Leader

Cell cycle regulators: mechanisms and their role in aetiology, prognosis, and treatment of cancer

R J A M Michalides

Division of Tumour
Biology, the
Netherlands Cancer
Institute, Plesmanlaan
121, 1066CX
Amsterdam,
Netherlands
R J A M Michalides

Correspondence to:
Dr Michalides.
email: rmichal@nki.nl

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Tumour cells have sustained derailments in growth control, have escaped from immunological clearing systems, and have acquired immortality as a result of an accumulation of genetic alterations that provide these cells with a selective advantage over others (fig 1). Tumour development is therefore a disease of the DNA and is heritable when these genetic alterations have occurred in the reproductive cells. Progression in tumour development is the result of a stepwise accumulation of genetic abnormalities. The exact number of abnormalities involved in each different tumour type is not known, but in the development of colonic cancer at least seven alterations are involved.^{1 2}

Over the last 20 years developments in cellular and molecular biology have revealed many such alterations in tumour cells. The variety among these would render it impossible to devise a general strategy for diagnosis and treatment that is based upon individual alterations, unless these alterations were to end up in a central control system which ultimately determines the fate of cell growth. Also for practical reasons it is essential for pathologists and clinicians either to distinguish common themes among the numerous genetic alterations or to recognise the most relevant ones. This overview is an attempt to search for such crucial alterations among the numerous possibilities trotted out by cellular and molecular biologists, and to seek support for them in recent reports on immunopathology and treatment. The overview is far from complete, and I shall only attempt to demonstrate that these common themes are starting to assist us in the diagnosis and treatment of cancer.

Control of cellular proliferation

Growth of cells is the net result of cell duplication, differentiation, and cell death, which are mutually regulated. Cells enter the cell cycle and commit to DNA synthesis in response to external factors, including growth factors and cellular adhesion. During the first phase of the cell cycle, G1, cells are responsive to these

Figure 1 Molecular biological and morphological tumour progression.

Figure 2 Cell cycle progression. cdk, cyclin dependent kinase; MC, mitotic checkpoint.

Figure 3 (A) Activation of signalling pathways leads to transcription of cyclin D1. (B) Characteristics of the promoter, mRNA, and the protein of cyclin D1. MAP, mitogen activated protein; TF, transcription factor.

mitogenic stimuli and are dependent on them in order to reach a critical restriction point, termed R, at the end of G1.³ Beyond the restriction point R, cell cycle transition becomes autonomous. Transition through the cell cycle is regulated by the activities of cyclin dependent kinases (cdk) and their inhibitors, cdk associated inhibitors (cki) (fig 2).

A cdk is active as a serine/threonine kinase if it associates with a cyclin protein and becomes activated by phosphorylation and dephosphorylation through cyclin activated kinase (CAK), weel-kinase, and cdc25 phosphatase.^{4,5} This complex cdk activation mechanism provides multiple levels of control. Cyclin proteins are only present during particular phases of the cell cycle. This is the result of specific induction and elimination of these proteins in the cell cycle. Induction of cyclins starts when cells enter the cell cycle from a quiescent state. It requires growth factors and adherence of cells to extracellular matrix components such as collagen or fibronectin. Binding of growth fac-

tors to receptors, either at the surface of the cells (for instance for epidermal growth factor) or in the cytoplasm (in the case of steroids), triggers a cascade of events by which the signal of the ligand binding to the receptor is transmitted into the nucleus, resulting finally in the activation of transcription factors (fig 3A). The number of intermediates in each of these signal transduction pathways varies, and mutations in their genes provide ample opportunities for continuous stimulation.

All these signal transduction pathways finally activate transcription factors that are responsible for transcription of, among other things, the cyclin D gene, coding for the first cyclin protein acting in G1. Three different cyclin Ds have been identified (D1–3); their distribution is more or less cell type specific, with cyclin D1 being expressed in most epithelial and fibroblast cells and cyclin D3 in lymphoid cells.⁶ The promoter of the cyclin D1 gene contains multiple DNA binding sites for transcription factors which are either induced or activated by growth factors to regulate its expression (fig 3B).^{7–13} Adhesion of cells onto extracellular matrix components also induces transcription of cyclin D1, most probably through the mitogen activated protein kinase (MAPK) pathway, which becomes activated when integrin receptors bind to extracellular matrix components.^{14–17} D type cyclins are rate limiting for cell cycle progression, since enforced overexpression of cyclin D1 accelerates G1 transition, whereas, conversely, inactivation of cyclin D1 by microinjection of antibodies or antisense DNA constructs induces a G1 arrest.^{18–20} Cyclin D1 protein associates with cdk4 or cdk6, and this complex is then positively regulated by CAK and cdc25 and negatively by weel/mik kinase and cyclin kinase inhibitors (see below). The final target of an activated cyclin D:cdk4 or cdk6 kinase complex is the retinoblastoma protein, pRb.²¹ Phosphorylation of this protein releases E2F transcription factors which are required for the transcription of E2F responsive genes, including those with proteins involved in DNA synthesis and mandatory for S phase progression.²¹

