Alpha-Interferon and its Effects on Signalling Pathways within Cells

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FINAL

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Abstract: Interferon-α (IFNα) is a recombinant protein widely used in the therapy of several neoplasms such as myeloma, renal cell carcinoma, epidermoid cervical and head and neck tumours and melanoma. IFNα, 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 tumour cell growth is directly suppressed by IFNα 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 IFNα and the supposed mechanism of apoptosis induction by IFNα. We will discuss data obtained by us and others on the triggering of the stress-dependent kinase pathway and on the modulation of protein synthesis machinery induced by IFNα and their correlations with the apoptotic process. Until today, inconsistent data have been obtained regarding the clinical effectiveness of IFNα in the therapy of solid tumours. In fact, the benefit of IFNα treatment is limited to some neoplasms while others are completely or partially resistant. The mechanisms of tumour resistance to IFNα have been studied *in vitro*. The alteration of JAK- Signal transducer and activator of transcription components of the IFNα-induced signalling, can be indeed a mechanism of resistance to IFN and cross talks between IFNα and survival signals has been also described. However, we have recently described a reactive mechanism of protection of tumour cells from the apoptosis induced by IFNα dependent on the epidermal growth factor (EGF)-mediated Ras/extracellular signal regulated kinase (Erk) signalling. The involvement of the Ras->Erk pathway in the protection of tumour cells from the apoptosis induced by IFNα 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 IFNα against human tumours based on the new mechanistic insights achieved in the last years.

Key words: Interferon α, STAT, SOCS, ubiquitin, EGF-R, ras, eIF5A, hypusine.

THE INTERFERON SUPERFAMILY

The interferons (IFNs) represent proteins with antiviral activity that are secreted from cells in response to a variety of stimuli (see Refs. 1–4 for detailed reviews). There are at least 5 classes of IFN alpha, beta, gamma, tau and omega. The interferons are divided into two groups designated type I and type II interferons. IFNγ is the only type II interferon, whereas the type I interferons consist of four major classes: IFNα, IFNβ, IFNω, and IFNτ. There is only one Hu-IFNω and one Hu-IFNβ, but a family of multiple IFNα species exists. It is unlikely that any human IFNτ exists. In general, exposure of cells to viruses and double stranded RNAs induce the production of IFNα and IFNβ species. The classical function of these proteins is the protection against viral infections. However, it emerged soon the ability of IFN of inhibiting tumour cell proliferation 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, therefore, clear 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 anti-cancer 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 has given emphasis on the molecular pathways regulated by IFN in the view of potentiating its antitumour activity [5].

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Interferon Alpha and Signal Transduction

The IFN Receptor Family

The IFN-αR1, IFN-αR2, CRFB4, IFN-γR1, and IFN-γR2 chains are members of the cytokine type 2 receptor family as described by Bazan and by Thoreau et al. 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 [6-8].

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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 [9]. These homologies relate the interferon receptor components to the fibronectin type III structure, which in turn relates all these structures to the immunoglobulin superfamily.

IFNRs tranduce their signals after activation via stimulation of two tyrosine kinases associated to the cytoplasmic tail of the receptor: Tyk2 and Jak1. These kinases after activation mediate the tyr phosphorylation of the signal transducer and activator of transcription (STAT) 1 and 2. STAT proteins are a family of latent cytoplasmic transcription factors involved in cytokine, hormone, and growth factor signal transduction [10-16] (Fig. **1**). 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 [17-19] (Fig. **1**). The final biological effect of the interaction of STAT with DNA, and the consequent induction of gene transcription, is the growth inhibition. One of the mechanisms by which IFNα induces growth inhibition is the triggering of apoptosis.

MECHANISMS OF CELL GROWTH INHIBITION BY IFNα: APOPTOSIS

Apoptosis plays an important role in the control of many normal physiological processes, such as embryonic development, immune regulation and maintenance of tissue homeostasis [20]. 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 out-growth of malignant clones and may also explain a variable susceptibility to various anti-cancer drugs [21,22]. Induction of apoptosis is thus a highly attractive mechanism for IFNα's antitumoural activity, and it could also play a role in the clearing of virus-infected cells.

IFNα can indeed induce apoptosis in some transformed cell lines as well as in primary tumour cells [23-26]. Furthermore, in myeloma, as well as in glioma cell lines,

