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PROSPECT

Cytokine Triggered Molecular Pathways That Control Cell Cycle Arrest

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Abstract Recent progress has been made concerning the understanding of the molecular pathways that mediate the growth suppressive effects of inhibitory cytokines. Interferons, interleukin-6 and transforming growth factor-β were investigated in these studies. Cell lines that display growth sensitivity to all three cytokines and growth resistant derivates provided a suitable genetic background to determine whether common or unique post-receptor elements mediate the effects of each cytokine. Three nuclear genes, c-*myc*, RB, and cyclin A were found to be common key downstream targets along the cytokine induced growth suppressive pathways. Genetic and pharmacological manipulations proved that these molecular responses fall into few complementary pathways that function in parallel to achieve the cytokine mediated G0/G1 arrest. New strategies, such as knock out anti-sense gene cloning were developed and they currently provide powerful tools for the isolation of genes along the signaling pathways of growth arrest. © 1992 Wiley-Liss, Inc.

Key words: interferons, interleukin-6, TGF-β, cyclin A, c-*myc*, pRB, G0/G1 arrest

The realization that loss of function aberrations of growth suppressor genes contributes to tumorigenesis led part of recent cancer research interests into the study of the negative aspects of growth control. However, only a few members of this set of growth constraining genes have been identified so far, such as RB, p53, DCC, NF-1, WT-1, erbA, and K-rev-1 [reviewed in 1–3]. While the most fruitful direction has been the cloning and identification of these genes through the study of rare familial cancers, very little has been achieved by using functional approaches mainly due to the initial lack of convenient biological and molecular tools. In our opinion, a breakthrough step in designing new functional approaches stems from the understanding that growth arrest is controlled by extracellular signals and these are often generated by the interaction of diffusible polypeptides with their cell surface receptors [4–6]. Therefore, in analogy to the well-studied growth stimulatory pathways, the main genetic elements associated with negative growth circuits may also consist of receptor generated second messengers and transcription factors that transduce the negative signals of the extracellular polypeptides (designated growth inhibitory cytokines). Thus, the study of the mode of action of cytokines may provide new functional approaches to analyze the intracellular molecular pathways that suppress cellular growth. The intention of this overview is to discuss the potential use of growth inhibitory cytokines to 1) identify new growth suppressor genes and 2) analyze specific interactions with three cell cycle controlling genes and organize the molecular modifications along biochemical pathways that restrain cell proliferation.

Several cytokines are capable of inhibiting the proliferation of certain target cells growing in culture or in in vivo experimental systems. The list consists of a group of structurally unrelated families of secreted proteins. Some of the cytokines in this group, such as interferons (IFNs) (α, β, γ), transforming growth factor-β (TGF-β) (β_1, β_2), tumor necrosis factors (TNF-α, TNF-β), oncostatin M, and amphiregulin [7–12], restrict the proliferation of a large spectrum of target cells. Others, such as interleukin-1 (IL-1) and interleukin-6 (IL-6), function as growth inhibitors in a small number of cell systems [13]. The molecular cloning of these cytokines has been followed by the identification of the respective cell surface receptors but the most challenging unresolved issue remained the molecular basis of their antiproliferative mode of action.

This overview will focus on three main cytokines—IFNs, TGF-β1, and IL-6—and will re-

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late to some of the initial progress that has been recently achieved in several laboratories concerning the identification of the post-receptor genetic elements that transduce their growth suppressive effects. An important issue addressed here is whether different cytokines share common post-receptor growth suppressive elements. Moreover, do these elements generate a single linear cascade of events or alternatively lie along a few biochemical pathways that function in parallel?

DESCRIPTION OF STRATEGIES TO STUDY THE ANTIPROLIFERATIVE MODE OF ACTION OF CYTOKINES

The choice of the appropriate sensitive cell lines to study the molecular mode of the action of cytokines turned out, retrospectively, to be a crucial step in this issue. It was influenced by cell cycle studies in the mammalian and the yeast cell systems that proved the importance of major control points in the G1 phase [14–16]. A corollary of these studies was that growth sensitive cell lines should be chosen according to their ability to arrest in response to the cytokine in the G0/G1 phase of the cell cycle. Several hematopoietic and epithelial cell lines that respond to IFNs, TGF-β1, and IL-6 by that manner have been chosen for the subsequent molecular work. Burkitt lymphoma cell lines [17,18] as well as the HeLa epithelial cell line [19,20] were chosen for studying the IFN mode of action (α + β and γ species, respectively). As for TGF-β1, mink lung epithelial cells and human or murine keratinocytes were mainly investigated [21–24]. Another cell system that was extensively used in these studies was the M1 myeloblastic cell line that develops the G0/G1 specific block in response to IFNs, TGF-β1, or IL-6 [25]. The latter cell system provided the opportunity to compare the post-receptor elements that mediate the effects of three different cytokines within the genetic background of a single cell.

