGase was not due to the induction of its transcription, but to a decrease of its degradation via a proteasome-dependent pathway. Therefore, IFN α modulates apoptosis through the regulation of the degradation of intracellular proteins involved in the triggering of apoptotic process. Not only the target of the IFN α -dependent signaling but also the components of the pathway activated by the cytokine itself can be subjected to regulation via proteasome-dependent degradation. In fact, Mumps virus, a common infectious agent of humans, causing parotitis, meningitis, encephalitis, and orchitis, induces degradation of STAT3 mediated by its ubiquitination and subsequent proteasome-dependent degradation (Ulane et al., 2003). The latter could be a mechanism by which viruses protect themselves by the anti-viral action of IFN α . Finally, one of the modes used by SOCS to turn off the IFN α -dependent signaling is the delivery of the transductional components to the degradative proteosomal machinery (Larsen and Ropke, 2002).

ESCAPE MECHANISMS TO ANTI-PROLIFERATIVE EFFECTS OF IFN α

Until today inconsistent data have been obtained regarding the clinical effectiveness of IFN α in the therapy of solid tumors. In fact, the benefit of IFN α treatment is limited to some neoplasms while others are completely or partially resistant. The mechanisms of tumor resistance to IFN α have been studied in deep in vitro. The alteration of JAK-STAT components of the IFN α -induced signaling, can be indeed a mechanism of resistance to IFN α . In fact, an old issue that has been associated to the resistance of tumour cells to the biological effects of IFN α is the disruption of its signal transduction pathways based on the altered expression of STAT proteins in several cancer cell types (Wong et al., 1997; Landolfo et al., 2000; Yamauchi et al., 2001; Brinckmann et al., 2002). In details, it has been shown that melanoma cell lines refractory to the antiproliferative effects of IFNs are deficient in STATs and that the expression of STATs can be restored by in vitro gene therapy (Wong et al., 1997). Analogous effects were demonstrated on myeloid leukemic and renal carcinoma cells (Yamauchi et al., 2001; Brinckmann et al., 2002).

However, recent data have demonstrated that the JAK/STAT pathway is not sufficient to sustain the antiproliferative response in an interferon-resistant human melanoma cell line. Additional studies confirm that STAT1 and STAT3 expression and IFN α induction and activation are not altered between both variants. (Jackson et al., 2003). DNA microarrays performed on two T cell lymphoma lines (resistant or sensitive to IFN α) showed that resistance to IFN α is consistently associated with changes in the expression of a set of 39 genes, involved in signal transduction, apoptosis, transcription regulation, and cell growth (Tracey et al., 2002). These results highlight the likely heterogeneity

in the mechanisms leading to interferon resistance both in cell lines and tumours.

Beside these mechanisms of resistance towards the growth inhibitory and apoptotic activity of IFN α , also the triggering and/or hyperactivation of survival and proliferative pathways can be supposed in cancer cells. This hypothesis is furtherly supported by the evident dysregulation of proliferative signaling in transformed cells. In this view, we have reported that IFN α increases the expression and function of the EGF-R at the surface of human epidermoid carcinoma cells (Budillon et al., 1991; Caraglia et al., 1995). On the basis of these findings, we have hypothesized that increased EGF-R expression and function could be part of an inducible survival pathway, which is activated in the tumour cells by the exposure to IFN α (Tagliaferri et al., 1994). Moreover, we have found that the addition of EGF to IFN α -treated KB cells completely antagonized apoptosis induction suggesting that the EGF-R signaling suppresses apoptosis (Caraglia et al., 1999) (Fig. 2). These results appear also in line with the recent findings demonstrating the involvement of growth factor-dependent pathways in the protection from caspase activation induced by Bad overexpression (Jan et al., 1999). Moreover, it has been demonstrated that the EGF-R-dependent pathway controls keratinocyte survival and the expression of the pro-apoptotic bcl-x_L through a MEK-dependent pathway (Jan et al., 1999).