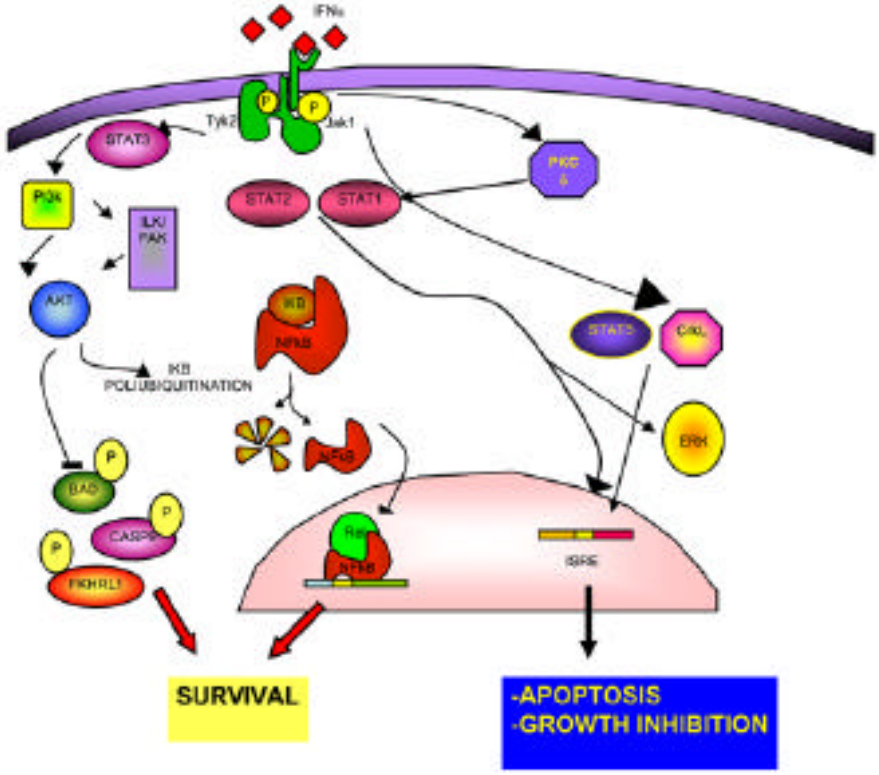

Fig. (1). Signal transduction pathways activated by IFNα.

IFNα, after the interaction with its receptor, activates the tyr kinases Jak-1 and Tyk-2 that are responsible for the activation of the cytoplasmic targets of IFNα. (*Right part*) The tyr phosphorylation of the targets causes the translocation to the nucleus of STAT1 and 2 hetero and homodimers, of STAT5-CrkL heterodimers that migrate to the nucleus and binds to DNA mediating the transcription of apoptotic proteins. Jak-1 and Tyk-2 can also phosphorylate and activate PKCδ that, in turn, phosphorylates STAT2 on Ser and enhances the activity of the latter. (*Left part*) On the other hand, IFNαR can also activate PI3K via STAT5 and consequently it can stimulate Akt that, in turn, provides survival signals via FKHRL1, BAD, Caspase 9 and NF-kB. STAT1/2 dimers can also activate ERK, but the functional meaning of this interaction is still uncertain. The IFNα-mediated signalling generates also a feed-back mechanism mediated by SOCS1/3 and PTPs (Shp1 and 2) that switch off the signal. → Stimulating activity. ⊣ Inhibiting activity.