E2F represents a family of at least five different transcription factors which bind to hypophosphorylated pRb or to related p107 and p130 proteins.²² pRb appears unique among these E2F binding proteins, since elimination of pRb alone is sufficient to liberate abundant E2F activity to render growth of cells growth factor independent.²³ Once activated, each of these E2Fs acts as a transactivator and mediates transcription of E2F responsive genes, among which are cyclins D1, E, and A. The promoters of cyclins D1, E, and A contain E2F binding sites.^{8,24} Thus once cyclin D1:cdk4 is active it generates more cyclin D1 by a positive feedback mechanism. Further progression through G1 to restriction point R requires activity of cyclin E, and passage through the S phase demands cyclin A activity, each in a complex with cdk2. Induction of these two cyclins is depending on E2F,^{25,26} implying that once cyclin D1:cdk4 activity has

Figure 4 Activation and destruction of the cyclin D1:cdk4 kinase complex.

set the G1 regulatory system into motion, cyclin E:cdk and cyclin A:cdk activities are induced and cell cycle transition may occur (fig 2). Each of these G1 cyclin:cdk complexes phosphorylates pRb, however, at different sites²⁷ and with different effects.^{28–29} The different effects may result from the release of different E2F members from the pRb or pRb family members^{30–32} or be due to specific activities of each of the cyclins (see below), or both of these.

Activity of G1 cyclins is directly linked to DNA synthesis, since cdc6 protein, which is essential for DNA replication in S phase, is induced by E2F that is released by cyclin D or cyclin E:cdk activity, but not by cyclin A kinase activity.^{33–35} The latter is more likely to be involved in the release of restrictive components from the DNA replication complex during S phase.³⁶ Activation of G1 cyclins results in induction of cyclin B during G2, which becomes associated with cdk1 that also binds to cyclin A. Their combined activities mediate transition through G2/M phase of the cell cycle.^{37–38}

Degradation of cyclin D either results from specific degradation of its mRNA³⁹ or from degradation of the protein by proteases after recognition of degradation specific PEST sequences at the C terminus (fig 3B) or through ubiquitin dependent proteasomal degradation (fig 4). Cyclins serve as a substrate of their own cyclin:cdk complex; phosphorylation of cyclins creates a target for ubiquitin dependent, protease mediated degradation of cyclins D1, E, A, and B.^{41–43} This degradation of cyclins terminates their action and provides a one way direction to the cell cycle.

Thr-156 is the phosphorylation site of cyclin D1 by a yet unknown kinase, which is mandatory for phosphorylation of Thr-172 by CAK and for entry into the nucleus.⁴⁰

Stimulation of growth is mediated by the action of the cyclin:cdk, whereas inhibition of growth is imposed by the cki suppressor proteins (fig 5).^{44–46} The cki include two families: first, the INK4 family which encompasses p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, and p19^{INK4D},

all of which inhibit cyclin D:cdk4 kinase activity by binding to the cdk4 site which associates with cyclin D⁴⁷; second, the CIP/Kip family members—including p21, p27, and p57—which bind to all cyclin:cdk complexes and inhibit their activity. There is regulation of the cki at various levels, by transcription, by post-translational modulation, and by a shift in their cyclin:cdk targets. When cells enter the cell cycle from quiescence, growth factors induce cyclin D1 expression and formation of the cyclin D:cdk4 complex. This increased complex formation will absorb cki-p27 until a point is reached where excess cyclin D:cdk4/6 complex has titrated out all available p27. From this point on, cyclin D:cdk4/6 will function as an active kinase and generate free E2F, which induces transcription of cyclin E. The story is now reiterated: increasing cyclin E:cdk2 levels titrate out p27 by which unbound, active cyclin E:cdk2 is generated.^{48–49}

CIP-cki complexes inhibit cyclin:cdk with different efficiency and affinity: p27 inhibits cyclin A:cdk2 more efficiently than cyclin D:cdk,⁵⁰ whereas p21 inhibits both with equal efficacy; cki-p27 binds to cyclin A:cdk2 with an approximately 10-fold higher affinity than to cyclin D2:cdk4, and p21 has a higher affinity for cyclin D2:cdk4 than for cyclin E:cdk2.⁵¹ In addition, cellular levels of cki affect inhibition: p21 and p27 in low concentrations stabilise a cyclin D:cdk complex, whereas they inhibit its kinase activity when present in high concentrations.⁵² These data suggest that cyclin D:cdk complexes may function as a catcher for p27 or p21. When cyclin D1 levels increase, cyclin D:cdk complexes absorb more p27/p21 at the expense of its binding to cyclin:cdk2 complexes. This results in G1 transit, and vice versa. Such a shift in targets of cki is the basis of action of transforming growth factor β (TGF- β), anti-oestrogens, or rapamycin: TGF- β stabilises cki-p15,⁵³ which preferentially binds to cyclin D:cdk. This causes a displacement of cki-p27 from cyclin D1:cdk4 which now becomes associated with cyclin E:cdk2, causing a G1 arrest in epithelial cells. In addition,

Figure 5 Positive and negative regulators of the cell cycle: the crucial role of E2F

arrest of oestrogen receptor positive cells by anti-oestrogens reduces protein levels of cyclin D1, whereby cki-p27 is released from cyclin D1:cdk4 complexes; it now becomes associated with cyclin E:cdk2, thereby causing a G1 arrest.^{54 55} Also, rapamycin delays accumulation of cyclin D1 mRNA, which leads to impaired formation of cyclin D1:cdk4 complexes. This results in a retargeting of cki-p27 to cyclin E:cdk2, thereby causing a rapamycin induced G1 arrest.⁵⁶