Furthermore, the EGF- and Ras-dependent MAPK cascade is hyperactivated in IFN α -treated cells and could be further stimulated by the addition of EGF. In these experimental conditions, an increased activity and responsiveness to EGF stimulation of Ras, Raf-1, and Erk-1 and 2 was found in KB cells exposed to IFN α (Caraglia et al., 2003a,b). These findings suggest that the EGF-R function is preserved in IFN α -treated cells. We have previously described that other anti-proliferative agents, such as cytosine arabinoside, 5aza-2'-deoxycytidine and 8-chloro-cAMP (8ClcAMP), also increase EGF-R expression on KB cells (Caraglia et al., 1993, 1994; Budillon et al., 1999). On this basis, we have hypothesized that the up-regulation of growth factor receptors is a common event in growth inhibited tumor cells and could represent a protective response towards the antiproliferative stimuli (Tagliaferri et al., 1994). Also in the case of 8ClcAMP, the EGF-induced MAPK signaling is amplified likely as a consequence of the increased expression of EGF-R (Budillon et al., 1999). However, MAPK activity is reduced in 8ClcAMP-treated KB cells suggesting a selective inhibition of Erks or of a still unknown upstream activator induced by the drug (Budillon et al., 1999). The involvement of the Ras→MAPK pathway in the protection of KB cells from the apoptosis induced by IFN α is further demonstrated by both Ras inactivation by RASN17 transfection and MEK-1 inhibition by exposure to PD098059 (Caraglia et al., 2003a,b). In fact, the transfection of RASN17 in KB cells caused apoptosis suggesting that the integrity of Ras function is necessary to produce an anti-apoptotic signal that mediates a survival response in cells exposed to IFN α via Erk-1 and 2 activation. In fact, we have demonstrated that Ras-dependent survival signaling targets Erk-1/2 since the reduction of MAPK activity by PD098059 enhanced apoptosis caused

by IFN α . An additional important finding is that PD098059 specifically abrogated the recovery from apoptosis induced by EGF in IFN α -treated cells. Therefore, our results suggest that the activation of Ras→Raf-1→Mek1→Erk-1/2 signaling has a prominent role in the anti-apoptotic effects exerted by EGF in epidermoid cancer cells exposed to IFN α providing evidence of the potential benefits of the molecular interference with this pathway (Caraglia et al., 2003a,b) (Fig. 2). However, the occurrence of other survival pathways will warrant further investigations and we can not presently completely exclude a role of Akt pathway in the modulation of apoptosis of KB cells. We have recently found a cross-talk between ras→erk-dependent pathway and protein synthesis machinery. In details EGF induces increased ras and erk activity and enhanced hypusine synthesis. IFN α , on the other hand, reduces the intracellular hypusine levels and this effect is antagonized by EGF (Caraglia et al., 2003a,b). The involvement of erk in the antagonizing effect of EGF is demonstrated by the concomitant addition of the erk inhibitor PD098051 that, alone, induces apoptosis and reduces hypusine levels and when used in combination with IFN α , synergizes with the latter in inducing such biological and biochemical effects. Therefore, the regulation of eIF-5A activity and, consequently, of the efficiency and specificity of protein synthesis machinery could represent a further mechanism by which ras→erk-dependent pathway counteracts apoptotic and antiproliferative effects induced by IFN α in cancer cells (Caraglia et al., 2003a,b) (Fig. 2). Other mechanisms of resistance can be supposed to be based on the intrinsic properties of the IFN α -dependent signal transduction pathway and on its capacity to interact with other signal transduction pathways often involved in cell survival. In fact, as described above, IFN α can activate Akt via STAT3 and PI3K and the consequent survival signaling that leads to the activation of NF κ B in lymphoma cells (Constantinescu et al., 1994; Mullersman and Pfeffer, 1994; Franke et al., 1995; Pfeffer et al., 1997; Yang et al., 1998). Moreover, a hyperactivation of the feed back mechanisms could occur in cancer cells and induce the occurrence of resistance to IFN α .