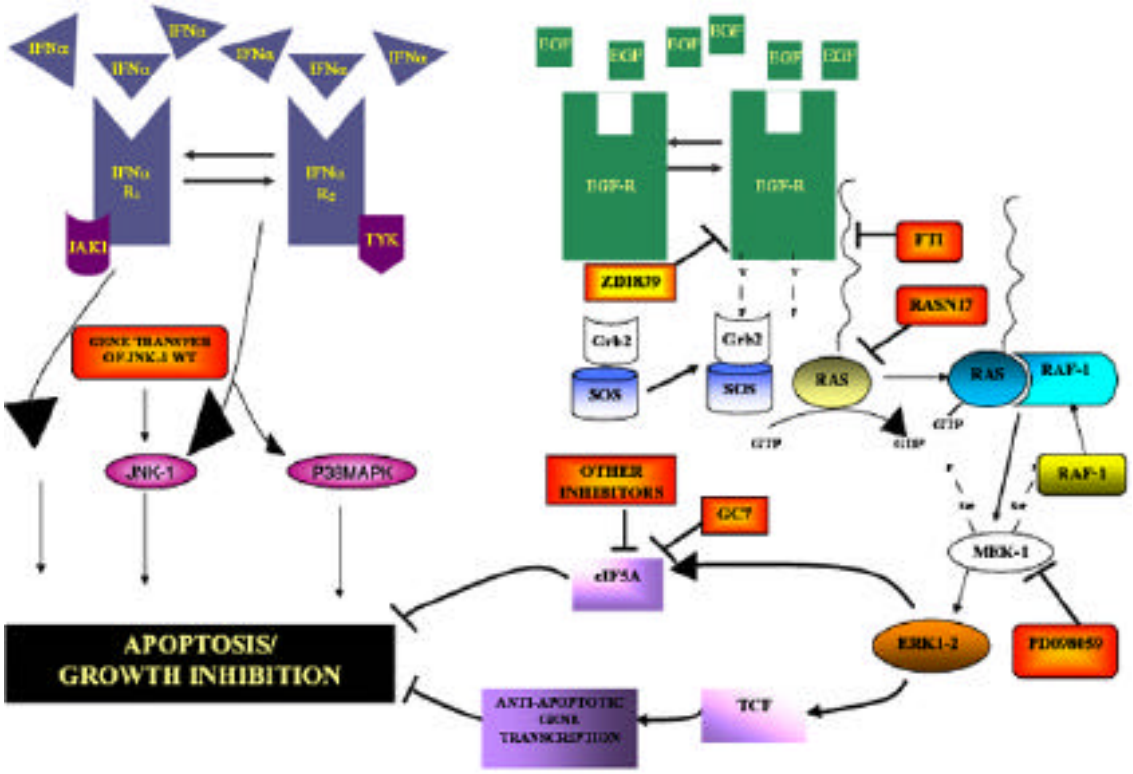

Fig. (2). Escape mechanisms to anti-tumour effects by IFNα and proposed overcoming strategies.

(*Left*) After the engagement of IFNα with its receptor and the consequent activation of the STAT-dependent signalling IFNα induces apoptosis likely through the activation of caspase cascade mediated by JNK-1 and/or p38MAPK stimulation and through the mitochondrial involvement. The final mediators of the effect of the cytokine on caspase and mitochondria are not still known (*Right*) EGF triggers a ras-dependent ERK-1/2 activation that inhibits IFNα−induced apoptosis probably counteracting on caspase cascade activation. Moreover, ERK-1/2 has also a stimulating action on the activity of eIF5A 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 IFNα. Moreover, the selective inhibition of eIF5A with the hypusine synthesis inhibitor GC7 or with other specific inhibitors to be found could enhance the apoptotic properties of IFNα. The inhibition of the feed back mechanisms of the IFNα-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 IFNα. 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; eIF5A, eukaryotic initiation factor of protein synthesis 5A; GC7, GC7, 1-guanyl-1,7-diaminoheptane; JNK-1, Jun kinase-1; p38 MAPK, p38 mitogen activated protein kinase.

long term treatment with IFN has been suggested to sensitize the cells to Fas-induced apoptosis [27,28]. Moreover, The Fas ligand (FasL)/ Fas receptor (FasR) system may mediate effects of IFNα2 in basal cell carcinoma [29]. In fact, after injection of IFNα2 into basal cell carcinomas, FasR and apoptosis were induced, and tumours regressed. However, Chawla-Sarkar et al. have recently demonstrated that IFNβ is a stronger FasL/Fas and apoptosis inducer than IFNα in melanoma cells [30]. Similar data were obtained by Sance Âau et al. in sarcoma cell lines in which IFNβ induces p38 MAPK-mediated Ser 727 STAT1 phosphorylation and apoptosis more efficiently than IFNα [31]. Despite these biological differences the molecular basis of the diversity between IFNα and β in the induction of apoptotic events is still unknown.

Mechanisms of Programmed Cell Death (Apoptosis) Induction by IFNα

The Caspase and Mitochondrial Involvement

The detailed molecular background to IFNα-induced apoptosis remains unclear, but it was recently shown that it involves an ordered activation of caspases and the mitochondrial pathway, for example, cytochrome c (cyt c) release, loss of mitochondrial membrane potential (DC) as well as caspase-9 activation in hematopoietic tumour cell lines [26]. Moreover, Thyrrell et al. have demonstrated, in the same experimental system, that IFNα-induced apoptosis is not inhibited by antagonistic antibodies to the Fas-receptor and, thus, it is a Fas-independent effect [26]. Panaretakis et al. have demonstrated, in a similar experimental model, that IFNα-induced apoptopsis occurs together with the activation

of the pro-apoptotic Bcl-2 related proteins Bak and Bax [32]. In fact, they found that IFNα induces activation of the two proapoptotic Bcl-2 family members, Bak and Bax and showed that apoptotic cells always had high levels of activated Bak, and that the majority of apoptotic cells contained a high level of Bax in its active conformation suggesting their direct involvement in IFNα-induced cell death [32]. However, Bak activation occurred early in the apoptotic response, prior to the cyt c release and loss of DC, whereas Bax activation followed these events [32]. 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 IFNα [33] (see also Table **1**). At this regard, it was reported that IFNα, similarly 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 IFNα induces growth inhibition of other multiple myeloma cells [34] (Table **1**). Taken together these results suggest a mitochondrial involvement in the apoptosis triggered by IFNα.