As well as G1/S, G2/M transition is also influenced by cki p21, depending on the status of Rb. In Rb+/+ cells, p21 expression causes a G1/S arrest, whereas a G2 arrest by p21 is more prominent in Rb-/- cells.^{57 58} Induction of p21 occurs in both a p53 dependent and a

p53 independent manner, where the p53 protein is a transcription factor induced upon DNA damage. Expression of genes involved in growth arrest (cki-p21) or apoptosis (bax) is promoted by p53.^{59 60} The specific response of p53 depends on Rb status: in Rb-wt cells, p53 induction leads to cell cycle arrest by induction of p21, whereas Rb deficient cells bypass the G1 checkpoint and undergo apoptosis.

Links between positive and negative regulatory circuits

The regulatory circuits in cell cycle control are self restrictive in the way that once a pathway becomes activated, a restraint mechanism is induced in order to terminate the original stimulus. Growth factor stimulation induces expression of cyclins which, after fulfilling their function, induce self mediated destruction. Besides this self fulfilling restraint, growth factors induce expression of cki or of its regulators, thereby preventing excessive activation of the Rb pathway. These different regulatory circuits provide multiple levels of control in normal cells, but also multiple possibilities for distortion in tumour cells.

The p19^{ARF}-p53 connection

Action of p53, the "guardian of the genome"⁶¹ or "gatekeeper" of Rb,⁶² is regulated in three ways (fig 6): by DNA damage, by E2F, and in a negative feedback loop by mdm-2/p19^{ARF}.

When normal mammalian cells are subjected to stress signals, oxygen deficiency, radiation, DNA damage, or chemotherapeutic drugs, p53 is activated, leading to p53 mediated induction of programmed cell death (for example through bax) or cell cycle arrest (through p21), or both.⁶³ The p53 response to stress may be mediated by DNA dependent protein kinase⁶⁴ or by the ATM kinase, encoded by the gene responsible for ataxia telangiectasia.⁶⁵ E2F also induces p53 expression⁶⁶ which results in cell cycle arrest in Rb-wt cells, and in p53 mediated apoptosis in Rb-/- cells.^{67 68} Furthermore, p53 also induces mdm-2 which subsequently binds and destabilises p53 in a feedback loop control mechanism.⁶⁹ The mdm-2 mediated degradation of p53 is controlled by protein p19^{ARF}, one of the two tumour suppressors encoded for by the INK4A locus on chromosome 9p21; p19^{ARF} neutralises mdm2⁷⁰ and thereby stabilises p53. Expression of p19^{ARF} is activated by E2F1,⁷¹⁻⁷⁴ by which mechanism the two tumour suppressors pRb and p53 become linked.⁷⁵ E2F, which is released from pRb by cyclin action upon growth factor stimulation, thereby induces p53, which leads to cki-p21, bax, or other apoptosis promoting proteins, and to mdm2 which halts p53 action. At the same time, growth factors induce p19^{ARF}, whereby mdm2 action is prevented. In this way, acting through p19^{ARF}, E2F-1 protects cells from oncogenic changes that result in abnormal proliferation, which is independent of DNA damage (fig 6).

It may now become evident how deregulated E2F expression, either by overactivity of cyclin D1:cdk4 or by abortion of pRb, may lead to p53 associated arrest or apoptosis.^{66 76-78} This

Figure 6 The E2F-p19^{ARF}-p53 connection between growth control, growth arrest, and apoptosis.

Figure 7 Cooperation between cell adhesion and growth factor stimulation in induction of cyclin D1. FAK, focal adhesion associated kinase; GF, growth factor; LPA, lysophosphatidic acid.

dual pathway explains also how p53 induced arrest, or apoptosis by cytostatic drugs, taxol,⁷⁹ or irradiation, becomes enhanced by E2F, either directly by cyclin D1:cdk4 activity or Rb mutation,^{80–83} or indirectly by p16 mutation.⁸⁴ In this way E2F stimulation results in enhanced chemo- and radiosensitivity.

Growth factors as inhibitors

Growth factors do not only stimulate cell proliferation, but they may also act as growth inhibitors, depending on the cell type and on the stimulatory pathway that is involved. TGF- β is such an example, being a growth stimulator in fibroblast cells with receptors for TGF- β , but a negative regulator in epithelial cells.⁸⁵ Not only may the growth factor have different actions, but the pathway activated by a growth factor may also have dual endpoints: E2F activation leads to induction of cyclins, promoting cell cycle transition, as well as to p19^{ARF} which halts cell cycle transition. In fact, expression of most of the cki is influenced by dual growth regulation, since expression of cki p16 and p21 is dependent on E2F activity,^{86–92} and that of p27 is enhanced by cyclin D1:cdk4 activity and is thus likely also to be dependent on E2F.⁹³ This ambiguity explains the p53 dependent and independent expression of p21 during cell growth, differentiation, and DNA damage.⁹⁴ The increased expression of p21 or p27 by cyclin D1 does not always result in cell cycle arrest, since the increased level of cyclin D1:cdk4 can absorb more cki,^{88–95} or may otherwise be associated with differentiation.⁹² Increased expression of p16 by E2F, however, results in displacement of cyclin D from cyclin

