The pharmacology of cell differentiation
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Mechanisms of action of p53

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Introduction

The p53 gene and its protein product, p53, have been the subject of intensive research over the last few years (for recent reviews see 1-7). These studies have indicated that p53 is a tumor suppressor whose inactivation may be a crucial event in the development of many types of human cancer. Alterations in the p53 gene have been observed in a large variety of cancer cells, of both human and rodent origin. In fact, the p53 gene now appears to be the most frequent target for genetic alterations in human cancer; as many as 50-70% of all individual human tumors may carry some sort or another of p53-related aberration. Most often, this entails the acquisition of point mutations within the protein coding region of the p53 gene, giving rise to the production of a mutant polypeptide. Such mutant p53 proteins are generally believed to have become functionally defective. Thus, the main purpose of these mutations is most probably to abrogate tumor suppressor activities inherent to the wild type (wt) p53 protein. Yet, certain mutations may also provide the resultant protein with overt oncogenic properties (see 8 for detailed discussion). In addition to point mutations, there are a number of other genetic mechanisms whereby wt p53 expression may be abolished in the course of tumor progression. These include gross deletions in the p53 gene, sometimes eliminating almost an entire copy of the gene, splice-site mutations which can lead to the synthesis of severely truncated or internally deleted proteins, or events which lead to the absence of any detectable p53 transcripts, presumably through promoter inactivation. In addition, cells may be deprived of wt p53 activity through the expression of other proteins which affect the functionally or the stability of the p53 polypeptide. The best studied examples involve proteins encoded by various DNA tumor viruses, which specifically target wt p53 (reviewed in 1,3). Recently, it has become apparent that wt p53 activity may also be quenched through the action of other cellular proteins, such as the one encoded by the putative oncogene mdm2 (9,10). Moreover, certain tumor cells may override the activity of resident wt p53 by preventing it from entering the nucleus (11), the presumable site of its normal action; the underlying molecular mechanism is as yet unclear.

Strong experimental support for the notion that p53 is a tumor suppressor product has been provided by the ability of wt p53-encoding expression plasmids to inhibit oncogene-mediated transformation *in vitro* (12,13), as well as to abrogate the tumorigenicity of a number of different cell lines in experimental animal models (14-17). Attempts to define the biological and biochemical activities which underlie the ability of wt p53 to act as a potent tumor suppressor have been carried out in a large number of laboratories. These studies typically involved the use of p53-specific expression vectors, and

have led to a much better understanding of the normal functions of wt p53 and of the significance of the p53 mutations encountered in tumor cells.

p53 can block cell cycle progression.

In most cell types studied so far, the most pronounced effect of the forced expression of wt p53 was the induction of a growth arrest. This was seen not only in mammalian cells (18-22), but even in yeast cells that had been made to express wt human p53 (23,24). Typically, the arrested cells exhibited a G1 DNA content. Thus, the expression of wt p53, at least at the relatively high levels obtained in most experiments, has a potent anti-proliferative effect in cells which can respond to the appropriate signals. The data further suggest that p53 acts preferentially at a specific point in the cell cycle, presumably in late G1 or at the G1 to S transition.

p53 can induce apoptosis independently of a G1 arrest.

While the forced expression of wt p53 in transformed cells often gives rise to a growth arrest, this is not always the case. A very different situation was, in fact, encountered when such a genetic manipulation was attempted in cells of the M1 line, clone S6 (25). These cells originate in a murine myeloid leukemia, and are devoid of any detectable p53 expression. One of the distinctive features of these cells is their ability to undergo differentiation into monocytes upon incubation in the presence of interleukin 6 (IL-6). M1 clone S6 cells were transfected with an expression plasmid encoding a temperature-sensitive (ts) mutant of murine p53, p53val135. The p53val135 polypeptide has properties indistinguishable from those of other p53 mutants when the cells are kept at the restrictive temperature of 37.5°C (21,22,26,27). However, upon exposure to the permissive temperature of 32.5°C, the protein assumes properties very similar to those of authentic wt p53. This activation process entails a conformational switch, which can be monitored through the use of conformation-specific monoclonal antibodies against p53 (28). In fibroblasts, the consequence of this activation is the induction of a growth arrest, which is in line with the effects of authentic wt p53 in many other cell types. However, when M1 cells stably transfected with the ts p53 were shifted to 32.5°C, the outcome was more dramatic. Rather than entering a reversible growth arrest, the cells rapidly lost viability; typically, most of the population died within 24-48 hours of being shifted down to the permissive temperature (25). The process responsible for the death of these cells had the characteristics of apoptosis. This was reflected in the morphology of the dying cells (Fig. 1), which included prominent chromosomal condensation, a reduction in cell volume, and subsequently the fragmentation of the nucleus and of the cells as a whole. In addition, internucleosomal fragmentation of the cellular DNA, manifest as a characteristic DNA "ladder", became evident rather shortly after the activation of the transfected p53 at 32.5°C (25; Yonish-Rouach et al., submitted). Hence, in these leukemic cells wt p53 activity gave rise to apoptotic cell death rather than to a growth arrest. Notably, IL-6 greatly inhibited the apoptotic effect of p53 in this system (25).