The Stress Kinase Cascade Involvement

In eukaryotic cells enzymatic isoforms of MAPK, such as Jun kinase-1 (JNK1) and p38 kinase, which 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 [35] and the p38 MAPK (that belongs to the stress-activated kinases) [36-38] (Table **1**). It was recently shown that activation of p38 is required for transcriptional activation of IFN-sensitive genes [36-38]. 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 [38], apparently involving a STAT-independent nuclear mechanism [36-38]. It was, moreover, found that p38 MAPK pathway is engaged in type I IFN signalling in primary human hematopoietic progenitors and its function is required for the generation of the suppressive effects of interferons on normal hematopoiesis [39]. Moreover, p38 MAPK, is involved in the generation of the antileukemic effects of IFNα in break cluster region (BCR)-ABL-expressing cells of acute myeloid leukemia [40]. We have reported that IFNα induces apoptosis and increases the expression of the epidermal growth factor receptor (EGF-R) at the surface of human epidermoid carcinoma cells [41]. We have moreover found that apoptosis induced by IFNα is completely antagonized by EGF and that IFNα enhances the activity of EGF on these cells [42]. 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 IFNα [43]. Indeed IFNα increases the expression of HSP27, HSP90 and HSP70 inducible forms while does not change the levels of the constitutive form of HSP70 [44]. At the same time, IFNα induces an about 5-fold increase of activity of JNK-1 and p^{38}MAPK and the transfection of KB cells with a plasmid encoding for a wild type form of JNK1 ($JNK1^{wt}$) induces per se apoptosis and enhances the apoptosis induced by IFNα [44]. All these effects were antagonized by the addition of EGF to IFNα-treated cells suggesting again a counteracting role of the EGF-R-mediated pathway on the stress-activated IFNα-induced signalling (Table **1**).

A tumour suppressor gene specifically activated after a genotoxic stress is p53. Takaoka *et al.* have recently shown

Table 1. Modes by Which IFNα Induces Apoptosis

General mechanism	Molecular target	Ref.
Mitochondrial involvement		
	FasR/FasL	*126-129*
	Cyt-c release and loss of mitochondrial membrane potential	*125*
	Fas-independent Caspase 9 activation	*131*
	Bak and Bax activation	*132*
Stress kinase cascade		
	P38 kinase and consequent activation of MapKapK-2	*134-137*
	JNK-1 and P38 kinase	*141*
	P53	*143*
	STAT3->NF-kB->Akt	*86*
	- Sensitization to TRAIL - DR5 increase->NF-kB inactivation	*144,145*

that transcription of the p53 gene is induced by IFNα/β, accompanied by an increase in p53 protein level. IFNα/β signalling itself does not activate p53; rather, it contributes to boosting p53 responses to stress signals. In these experimental conditions p53 gene induction by IFNα/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 [45]. The role of NF-kB in the apoptosis induced by IFNα is controversial. In fact, it has been demonstrated that IFNα can activate NF-kB through STAT3 and via phosphatidylinositol 3 kinase (PI3K) and Akt activation in lymphoma cells and promotes survival of human primary B-lymphocytes via PI3K [46]. Other studies demonstrate that IFNα sensitizes human hepatoma cells to TRAIL-induced apoptosis through DR5 upregulation and NF-kB inactivation or suppresses the antiapoptotic effect of NF-kB and sensitizes renal cell carcinoma cells *in vitro* to chemotherapeutic drugs [47,48] (for a summary see Table **1**).

THE PROTEIN SYNTHESIS AS A TARGET OF IFNα 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 49].

PKR

IFNs regulate at the transcriptional level more than 200 gene products that determine their responses. The protein kinase dependent from dsRNA, PKR, involved in protein synthesis regulation, is one of the best studied of these genes. Human PKR is a serine-threonine kinase of 551 amino acid residues, with two dsRNA binding motifs (DRBM) at its N terminus and a conserved kinase domain at its C-terminus [50]. Upon dsRNA binding or by protein-protein interaction with the newly discovered activator protein PACT/RAX,4;5 PKR dimerizes, later autophosphorylates and thus become activated (for a review see 51]. Once activated, PKR phosphorylates exogenous substrates. The best characterized substrate of PKR is the small subunit of the eukaryotic initiation factor 2 (eIF**2)**. PKR inhibits translational initiation by phosphorylating eIF2 on serine [52]. Additionally, PKR also regulates the action of several transcription factors such as NF-kB, IRF-1, p53, STAT1, NF-90 and it has been proposed to phosphorylate DRBP76. Modulation of all of these different targets allows PKR to control diverse cellular processes, such as cell growth [53], differentiation [54] and to exert antitumour activity [55,56]. In addition, PKR is differentially regulated during cell cycle [57] and also induces apoptosis in a manner strictly dependent of its kinase catalytic activity [58]. The biological importance of PKR function is further remarked by the existence of a multitude of cellular and viral inhibitors of PKR action. Among cellular inhibitors of PKR action we can mention p58 IPK, a protein induced during influenza virus infection and by heat shock [59]; the eIF-2-associated glycoprotein p67 [60], and the Tar RNA binding protein (TRBP) [61], a cellular protein that binds to the DRBM of PKR independently of dsRNA.

PKR has been involved in apoptosis induced by many different stimuli. In the case of poxviruses, the stimuli resulting in PKR activation upon its expression from viruses (VV) is most probably dsRNA produced as a result of symmetrical transcription from late genes during VV infection [62].