D:cdk complexes and in a rapid proteolysis of this protein, which leads to reduced levels or absence of cyclin D1. In this way mutation of Rb leads to absence of cyclin D1.^{96–97}

Adhesion mediated growth control

Most cell types require mitogenic growth factors as well as adhesion of cells onto extracellular matrix components for proliferation.¹⁵ Adhesion to extracellular matrix is mediated by integrins, which are transmembrane proteins that dimerise and undergo conformational changes by binding to extracellular matrix and thereby activate the integrin mediated signal pathway.⁹⁸ This involves many yet undissolved steps and includes activation of focal adhesion associated kinase (FAK), which is connected through src and sos to the MAP kinase pathway (fig 7).^{9–17}

Activation of integrins also leads to activation of Rho family members, including GTPases rac, cdc42, and rho, which are involved respectively in ruffling, formation of filopodia, and stress fibre formation—processes associated with rearrangement of the actin cytoskeleton that goes along with spreading of cells upon attachment to extracellular matrix.⁹⁸ The GTPases ras and rho interact to regulate expression of cki-p21, where p21—as was mentioned above—is induced by ras, and rho negatively interferes with the induction of p21 by ras⁹⁹ (fig 7). Growth factor stimulation then leads to two events: to activation of ras and thereby to p21 through growth factors in the medium; and to activation of rho through adherence to extracellular matrix in conjunction with serum factors, of which

Figure 8 The cadherin-β-catenin-Tcf-myc-telomerase pathway in tumour cells. APC, adenomatous polyposis coli suppressor; GSK, glycogen synthetase kinase.

lysophosphatidyl acid (LPA), is the most relevant factor. The other two GTPases, rac and cdc42, activate—most probably through activation of p65^{PAK}—the JunK pathway which leads to cyclin D1 expression and E2F dependent transcription.¹⁰⁰ These recent findings indicate that adhesion independent growth of tumour cells may be caused by disruption of the Rb pathway,^{101–103} as is frequently encountered in tumour cells of advanced stage.¹⁴ Since sustained detachment of normal cells from the extracellular matrix usually leads to apoptosis, also in the presence of growth factors,^{104–105} this is a relevant obstacle on the road to anchorage independent growth. Activation of FAK kinase and of the GTPases rac and cdc42 requires cellular attachment and spreading,¹⁰⁶ and the latter demands dimerisation of integrins. Dimerisation of integrins may then be the crucial step in anchorage independent proliferation, which in Rb wild-type cells will not be overcome by overexpression of cyclins alone.

Not only does adhesion onto the extracellular matrix affect cell cycle regulation, but intercellular adhesion through cadherins also influences cell cycle regulation by cki-p27 and by the β-catenin-Tcf pathway. In normal cells intercellular binding through E-cadherin molecules results in binding of β-catenin to E-cadherin on the cellular membrane or to destruction of β-catenin by an APC/GSK3 complex formation (fig 8).¹⁰⁷ In both cases entry of β-catenin into the nucleus is prevented. Mutation of APC or β-catenin, or either mutation or downregulation of E-cadherin, prevents degradation of β-catenin, which now moves to the nucleus and coactivates transcription factor Tcf to mediate transcription of, among other things, transcription factor myc¹⁰⁸; myc induces transcription of telomerase,¹⁰⁹ providing immortality to cells

(see below), but also induces a yet unidentified factor which releases p27 mediated inhibition of cyclin E:cdk2 activity, thereby releasing p27 mediated growth inhibition.¹¹⁰

What else is up or down?

Is this all there is to cell cycle control? A weekly glance in the journals might tell you that tumour cells have more options in store than we might think of. The emphasis in this review is on control over the G1/S phase of the cell cycle, since in this phase the cells are extremely sensitive to regulation by external factors. Once cells have traversed restriction point R (fig 2), cell cycle transition is autonomous—not that matters may not go wrong there as well! In fact, aneuploidy—the form of genetic instability characteristic of a more advanced tumour phenotype—is associated in colonic cancer, and probably in other tumour types as well, with loss of control over mitosis by mutational inactivation of the BUB1 gene, which in normal cells takes care of the proper segregation of the chromosomes.¹¹¹ Furthermore, in a small number of tumours genetic instability is also caused by an increased mutation rate at nucleotide level, leading to microsatellite instability as a result of defective repair of DNA damage.^{112–113}

Tumour development requires escape from clearing systems. To do this, tumour cells have to arrest apoptosis as well as downmodulate immunological defence systems. Escape from apoptosis is not only mandatory in order to escape from chemotherapy and radiotherapy, but tumour cells also have to overcome “natural” apoptosis invoked by hypoxia¹¹⁴ or detachment from extracellular matrix components.¹⁵ As more and more apoptosis regulatory pathways, and the genes involved, become known,¹¹⁵ deregulation of each of these genes may be required to prevent apoptosis in various

Figure 9 Deregulation of the Rb-cyclin D1-cdk4-p16 pathway in cancer.

types of tumours. Studies on transformation of cells by adenoviruses have shown how transforming proteins downregulate expression of HLA on the cell surface, thereby drastically reducing putative targets for immunological attack, in order to escape from such attack.¹¹⁶

However, even tumour cells would not escape from inevitable aging if they had not found a way of preventing ordinary mortality. The number of telomeric DNA repeats at the end of a chromosome is an unmistakable indication of the proliferative history of cells, since telomeres shorten progressively with each succeeding cell division owing to the switching off of telomerase activity.¹¹⁷ This telomerase activity becomes reactivated in tumour cells by, among other things, the activity of the myc transcription factor.¹⁰⁹ In this respect, it is noteworthy that overexpression of myc often occurs in different tumour types, including colon, breast, head and neck, and lung cancer, and is associated with advanced stage of disease.¹¹⁸

In further progression of tumours, (re)activation of cell migration is a requirement for invasion and metastasis of tumour cells, which touches on regulation of adhesion, cell movement, and secretion of proteases in order to dissolve surrounding membranes. Derangement of expression of these genes adds to the genetic acrobatics in tumour cells.

