

ADENOVIRUS SUBVERSION OF IMMUNE SURVEILLANCE, APOPTOTIC AND GROWTH REGULATORY PATHWAYS: A MODEL FOR TUMORIGENESIS*

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The adenovirus system provides a novel model for evaluating the roles of multiple factors involved in tumour progression. In common with other DNA tumour viruses, adenovirus employs a variety of strategies to evade immune surveillance and perturbs cellular apoptotic and growth regulatory pathways to ensure efficient replication of progeny virions. Such subversion of cellular networks is also found in tumour cells. The mechanism behind the avoidance of immune surveillance and the extent of cellular network interference achieved by adenovirus is still being uncovered and is predicted to have ramifications for the design of cancer therapeutics.

Keywords: adenovirus, immune surveillance, a model for tumorigenesis

Viruses have evolved many strategies to prevent recognition of infected cells by the host immune system. Virally infected cells are usually recognised by T lymphocytes that detect fragments of viral proteins displayed on the surface of the infected cells by major histocompatibility complex (MHC) molecules. The synthesis of viral proteins within the host cell is essential for viral replication and normally T cells can control rapidly replicating viruses that give rise to acute viral disease. However, this immune recognition can be thwarted by viruses with quite serious complications for the host organism: for example, it has been known for over

* Dedicated to Professor Ilona Béládi on the occasion of her 80th birthday.

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40 years that the DNA tumour viruses can induce tumours in animals and many are now associated with human cancer. Failure of the immune system to recognise cells infected by DNA tumour viruses permits viral genes involved in oncogenesis and replication to be expressed. Consequent subversion of cellular checkpoints and normal host cell protein expression by proteins encoded by the DNA tumour virus transforms the phenotype of primary cells *in vitro*. Such transformation is termed neoplastic if subsequent delivery of transformed cells gives rise to a tumour *in vivo*. Some DNA tumour viruses are capable of both transformation and neoplastic transformation depending on the serotype. For example, adenovirus (Ad) and human papillomavirus (HPV) have been classified according to their oncogenic potential; essentially, the immune system is seeing each virus as a distinct organism.

**Oncogenicity of adenovirus is dependent on serotype and host species
– key factor is cellular immunity**

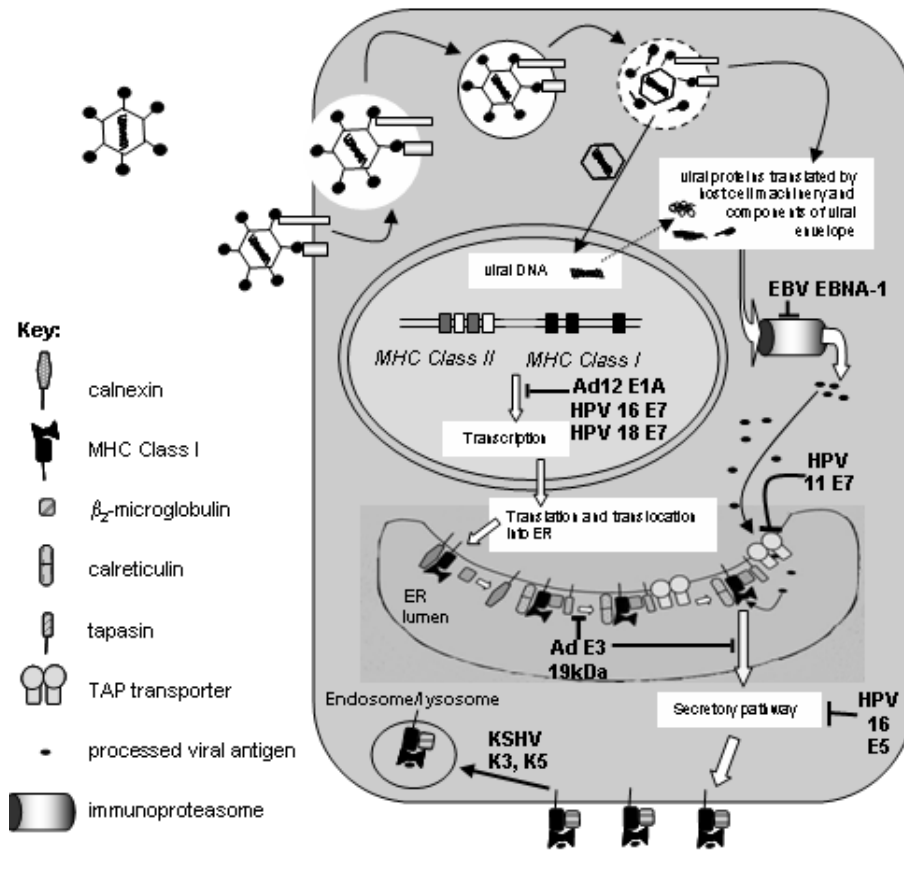
Adenoviruses are widespread in nature and infect birds (*Aviadenovirus*) and mammals (*Mastadenovirus*). There are at least 51 human adenovirus serotypes that are classified into 6 subgenera or species (A to F) based on oncogenicity in newborn hamsters and nucleotide sequence homology. Although both species A (Ad serotype 12) and C (Ad2 and Ad5) can transform primary rodent cells *in vitro* with equal efficiency [1, 2] only adenoviruses classified as species A and cells transformed by species A Ads are tumorigenic in immunocompetent rodents [3, 4], meaning that they give rise to tumours in every animal within two months. In contrast, viruses of species B (such as Ad3 and Ad7) are weakly oncogenic; after a year of observation tumours were observed in only a few animals. In humans, adenoviruses cause persistent infections and are commonly associated with respiratory, ocular and gastrointestinal infections depending on the serotype; however, Ads have not, to date, been found to be associated with cancer in humans. Why Ads are not oncogenic in humans is an unsolved mystery; based on clinical evidence, compromised cell-mediated immunity seems to be a key requirement for fatal or disseminated Ad infection [5–10]. Further evidence for the importance of cell-mediated immunity comes from studies in rodents suggesting that oncogenicity of cells transformed with adenovirus serotypes of species A or the usually non-oncogenic species C is markedly increased in immunosuppressed animals [11–13].