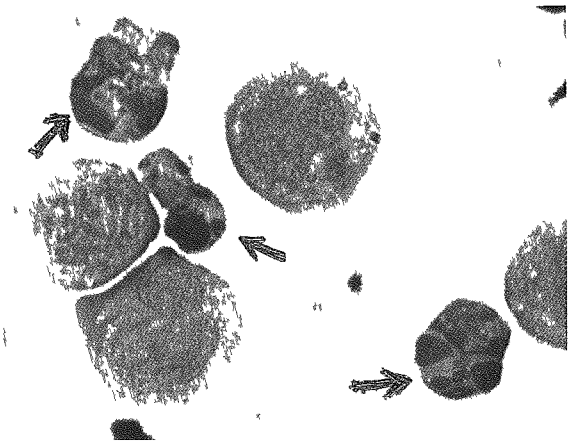

Figure 1. Morphological changes induced by wt p53 activity in M1 cells. M1 clone S6 cells were stably transfected with a plasmid encoding the ts p53 mutant p53val135. Cells of a clone expressing the ts p53 protein were shifted to 32.5°C for 12 hours, at which time they were fixed and stained. Arrows indicate cells with typical apoptotic morphology, which includes a reduction is size and the formation of areas of condensed chromatin in the nucleus.

In a number of factor-dependent myeloid cell lines, induced to undergo apoptosis upon withdrawal of the essential factor, cell death is preceded by a G1 growth arrest (29,30). It could therefore be speculated that such cells are programmed to enter the apoptotic pathway once arrested for a sufficiently long period in G1. The observed lethal effect of wt p53 in M1 cells could then simply be due to the induction of a prior G1 arrest, without implying any direct relationship between p53 and apoptosis. To address this concern, we looked at the effects of wt p53 activation on cell cycle progression in M1 cells. When the cell cycle distribution pattern of M1-p53 transfectants was monitored at various times following the shift-down to 32.5°C, it was found to be indistinguishable from that of the same cells maintained at 37.5°C; this was true even at late times after the activation of the transfected p53, when the cells were already in advanced phases of apoptosis. Furthermore, the cells were found to be engaged in ongoing DNA synthesis, as assessed by the measurement of tritiated thymidine incorporation as well as by fluorocytometric determination of bromodeoxyuridine incorporation (Yonish-Rouach et al., submitted). These observations indicated that the activation of wt p53 did not cause any measurable growth arrest, and that cells were still capable of going into the S phase even when the apoptotic process was well on its way. To establish more firmly that there was no growth arrest, M1-p53 cells actively proliferating at 37.5°C were subjected to centrifugal elutriation. This allowed the isolation of sub-populations at different phases of the cell cycle. Each population was then shifted to 32.5°C, and cell cycle parameters were monitored at various times after wt p53 activation. It was found that, with increasing incubation periods

at the permissive temperature, cells initially isolated as being in G2/M continued their transit through G1 and into S without any apparent arrest. The same was true also for cells corresponding to other phases of the cycle at the time of elutriation. Thus, wt p53 activation can lead directly to apoptosis, without any obvious coupling to a growth arrest. These data strongly argue that p53 may have a direct role in the regulation of cell survival.