Essential derailments in cancer

Tumour cells have undergone distortions in control over growth, apoptosis, and aging. The essential pathways in these are, respectively, the Rb pathway, p53, and telomerase (re)activation. Derailments in these pathways provide increasing autonomy for tumour cells and a selective advantage over other cells. For example, overexpression of cyclin D1 protein in MCF7 breast cancer cells renders these cells capable of proliferating under reduced serum concentrations by affecting the proportion of cells in the G₀, quiescence state.⁹⁵ Overexpression of cyclin D1 prevents cells from entering quiescence and therefore enhances the proliferative fraction of tumour cells. Since tumour cells in vivo will encounter various growth factor conditions, overexpression of cyclin D1 most probably allows tumour cells to continue

to proliferate in low growth factor conditions in vivo as well.

Distortion of the Rb pathway is a common phenomenon in cancer (see below), and is achieved by disruption of Rb, overexpression of cyclin D1 or cdk4, or downregulation of cki-p16 (fig 9). Various mechanisms may account for these deregulations:

For Rb: point mutation, deletion, and functional inactivation by the binding of viral proteins at the site of interaction with cyclin:cdk.

For cyclin D1: amplification of the gene, inter- and intrachromosomal recombination, loss of destabilising sequences in the mRNA by deletion¹¹⁹ or alternative splicing,^{120 121} or overexpression as a result of aberrant growth factor stimulation.

For p16: most often by deletion, infrequently by point mutation and by methylation of promoter sequences.

For cdk4: by amplification¹²² and by mutation of its interaction site with p16.^{123 124}

When one adds up all these possibilities and examines series of breast, lung, head and neck, or melanoma cancers for any of these alterations, one is likely to find that all tumours have undergone some distortion in the Rb pathway.¹²⁵ The same accounts for disruption of the p19^{ARF}-p53 pathway which prevents apoptosis from occurring, whereas most advanced carcinomas also show telomerase activation in order to become immortal.^{126 127}

Clinical relevance of alterations in Rb, p53, and telomerase pathways

Are the particular aberrations in these pathways relevant for the process of tumour progression and for treatment? To answer this, one would like to discern different stages of tumour progression according to genetic alterations in the different tumour types. This was ideally encountered in the case of progression of colonic cancer, where the different stages of progression from initial lesion to invasive phenotype can be studied within the same neoplasm.² Here, progression by morphological criteria is paralleled by a stepwise accumulation of genetic alterations. Where this ideal situation is not met, as is the case in most other cancer types, cumulative association between frequency of genetic alterations and increased tumour stage yields a similar ranking of genetic alterations for progression of head and neck cancer,^{128 129} whereas single associations underline (or undermine) the relevance of a particular genetic alteration in other tumour types.

Studies in which alterations in the primary tumours (without obvious dissemination) are associated with disease-free interval or with disease associated survival have many pitfalls, but are often used for want of other options. If a certain association turns out to be significant, one might wonder about its specific effect. For instance, overexpression of cyclin D1 protein is consistently associated with shortened disease-free interval in squamous carcinomas of the head and neck (see below). In this tumour type, one hardly ever finds Rb mutation¹³⁰ but rather often finds p16 mutations.^{131 132} Furthermore,

Figure 10 Alternative activation of oestrogen receptor (ER) by cyclin D1 or cyclin A:cdk2.

most of these tumours lack a functional p53. Overexpression of cyclin D1 would only provide a selective advantage to cells when they are normal for Rb and lack expression of p16. On the other hand, expression of p16 would have no effect on cyclin D1 in Rb mutated cells. If one presumes that all lesions in squamous carcinoma of the head and neck originate from smoking or alcohol abuse, and that these neoplasms have circumvented p53 mediated arrest or apoptosis by mutation of p53, then a growth advantage would be acquired in Rb mutant cells or in cyclin D1 overexpressing cells with a mutation or reduced expression of p16. These tumour cells would be less dependent on growth factor stimulation or on adhesion onto extracellular matrix components. Why this reduced dependency in head and neck cancer is mainly achieved by overexpression of cyclin D1 and not by direct deregulation of Rb remains an enigma.

This example demonstrates that the aim of discerning different stages of tumour progression according to genetic alterations is not likely to be fulfilled by examining just a single alteration, or only a few, but that a hierarchy of genetic alterations must be taken into account. In the Rb pathway this hierarchy includes: disruption of pRb, overexpression of cyclin D/disruption of p16, and overexpression/mutation of cdk4. These possibilities should be examined in conjunction with disruption of the p19^{ARF}-p53 pathway, and other not yet fully explored major pathways of apoptosis induction, and with reactivation of telomerase. Only then may pathology based on gain or loss of functions provide a meaningful and conceptual replacement of an established pathology based on morphology, which is highly effective but lacks the power to predict the outcome of most lymph node negative tumours. It is not difficult to predict that rolling stones will descend from the mountain; it is a challenge to predict which ones will start to roll.