How tumours can escape immune surveillance

A large deciding factor governing whether a tumour arises or not is the ability of the immune system to recognise and destroy tumour cells; a process termed immune surveillance by Sir Macfarlane Burnet and Lewis Thomas in the 1950s [reviewed in 14]. There are three main ways in which a tumour can escape the immune system. Firstly, tumours can be of low immunogenicity if they lack peptide: MHC ligands, adhesion molecules or co-stimulatory molecules required to trigger a primary T cell response. Secondly, antigenic variation can occur; antigens expressed by a tumour can be lost by antibody-induced internalisation or antigenic variation. Finally, the tumour itself can produce factors such as TGF- β that directly inhibit T cells. A major factor in the tumorigenicity of Ad12-transformed cells in immunocompetent rodents is reduction of MHC Class I antigen presentation to CD8⁺ T cells [15, 16].

Subversion of the MHC Class I pathway

It is becoming clear that many viruses avoid immune surveillance by interfering with the canonical MHC class I pathway as outlined in Figure 1 by a variety of methods [reviewed in 17]. In the absence of viral infection, the peptides presented by cell surface MHC Class I molecules are derived from general cellular protein turnover [reviewed in 18]. When infected by a virus, this peptide 'fingerprint' changes with the presentation of viral peptides to allow recognition of the virally infected cell by CD8⁺ T cells. It is advantageous to the virus to block the presentation of viral peptides by MHC class I molecules and so prevent the signal to CD8⁺ T cells to kill the infected cell. For example, the adenovirus E3 19kDa protein is an N-linked glycoprotein that is localised to the ER membrane, where it binds MHC Class I $\alpha 1$ and $\alpha 2$ domains thereby preventing transport from the ER to the cell surface and hence CD8⁺ T cell recognition of antigens [19]. However, it has been found that CD8⁺ T cell recognition of Ad-infected cells differs depending on the strain of mice; it is governed by the affinity of Ad E3 19kDa for individual class I alleles [20]. It can be seen from Figure 2 that mice, humans and rats possess various classical MHC molecules; the high amount of allelic diversity associated with each locus (for example, HLA-A has roughly 250 known alleles) means that E3 would have to be capable of associating with a wide range of MHC Class I heavy chains. This represents a formidable design challenge; not surprisingly, direct targeting of MHC Class I heavy chains at the protein level is not a common strategy employed by viruses.



The gene encoding for the E3 19kDa protein is absent from the adenovirus 12 genome [21], and E3 genes are not usually expressed in Ad-transformed cells. This suggests that an alternative strategy for MHC Class I pathway subversion can be employed by adenoviruses in viral transformation and, in the case of species A Ads, in viral infection. It has recently been reported that levels of MHC Class I are down-regulated on the surface of human cells transformed with adenovirus 12 compared to human cells transformed with adenovirus 5 [22]. So, how does the down-regulation of MHC Class I on the surface of human cells transformed with the oncogenic adenovirus 12 occur in the absence of the E3 19kDa protein?

The oncogenicity of Ad-transformed cells is determined by the serotypic origin of the E1A gene [26]. It has been known for some time that Ad12 E1A is

Figure 1. The MHC Class I pathway is inhibited by DNA tumour viruses. Ideally, viral proteins derived from transcription and translation of viral DNA and from the plasma membrane (such as viral envelope proteins) would be processed by the immunoproteasome. The immunoproteasome differs from the normal proteasome due to the presence of three IFN- γ inducible subunits thought to be active proteases; low molecular weight protein (LMP) 2 and LMP7 (encoded in the MHC Class II region and indicated in dark grey in the nucleus) and MECL-1 which is not encoded within the MHC. Processed viral antigens are then transported into the ER by the transporter associated with antigen processing (TAP); a heterodimeric ATP-binding cassette (ABC) transporter consisting of TAP-1 and TAP-2 encoded within MHC Class II region (shown in light grey in the nucleus). Newly synthesized, partially folded MHC Class I heavy chains are retained in the ER lumen by binding to the chaperone protein calnexin. Binding of β 2-microglobulin to MHC Class I heavy chain triggers dissociation from calnexin and the partially folded heterodimer then binds to a complex of proteins that include calreticulin and the TAP-1 associated protein, tapasin. Encoded by a gene lying within the MHC Class II region, tapasin forms a bridge between MHC Class I and the TAP-1 subunit of the transporter. Binding of peptide to the partially folded MHC Class I heavy chain; β 2-microglobulin heterodimer triggers release from the TAP complex. The fully folded MHC Class I molecule leaves the ER and is exported through the Golgi apparatus to the cell surface via the secretory pathway. Both adenovirus and human papillomavirus encode various proteins that interfere with this pathway resulting in down-regulation of surface MHC Class I expression, as discussed in the text. Other DNA tumour viruses also encode proteins that interfere with the MHC Class I pathway. For example, KSHV K3 and K5 proteins use E3 ligase activity to tag the cytosolic tail of MHC Class I molecules for ubiquitination promoting endocytosis of MHC Class I from the plasma membrane [reviewed in 17]. EBV EBNA-1 contains a glycine-alanine repeat (GAR) domain that prevents proteasomal degradation of the full-length protein and functions to limit transcription of defective translation products that would otherwise be processed [reviewed in 17]. Figure adapted from [17]

sufficient for the down-regulation of surface MHC Class I in rodent cells transformed with adenovirus 12 compared to adenovirus 5 and that this repression is achieved at the level of transcription [27–29]. Although there is much more to learn about the mechanism of differential transcriptional regulation of MHC Class I-related genes, current evidence points to E1A affecting factors that bind to the MHC Class I heavy chain conserved enhancer sequence known as the class I regulatory element (CRE) (Fig. 3). In Ad12-transformed cells, binding of the NF- κ B transcription factor heterodimer p65:p50 (NF- κ B1) to CRE region I is decreased [30–33] and conversely binding of the chicken orphan upstream promoter-transcription factor (COUP-TF) II to CRE region II is increased [34–37].