How does wt p53 activation elicit an apoptotic response? It is generally believed that myeloid progenitors, from which M1 cells are derived, are constantly dependent on specific factors for survival in the body. In the absence of such factors these cells will die, presumably through an apoptotic process. This notion gains support from many studies in which myeloid progenitors were shown to undergo apoptosis in response to survival factor withdrawal *in vitro*. It is therefore tempting to speculate that p53 is somehow required for maintaining a tight dependence on such survival factors. Consequently, when wt p53 expression is abrogated (as is the case in M1 cells), the cell will gain the ability to survive illegitimately in the absence of these factors. Once wt p53 activity is restored, the cells will become again factor dependent for survival, and will undergo apoptosis unless provided with an appropriate factor. The fact that IL-6 can spare M1 cells from p53-mediated apoptosis (albeit only for a limited period) argues in favor of this conjecture. Yet, IL-6 is not considered a physiological survival factor for myeloid progenitors, whereas IL-3 is. Unfortunately, M1 cells normally fail to express receptors for IL-3, and it was thus not surprising to find that IL-3 could not rescue them from p53-mediated death (data not shown). However, M1 cells can be made to express IL-3 receptors upon prolonged exposure to IL-6 (31). We therefore asked whether IL-3 could act as a protective agent in this system once expression of its cognate receptor was turned on. To that end, M1-p53 transfectants were incubated for 3 days at 32.5°C in the presence of IL-6. Viable cells were then purified over a ficoll gradient, and placed again at 32.5°C in the presence of various cytokines. In the absence of any added factor, the cells rapidly lost viability. Hence, the requirement for a survival factor is still maintained even after extended incubation with such factor. As expected, the readdition of IL-6 again spared a significant fraction of the cells from p53-mediated cell death. More importantly, a comparable degree of extended survival could now be elicited also by IL-3. Thus this cytokine, known to act as a potent survival factor for normal and non-leukemic myeloid progenitors, could also provide a similar function to myeloid leukemic cells exposed to the action of wt p53. The latter finding strengthens the conjecture that wt p53 may indeed play an important role in maintaining the dependence of myeloid progenitors, and perhaps additional cell types, on survival factors. Experiments aimed at confirming this hypothesis in a more physiological context are currently underway.

Transcriptional regulation by p53.

The fact that p53 is essentially a nuclear-acting protein, possessing structural features of a transcription factor, is consistent with the notion that it may act through regulating the expression of one or more genes which are directly involved in cell cycle progression. We therefore looked at the effect of p53 activation at 32.5°C on the expression levels of a number of endogenous

genes in cells overproducing the ts p53. Indeed, it could be demonstrated that both c-*fos* (32) and c-*myc* (Fig. 2) expression were down-regulated by activated wt p53. In order to find out whether the effect of p53 was exerted at the transcriptional level, various cell types were transiently co-transfected with a p53 expression plasmid and a reporter plasmid in which the bacterial CAT gene was placed under the control of various promoters. These experiments demonstrated unequivocally that wt p53, but not various biologically inactive mutants thereof, can repress the transcriptional activity of many of the tested promoters (32). Similar findings were reported by a number of other laboratories (33-36).

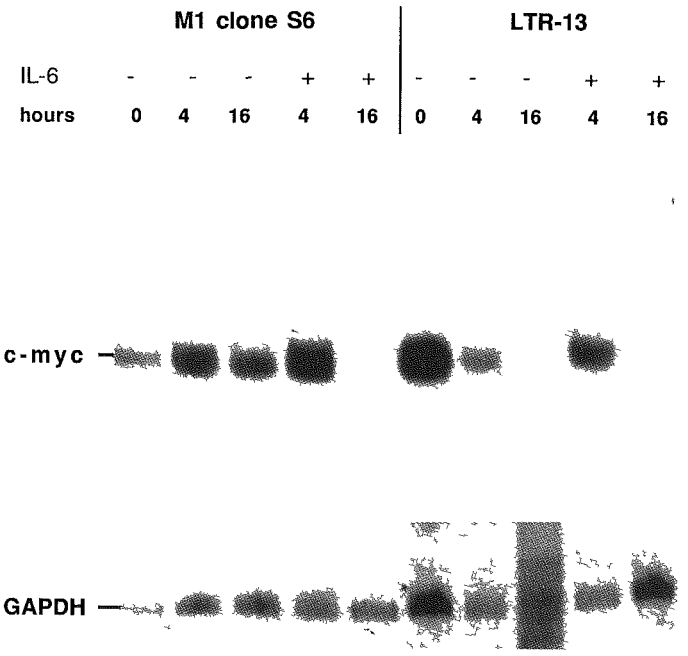

Figure 2. Effect of wt p53 overexpression on c-*myc* mRNA levels. Cells of the parental (non-transfected) M1 clone S6 or of clone LTR-13, derived by transfection of M1 cells with the ts mutant p53val135, were maintained at 32.5°C for the periods indicated above each lane. When indicated, IL-6 was added to the culture medium at the time of the shift down to 32.5°C. Cytoplasmic RNA was extracted at each of the time points, and 20 μg of each preparation was subjected to Northern blotting and sequential hybridization with a mouse c-*myc* probe and a mouse GAPDH probe.