Two different strategies have been developed in recent years in order to identify the genes that lie along the growth suppressive pathways of inhibitory cytokines. One strategy starts from a systematic study of known genes that have a central role (positive or negative) in cell cycle control. In the first stage it is tested whether the expression or the function of those genes is selectively modified by the cytokine in the growth sensitive cells. Next, genetic or drug manipulations that specifically prevent one of the gene responses are made in order to find out whether they partially relieve the cell cycle block thus proving directly causal-effect relationships. Finally, the receptor triggered mechanisms that modify these cell cycle controlling genes are studied and their organization along biochemical pathways determined. Few cell cycle controlling nuclear proteins have been investigated in this manner, among which the c-*myc*, RB, and cyclin A genes will be discussed here in detail.

The second strategy developed for cloning new genes that operate along the signalling pathways of cytokines has been based on functional positive growth selection. This strategy consists of exploiting random gene inactivation by cDNA libraries cloned in anti-sense orientation, combined with exposure of cells to the inhibitory cytokines. The goal is to isolate genes whose inactivation is rate limiting in the transduction of negative growth signals and therefore confer growth resistance to the inhibitory cytokine.

c-*myc* STUDIES

It is well established that IFNs (α, β, or γ), IL-6, and TGF-β1 selectively reduce, within few hours, the c-*myc* mRNA levels in growth sensitive hematopoietic and epithelial cell lines. The IFN/c-*myc* interactions have been extensively studied in the Burkitt lymphoma sensitive cell lines [17,18] in HeLa epithelial cells [19] and in the M1 myeloblastic cells [26]. In the latter cell system IL-6 and TGF-β1 also reduce c-*myc* mRNA and protein leading to comparative studies of c-*myc* responses to different cytokines in a single cell line [25,26]. The main molecular work concerning TGF-β1/c-*myc* interactions has been performed, however, in skin keratinocytes where the most rapid decline in c-*myc* mRNA levels have been detected [23]. In all cases (except for the effect of TGF-β1 in the M1 cells) nuclear run on experiments illustrated that the cytokines reduced the initiation of c-*myc* transcription [17–19,23,26]. The reduction of c-*myc* mRNA by both IFN and TGF-β1 was abrogated by cycloheximide, suggesting that it does not belong to the immediate early gene responses but rather depends on new protein synthesis [19,27]. A systematic screen of the 5′ cis regulatory sequences of the c-*myc* gene responsive to TGF-β1 resulted in the identification of a region located between nucleotides −100 and +71 (relative to P1 transcription site) that is required for the negative responses to TGF-β1 in the keratinocyte cell system [23]. In this particular cell system the TGF-β1 mediated suppression of