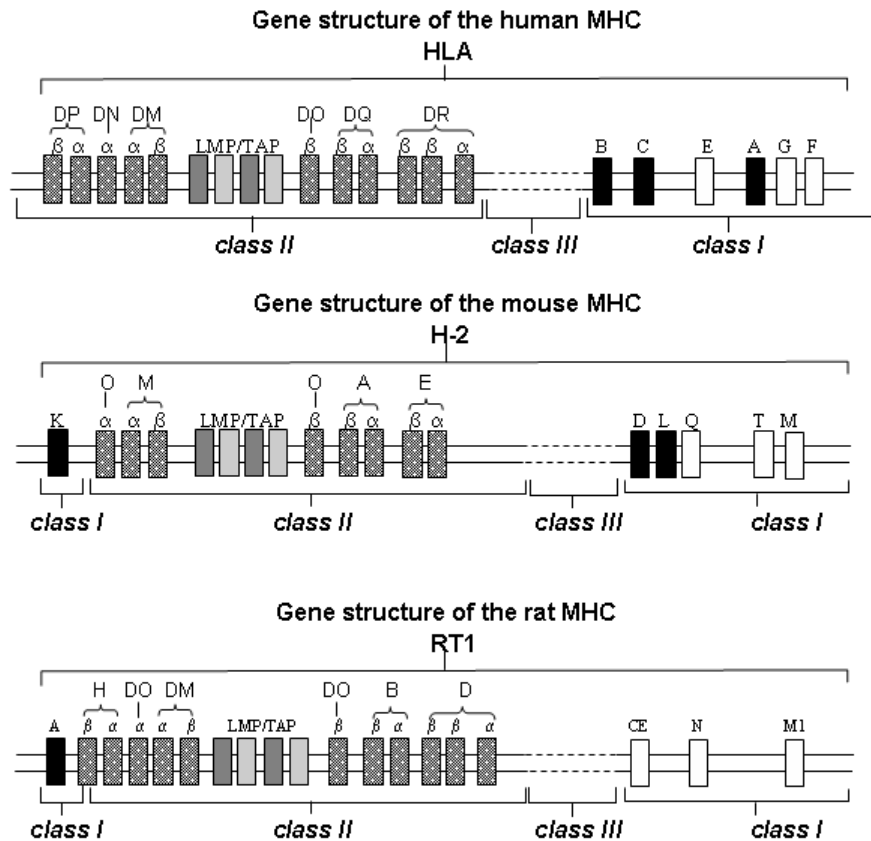


Figure 2. The organisation of the major histocompatibility complex (MHC) region in human HLA, mouse H2 and rat RT1. The genes encoding MHC Class I heavy chains (shown in black) and MHC Class II α and β chains (denoted by polka dots) are highly polymorphic. In contrast, the genes encoding the non-classical MHC Class I (shown in white) have low polymorphism. The MHC Class II region also contains the transporter associated with antigen processing genes (TAP1 and TAP2) and the LMP genes encoding IFN- γ inducible subunits of the immunoproteasome. Various alleles of the MHC Class I heavy chains exist at each locus (shown in black) which accounts for the polymorphic nature of the α_1 and α_2 regions of the MHC Class I heavy chain that form the peptide-binding cleft. Such polymorphism means that an individual has a sufficient number of allelic variants of MHC molecules to recognise peptides and could affect affinity of Ad E3 19kDa protein for MHC Class I molecules. Like MHC Class I heavy chain, TAP and LMP genes are polymorphic. Although no studies have been carried out on the effect of polymorphisms on processing of DNA tumour virus antigens, evidence suggests no influence of this polymorphism on viral antigen processing of the RNA virus Hepatitis C [23]. Figure based on [24, 25]