The kinetics of repression of the endogenous c-*myc* (Fig. 2; Yonish-Rouach et al., submitted) and c-*fos* (32) genes in cells harboring the ts p53 was relatively rapid. This could imply a direct effect of p53 on transcription. Equally, it could be a secondary effect of growth inhibitory processes, initiated rapidly after induction of wt p53 activity. To distinguish between these two mechanisms, we tested whether purified p53 protein could repress transcription in a cell-free system. To that end, we chose the c-*myc* promoter as a target, owing to the rapid kinetics of c-*myc* shut-off observed in ts p53-containing cells. Highly purified wt p53, generated in insect cells infected with a recombinant p53 baculovirus, was added to an *in vitro* transcription system programmed to transcribe from the c-*myc* promoter. With increasing concentrations of p53, there was a marked reduction in the amounts of c-*myc* transcripts made. The inhibitory effect of p53 was abolished by heat-inactivation, and was not observed with a similarly processed preparation from insect cells infected with a non-recombinant baculovirus (Ragimov et al., submitted). Furthermore, kinetic studies indicated that the transcriptional effect of wt p53 is exerted at the stage of assembly of the pre-initiation complex. Once the complex has been formed, addition of p53 has no effect. Thus, in this system p53 interferes with the pre-initiation step rather than with the polymerization process itself. One possible way in which this could be achieved is through interaction of p53 with some general transcription factor, such as TFIID. Experiments to test this notion are in progress.

The ability to repress the expression of genes required for ongoing cell cycle progression, such as c-*myc*, could provide an obvious explanation for the anti-proliferative effects of p53. Yet, there is a major drawback to this interpretation, which is the fact that p53 seems to repress a large number of promoters, some of which bear no clear relevance to growth control (32-34). One therefore wonders whether the observed effects may not simply constitute an artefact of overexpression, resulting in squelching of an essential general transcription factor in a rather non-specific manner. A possible way out of this problem could be by demonstrating that certain genes are much more sensitive than most others to transcriptional inhibition by p53. Specifically, one would expect such genes to be repressed in their native chromosomal configuration by the expression of relatively physiological concentrations of wt p53. To our best knowledge, such data is still missing. Hence, the relevance of this type of transcriptional repression for the normal tumor suppressor activities of wt p53 remains questionable.

An alternative mode of transcriptional regulation by p53, described recently, appears perhaps more relevant. Over the last year, a number of laboratories have reported the identification of DNA elements to which p53 can bind in a sequence-specific manner (37-41). It should be noted that, under the same conditions, the c-*myc* promoter failed to display any specific binding to p53 (A. Zauberman, unpublished observations). When tested in appropriate *in vivo* and *in vitro* assays, these p53-binding elements could indeed be shown to confer upon promoters the ability to be transcriptionally activated by wt p53 (40-43). These observations now make p53 appear as a *bona fide* transcription factor, which can activate transcription in a sequence-specific manner. It is thus very plausible that, if p53 indeed exerts its tumor suppressor function through transcriptional regulation, the pertinent targets ought to be looked for

amongst the genes which respond to p53 through DNA elements containing specific binding sites for this protein. So far, no such target has been clearly identified. The muscle creatine kinase (MCK) gene does contain a p53 binding region about 3Kb upstream to its transcription initiation site, and this region can confer the ability to be up-regulated by wt p53 (41,44). However, it is not clear whether MCK has anything to do with the biological activities of wt p53.

An unexpected potential target gene for positive regulation by wt p53 -the mdm2 gene- has recently been identified in our laboratory. The mdm2 gene was initially cloned from a highly tumorigenic derivative of mouse fibroblasts, and was subsequently shown to act as a potential oncogene *in vivo* (45). More recently, the protein product of the mdm2 gene was found to associate tightly with p53 (9). Overexpression of mdm2 effectively interfered with the biological activity of co-expressed wt p53, as measured in a transcriptional activation assay (9). The significance of this interaction was highlighted by the realization that certain human tumors contain amplified mdm2 genes, which apparently allow those tumors to undergo malignant progression without any need for p53 gene mutations (10).