Evidences pointing to PKR as playing a role in dsRNA-induced apoptosis are also found in studies carried out in 3T3 cells expressing a noncatalytic mutant PKR or using mouse embryonic fibroblasts (MEFs) derived from PKR 0/0 mice [63,64]. Evidences pointing to PKR as playing a role in dsRNA-induced apoptosis are also found in studies carried out in 3T3 cells expressing a noncatalytic mutant PKR or using MEFs derived from PKR 0/0 mice [61,64]. However, it must be noted that dsRNA-induced apoptosis is a complex process involving different pathways [65]. For example, the IFN-inducible 2-5A system, composed of dsRNA dependent 2-5A-oligosynthetases and the RNase L, is involved in apoptosis induction [66,67]. Clearly, both PKR and the 2-5A system can induce apoptosis by independent mechanisms. RNase L is able to induce apoptosis both in PKR +/+ and PKR 0/0 cells and this correlates with RNA breakdown and inhibition of protein synthesis [66]. Although expression of PKR induces activation of RNaseL through an unknown mechanism [68], expression of PKR is still able to induce apoptosis in RNAseL 0/0 cells (Gil *et al.,* unpublished observation). Moreover, studies carried out with cells derived from PKR 0/0 mice have shown that the PKR-defective MEFs are more resistant to apoptosis induced by lipopolysaccharide (LPS), tumour necrosis factor (TNF), and also dsRNA when compared with wild type MEFs [64]. A role for PKR in TNF-α-induced apoptosis had been previously suggested from experiments performed in U937 cells [69]. Other studies using 3T3 cells expressing the noncatalytic trans-dominant negative mutant of PKR (K296P), have also involved PKR in TNF-α-induced apoptosis and in apoptosis induced by serum starvation [63]. Interestingly, IL-3 withdrawal from the IL-3-dependent NFS/N1.H7 cell line, induces PKR autophosphorylation, eIF-2 phosphorylation and also correlates with increased cell death by apoptosis [70], thus suggesting involvement of the PKR pathway in growth factor withdrawal-induced apoptosis. Analysis of the role of PKR targets on mediating apoptosis induction has revealed a complex scenario. In fact, the role of eIF2α, NF-κB and p53 on PKR-induced apoptosis has been implicated by different studies. PKR activation regulates translational and transcriptional pathways (eIF2α and NF-κB-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 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 [71].

The Eukaryotic Initiation Factor-5A of Protein Synthesis (eIF5A)

The eukaryotic initiation factor 5A (eIF5A) belongs to a family of translational fators that are involved in the regulation of cell growth. In fact, it has been recently isolated and characterized a distinct isoform, called eIF5A2, that is often overexpressed and/or amplified in human ovarian and colon cancer cells. $eIF5A_2$ has a 84% sequence identity with eIF5A and, analogously, a function in regulation of both protein synthesis and cell growth has been supposed for it [72-74].

eIF5A and, likely, eIF5A2 are unique because their activities are modulated by a series of post-translational modifications that culminates in the formation of the unique amino acid hypusine. Hypusine [N^{ε}-(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 eIF5A precursor [75] and by the subsequent hydroxylation at carbon 2 of the incoming 4-aminobutyl moiety [76,77]. eIF5A probably acts in the final stage of the initiation phase of protein synthesis by promoting the formation of the first peptide bond [78]. Hypusine plays a key role in the regulation of eIF5A function because its precursors, which do not contain hypusine do not have activity [79]. These biochemical correlates make eIF5A peculiar. In fact, only the hypusine-containing eIF5A form is active and, consequently, the dosage of intracellular hypusine content measures also the activity of eIF5A since hypusine is contained only in this factor. The correlation between hypusine, and thus eIF5A activity, and cell proliferation [80,81] suggests that activated eIF5A might play a role in cell growth and differentiation [82]. More recently a correlation has been found between the polyamine-dependent modification of eIF5A and the triggering of apoptosis in tumour cells [83]. In fact, excess putrescine accumulation in hepatoma tissue culture DH23A/b cells induces apoptosis and suppresses the formation of hypusine-containing eIF5A [83]. Furthermore, we have evidenced an *in vitro* post-translational modification of eIF5A catalyzed by tissue transglutaminase (tTG) [84] 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 [84]. We have reported that IFNα induces growth inhibition and reduction of the activity of eIF5A in human epidermoid cancer KB cells [85]. The activity of eIF5A 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 [85]. Moreover, it has been suggested that eIF5A can function as a cofactor of HIV Rev protein that is related to the control of viral replication [86-88]. An additional nuclear localization of eIF5A has been recently described with the consequent export of the factor out of the nucleus even if the nuclear function of eIF5A is still unknown [89]. At the present, specific inhibitors of hypusine formation that act through the deoxyhypusine synthase inhibition such as N1-guanyl-1,7-diaminoheptane (GC7) and ciclopirox exist. It has been shown that these agents can induce growth inhibition and apoptosis in human cells [90, 91] and, therefore, could be useful in the specific targeting of eIF5A in integrated anticancer therapeutic strategies. All these data support the hypothesis of an involvement of eIF5A, another protein synthesis regulator, in the apoptosis induced by IFNα in human epithelial cells.