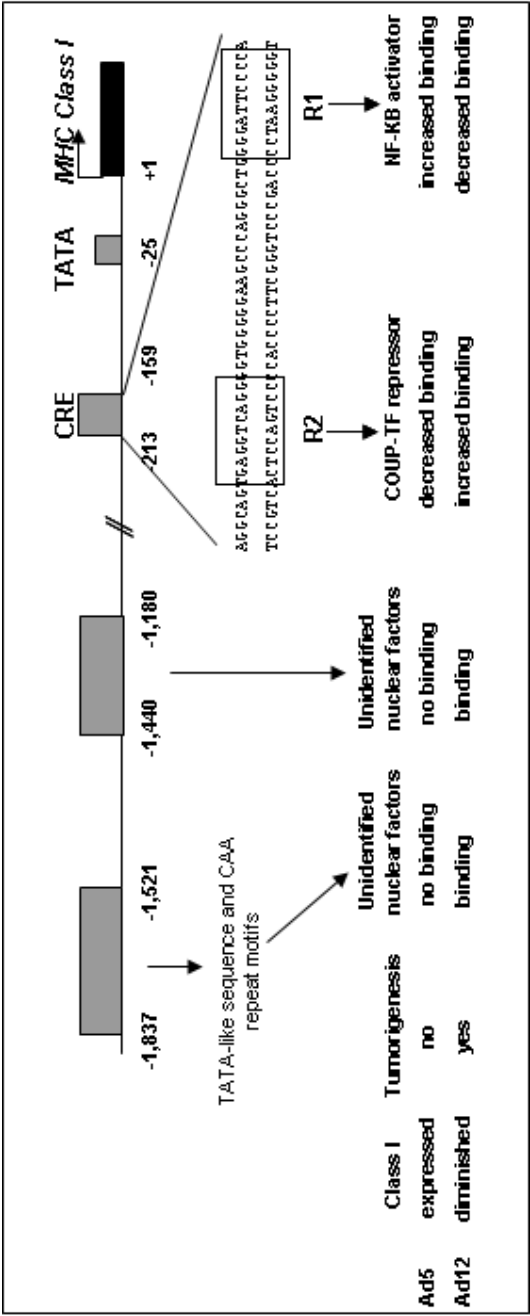


Figure 3. Transcriptional control of MHC Class I. The transcription of MHC Class I is under the control of the class I regulatory element (CRE) which binds the transcriptional activator NF-κB in region 1 (R1) and the transcriptional repressor COUP-TFII in region 2 (R2). Transcription from CRE is active in Ad5-transfected cells and vice versa in Ad12-transfected cells. A less-well characterised 260 bp region distal to CRE has been found to be sufficient for down-regulation of MHC Class I promoter and binds as yet unknown nuclear factors from Ad12- but not Ad5-transformed cells [48]. A further distal region encompassing 316 bp contains CAA repeat elements between -1,725 to -1,705 and -1,591 to -1,568; either CAA repeat in conjunction with the TATA-like sequence (located between -1,773 and -1,767) has been implicated in Ad12 E1A 13S mediated negative control of the H-2Kb promoter [49, 50]. However, this 316 bp region is not involved in Ad12-mediated negative control of the H-2Kb promoter [47]. Figure adapted from [47]

Transcriptional activation of MHC Class I gene is blocked by Ad12 E1A

The NF- κ B heterodimer is retained in the cytoplasm of resting cells by interaction with I- κ B, also termed p105. Various stimuli including viral proteins [reviewed in 38] can activate the I- κ B kinase complex to phosphorylate p105 on two serine residues thereby triggering ubiquitination and subsequent proteasomal degradation of p105 [reviewed in 39]. This releases the NF- κ B heterodimer which translocates to the nucleus where it can carry out transcriptional activation of MHC Class I heavy chain, amongst other target genes. It has been found that NF- κ B1 (p65/p50) does translocate to the nuclei of Ad12-transformed cells but somewhat unusually is incapable of DNA binding [31]. This is thought to be due to the abnormal hypophosphorylated state of the p50 subunit found in Ad12-transformed cells [40]. Recently, the DNA binding ability of p50 has been shown to depend on phosphorylation of Ser337 by the catalytic subunit of protein kinase A (PKAc) [41]. Ad12 E1A has been found to block this phosphorylation by interacting with both PKAc and p50 (Guan and Ricciardi, personal communication). It has also been suggested that disruption of the proteolytic processing of I- κ B (p105) by Ad12 E1A 13S alters the ratio of p50 to p105 in favour of p105, resulting in abnormally high levels of p105 that sequester p50 and p65 to the cytoplasm; the low concentrations of NF- κ B transcription factors could account for the decreased binding to CRE region I [reviewed in 42]. Gene activation is strongly associated with acetylation of the histone component of chromatin [43] and activation of NF- κ B responsive genes can be mediated by acetyltransferase enzymes downstream of TNF- α signalling [44–46]. However, although TNF- α treatment of Ad12-transformed cells increases the level of NF- κ B binding to DNA, it fails to relieve the transcriptional repression of MHC Class I heavy chains [32, 37].

Transcriptional repression of MHC Class I gene is achieved by Ad12 E1A

It has been found that the protein complex bound to CRE in Ad12-transformed cells comprises COUP-TFII, HDAC and Ad12 E1A [47]. HDACs are recruited to chromatin by DNA-bound repressor proteins where they act as transcriptional co-repressors by removing acetyl groups from histones [43]. In the currently proposed model for transcriptional repression of the MHC Class I heavy chain, COUP-TFII binds to CRE, recruits HDAC and mediates repression of MHC Class I gene expression through HDAC. Ad12 E1A is incapable of DNA binding,

so is thought to associate with the complex bound to CRE via protein:protein interactions; exactly how this is achieved remains to be elucidated.

In addition, less well-defined elements upstream of CRE are thought to mediate E1A repression of H-2K^b. Taking the transcriptional start site as bp position +1, the CRE region encompasses –213 bp to –159 bp; other sites implicated to be involved in Ad12 E1A repression of MHC class I heavy chain expression are a 260 bp region found at –1,440 bp to –1,180 bp [48] and a 316 bp area covering –1,837 bp to –1,521 bp [49–51]. Conflicting evidence exists regarding the 316 bp area; studies in this laboratory have found that this region is not implicated in repression of transcription from the mouse H-2K^b promoter [48]. The 260 bp region has been shown to be sufficient for Ad12 E1A-mediated down-regulation of the H-2K^b promoter; this down-regulation is thought to be achieved by the binding of nuclear factors from Ad12- (but not from Ad2- or Ad5-transformed cells) to more than one element within this region [48]. The same region is also implicated in H-2K^b repression by HPV18 E7 [52]. The identity of the elements within the 260bp region and the possible nuclear factors that bind to them are currently unknown but may represent a strategy for MHC Class I down-regulation that is shared by oncogenic Ad12 E1A and the high-risk HPV 18 E7 protein and possibly also the HPV 16 E7 protein that has been shown to repress H-2K^b promoter activity as efficiently as Ad12 E1A and HPV 18 E7 [52]. More recently, HPV 16 has been found to possess another strategy for down-regulation of MHC Class I surface expression; the HPV 16 E5 protein preferentially sequesters HLA-A and HLA-B molecules in the Golgi apparatus, thereby preventing transportation to the cell surface, but does not affect transport of HLA-C or HLA-E molecules [53]. However, the authors observed that as E5 is not often expressed in cervical carcinomas it is unlikely that the down-regulation of HLA class I observed in cervical carcinomas is due to E5 [53].