It might be clear from a limited and selected survey on some tumour systems given below that pathology is still in its infancy in making an inventory of these dysfunctions in tumours.

Other activities of cyclins

The major role of cyclin:cdk is to direct regulation of the cell cycle, but it is also becoming apparent that some cyclin:cdk complexes are also involved in other biological processes, which are indirectly associated with proliferation. Cyclin-H and cdk7, a cyclin:cdk complex which is not itself associated with a particular cell cycle transition, are part of the TFIIH nucleotide-excision complex, but are also identified as components of CAK.¹³³

Cyclin E:cdk2 is associated with the spliceosome associated protein SAP 155, which is efficiently phosphorylated by cyclin E:cdk2. This suggests that a fraction of cyclin E:cdk2 is involved in the splicing machinery of mRNA.¹³⁴

Cyclin D binds directly to a myb-like transcription factor, DMP1, which becomes activated by phosphorylation through cyclin D:cdk activity, suggesting that the latter may also regulate gene expression in an Rb independent manner.¹³⁵ Cyclin D1 also inhibits the activity of another transcription factor involved in myogenic differentiation, showing that it may affect transcription directly.¹³⁶

Other jobs on the side carried out by cyclin D1 and cyclin A might be relevant for activation of the oestrogen receptor (fig 10). Cyclin D1 stimulates activation of the oestrogen receptor through direct binding unhampered by anti-oestrogens.¹³⁷ This stimulation of the oestrogen receptor by cyclin D1 is cdk independent,^{137 138} in contrast with oestrogen receptor activation by cyclin A:cdk2, which requires cdk2 activity and is inhibited by cki-p27.¹³⁹ These findings suggest that transcriptional activation of the oestrogen receptor may occur in the absence of oestradiol in cells with overexpression of cyclin D1 or cyclin A. However, for cyclin D1 to stimulate the oestrogen receptor, it should be present in a vast excess and in an unbound state, a situation that is hardly met in tumour cells (see above), and which may only result in transient transfections, but is probably absent in vivo.

What does distortion of the Rb pathway do to cells?

The idea that progression of the cell cycle through G1 to S requires only phosphorylation of pRb and thereby releases E2F turns out to be too simple. Cyclin E can overcome a p16 induced arrest of the cell cycle without phosphorylation of pRb,¹⁴⁰ indicating that other targets of cyclin E:cdk2 activity determine the full transit through G1. It is not (yet) clear which other target this may be, but myc overexpression can do the same.¹⁴¹

In general, overexpression of cyclins D1 or E does lead to accelerated transition through G1, and overexpression of cyclin D1 does lead to reduced growth factor dependency and a reduced entry into G₀, quiescence. Activation of E2F by overexpression of cyclin D1 or cyclin E leads to p53 dependent apoptosis under reduced serum conditions.⁷⁶ In transformation assays, overexpression of cyclin D1 by itself is not sufficient to induce transformation, but it does so in conjunction with adenovirus E1A.¹⁴² In transgenic mouse models the effect of cyclin

D1 overexpression does depend on the promoter that is used in the transgene. Use of the EBV promoter yields squamous cell carcinomas,¹⁴³ that of immunoglobulin enhancer stimulates lymphoma development in conjunction with myc,¹⁴⁴ and those of keratin in epidermal hyperproliferation¹⁴⁵ and of mouse mammary tumour virus (MMTV) cause hyperplasia, ultimately leading to carcinoma development.¹⁴⁶ But cyclin E in a transgene under control of the promoter of β lactoglobulin also produces hyperplasia in the mammary gland.¹⁴⁷ These data show that overexpression of cyclin D1 and of cyclin E alleviates growth restriction, but does not itself lead to transformation. Additional genetic alterations are mandatory for full transformation.

The absence of cyclin D is more helpful in demonstrating the subsidiary roles fulfilled by cyclins D: mice lacking cyclin D1 have reduced body size, show symptoms of retinal deformation, and fail to undergo proliferation of breast cells upon pregnancy (steroid) stimulation.^{148 149} Female mice lacking cyclin D2 are sterile owing to the inability of ovarian granulosa cells to proliferate in response to follicle stimulating hormone (FSH), whereas mutant males display hypoplastic testes.¹⁵⁰ These data show that D-type cyclins are dispensable (mice lacking these D-type cyclins are still alive), and that other D-type cyclins most probably take over the role of the missing one. They further demonstrate that their subsidiary roles are not dispensable: for cyclin D1, this is activation of the oestrogen receptor in mammary gland development; for cyclin D2, it is activation of a yet to be determined pathway that is involved in testicular development.

The crucial role of Rb is underlined by the non-viability of Rb homozygous knock out mice, whereas heterozygotes display increased apoptosis and sensitivity to tumour development.¹⁵¹

A peculiar and somewhat overlooked aspect of overexpression of cyclin D1 is its ability to enhance gene amplification,¹⁵² which may well contribute to genomic instability.