transcription from the c-*myc* promoter/chloramphenicol acetyltransferase (CAT) constructs was blocked by the transient expression of genes encoding SV40 large T antigen, adenovirus 5 E1A and E7 of human papillomavirus type 16 [24]. The abrogation occurred by the intact gene products that bind few cellular proteins including the RB protein (pRB), but not by the mutated, binding defective versions of these viral proteins, suggesting that pRB could mediate c-*myc* suppression by TGF-β1 [24]. This was confirmed by transient expression of pRB in keratinocytes that repressed transcription of the human c-*myc* promoter as effectively as TGF-β1 through sequences that lie between positions −86 and −63 [28]. The study of the responsiveness of the c-*myc* promoter to IFNs and IL-6 has so far been focused on other regions at the 5′ flanking sequences of the gene. One mapped to a negative regulatory element (NRE) that lies between position −300 and −350 and contains TRE-like sequences [29]. IL-6 treatment of M1 cells increased jun B mRNA and protein expression and induced Jun binding to the TRE motif [30]. Another element is the E2F motif located between P1 and P2 between positions −65 and −58 (relative to P2 transcription site). Binding of free E2F and E2F complexed with pRB or cyclin A is strongly suppressed by IFN and IL-6 in M1 and Daudi cells [D. Melamed and A. Kimchi, in preparation]. Many tumor derived cell lines failed to reduce c-*myc* in response to IFNs in spite of the presence of functional cell surface receptors [17]. Growth resistant clones also failed to respond by induction of Jun binding to the TRE sequences and failed to reduce the binding of protein complexes to E2F motif [30 and unpublished data]. Moreover, c-*myc* responses were restored in some of those resistant cells by fusing them with IFN sensitive cells. This suggested that the resistant cells carried a recessive type of genetic deregulation that could be complemented in trans in the stable cell hybrids [18]. Together, those different lines of research indicate that the trans-acting negative elements of c-*myc* may be subjected to inactivation during tumorigenesis either by physical interaction with the viral nuclear oncoproteins [24] or by other recessive loss of function genetic aberrations [18].

The second step, once the correlation between growth sensitivity to cytokines and c-*myc* reduction had been established, was to move to causal relationships studies. The goal was to specifically prevent the reduction of c-*myc* expression by introducing into the M1 cells constitutive versions of c-*myc* constructs and to analyze the phenotypic outcome. The c-*myc* coding sequences were expressed from the SV40 early promoter that is not reduced but rather slightly stimulated by IFNs and IL-6 [26]. The expression data performed in many individual stable clones confirmed that the exogenous mRNA levels of c-*myc* failed to be reduced by IFN and IL-6 while the response of the endogenous c-*myc* as well as of other genes continued to take place normally. Thus, a scenario has been achieved where the c-*myc* protein levels in the treated cells remained constitutive (but not overexpressed), without interfering with other molecular responses to IFN and IL-6. A detailed analysis of the growth responses to IFN and IL-6 revealed that the cytokine induced G0/G1 arrest was completely abrogated in those transfected clones [26]. Yet the transfected cells did not display a complete resistant phenotype to IFN or IL-6 and eventually they ceased growth at different points along the cell cycle phases [26]. These aberrant types of growth responses occurred either immediately in the case of IFN or after few extra-cycles of division (at slower rate) in the case of IL-6 [26] and was followed by rapid cell death. These studies established that the reduction in the expression of c-*myc* is essential for generating the G0/G1 specific arrest point by cytokines. They also suggested that the *myc* suppression must operate in combination with other putative cytokine-induced molecular changes since its abrogation was not sufficient by itself to allow indefinite growth in the presence of the cytokine. In another work, M1 cells have been transfected with constitutive β-actin driven c-*myc* constructs and tested for IL-6 responses [31]. The G0/G1 arrest by IL-6 was abrogated and the cells acquired an intermediate phenotype that grew slower in IL-6, supporting again the concept of multiplicity of pathways [31]. As a supplement to these studies it is of interest to mention that in contrast to IFN and IL-6, TGF-β1 continued to reduce the expression of the exogenous c-*myc* mRNA and protein in the *myc* transfected M1 clones at the post-transcriptional level which is the main level that also reduces the endogenous c-*myc* expression by TGF-β1 [A. Yarden and A. Kimchi, unpublished data]. As a consequence, the cell cycle responses of the *myc* transfected M1 clones to TGF-β1 were not interrupted.

Support for the existence of multiple pathways in the mode of action of IFNs also comes from a set of experiments that tested whether protein kinase C (PKC) mediates growth inhibitory responses to IFN (α or β). The approach of depleting cells from PKC activity (the α, β, and γ species) by prolonged exposure to phorbol esters was undertaken. PKC depletion of two different hematopoietic cell lines (Daudi Burkitt lymphoma and M1 cells) was monitored on immunoblots and by direct measurements of the enzyme activity [32]. Desensitization of PKC generated the same partial growth resistant phenotype that was characteristic of the *myc* transfected cells. However, the c-*myc* mRNA and protein responses were not affected at all in the PKC depleted cells, and the extent of c-*myc* reduction by IFN was similar in naive cells and in cells that were pre-exposed to phorbol esters [32]. These data therefore strongly supported the existence of at least two independent molecular pathways that block cell cycle progression in G0/G1, one of which depends on PKC and the other involves the transcriptional suppression of c-*myc*. Each of these pathways is necessary but not sufficient alone to induce the specific block in cell cycle progression and gives rise to an intermediate phenotype that is partially resistant to the cytokine. PKC depletion did not interfere with the TGF-β1 mediated G0/G1 arrest of M1 cells [32] reflecting a second molecular difference between the mode of action of IFN and TGF-β1.