In an independent search for p53-binding cellular proteins, we identified a 95 kD polypeptide (p95) which was preferentially bound to p53 in cells overexpressing wt p53 (46). In fact, p53-p95 interactions were hardly detectable in cells expressing the ts mutant p53val135 at 37.5°C, whereas ample quantities of p53-p95 complexes were found in the same cells maintained at 32.5°C. This suggested that the p53-p95 association was tightly linked to the presence of activated wt p53, and perhaps played a role in the biological effects elicited by p53. In view of the identification of mdm2 as a p53-binding protein, and given the similarity between the reported apparent molecular masses of mdm2 (85-90kD, ref. 9) and of p95, it seemed quite likely that the two were identical. To address this possibility, antibodies were produced against mdm2 protein expressed in E. coli, and tested against extracts from various p53-transfected cells. The results demonstrated unequivocally that p95 was indeed a product of the mdm2 gene (Barak et al., submitted). Moreover, it was found that mdm2 protein levels were highly elevated in cells overexpressing wt p53 activity (ts p53 at 32.5°C), while hardly any mdm2 protein was present in the same cells at 37.5°C. On the other hand, a temperature down-shift failed to induce mdm2 expression in cells overexpressing a non-ts p53 mutant. Thus, the preferential detection of p53-mdm2 complexes at 32.5°C was simply due to the presence of much higher levels of the mdm2 polypeptide at the permissive temperature. In fact, virtually all the cellular mdm2 protein was bound to p53 in such cells.

Analysis of cellular mRNA revealed that the enhanced mdm2 protein levels at 32.5°C were caused by a marked elevation in the concentration of mdm2 transcripts. The increase in mdm2 mRNA levels was a rapid consequence of wt p53 activation, and could already be observed within the first two hours following the temperature down-shift. Moreover, this effect of overexpressed wt p53 could not be prevented by cycloheximide (Barak et al., submitted). Hence, no *de novo* protein synthesis is required. These findings strongly suggest that the induction of mdm2 expression is achieved by p53 directly, rather than through the activation of repression of another gene which serves as a mediator in this process. So far, we still do not have formal evidence that the

effect of p53 on mdm2 expression is at the transcriptional level; this is partly due to the fact that the mdm2 promoter has not been identified yet. Nevertheless, the data strongly suggest that the mdm2 gene is a target for specific up-regulation by wt p53, either transcriptionally or post-transcriptionally. Whether this reflects a negative feedback mechanism, intended to ensure tight control of p53-mediated signal transduction, or whether the resultant p53-mdm2 complexes possess any distinct biochemical activity, is presently a subject for further investigation.

Inactivation of wt p53 through a dominant negative mechanism can lead to transformation.

Various mutant forms of p53, of the types encountered frequently in tumors, can exert a potent oncogenic activity *in vitro*. The system that has been employed most extensively to demonstrate such oncogenic activities of p53 makes use of primary rat embryo fibroblasts (REF). When transfected with plasmids encoding mutant p53 alone, such cells can undergo immortalization; in combination with *ras*, mutant p53 overexpression causes REF to become transformed and tumorigenic (reviewed in 1,8,47). A variety of earlier observations have raised the possibility that the transforming potential of mutant p53 in these and other systems involves a dominant-negative mode of action (reviewed in 1,3,6,8). In this scenario, mutant p53 is believed to interfere with the normal biochemical and biological functions of the endogenous REF wt p53, thereby rendering the cells functionally p53-deficient. It was found that the resultant transformants contain complexes in which their endogenous wt p53 is associated with the transfected mutant p53. This has suggested that the negative dominance is achieved through the formation of functionally impaired oligomers, even though the precise nature of the functional lesion remained unknown.

To address this hypothesis more rigorously, we asked whether the mere formation of mixed oligomers between wt p53 and a non-functional counterpart was sufficient to cause REF transformation. To that end, a series of expression plasmids were generated, encoding short "miniproteins" comprising various overlapping fragments of the C-terminus of p53. It should be noted that these miniproteins were derived from wt p53, and did not contain any internal point mutations. The reason for choosing this strategy was the previous demonstration that the C-terminus of p53 is important for the formation of homo-oligomers (47,48). The ability of each miniprotein to oligomerize with wt p53 was tested by co-translating each pair in a reticulocyte lysate cell free translation system. Each miniprotein was then overexpressed in REF, in combination with various oncogenic plasmids.