ACTIVATION OF PROTECTIVE SURVIVAL SIGNALS BY IFNα: A SELF-LIMITING ACTIVITY PROCESS.

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 IFNα-dependent activation of STAT 3 in human peripheral blood-derived T cells and leukemic T cell line Kit225. In this experimental model the observation that IL-2 and IFNα activate JAK1 to a comparable degree, but only IFNα activates STAT1, indicates that JAK1 activation is not the only determining factor for STAT1 activation [92]. 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 [93, 94].

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 [95]. Thus, STAT3 acts as an adapter to couple another signalling pathway to the interferon receptor: the PI3K [96]. 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 [97]. Based on homology to the PKA and PKC family of protein kinases, Akt was also named protein kinase B and RAC-PK [98]. 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 [99]. Akt apparently promotes cell survival by phosphorylating multiple targets, including the Bcl-2 family member BAD [100], the apoptosis-inducing enzyme caspase-9 [101], and the Forkhead transcription factor (FKHRL)1 that regulates Fas ligand gene expression [102]. The recent results by Yang et al. has shown in lymphoma cell models that IFN activates Akt enzymatic activity and that kinase-dead Akt blocks IFN-promoted NF-kB activation, indicating that Akt is important for IFN-promoted NF-kB activation. Moreover, a constitutively active Akt construct promotes NF-kB activation. These data suggest that the main target of the IFNα–induced Akt activation is NF-kB 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 [46]. Recent data suggest a role of Akt activation induced by IFNα in the regulation of monocyte adhesion [103].

Also STAT5 has been demonstrated to be activated by IFNα in lymphoma and tumour cells [104]. A recent report has implicated STAT5 in the engagement of CrkL in IFN

signalling, 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 both IFNα and β-induced signalling [105]. Recent reports have suggested that STAT5 is involved in IFNα signalling also in myeloid cell lines and HeLa cells [108], and its activation has been observed in response to differentiation and growth arrest signals [105-107].

Controversial data are, on the other hand, available, on the interaction between IFNα and the extracellular signal-regulated kinase-dependent signalling. It was, in fact, found that the serine-threonine kinase mitogen-activated protein kinase (MAPK) [specifically, the 42-kilodalton 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 IFNβ induces tyrosine phosphorylation and activation of MAPK and caused MAPK and STAT1 to co-immunoprecipitate [35]. Furthermore, expression of dominant negative MAPK inhibits IFNβ-induced transcription. Other groups have shown that short-term treatment with IFNα can activate the mitogen extracellular signal regulated kinase (MEK)/ERK pathway [109,110] in haematological experimental models. Romerio et al. have recently demonstrated that long term exposure of leukemic and lymphoma cells to IFNα induces a decrease of the activity of MEK and ERK through a ras->raf-1-independent pathway. Moreover, the addition of a MEK inhibitor (and thus of MAPK activity) increases the growth inhibition induced by IFNα [111,112].

Another molecular target of type I IFN receptor is protein kinase C (PKC) δ, a member of the protein kinase C family of proteins that is activated during engagement of the Type I IFN receptor and, consequently, associates with STAT1. Such an activation of PKCδ appears to be critical for phosphorylation of STAT1 on serine 727, as inhibition of PKCδ activation diminishes the IFNα-or IFNβ-dependent serine phosphorylation of STAT1. In addition, treatment of cells with the PKCδ inhibitor rottlerin or the expression of a dominant-negative PKCδ mutant results in inhibition of IFNα- and IFNβ-dependent gene transcription via ISRE elements. Interestingly, PKCδ inhibition also blocks activation of the p38 MAP kinase, the function of which is required for IFNα-dependent transcriptional regulation, suggesting a dual mechanism by which this kinase participates in the generation of IFNα responses [113]. The complex signal transduction network activated by IFNα is summarized in (Fig. **1**).

Turning Off the Signal: the SOCS Family and the SH2-containing Protein Tyrosine Phosphatases

The family of suppressors of cytokine signalling, SOCS, consists of eight members (SOCS-1 to SOCS-7 and CIS) all sharing a central SH2 domain and a C-terminal SOCS box [114]. These family members have been implicated in the negative regulation of several pathways, particularly the JAK/STAT pathway, and since this signalling pathway is responsible for their induction, they form part of a classical negative feedback circuit. To date, at least three different modulating mechanisms have been demonstrated: 1) through the SH2 domain they bind to phosphotyrosines on the target protein, leading to inhibition of signal transduction by N-terminal inactivation of JAK, 2) by blocking access of STAT to the receptor sites, or 3) by SOCS box-targeting bound proteins to proteasomal degradation [115]. IFNα can specifically induce SOCS1 and 3 that are candidates for the specific inhibition and turning off the IFNα-dependent signalling [116,114].