pRB AND CYCLIN A STUDIES

The aforementioned information suggesting that the protein product of the retinoblastoma gene (pRB) may mediate some of the TGF-β1 growth suppressive effects [24,28] prompted further interest in this gene. First, the nature of pRB modification by cytokines was studied by testing possible changes in the phosphorylation state of the protein. This stemmed from extensive work that investigated the cell cycle dependence of pRB phosphorylation and suggested that the underphosphorylated form of pRB may be the functional growth suppressive form [33–38].

The first data on pRB modification by cytokines came from the laboratory of J. Massagué using TGF-β1 and the growth-sensitive mink lung epithelial cells. TGF-β1 prevented the phosphorylation of pRB that takes place at the G1/S boundary [21]. Also in HaCaT human keratinocytes TGF-β1 converted pRB into the fast migrating underphosphorylated forms of the molecules [39]. A crucial question is whether TGF-β1 interferes with pRB phosphorylation as part of its antiproliferative mode of action or alternatively this molecular event is the indirect consequence of the fact that TGF-β1 blocked cells in G1 phase by other mechanisms.

Recent work specifically addressed the issue of causal-effect relationships by using the manipulations described above that destroyed the phase specific arrest either by a single gene replacement (c-*myc*) or by the enzymatic inactivation of PKC. It was found that IL-6 continued to suppress pRB phosphorylation in the *myc* transfected clones with almost maximal efficiency, while the decline in c-*myc* expression was prevented and the cell cycle distribution was not changed [25]. This proved that the modification of pRB by the cytokine occurs in response to the external stimulus and is not a simple reflection of the G0/G1 arrest. Also, these studies illustrated that the suppression of phosphorylation is not influenced by the loss of c-*myc* responses and that the pRB dephosphorylation responses are not sufficient by themselves to induce G0/G1 arrest when c-*myc* expression remains constitutive.

A second manipulation that was then used for the analysis of pRB responses was the depletion of PKC from cells which also abrogates the IFN mediated G0/G1 arrest (see above). A significant portion (50%) of pRB molecules remained phosphorylated after IFN treatment of the PKC desensitized cells, suggesting that part of pRB conversion into the underphosphorylated forms depends on active PKC [25].

M1 cells blocked in S phase by hydroxyurea were then treated with IFNs, IL-6, or TGF-β1. Each of the three cytokines failed to convert the hyperphosphorylated forms of pRB that accumulated in this phase of the cell cycle into the underphosphorylated forms, suggesting that suppression of pRB phosphorylation is phase specific. In contrast, other molecular responses to IFN and IL-6, including the reduction of c-*myc* expression, continued to take place in S phase-blocked cells, suggesting that they develop in the absence of detectable underphosphorylated forms of pRB [27]. Taken together, the selective reduction of c-*myc* expression and the suppression of pRB phosphorylation develop simultaneously and independent of each other in response to growth inhibitory cytokines and both

are required for transducing the specific arrest of cell cycle progression in the G0/G1 phase.

If dephosphorylation activates the function of pRB as a transcription factor, then a spectrum of genes, other than c-*myc*, should exist that will be exclusively modulated (up or down) by the cytokine-induced underphosphorylated pRB forms. Other promoters in addition to c-*myc* have been shown by previous reports to be trans-repressed or activated by the pRB protein in cotransfection experiments such as c-*fos* [40] and the TGF-β_1 [41] promoters. Analysis of the cytokine effects in the genetically and drug manipulated cell systems discussed above should provide convenient tools to identify those downstream target genes. Further, if pRB mediates the rapid suppression of c-*myc* by TGF-β in human keratinocytes [24], then there should exist another unidentified cytokine-induced mechanism that may activate the function of pRB in trans-suppressing c-*myc* without involving phosphorylation-dephosphorylation events. The identification of such possible alternative routes of pRB modification are of great interest, especially considering the association of pRB with the E2F transcription factor [42–45] and the strong reduction in the binding of these complexes to the E2F site within the c-*myc* promoter irrespective of the phosphorylation state of pRB [D. Melamed and A. Kimchi, in preparation].