PERSPECTIVES AND FUTURE DIRECTIONS

More germane to clinical practice is the possibility that IFN α treatment could be improved by the concomitant administration of agents known to enhance JAK-STAT responses; the use of retinoids in combination to IFN α in cancer therapy is a salient example (Harvat et al., 1997; Ransohoff, 1998). However, on the basis of our previous findings, three different therapeutic strategies are under preclinical investigation in order to increase the anti-cancer activity of IFN α . On the basis of the involvement of stress kinases in the apoptotic effects triggered by IFN α , experiments are in progress in order to construct viral vectors of JNKs to be used in combination with the cytokine in experimental preclinical models (Caraglia et al., 1999) (Fig. 2). Moreover, we have also identified in epidermoid cancer cells a specific pathway that is activated in response to apoptotic stimuli induced by IFN α . In details, we have demonstrated that the hyperactivation of ras and erk pathway dependent from tyrosine kinase activity of EGF-R is a

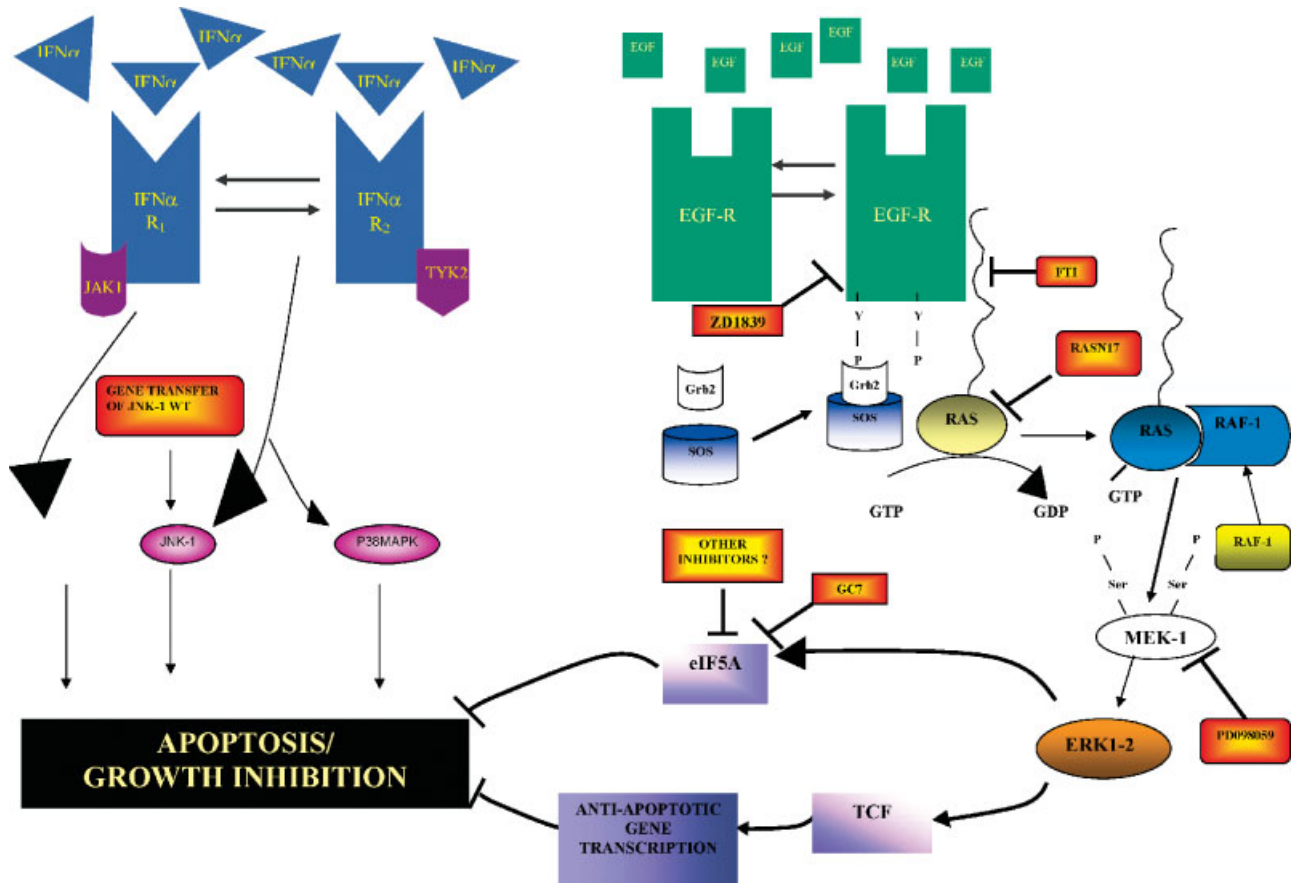


Fig. 2. Escape mechanisms to anti-tumor effects by IFN α and proposed overcoming strategies. **Left:** IFN α induces apoptosis likely through the activation of caspase cascade mediated by JNK-1 and/or p38MAPK activation and through the mitochondrial involvement. **Right:** EGF triggers a ras-dependent ERK-1/2 activation that inhibits IFN α -induced apoptosis probably counteracting on caspase cascade activation. Moreover, ERK-1/2 has also a stimulating action on the activity of eIF-5A that displays anti-apoptotic activities. The inhibition of this pathway through the use of the EGF-R-associated tyrosine kinase inhibitor ZD1839 or the dominant negative ras RASN17 or FTIs or the MEK-1 inhibitor PD098059 induces the release of this anti-apoptotic pathway with the subsequent potentiation of the apoptosis induced by IFN α . Moreover, the selective inhibition of eIF-5A with the hypusine synthesis inhibitor GC7 or with other specific

inhibitors to be found could enhance the apoptotic properties of IFN α . The inhibition of the feed back mechanisms of the IFN α -dependent pathway could be an additional strategy in order to enhance anti-neoplastic effects of the cytokine. — Stimulating activity. —| Inhibiting activity. Red squares show the possibilities of therapeutic interventions in order to increase the antiproliferative activity of IFN α . EGF, epidermal growth factor; EGF-R, EGF receptor; FTI, farnesyltransferase inhibitor; RASN17, dominant negative ras plasmid; TCF, ternary complex factor; Erk, extracellular signal regulated kinase; Mek, mitogen extracellular signal regulated kinase; eIF-5A, eukaryotic initiation factor of protein synthesis 5A; GC7, 1,7-diaminoheptane; JNK-1, Jun kinase-1; p38 MAPK, p38 mitogen activated protein kinase.