An additional mechanism of feed-back of the signalling induced by the cytokine is the activation of specific phosphatases that dephosphorylate and inactivate the STAT and IFNα-receptor associated kinases. Several studies showed that inhibition of protein tyrosine phosphatase (PTP) activities can interfere with the IFN-induced Jak/STAT pathway in a positive or a negative manner [117-119]. In particular, two mammalian SH2-containing cytoplasmic PTPs, Shp-1 and Shp-2, have been implicated in the regulation of IFN signalling [120,121]. Enhanced tyrosyl phosphorylation of JAK1 but not TYK2 was observed upon IFN treatment of Shp-1-deficient macrophages isolated from moth-eaten (me) mutant mice, which was accompanied by a significant increase in the amount of IFN-induced STATs bound to the gamma response region (GRR) DNA probe [120]. After IFN stimulation, Shp-1 was reversibly associated with IFNα receptor complex that contains JAK1 and TYK2 as well. Thus, Shp-1 appears to participate in a negative control of distinct components in IFN-stimulated JAK/STAT pathways in macrophages, although the biological significance remains to be determined [120]. In contrast to the predominant expression of Shp-1 in hematopoietic cells, Shp-2 is a ubiquitously expressed enzyme that appears to be involved in multiple signalling pathways downstream of a variety of growth factors and cytokines [122,123]. Shp-2 tyrosine phosphatase functions as a positive regulator in mitogenic stimulation of Erk [124,125]. Furthermore, Shp-2 plays a critical role in the control of cell spreading, migration, and focal adhesion, by working in concert with focal adhesion kinase (Fak) [126]. In another study, Shp-2 was shown to bind the IFN-α/β receptor *in vitro* with a purified glutathione S-transferase fusion protein containing the intracellular part of the receptor. This interaction *in vitro* was not affected by IFN occupation of the receptor [121]. Treatment of cells with IFN induced tyrosyl phosphorylation of Shp-2, and transient expression of a catalytically inactive mutant of Shp-2 had a dominant negative effect on IFN-stimulated luciferase activity under the control of an IFN-stimulated response element (ISRE). These results suggest that Shp-2 might be involved in the IFN-initiated signalling pathway. Moreover, it has been demonstrated that Shp-2 is involved in protection of cells from the cytotoxic effect of IFNs and that Shp-2 acts as a negative effector in mediating the activation of STATs induced by IFNα. More recently, the T cell protein tyrosine phosphatase (TCPTP), involved in hematopoietic maturation, has been identified as physiological inactivator of JAK1 and JAK3 [127].

ESCAPE MECHANISMS TO ANTI-PROLIFERATIVE EFFECTS OF IFNα

Until to day inconsistent data have been obtained regarding the clinical effectiveness of IFNα in the therapy of

solid tumours. In fact, the benefit of IFNα treatment is limited to some neoplasms while others are completely or partially resistant. The mechanisms of tumour resistance to IFNα have been studied in deep *in vitro*. The alteration of JAK-STAT components of the IFNα-induced signalling can be indeed a mechanism of resistance to IFNα. In fact, an old issue that has been associated to the resistance of tumour cells to the biological effects of IFNα is the disruption of its signal transduction pathways based on the altered expression of STAT proteins in several cancer cell types [128-131]. 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 [131]. Analogous effects were demonstrated on myeloid leukemic and renal carcinoma cells [129,130].

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 IFNα induction and activation are not altered between both variants. [132]. DNA microarrays performed on two T cell lymphoma lines (resistant or sensitive to IFNα) showed that resistance to IFNα is consistently associated with changes in the expression of a set of 39 genes, involved in signal transduction, apoptosis, transcription regulation, and cell growth [133]. These results highlight the likely heterogeneity in the mechanisms leading to interferon resistance both in cell lines and tumours.

It has been more recently reported that constitutive expression of SOCS3 confers resistance to IFNα in chronic myelogenous leukemia cells [134].

Cross-talks between different cytokines and the IFNα-dependent pathway have been described *in vitro* and *in vivo*. In fact, it was described that administration of IL-1β *in vivo* attenuated IFN-α-induced STAT1 tyrosine phosphorylation in the liver but not in the spleen of mice [135].

Beside these mechanisms of resistance towards the growth inhibitory and apoptotic activity of IFNα, also the triggering and/or hyperactivation of survival and proliferative pathways can be supposed in cancer cells. This hypothesis is further supported by the evident disregulation of proliferative signalling in transformed cells. In this view, we have reported that IFNα increases the expression and function of the EGF-R at the surface of human epidermoid carcinoma cells [41, 42]. 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 IFNα [43]. Moreover, we have found that the addition of EGF to IFNα-treated KB cells completely antagonized apoptosis induction suggesting that the EGF-R signalling suppresses apoptosis [44]. 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 [205]. Moreover, it has been demonstrated that the EGF-R-dependent pathway controls keratinocyte survival and the expression of the pro-apoptotic bcl-x_L expression through a MEK-dependent pathway [136].