Derailments in tumors in vivo

Many reviews have recently appeared describing distortions of the Rb and p19^{ARF}-p53 pathway in different types of tumours.^{129 153-156} The studies quoted below are only given to complement these and to illustrate novel tendencies.

Bladder cancer—In separate studies, prognosis of patients with bladder cancer was significantly poor when the primary tumour was less positive for Rb,¹⁵⁷ or showed overexpression of cyclin D1,¹⁵⁸ indicating that distortion of the Rb pathway contributes to poor prognosis.

Breast cancer—All of the disturbances in the Rb/cyclin D1/p16 pathway are encountered in breast cancer: abnormal (reduced or absent) pRb^{159 160} in 20% of non-invasive cancers, which was associated with increased proliferation rate and oestrogen receptor negativity; no loss or mutation of cki-p16 in primary breast cancer¹⁶¹ but frequent loss of p16 expression in approximately 45% of all breast cancer

cases.^{159 162 163} Overexpression of cyclin D1 in 40–50% of all cases,^{154 155 164} of which half is due to amplification of cyclin D1 on chromosome 11q13. This amplification, and corresponding overexpression, is associated with a more aggressive tumour phenotype and a worse prognosis.¹⁶⁵⁻¹⁶⁸ Overexpression of cyclin D1 protein or mRNA is noticed in high grade ductal carcinomas in situ, which show more frequent recurrences than low grade tumours,^{169 170} and it occurs in the vast majority of invasive lobular carcinomas, but not in non-invasive lobular carcinomas.¹⁷¹ However, overexpression of cyclin D1 protein was not by itself indicative of prognosis in large series of patients with a low stage of breast cancer.^{164 172-174} This apparent contradiction may be explained by the fact that only approximately half of all cases with overexpression of cyclin D1 protein can be accounted for by amplification of the cyclin D1 gene, which is the most frequent genetic aberration and which is linked to poor prognosis. Since overexpression of cyclin D1 protein is highly significantly linked to oestrogen receptor,¹⁷³⁻¹⁷⁵ and since cyclin D1 is turned on by activated oestrogen receptor, the other half of the cases with overexpression of cyclin D1 protein in breast cancer may result from “normal” stimulation by oestradiol, the impact of which on breast cancer development remains to be investigated.

Co-overexpression of cyclin D1 together with epidermal growth factor receptor or pRb is more indicative of poor prognosis than expression of cyclin D1 alone.¹⁷⁶

Aberrations in other cell cycle regulators are also becoming apparent in breast cancer. Cyclin E overexpression in 20% of non-invasive cancer is associated with a more aggressive phenotype,^{177 178} in particular in conjunction with low cyclin D1 expression.¹⁷⁴ Cyclin E overexpression is prevalent in oestrogen receptor negative breast cancer cases, whereas Rb deletions occur only in this group of breast cancers.^{174 177}

Reduction of cki-p27 expression, whether or not in conjunction with overexpression of cyclin E, is indicative of poor prognosis in node negative breast cancer patients,¹⁷⁹⁻¹⁸² and also in colonic cancer¹⁸² and prostate cancer.^{183 184}

Inactivation of E-cadherin, which may lead to raised myc and activation of telomerase (see above), is often found in infiltrating lobular breast cancer, but not in other breast cancer types,¹⁸⁵ promoting two steps in tumour progression: adherence independence and immortality.

Mutations in p53 releasing apoptotic control were found in 69 of 316 primary breast cancer cases and were significantly associated with poor prognosis.¹⁸⁶⁻¹⁸⁸ These studies also indicated that direct analysis of mutation has substantially better prognostic value than immunohistochemical overexpression of p53.

Only a few studies have dealt with interactions between deregulated cell cycle regulators, and have confirmed the experimental findings described above: the inverse relation between pRb and p16,^{159 174} the positive interaction between cyclin D1 and p21,^{92 189 190} and the

Figure 11 Molecular biological model for breast tumour progression.

absence of cyclin D1 in Rb negative tumours,^{160 174 189} which now, however, do prevalently overexpress cyclin E.¹⁷⁴

When one considers the model of tumour progression associated with deregulation of growth stimulation (the Rb/p16/cyclin D1 pathway) and of apoptosis (among others, the p53 pathway), then none of these investigations examined whether combinations of such deregulations contribute to a more advanced stage, or whether they occur in more advanced stages of breast cancer. Interesting in this respect is the finding that overexpression of cyclin D1 did not coincide with mutation of p53 (measured by immunohistochemistry) in a series of 248 breast cancer patients of low stage.¹⁷³ One would presume that progression of breast cancer in earlier stages may go along with either disturbed growth stimulation or disrupted apoptosis, and that more advanced breast cancer stages would represent mutations in both of these pathways and reactivation of telomerase. From this summary of published reports, a molecular biological model for progression of breast cancer may be derived, which is presented in fig 11.

In this model, morphological hyperplasia may coincide with either loss of growth control by amplification of cyclin D1, by loss of Rb function, or by loss of p16; or with loss of control of apoptosis by loss of p53 function or by loss of other apoptosis regulating genes; or with loss of senescence control by reactivation of telomerase activity through upregulation of myc as a result of reduction of E-cadherin or mutation of β -catenin or APC. When two of these regulatory pathways have been demolished, these neoplasias may present as limited carcinoma in situ, whereas where all three pathways are affected, they may present as morphological carcinoma. When cells have undergone one particular mutation resulting in loss of growth control, there is no further selective advantage for additional mutations leading to loss of growth control. Mutations within one regulatory unit—either growth control, apoptosis, or senescence—are therefore usually mutually exclusive.