How do the cytokines reduce the phosphorylation of pRB? One possibility is that the cytokines directly inhibit the activity of the pRB kinase(s). Several recent lines of evidence suggest that the major pRB kinases are the $p34^{cdc2}$ or the products of closely related genes that belong to the cyclin dependent kinases (cdks) [46,47]. The latter could function in complex with cyclin A [48] or with the recently identified G1 cyclins [49–51] to phosphorylate pRB at G1/S boundary and during S and G2 phases. Along this line, each of the cytokines could either inhibit the synthesis, reduce the stability, or induce post-translational inhibitory modifications of the putative subunits that generate the active cdk complexes. Recent work has demonstrated that in the synchronized mink lung epithelial cells, TGF-β1 blocks $p34^{cdc2}$ phosphorylation and H1 histone kinase activity if added before the G1/S phase boundary [22]. Similar interactions have been detected concerning IFN-α in the Daudi cell system [52]. However, it was not possible to distinguish in the aforementioned studies between the direct effect of TGF-β1 or IFN and indirect consequences of the G1 arrest. Recent work showed that IFNs, IL-6, and TGF-β1 sharply reduced the cyclin A protein levels in hematopoietic cell lines [25]. In these studies the manipulations that discriminate between the direct effect of the cytokine and indirect consequences of the G1 arrest (discussed for the pRB) were applied showing that all the three cytokines interacted with cyclin A gene expression. In HaCaT human keratinocytes TGF-β1 reduced cyclin A protein levels [39]. The reduction occurred due to selective inhibition by TGF-β1 of the mRNA levels, and resulted in the suppression of cyclin A directed protein kinase activity measured in immune complexes [39]. The depletion of PKC from cells that partially interfered with pRB dephosphorylation completely rescued the cyclin A responses to IFN [25]. Thus the reduction of cyclin A could be responsible for part of the suppression in pRB phosphorylation, a possibility that is currently examined in a direct approach that is based on genetic manipulations with constitutive versions of the cyclin A gene.

THE POWERFUL TOOL OF GROWTH RESISTANT MUTANTS

Stable cell variants that are growth resistant to the antiproliferative effects of cytokines can serve as powerful tools to further extend the available molecular studies on the cytokine mode of action, providing that the genetic or the epigenetic defect that they carry is at the post-receptor level. The most interesting are the specific cell variants derived from parental cells that display growth sensitivity to a few different cytokines. The latter resistant variants can be tested for their sensitivity to the other cytokines, not selected for, and thus be used for studying the issue of cross resistance between cytokines.

For those purposes, we have recently isolated growth resistant cell variants to IFN (α, β), IL-6, or TGF-β1 from the M1 myeloblastic cells. The clones were isolated by a direct selection, by growing the cells continuously in the presence of each of the cytokines. They appeared at a frequency of 10^{-5} for IFN and 10^{-4} for IL-6 and TGF-β1. The clones chosen for further study displayed a stable phenotype and expressed functional cell surface receptors. The latter was tested by measuring receptor generated molecular events that are not associated with the growth

signalling and choosing the clones that continue to respond normally by those parameters. Such measurements should score for mutations that are located downstream to the very early steps of receptor signalling and therefore could be restricted to the growth pathways of the cytokine. In this respect the IFN resistant clones were tested for the induction of the 2-5A synthetase gene as was done before for Daudi growth resistant mutants [17] leading to the identification of eight positive independent clones that were classified as IFN growth resistant, receptor positive cells (Table I). As for IL-6, five growth resistant M1 clones that continued to respond to the cytokine by induction of the membranal antigens, Fc receptor and Mac1 (characteristic of differentiation towards monocytes that is triggered by IL-6), were identified. From the three TGF-β growth resistant M1 clones that have been isolated, one was definitely classified as carrying functional receptors that transduce some molecular responses, and the two other stable clones that were positive in the radiolabelling assay of TGF-β receptors [55] are being further analyzed (Table I).