The results (Fig. 3) indicated that a number of different miniproteins could efficiently transform REF when expressed in concert with activated *ras*. Moreover, the same miniproteins could also significantly enhance the ability of a combination of *myc* and *ras* to transform REF (49). In both assays, the miniproteins actually transformed more efficiently than full length, tumor-derived mutant mouse p53. Most importantly, a miniprotein containing a small internal deletion could neither bind to wt p53 nor exert any transforming effect on REF. The data is summarized in Fig. 4. Hence, small fragments of p53 can be highly transforming, as long as they can form tight associations

with co-expressed endogenous wt p53. These data strongly support the conjecture that the formation of inactive mixed oligomers is indeed sufficient for effectively making cells "desuppressed", through the abrogation of the activity of the resident wt p53 in these cells.

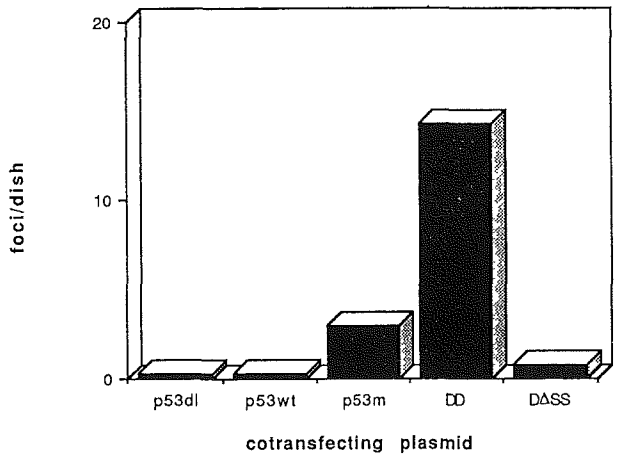

Figure 3. C-terminal p53 miniproteins can cooperate with *ras* in the REF cotransformation assay. REF were transfected with a combination of plasmids encoding mutant Ha-ras and each of the indicated p53 species. Transformed foci were scored 12 days later. p53dl- vector control (no p53 made); p53wt- wt p53; p53m- p53 mutant derived from Meth A cells; DD and DΔSS- C-terminal p53 miniproteins (see Fig. 4 for details).

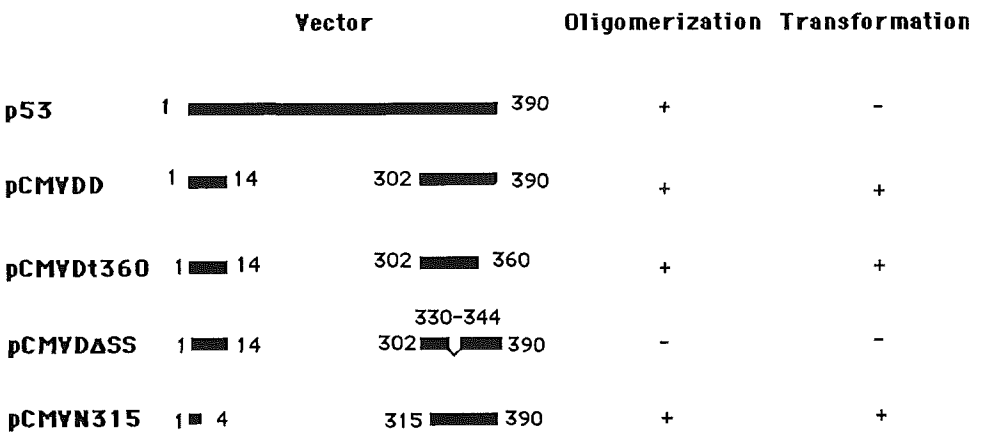

Figure 4. Schematic description of the various p53 miniproteins and their activities. Numbers indicate amino acid positions of mouse p53. For further details see ref. 49.