On top of this, additional mutations leading to aberrant expression of proteases or cell adhesion molecules affect the metastatic behaviour of tumour cells, resulting in invasive carcinomas.

Mantle cell lymphoma—Mantle cell lymphoma comprises approximately 10% of all non-Hodgkin lymphomas and represents a generalised disease with poor prognosis. More than 90% of these mantle cell lymphomas show overexpression of cyclin D1 as a result of t(11;14) chromosomal translocations.^{191 192} This is a remarkable example of a sporadic tumour type in which almost 100% of the cases are consistently associated with a particular genetic aberration, namely overexpression of cyclin D1. This unique association allows the use of overexpression of cyclin D1 in the rather difficult morphological diagnosis of mantle cell lymphoma.

Squamous cell carcinomas of the head and neck and oesophagus—Amplification and overexpression of cyclin D1 or loss of cki-p16 is significantly associated with a more advanced tumour phenotype and with poor prognosis.^{129 193–198} In particular in tumours with pRb expression, overexpression of cyclin D1 is indicative of poor prognosis.^{196 199} Inactivation of either the apoptotic pathway (through p53) or the growth promoting pathway (through overexpression of cyclin D1) is associated with poor prognosis, whereas derailments of both these pathways are more prevalent in more advanced tumours (T4, stage IV).¹⁹⁵ This indicates again that tumour progression coincides with cumulative deregulation of pathways controlling growth and apoptosis.

Immunohistochemical studies also confirm the experimental findings that overexpression of cyclin D1 is associated with increased expression of cki-p27²⁰⁰ and in a p53 independent manner with cki-p21.²⁰¹

Lung cancer—Whereas in small cell lung cancers Rb is often mutated, in non-small-cell lung cancer p16 and p27 status is a significant prognostic factor, and the lack of expression of these cki is associated with poor prognosis.^{202–206} Loss of p19^{ARF} is more common in small cell lung cancer than in non-small-cell lung cancer.²⁰⁷ The impact of p19^{ARF} mutations in cancer will undoubtedly become a new fishing pond.

Other types of tumour—Overexpression of cyclin D1 and mutation of p53 (assessed by immunohistochemistry) are independent prognostic factors in *colonic cancer*,^{208 209} whereas overexpression of cyclin D1 is more prevalent in advanced stages²¹⁰ and is associated with intestinal adenomas in patients with familial adenomatous polyposis.²¹¹ Lack of p16 expression or overexpression of cyclin D1 is associated with poor prognosis in *pancreatic cancer*.^{212 213} Overexpression of p53 and disturbances in the Rb pathway, either lack of Rb and p16 expression or overexpression of cyclin D1, were markers of poor prognosis in *ovarian cancer*.^{214 215} Remarkable was the high expression of p53 and p16 in Rb negative tumours (acting through E2F?).²¹⁴ Furthermore, overexpression of cyclin D1 is indicative of poor

prognosis in *prostatic adenocarcinoma*.²¹⁶ In the light of the specific side effects mentioned above, aberrant expression of cyclin D2 is particularly associated with *testicular tumour* development.²¹⁷

This incomplete list of distortions in the pRb and p53 pathways in different types of cancer shows that deviations in these pathways are linked to tumour progression. Because in most studies only one or a few of the players have been examined, it is not yet always clear which of these disturbances (if any) are the most informative indicators of poor prognosis, and why. The mass of evidence that one or other of these deregulations contributes to a poor prognosis in different types of cancer suggests that a modern approach to risk assessment should involve an evaluation of these markers in prospective studies.

Cell cycle regulators and sensitivity to cytostatic agents and radiation

Since cell cycle regulators also influence sensitivity to agents which either cause arrest of the cell cycle or induce apoptosis (see above), one might presume an intrinsic dependency between these. Sensitivity of cells towards cytostatic agents or radiation has until now mostly been studied with respect to p53 status. Loss of p53 function in tumour cells indeed confers increased resistance to chemotherapy or radiation.^{218–221} The connection between E2F and p53 by way of p19^{ARF} has now been revealed, and both of them induce either p21 or apoptosis; thus a costimulatory effect is most likely to occur when cells with a normal p53 and Rb are being treated with growth factors together with cytostatic agents, as has indeed been reported for taxol and radiation.^{82–83, 222} One might predict that deregulation of the pRb pathway would render tumour cells with intact p19^{ARF}-p53 more sensitive to these agents, whereas mutation of p19^{ARF} alone would eliminate any costimulatory effect of growth factors by way of E2F. Inventories of this kind have hardly begun to be made, but might well turn out to be essential for assessment of a more effective form of drug treatment.

Conclusion

This overview indicates that an inventory of the essential deregulations in the cell cycle regulators adds to a better understanding of the genesis of different types of cancer. Data are accumulating that these deregulations are associated with the development of specific types of cancer and with their clinical behaviour. This may well lead to the development of specific cancer treatments and to improved efficacy of existing treatments, and should therefore become a must in molecular pathology.

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