strong antiapoptotic pathway in cancer cells exposed to IFN α (Caraglia et al., 2003a,b). Therefore, the selective inhibition of a suspected target of this pathway could be an interesting strategy in the chemoprevention of human tumor. In this view, we have preliminarily found that the specific EGF-R-associated kinase inhibitor ZD1839 (IRESSA), already used in phase II/III clinical trials in the therapy of lung epidemoid cancer, synergizes with IFN α in inducing the growth inhibition and apoptosis of several human epidermoid cancer cell lines which is coupled to complete inhibition of ras and erk activity (Budillon et al., manuscript in preparation). Moreover, the selective inhibition of ras with gene transfer therapeutic strategies based on the delivery of dominant negative forms of ras such as RASN17 or with agents that block ras farnesylation such as the farnesyltransferase inhibitors (FTI) could be also considered in

order to enhance the antiproliferative action of IFN α . MEK-1 and consequently the activation of ERK-1/2 could be also evaluated as additional target through the use of selective inhibitors such as PD098059. Finally, on the basis of the previous findings, we can also hypothesize that the selective interference on eIF-5A activity could be an additional target in order to potentiate the antitumor efficacy of IFN α . In fact, we have found that the hypusine synthesis inhibitors, and thus eIF-5A inactivator, GC7 synergizes with the cytokine in the induction of cell growth inhibition and apoptosis (Caraglia et al., 2003a,b). We have recently performed a computer-based prediction of the three dimensional structure of eIF-5A in order to define the structure of the hypusine-containing site (Facchiano et al., 2001). We are now planning a pharmacological screening of drugs with potential eIF-5A-inhibiting properties. The inhibi-

tion of eIF-5A either through the blocking of hypusine synthesis (mediated by agents similar to GC7) or the selective binding with the hypusine-containing site could represent a new scenario of intervention in anti-cancer therapy based on IFN α administration (Fig. 2).

In conclusion, the understanding of the molecular mechanisms regulating the signal transduction pathway mediated by IFN α and of the escape mechanisms activated in cancer cells could be useful in the design of new therapeutic strategies based on the use of IFN α and in order to widen the therapeutic window of this cytokine (Fig. 2).

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