Other strategies used to disrupt the MHC Class I pathway; targeting of LMP and TAP

Aside from down-regulation of the MHC Class I heavy chain gene, it is also known that levels of TAP1 and LMP2 mRNAs are down-regulated in Ad12-transformed rat cells and evidence suggests that this down-regulation is determined by E1A [54]. Both the CRE region II and the TAP1/LMP2 bidirectional promoter contain an NF- κ B binding site (Figs 3 and 4) and it has been suggested that E1A exerts an effect on components of the MHC Class I pathway by targeting NF- κ B [54]. The importance of TAP1 expression is apparent on consideration of the

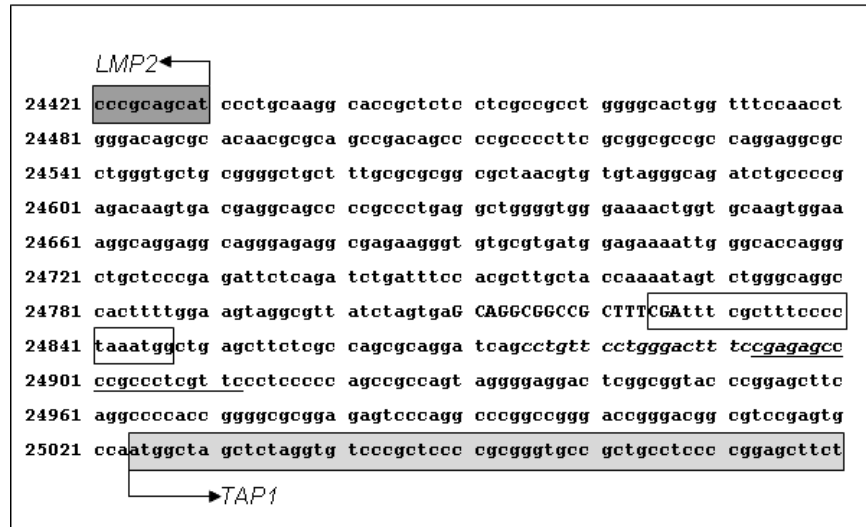


Figure 4. Transcriptional control of the LMP2/TAP1 genes is achieved by various factors that bind to the bidirectional promoter. The intergenic region between TAP1 (boxed in light grey) and LMP2 (boxed in dark grey) contains a bidirectional promoter that contains five cis-regulatory sequences. The source of the sequence is: BLAST record gi 34634 emb X66401.1 *H. sapiens* genes TAP1, TAP2, LMP2, LMP7 and DOB (residues 24421 to 25080). The interferon consensus sequence (ICS)-1 shown in upper case is required for constitutive expression of TAP and overlaps the ICS-2/GAS site. The overlapping ICS-2 and GAS binding site (boxed) binds unphosphorylated STAT1 to achieve constitutive expression of LMP2. Once phosphorylated by Janus kinases downstream of IFN- γ , STAT1 binding to the IFN- γ activated sequence (GAS) sequence results in rapid induction of the TAP1 gene, presumably to provide extra transport of viral peptides. An NF-KB binding site (indicated in italics) is sufficient for TNF- α upregulation of TAP1 gene expression; however, increased LMP2 expression requires binding to both NF-KB and SP-1 sites. The SP-1 site (underlined) overlaps the NF-KB site and is required for the constitutive expression of LMP2 and TAP1 [66, 67]. Polymorphism of the bidirectional promoter controlling expression of LMP2 and TAP1 has been frequently found in human tumours of various origins; however, this does not affect transcriptional activity of the LMP2/TAP1 promoter [68] and the polymorphism does not occur within any transcription factor binding site so far identified [69]. Levels of TAP1 and LMP2 are down-regulated in Ad12-transformed cells compared to Ad5-transformed cells by an unknown mechanism. It is tempting to speculate that transcriptional control of both CRE and the LMP2/TAP1 bidirectional promoter could be adversely affected by hypophosphorylated state of the p50 subunit found in Ad12-transformed cells [40]

MHC Class I pathway as outlined in Figure 1; in the absence of TAP1, no peptides can be transported across the ER membrane which would result in unstable MHC Class I molecules, reduced levels of cell surface MHC Class I molecules and increased likelihood of avoidance of CD8⁺ T cell recognition of infected cells. Further evidence for the importance of TAP1 comes from the observation that down-regulation of TAP1 expression in many cancer cells at the mRNA level results in avoidance of CD8⁺ T cell clearance of malignant cells [55–57]. In addition,

evidence from studies of metastatic murine carcinomas suggests that TAP down-regulation is at the level of transcription initiation caused by a decrease in the activity or expression of trans-activating factors that regulate TAP1 promoter activity [58]. As well as down-regulation of TAP1, impaired expression of the IFN- γ inducible proteasome subunits LMP2 and 7 have been reported in a number of tumour lesions and tumour cell lines [59–65]. The precise mechanism of transcriptional down-regulation of TAP1 and LMP2 in Ad12-transformed cells compared to Ad5-transformed cells has not been defined. However, it is tempting to speculate that the transcriptional control of both the MHC Class I heavy chain promoter and the LMP2/TAP1 bidirectional promoter could be adversely affected by the hypophosphorylated state of the p50 subunit found in Ad12-transformed cells [40].

It has been reported that the high risk HPV 18 E7 protein is capable of repressing the TAP1 and LMP2 promoters as efficiently as Ad12 E1A [52]. Low levels of TAP1 are found in both HPV 16 and 18 induced cervical cancers [70] so at first glance it seems odd that HPV 16 E7 does not affect expression from the TAP1/LMP2 bidirectional promoter [52]. However, it has been suggested that such conflicting activity could contribute to the apparently distinct patterns of carcinogenesis displayed by HPV 16 compared to HPV 18; for example, the latter serotype is associated with more advanced tumours and poorer prognosis [52]. Low risk HPV serotypes also target TAP1; for example, the E7 protein of HPV 11 which is associated with benign tumours of the trachea and larynx can bind TAP1 and block peptide transport [71], as indicated in Figure 1.