It was of importance to try to identify the biochemical activities of wt p53 which are lost upon formation of such mixed oligomeric complexes. In view of the reports that p53 possesses a sequence-specific DNA binding activity, and that the loss of this activity seems to be the most common feature of all tumor-derived p53 mutants tested thus far (37-40), it was attractive to speculate that the miniproteins may prevent wt p53 from engaging in such sequence-specific DNA interactions. Therefore, we tested whether the presence of various miniproteins affected the ability of wt p53 to bind a defined specific DNA target. This was done with *in vitro* translated proteins as well as with extracts from cells harboring each miniproteins together with endogenous wt p53. As a specific DNA target, we utilized either the element previously described by Kern et al. (38), or one recently isolated in our laboratory from a mouse genomic DNA library (A. Zauberman et al., in preparation). In all cases, it was found that the overexpression of oligomerization-competent miniproteins greatly reduced the ability of co-expressed wt p53 to interact successfully with target DNA (49). On the other hand, the internally deleted miniprotein p53DDSS, which was incapable of forming mixed oligomers (Fig. 4), also totally failed to interfere with this activity of wt p53. The strict correlation between the abilities to oligomerize, to prevent sequence-specific DNA binding by wt p53 and to transform REF, strongly argues that transformation by biochemically inactive p53 (miniproteins or full length mutants) can occur through a dominant negative mechanism, involving the formation of DNA binding-incompetent oligomers. This has important implications for the role of p53 mutations in the development of cancer.

Conclusion.

The p53 gene now appears to be a key player in cancer. The growing interest in p53 has led to a very rapid increase in the understanding of its biology and biochemistry, and this rapid rate of progress is likely to continue at least within the near future. The main conclusions so far have been that p53 is likely to act as a sequence-specific transcription factor, and that it may be involved in the regulation of cell cycle progression, differentiation and cell death, at least in transformed cells.

All these conclusions need to be evaluated in the context of two seminal p53-related findings made in the course of the last year. One of these findings is that mice can undergo apparently normal development without any p53. Thus p53 "knock-out" mice, generated through homologous recombination, did not display any measurable defects at birth and during the first weeks of post-natal development, even though many of them subsequently came down with early onset tumors (50). Therefore, even if p53 plays a role in such central processes as cell proliferation, apoptosis and differentiation, it is clearly not absolutely essential for any of these processes in fully normal cells. It is conceivable that the products of other genes can carry out efficiently all of those processes, perhaps substituting for p53 in its absence. The second central finding is that wt p53 is probably involved in the maintenance of genomic stability (51-53). There are now data which support strongly the possibility that p53 is required for the cell to respond properly to DNA damage. In the presence of active wt p53, exposure to DNA damaging agents results in a transient G1 growth arrest, during which the lesions are repaired before any damaged DNA can be

replicated. When p53 is defunct or absent, the cells continue uninterruptedly into S phase; the damaged DNA is then replicated and the resultant genomic aberrations are perpetuated. The two findings may in fact be related, and may predict that the contribution of p53 to development or to any other normal process will become evident only after some sort of DNA damage has occurred. Whether or not this turns out to be the case, it is obvious that a full elucidation of the importance of p53 will require a much better understanding of its biochemistry, and particularly a definitive identification of its molecular targets.

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Discussion - MECHANISMS OF ACTION OF p53

E. Dmitrovsky

I was wondering if you have some reagents available to investigate whether apoptosis is a one-way street, what if you reshift shifted cells, have you done those sort of experiments?

M. Oren

People had done a lot of work in apoptosis, in general, before we had anything to do with it and there's a certain point which is considered to be a no return point. The belief is that the final no return point is when you start getting DNA fragmentation and that is very obvious. If you start breaking the DNA down, there is nothing to go back from and in our system that seems to be really a no return point. Earlier than that, before we start seeing DNA fragmentation, let's say within the first three to six hours when there is hardly any DNA fragmentation, we can shift back. The cells recover nicely.

M. Crescenzi

Given the recent evidence that myc when inappropriately expressed can cause apoptosis, do you have any evidence that myc is involved in your system?

M. Oren

If anything, in this particular system we have evidence against it - disappointingly, but that is the fact. When you turn on wild type p53 those cells have high levels of myc, so we were hoping that the answer is going to be that myc is the counterpart that contributes the negative signal. However, when you activate p53 in those cells one of the first things you see is a very rapid decrease in myc expression. p53 is down regulating myc expression, and that has been shown by a number of investigators, in a number of cell types. Within two to three hours myc levels are down to very low levels. Since the no return point, as I just mentioned before, is at a stage in which myc is almost non existent, probably myc is not the key culprit. One possible candidate for this role is myb, by the way.