The members of each group of cell variants were tested for growth sensitivity to the other two cytokines, not selected for, using different growth parameters including colony formation in semi-solid medium, long-term cell counts of growth in suspension, and cell cycle distribution measurements. As summarized in Table I, no cross resistance was found among the three cytokines, and growth resistant clones to one cytokine continued to display full sensitivity to the other two cytokines. The lack of cross resistance suggests that the genetic or epigenetic changes in all the 16 cell variants affected post-receptor elements that are unique for each cytokine. This further implies that while the three cytokines interact with few common target genes (c-*myc*, pRB, cyclin A) they might use different mechanisms to modify them. Indeed we found, for example, that some of the IFN resistant clones that failed to reduce c-*myc* in response to IFN continued to reduce c-*myc* by TGF-β and IL-6 and vice versa. Finally, it should be mentioned that the cross responses studies led to another interesting observation concerning the growth behaviour of IFN resistant clones in IL-6 (Table I). Those clones displayed an aberrant and unique profile of cell cycle arrest in IL-6 in which part of the cells were blocked in the G2 phase at the expense of the G1 arrest. Since terminal growth arrest in M1 cells involves the autocrine production of IFN-β [53] these data reflect the contribution of the endogenous IFN loop to the system of irreversible growth arrest induced by IL-6.

TABLE I. Lack of Cross Resistance Between Cytokines

Cytokine / M1 clones[a]	IFN (α, β)	TGF-β	IL-6
Parental	S[b]	S	S
IFN^R (8/8)	R[b]	S	S[c]
TGF-β^R (3/3)	S	R	S
IL-6^R (6/6)	S	S	R

[a]The resistant clones were isolated by direct selection in the presence of either IFN (α + β) (200 u/ml), TGF-β1 (0.7 ng/ml), or IL-6 (150 u/ml).
[b]Sensitive phenotype (S) and resistant phenotype (R) were scored according to cytofluorimetric analysis, growth in suspension, and colony formation in semi-solid medium.
[c]Aberrant arrest in G1, G2 phases.

ANTI-SENSE APPROACH FOR CLONING NEW GENES ALONG GROWTH SUPPRESSIVE PATHWAYS

The approach is based on random inactivation of genes by an anti-sense cDNA expression library prepared from sensitive cells followed by direct selection for growth in the presence of inhibitory cytokines. The assumption is that a specific inactivation of a gene along the signalling pathway of a negative cytokine would convey growth advantage in cells that are exposed to the cytokine. This growth advantage is the powerful forward selection that is used to isolate the desired inactivation event from all the other random inactivation events. We have developed for this project an expression vector (pTKO-1), a derivative of EBV shuttle vector, that replicates as autonomous episome and therefore can be easily rescued from transfected cells [20]. The vector gives rise to a high frequency of stable transfectants thus allowing expression of the entire cDNA library in a single transfection process. The transcription unit that drives the cDNA expression was designed to give high expression levels in the continuous presence of IFNs [20]. The first system that was chosen consisted of HeLa cells and IFN-γ taking advantage of the high growth sensitivity of these cells to this cytokine. The cDNA library was prepared from a mixture of RNAs harvested before and at different time points after IFN-γ (to include both

constitutive and IFN induced mRNAs) and was cloned in anti-sense orientation in the EBV expression vector. After transfection the cells were subjected to the selection drug (Hygromycin B) in combination with IFN-γ treatment. Stable transfectants that grew in the presence of IFN-γ were pooled, and the episomal DNA fraction was further rescued from these cells and shuttled into bacteria. From the first group of 20 bacterial clones a single episome that conferred growth resistance to IFN-γ was isolated and tested for its biological function. In cells transfected with a control clone the background of cells growing in the presence of IFN-γ was very low; it increased by a factor of 100–1,000 in the cells that were transfected with the isolated anti-sense cDNA. The insert was 350 bp in size and hybridized to a single mRNA transcript, 500 bp long, present in HeLa cells and in human keratinocytes in constitutive levels [20]. Sequence analysis revealed an open reading frame of 300 nucleotides that completely matched to the human thioredoxin gene [20].