The ‘missing self’ hypothesis

The down-regulation of MHC class I in Ad12-transformed human cells leads to avoidance of recognition of the virally infected cell by CD8⁺ T cells. However, according to the ‘missing self’ hypothesis (Fig. 5) the absence or reduction in MHC Class I expression renders a cell susceptible to natural killer (NK) cells so Ad12-transformed human cells should still be recognised and destroyed by the immune system [reviewed in 72]. Further research since the initial proposal of the ‘missing self’ hypothesis uncovered more details of the interaction between the NK cell and the target cell and several NK cell activating and inhibitory receptors and host cell ligands have now been defined [reviewed in 73].

Recent observations in this laboratory seem to support the ‘missing self’ hypothesis in that, compared to Ad5-transformed cells, Ad12-transformed human

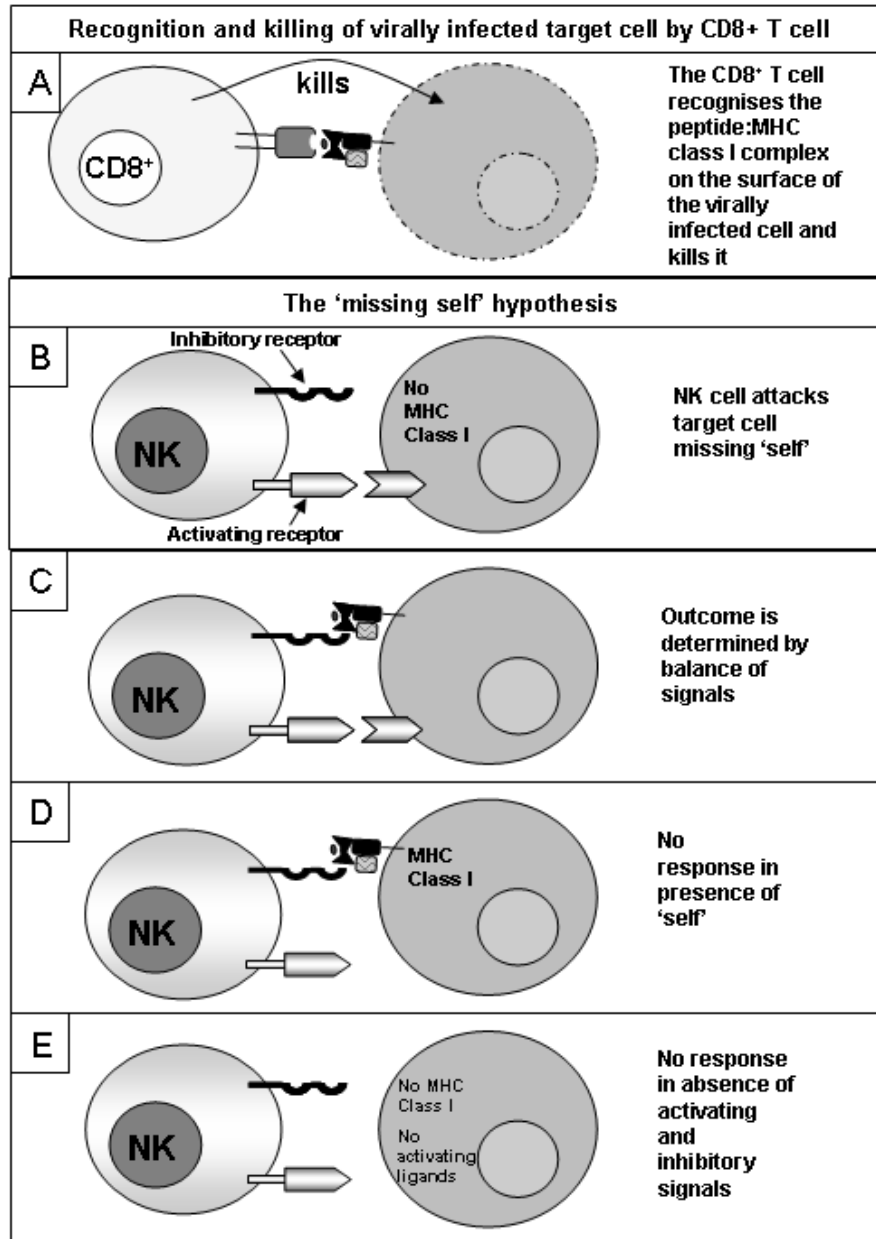


Figure 5. CD8+ killing, NK-cell self tolerance and the 'missing self hypothesis'. The down-regulation of MHC Class I by DNA tumour viruses leads to avoidance of the normal CD8+ T cell recognition of virally infected cells (panel A). But the cell has a backup response (panel B); cells deficient in MHC Class I surface expression are subject to NK cell-mediated killing because the activating signal is unchecked and NK cell release of cytotoxic granules and cytokines (TNF- α and IFN- γ) result in target cell lysis. However, the situation is complicated by the outcome being dependent on the balance of inhibitory and activating signals received by the NK cell (panel C). In the presence of MHC Class I and absence of activating ligands, the cell is recognised as self and not subject to NK cell-mediated killing (panel D). However, not all inhibitory signals are sent by MHC Class I recognition; other ligands expressed by the target cell can fulfil this role. In the absence of both activating and inhibitory signals no NK cell response is engaged (panel E). Figure based on [80]

cells were found not only to display reduced levels of surface MHC Class I expression but also were more susceptible to killing by lymphocyte-activated killer (LAK) cells (NK cells activated by IL-2) [22]. However, in contrast, the susceptibility of Ad5-transformed rodent cells to NK cell-mediated killing is greater than that of Ad12-transformed rodent cells [74]; one possible reason for this was proposed by Bottley et al. (2005) to be due to the NK cell receptor molecule differences between rodents and humans [reviewed in 75]. Interestingly, it was previously shown that, while infection of human cells with Ad2 or Ad5 did not induce increased NK cell susceptibility, transformation of human cells with Ad2 or Ad5 E1A does [76]. It has been proposed that this differential response to NK cell-mediated killing could involve the E3 14.7 kDa protein [42]. The E3 14.7 kDa protein is expressed during viral infection but not in transformed cells and inhibits the cytolytic effects of TNF- α [77, 78].