Furthermore, the EGF- and Ras-dependent MAPK cascade is hyperactivated in IFNα-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 IFNα [137]. These findings suggest that the EGF-R function is preserved in IFNα-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 [138-140]. On this basis, we have hypothesized that the up-regulation of growth factor receptors is a common event in growth inhibited tumour cells and could represent a protective response towards the antiproliferative stimuli [43]. Also in the case of 8ClcAMP, the EGF-induced MAPK signalling is amplified likely as a consequence of the increased expression of EGF-R [140]. However, MAPK activity is reduced in 8Cl-cAMP-treated KB cells suggesting a selective inhibition of Erks or of a still unknown upstream activator induced by the drug [140]. The involvement of the Ras->MAPK pathway in the protection of KB cells from the apoptosis induced by IFNα is further demonstrated by both Ras inactivation by RASN17 transfection and MEK-1 inhibition by exposure to PD098059 [137]. 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 IFNα via Erk-1 and 2 activation. In fact, we have demonstrated that Ras-dependent survival signalling targets Erk-1/2 since the reduction of MAPK activity by PD098059 enhanced apoptosis caused by IFNα. An additional important finding is that PD098059 specifically abrogated the recovery from apoptosis induced by EGF in IFNα-treated cells. Therefore, our results suggest that the activation of Ras->Raf-1->Mek1->Erk-1/2 signalling has a prominent role in the anti-apoptotic effects exerted by EGF in epidermoid cancer cells exposed to IFNα providing evidence of the potential benefits of the molecular interference with this pathway [137]. 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 fact, we found that IFNα is able to induce a strong inhibition of eIF5A activity since a reduction of hypusine synthesis is recorded with a parallel increase of eIF5A protein expression. This finding suggests a further reduction of the active fraction of eIF5A (hypusine-containing eIF5A:total eIF5A ratio). On the other hand, when EGF antagonized the apoptosis induced by IFNα a restoration of hypusine synthesis caused by the cytokine and an increase of Erk activity are recorded in cancer cells [141]. The involvement of Erk in the antagonizing effect of EGF is demonstrated by the concomitant addition of the Erk inhibitor PD098059 that, alone, induces apoptosis and reduces hypusine levels and when used in combination with IFNα, synergizes with the latter in inducing such biological and biochemical effects. On the basis of these results we have investigated if eIF5A could be really critical for the biological effects induced by IFNα. In this view, we have used the specific inhibitor GC7 and [142] we have found that this agent synergized with

IFNα in inducing cell growth inhibition and apoptosis suggesting a critical role for eIF5A in the modulation of cell proliferation induced by IFNα in human epidermoid cancer cells [141]. Therefore, the regulation of eIF5A 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 IFNα in cancer cells [141]. Other mechanisms of resistance can be supposed to be based on the intrinsic properties of the IFNα-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, IFNα can activate Akt *via* STAT3 and PI3K and the consequent survival signalling that leads to the activation of NFkB in lymphoma cells [93-97]. Moreover, a hyperactivation of the feed back mechanisms could occur in cancer cells and induce the occurrence of resistance to IFNα.

PERSPECTIVES AND FUTURE DIRECTIONS

More germane to clinical practice is the possibility that IFNα treatment could be improved by the concomitant administration of agents known to enhance JAK-STAT responses; the use of retinoids in combination to IFNα in cancer therapy is a salient example [143,144]. 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 IFNα. On the basis of the involvement of stress kinases in the apoptotic effects triggered by IFNα, experiments are in progress in order to construct viral vectors of JNKs to be used in combination with the cytokine in experimental preclinical models [44]. Moreover, we have also identified in epidermoid cancer cells a specific pathway that is activated in response to apoptotic stimuli induced by IFNα. In details, we have demonstrated that the hyperactivation of ras and Erk pathway dependent from tyrosine kinase activity of EGF-R is a strong antiapoptotic pathway in cancer cells exposed to IFNα [137]. Therefore, the selective inhibition of a suspected target of this pathway could be an interesting strategy in the chemoprevention of human tumour. 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 IFNα in inducing the growth inhibition and apoptosis of several human epidermoid cancer cell lines and is coupled to complete inhibition of ras and Erk activity (A. 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 farnesyl-transferase inhibitors (FTI) could be also considered in order to enhance the antiproliferative action of IFNα (M. Caraglia *et al.*, manuscript in preparation). 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 eIF5A activity could be an additional target in order to potentiate the antitumour efficacy of IFNα. In fact, we have found that the hypusine synthesis inhibitors, and thus eIF5A inactivator, GC7 synergizes with the cytokine in the induction of cell growth inhibition and apoptosis [141]. We have recently performed a computer-based prediction of the three dimensional structure of eIF5A in order to define the structure of the hypusine-containing site [145]. We are now planning a pharmacological screening of drugs with potential eIF5A-inhibiting properties. The inhibition of eIF5A 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 IFNα administration. Another still unexplored possibility is the selective interference with the molecules of the feed-back mechanisms of the IFNα-mediated signalling such as SOCS family proteins and SH2 containing PTPs. In fact, the regulation of the activity of these proteins could allow the correct action of IFNα in tumour cells that, on the other hand, can display a constitutive increased activity of the feed-back signals.

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

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