Thioredoxin is a dithiol reducing enzyme that contains highly conserved active cysteine disulfide rings composed of two cysteines separated by proline and glycine amino acids. It functions as hydrogen donor in many regulatory processes including the stabilization of glucocorticoid receptors and increase in the binding activity of Jun/Fos transcription factors to the AP-1 site [54]. It is concluded that the inactivation of this gene is rate limiting in the transduction of IFN-γ signals, and as a consequence the cells lose their antigrowth sensitivity to the cytokine. Dithiol reduction processes therefore may play important role in the IFN-γ signalling of growth arrest. Current attempts are aimed at identifying the specific genes that lie along the studied pathways and might be modified by thioredoxin. Obviously the method will be used for the isolation of additional post-receptors elements of IFNs as well as of other cytokines, and in the broader sense for the isolation of genetic elements that transduce any other extracellular negative signals such as contact inhibition or cellular senescence.

CONCLUSIONS AND FUTURE DIRECTIONS

It is clear that the identification of the genes that transduce the cytokine effects and lead to G0/G1 arrest is far from being completed. However, the limited information available so far excludes a simple possibility that those genes form a single linear cascade of events that couples the cell surface receptors to the specific block in cell cycle progression. Studies support the existence of multiple pathways and organize the involved genes into different complementation groups. For example, the pathway that turns off the expression of a key growth accelerator gene, c-*myc,* complements at least one additional wave of signalling that interferes with the phosphorylation and probably activates the function of another nuclear protein, pRB. Moreover, the pRB responses may reflect part of a broader process that involves direct interactions between growth inhibitory cytokines and components of the cyclin dependent kinases (cdks). This possibility is exciting considering the information on the multiple mechanisms that mediate the G1 arrest by mating pheromones in the budding yeast which all interfere with the expression of the G1 cyclins (CLNs) [16]. The reduction by the cytokines in cyclin A protein levels as well as the inhibition of $p34^{cdc2}$ phosphorylation support such a possibility and may represent the first halmark of cytokine/cdk interaction.

Another important conclusion from these studies is the existence of redundancy in the mechanisms that modify the key genes that have been studied so far. All the three cytokines, IFNs, TGF-β, and IL-6, interact with the same target genes, c-*myc,* pRB, and cyclin A. Several lines of evidence indicate that they may use different ways to modify these genes within a given cell. The lack of cross resistance between cytokines in the 16 M1 resistant cell variants analyzed so far supports this notion. Also, the initial molecular analysis shows, for example, that while IFN and IL-6 reduce c-*myc* expression in M1 cells at the transcriptional level, TGF-β1 induces in the same cells post-transcriptional mechanisms to reach the same goal. In contrast, transcriptional mechanisms are triggered by TGF-β1 in keratinocytes [23] while in another IFN sensitive clone of Daudi Burkitt lymphoma post-transcriptional mechanisms reduce c-*myc* expression [56]. Thus, the redundancy in mechanisms relates also to the mode of action of a single cytokine and the choice of which mechanism will be used probably depends on the genetic background of the treated cells.

The redundancy in mechanisms modifying the same key genes and the dependence of the G0/G1 arrest on few complementation groups of genes might prevent from a single genetic deregulation to confer complete growth resistance to the

negative cytokines. Since the cytokines mentioned in this review represent some of the physiological modulators that dictate tissue growth arrest when necessary [4–6] redundancy and multiplicity of pathways may provide a safety barrier against complete loss of negative control by a single genetic hit. The oncoproteins carried by the adeno- and papovavirus groups should evolve to knock out a few parallel pathways in order to confer complete growth resistance to negative signals and the repertoire of cellular proteins that they bind could reflect various key elements that each belong to a different negative signalling pathway. The latter suggestion was supported by a recent report that used a combination of different E1A mutants defective in their ability to bind one of more of the four characterized cellular proteins. While complete growth resistance to TGF-β was obtained by the wild type E1A gene that binds pRB, p107, p60 cyclin A, and p300, the E1A versions that bound only part of those proteins conferred partial growth resistance to TGF-β [57].

The main future challenge in this field is to continue identifying the upstream genetic elements that couple receptors to c-*myc,* cyclin A, and pRB responses and study the downstream effectors of these genes. In parallel, additional complementation groups of genes that contribute to the G0/G1 arrest should be screened. The anti-sense approach described here, as well as the potential use of the growth resistant cell variants to rescue the genetic deregulation that caused resistance, are two directions that undoubtedly will contribute to these issues in the future.

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