Currently, the identities of the ligands that signal from virally-infected cells to NK activating receptors are unknown and other possible viral mechanisms of interfering with NK cells remain a mystery. It is thought that activating NK receptors could play the crucial role in NK cell recognition of virally-infected cells, as opposed to the inhibitory receptor signal [79]. This raises the possibility that the increased susceptibility of Ad12-transformed human cells to NK cell-mediated killing compared to Ad5-transformed human cells could be due to increased expression of an activating ligand by Ad5-transformed cells; for example, expression of ligands for NK group 2, member D (NKG2D) are known to be induced by transformation, amongst other stimuli [reviewed in 80]. Although little is known about how NKG2D ligand expression is regulated on the surface of tumour cells, it has recently been found that IFN- γ inhibits expression of N60 (one of the murine NKG2D ligands) concomitantly with MHC Class I up-regulation and reduces susceptibility to NK cell-mediated killing of 3'-methylcholanthrene (MCA) induced

sarcomas from mice [81]. The first ligand for NKR-P2 (the rat orthologue of human and mouse NKG2D) has recently been discovered: named rat RAE-1-like transcript (RRLT) this transmembrane protein is upregulated in many tumour cell lines and tissues, and induces NK cell activation and renders cells susceptible to NK cell-mediated killing [82].

Evidence suggests that the conundrum of why Ad12-transformed human cells are more susceptible than Ad5-transformed human cells to NK cell-mediated killing will involve factors other than just MHC Class I down-regulation. Indeed, MHC Class I down-regulation by DNA tumour viruses is probably not sufficient to explain tumorigenicity; rather, it is becoming clearer that a complex web of interactions with host cell pathways is involved. The remainder of this review will summarise some of these interactions.

The interferon response: a prime target

One of the first responses of a cell to viral infection is to launch an interferon (IFN) response. In fact, IFNs were so named as they were found to interfere with viral replication in cells. Type I IFNs; the α and β interferons, have three main functions; they induce resistance to viral replication in cells, increase MHC Class I expression and antigen presentation in cells and activate NK cells to kill virus-infected cells. Unsurprisingly, viruses have evolved mechanisms to circumvent the IFN response [reviewed in 83].

Type II IFN (IFN- γ) plays an important role once the adaptive immune response has begun; it is produced in conjunction with TNF- α by activated NK cells and the synergistic activities of these two cytokines has been found to be responsible for the majority of cytotoxic LAK cell activity directed against MCF-7 cells [84]. For a virus-infected target cell to respond to an extrinsic death signal delivered via TNF- α or IFN- γ , the receptors on the target cells must be able to respond to the cytokines. Adenovirus proteins interfere with both TNF- α and IFN- γ receptors; specifically, the E3 encoded receptor internalization and degradation (RID) proteins α and β form a RID complex that directs internalisation and lysosomal degradation of TNF receptors [reviewed in 85] and the Ad5 E1A protein inhibits the expression of the IFN- γ R2 chain of the IFN- γ receptor [85]; whether these activities have effects on NK cell-mediated killing of adenovirus-transformed or infected cells has not been investigated.

The down-regulation of various IFN- γ inducible components of the MHC Class I pathway seems to be pointing to a role for IFN- γ pathway modulation in the

affected cells. Accordingly, research has been carried out into the potential of IFN- γ treatment of patients with various cancers; however, responses have been both positive [87] and negative [88]. Such dichotomy has been suggested to be due to different IFN-receptor status of patients [89]. Interestingly, an adenovirus vector carrying the IFN- γ gene has recently been shown to display highly efficient anti-tumour activity, with oncolysis, anti-angiogenesis and immune response induction is triggered in cancer cells *in vivo* [90]. This underlines the diverse nature of IFN- γ signalling, which can affect the JAK/STAT, the MAPK and the PI3K/mTOR signalling pathways [reviewed in 91].

DNA tumour viruses and oncogenic signalling

Interfering with the response to extrinsic cytokine signalling is not the full story of how DNA tumour viruses exert their oncogenic effects; as can be seen in Figure 6 a number of other cellular proteins are targeted by viral proteins. In common with tumour cells, cells infected with DNA tumour viruses evolve to become what has been cleverly termed ‘insidious Dorian Grays’, in that they remain ‘forever young’ by avoiding permanent cell cycle arrest (i.e. senescence) and display apparently endless replication potential [92]. The main goal of DNA tumour viruses is to achieve replication; this is dependent on maintaining favourable conditions within the host cell.

Avoidance of apoptosis: required for both DNA tumour virus targets and cancer

It can be seen in Figure 6 that the master regulator of apoptotic, senescence and repair programmes is the cellular transcription factor p53. In 50% of human cancers, p53 is mutated and if wild-type p53 is present in a tumour then more often than not mutations in other genes (for example, ARF) that affect p53 function and/or activation are found [93]. Oncogenes activate the p53 checkpoint by inducing ARF; this prevents Mdm2 degradation of p53 [94]. Targeting of p53 is also a common strategy employed by DNA tumour viruses to prevent premature apoptosis of infected host cells; for example, both Ad and HPV express proteins from the E1B (in concert with E4-ORF6) and E6 regions, respectively, that bind to and degrade p53 [95–100]. Repression of p53-mediated transcription can also be affected by Ad E1B 55K in the absence of E4-ORF6 [101]. Interestingly, HPV E6 can also induce avoidance of p53-mediated apoptosis by inhibiting p300-mediated

acetylation [102]. Avoidance of apoptosis allows proliferation of the cell to continue unabated. In addition, it has recently been shown that p53 enhances the transcription of late adenoviral genes; this has led to the suggestion that p53 is 'highjacked' by adenovirus to assist with late gene expression prior to degradation [103]. The study also showed that Ad-induced cell lysis occurred more efficiently in cells expressing functional p53. These findings have important consequences for lytic cancer therapies: in particular, ONYX-015.