H.P. Koeffler

One always worries when one is using a retrovirus promoter, instead of the endogenous promoter, that you may be studying toxic effects. Normal myeloblasts don't die, they typically differentiate and probably would live six days or so until they became a granulocyte. Also if you take human acute myelogenous leukemic cell lines that do and do not have the p53 mutation or p53 that is not intact, they also don't die, they live probably about as long as their media is replenished. Why do you think when you transfect, or in this case bring the temperature down to 32.5 C , that the cells die in less than 24 hours.?

M. Oren

M1 cells are not factor - dependent, for a number of reasons, presumably, some of which I don't know. One of these reasons, probably, is p53 loss, among other things. What I am suggesting is that re-addition of p53 makes them factor - dependent and then once they have p53 they can be compared to factor dependent - myeloblasts, upon factor withdrawal, and I think there the kinetics are similar. This is not evidence, it is just making comparisons. That was one aspect. The other one is that it is true that the levels of p53 we get here are high. That may have some effect on the kinetics of cell death, even though when we compare cell lines which have, I would say a ten fold difference or at least a five fold difference, in levels of activated p53 we don't see a big difference in the kinetics of cell death. So it is probably not that the more p53 they have, the faster they die. Above a certain threshold probably cells die as fast as they can and that is it. I don't really know what exactly are the normal counterparts of M1, so you can take guesses, and levels of p53 vary depending on what stage of myelodifferentiation you look at.

With regard to cell lines with and without p53 dying similarly, that just goes back to the fact that even if p53 is involved in mediating the signal, as we would like to believe, it cannot be the only mediator, because mice deprived of p53 develop normally. All we could say is that there may be a number of redundant genes, all of

which are eliminated by the time you get to something like M1 which is totally factor independent. If you restore any of them, then you turn on the dependence, at least partially, and you get a phenotype. I think that is fairly similar to what people see with tumor suppressor genes in general.

G.E. Francis

I think it is important to appreciate the impact of having a cell survival function and the ability to modulate death at progenitor cell stages on the feedback control of the whole system. It is exquisitely difficult to maintain the proliferation / differentiation balance within the tolerances required to get steady state, particulary at stem cell and early progenitor level. Having the ability, on withdrawal of one feedback loop component, like a GMCSF or an IL-3, to have death occur at that point, stops the system having a very coarse oscillatory behaviour, so this ability on withdrawal of a factor to kill a cohort of progenitor cells actually allows the system to much more sensitive to the signals that are regulating it, in terms of the rapidity of changes in amplification and direction of the system. So I think what we are seeing is an important component of normal regulation and not necessarily just a system to sense gross proliferation, differentiation, imbalance and gross trouble. I think this is going to turn out to be a key component when we try to model these systems. We have to put in a death function unless we postulate completely coupled proliferation and differentiation.

K. Nasmyth

If it were that survival factors act via p53, you might expect to see an effect, in normal cells, of survival factors on p53 by chemistry, physiology or cytology. For example, p53, is going in and out of the nucleus, and it looks as though in order to be active it must be in the nucleus. Is there any evidence that survival factors cause p53 in normal cells to accumulate in cytoplasm?

M. Oren

There are some reports in the literature which can be interpreted this way, even

though they have not been presented this way, and there is now the whole growing concept of conformational modulation of p53. p53 seems to be more modulated at the level of conformation, presumably something to do with phosphorylation, although that's just being worked out. There have been a couple of reports, including studies on myeloid cells which suggest that proliferating myeloid cells taken directly out of the bone marrow seem to have p53 in a conformation which some people would consider inactivated.

G. Capellá

I want to make a comment regarding the relationship between p53 and apoptosis, and this refers to the increasing observation by people trying to establish cell lines derived from epithelial tumors, specially from pancreatic and thyroid tumors, that a mutant p53 seems to be a requirement for establishing the cell line, and that could make good sense with your observations. In the case of thyroid tumors, the only one or two cell lines established do carry p53 mutations. The same happens with pancreatic cancer. In this case, most of the cell lines carry p53 mutations while only about 30% of the primary tumours carry the mutations.

M. Oren

I think that is a very important observation that can be extended also to other systems. One of the best studied is breast cancer where it is extremely hard to get really stable cell lines. If you look at mutations in breast cancer by really looking at the structure of the gene itself, p53 mutations are present only in a minority of primary tumors, while almost all cell lines, with a single exception that I am aware of, carry point mutations in p53.