Originally described in the last decade, ONYX-015 is an adenovirus lacking the E1B-55K open reading frame which inactivates the host cell p53 gene product. The initial theory was that infection of normal cells with ONYX-015 would lead to p53-mediated cell cycle arrest as the E1B-55K mediated anti-p53 activity would be absent. In contrast, infection of tumour cells with ONYX-015 should lead to cell lysis due to the lack of p53; viral progeny would be released and go on to infect and ultimately destroy the entire tumour. However, interest in ONYX-015 began to wane following several reports that ONYX-015 was not specific for p53-null cells [reviewed in 104]. The tendency of ONYX-015 to kill cells with wild type p53 as opposed to mutant p53 could be explained by p53 substituting for E1B-55K to promote viral late gene expression and hence replication [103]. However, the field of oncolytic virus research is still active particularly in China where H101, a genetically modified adenovirus based on ONYX-015 but with a slightly larger deletion in the E3 region, is expected to be marketed by Shanghai Sunway Biotech this year for the treatment of head and neck cancer [105].

Apoptotic mechanisms that operate independently of p53 also exist; for example, apoptotic pathways controlled by cell surface death receptors Fas and TNF. A decoy receptor for the Fas ligand (FasL) is up-regulated in many tumour cell lines, which has the effect of titrating FasL away from the actual death receptor [106]. Also, Fas cell surface receptors are up-regulated on activated T cells; this results in increased susceptibility to apoptosis following interaction with tumour cells expressing the FasL [107]; this has been proposed as a possible mechanism for immune escape in cervical carcinoma [108].

Deregulated cell cycle entry: a common feature in cancer and viral replication

Unchecked proliferation is possible due to disruption of growth and anti-growth signalling networks, most of which have the retinoblastoma family of tumour suppressor proteins (RB, p107 and p130) as central players, as indicated in

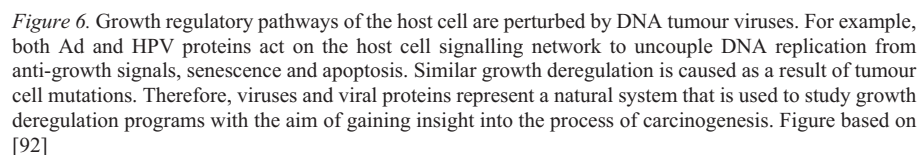


Figure 6. At least one third of human tumours have either lost or contain mutated RB; or, if wild-type RB is present other components of the pathway are deregulated [reviewed in 93]. DNA tumour viruses also make infected cells impervious to anti-growth signals. For example, Ad E1A and HPV E7 bind to the pocket region of RB/p107/p130 via a homologous LXCXE motif resulting in E2F activation [reviewed in 109]. E1A and E7 can also both bind to histone acetyl transferases (HATs) such as p300 [110] and E1A binding to either RB or p300 is sufficient for S phase entry of quiescent cells [111].

**Dysregulation of a highly complex web of interactions at play
in both cancer and DNA tumour virus infection:
the usefulness of the adenoviral model**

From just the simplified examples already discussed it should be apparent that the oncogenic potential of adenoviruses is governed by dysregulation of diverse pathways within host cells. Understanding these perturbations is worthwhile, as it could provide key insights into the rewiring of cells acquired during tumorigenesis. Although many valuable insights have come from mouse models, it is not practical to study spontaneous naturally occurring tumours in mice. Viral proteins can act as biochemical probes to identify critical cellular players in the tumorigenesis process [92]. The adenovirus model is particularly useful; although both human adenovirus species A and C can transform cells, only species A is oncogenic in immunocompetent rodents; therefore studying cellular networks affected by adenoviral proteins from both serotypes could provide clues to the pathological rewiring acquired during tumorigenesis. It has been suggested that if a particular region of E1A was involved in both MHC Class I down-regulation and oncogenesis this could provide a connection between the two processes [42]. Microarray studies of adenovirus-transformed cells performed in both this laboratory (unpublished data) and others [112, 113] as well as microarray studies of adenovirus-infected cells [114, 115] indicate that numerous target genes exist for Ad E1A; recently, Ad 12S E1A has been found to target most members of the urokinase-plasminogen activation system which possibly correlates with the anti-metastatic effect of Ad E1A in human cells [116].

Concluding remarks

The post-genomic era has seen huge advances in technology that have revealed many differences between tumour and normal cells; however, it is debatable whether all of these differences are relevant for creation or maintenance of the neoplastic phenotype. Studying how DNA tumour viruses such as adenovirus subvert host cell mechanisms could bring clarity to this. DNA tumour viruses such as adenovirus manipulate complex cellular pathways and avoid immune surveillance in a targeted fashion using a limited repertoire of viral proteins encoded by a relatively small genome size.

Current evidence points to the serotypic origin of the Ad E1A oncogene being the determining factor in the down-regulation of components of the MHC Class I pathway at the level of transcription: namely; TAP, LMP and MHC Class I. Adenovirus-transformed cells also display oncogenicity-dependent differential susceptibilities to NK cell mediated killing; whether this is due to E1A remains to be defined. Unravelling exactly how oncogenic Ad12 evades immune surveillance represents a major challenge. The down-regulation of MHC Class I may contribute to the escape from immune surveillance; however, given the diversity of cellular targets that adenovirus is capable of subverting a complex web of interactions are likely to be involved.

The persistence of both DNA tumour virus-infected cells and tumour cells is dependent not only on the avoidance of the immune system but also on dysregulation of a complex network of cellular pathways. A number of critical players in tumorigenesis such as p53 and RB have already been identified based on studies using DNA tumour viruses. The challenge is to fill in the rest of the cellular networks that are dysregulated and gain a better understanding of how the immune response is avoided. Such knowledge will enable the discovery of novel tumour targets and the rational design of oncolytic viruses and combination anti-cancer therapeutics.

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