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Expression of JC virus regulatory proteins in human cancer: Potential mechanisms for tumourigenesis

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Abstract

JC virus (JCV) is a human polyomavirus that is the etiologic agent of the fatal demyelinating disease of the central nervous system known as progressive multifocal leukoencephalopathy (PML). JCV is also linked to some tumours of the brain and other organs as evidenced by the presence of JCV DNA sequences and the expression of viral proteins in clinical samples. Since JCV is highly oncogenic in experimental animals and transforms cells in culture, it is possible that JCV contributes to the malignant phenotype of human tumours with which it is associated. JCV encodes three non-capsid regulatory proteins: large T-antigen, small t-antigen and agnoprotein that interact with a number of cellular target proteins and interfere with certain normal cellular functions. In this review, we discuss how JCV proteins deregulate signalling pathways especially ones pertaining to transcriptional regulation and cell cycle control. These effects may be involved in the progression of JCV-associated tumours and may represent potential therapeutic targets.

Keywords: JC virus; Neoplasms; Transcription; Genetic; Transcription factors; Leukoencephalopathy; Progressive multifocal; Cell transformation; Viral

1. Introduction

For any particular cancer, a knowledge of the genetic changes that have occurred and the signal transduction pathways that have become deregulated as the tumour developed is important for formulating a treatment for that cancer, whether by gene therapy or by small molecule inhibitors. Multiple successive genetic changes or "hits" are thought to be involved in the development of cancer from normal cells to fully malignant metastatic tumours. Despite the small size and simple organization of the genomes of polyomaviruses, such as the human JC virus (JCV), they encode powerful multifunctional transforming proteins that have been extensively studied as a model system for malignant transformation. We suggest that these viral proteins can provide several "hits" at once to cells and thus circumvent steps that are necessary for malignant transformation during non-viral tumourigenesis. In this short review, we will first consider the types of genetic changes that cells undergo as they progress from normality to becoming fully malignant. We will briefly describe what is known about the life cycle of JCV and the evidence, both experimental and clinical, that it has a role in human cancer. Finally, our current understanding of the mechanisms of action of the JCV regulatory proteins on cellular processes is the major topic of this review.

2. Genetic changes associated with cancer

A cancer cell can be defined as a cell that has accrued sufficient genetic changes to gain the ability to grow pro-

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gressively *in vivo* and kill the host. Mutations have been reported to occur in a great many genes that are associated with the multi-step development of various cancers. These can be classified into three broad categories: oncogenes, tumour suppressors and mutators.

Oncogene mutations typically subvert the machinery whereby extracellular proliferative stimuli are transduced to the nucleus to induce changes in transcription that are associated with cell growth. This enables the cell to undergo proliferation inappropriately in the absence of a physiological cue. Oncogenic mutations may occur in genes that encode growth factor receptors [1]; plasma membrane molecular switches such as Ras [2,3]; protein and lipid kinases that are constituents of signal transduction pathways in the cytoplasm such as Raf/MEK/Erk [4] and PI3K/Akt [5]; apoptotic regulators [6]; and transcription factors [2]. Mutations in the Ras and Raf proto-oncogenes are very common in human cancers [7–9].

The second category of genetic changes that are associated with cancer involves the tumour suppressor genes (anti-oncogenes). Tumour suppressors are negative regulators of cell growth that function to co-ordinate signalling pathways and prevent inappropriate proliferation. Mutations that inactivate tumour suppressors are a common feature of all cancers. The p53 tumour suppressor gene is mutated in most human cancers [10]. p53 is the central regulator of a signalling network that is turned on when cells become stressed or damaged thereby inhibiting cell proliferation and promoting apoptosis. This serves to eliminate damaged cells that could develop into a tumour explaining why p53 is often mutated in cancer cells [10]. The pRb tumour suppressor (retinoblastoma gene product) is the central coordinator of cell cycle progression and is regulated by multiple phosphorylation events [2]. pRb sequesters the E2F transcription factor which is required for expression of genes whose products are necessary for cells to enter S-phase [2].

The third category of mutation that can be found in tumours does not affect cell growth *per se* but rather promotes cancer by inducing genetic or karyotypic instability. Impairment of DNA repair genes can lead to a mutator phenotype that predisposes cells to incur mutations at a faster rate than normal cells thus promoting tumour progression. Examples of intracellular mechanisms that are involved in DNA damage repair, and can lead to a mutator phenotype when mutated, include nucleotide excision repair, base excision repair and mismatch repair. Hereditary cancer predisposition syndromes are characterized by defects in a particular DNA repair gene, *e.g.*, hereditary non-polyposis colorectal carcinoma (HNPCC), Bloom syndrome, ataxia-telangiectasia, and Fanconi anemia [11]. HNPCC is associated with a defect in a mismatch repair gene [12,13].

Cancer is a multi-step process involving many successive "hits". The paradigm for this process of tumour evolution is provided by the colorectal adenoma-carcinoma sequence [14]. Mutations in all three of the categories outlined above are involved in the multiple steps of tumour progression. It is possible that tumourigenesis associated with polyomaviruses, such as JCV, is enhanced because they express proteins that are highly multifunctional and hence can deliver the equivalent of many hits at once. The multiple functions of these proteins will be described below and fall into all of the three categories. Indeed, in the case of the polyomavirus SV40, the large-T oncoprotein can effect the direct tumourigenic conversion of normal human diploid epithelial and fibroblast cells when expressed ectopically together with H-ras and telomerase [15].

3. JCV

JCV is a polyomavirus, a family of viruses that are non-enveloped, have icosahedral capsids and contain small, circular, double-stranded DNA genomes. Multiple animal species harbour polyomaviruses and these typically have a very limited host range and do not productively infect other species [16,17]. Mouse polyoma virus [18] and simian vacuolating virus 40 (SV40) [19] were the first to be discovered and have been intensively studied as model systems for investigating the basic eukaryotic molecular biology of processes such as DNA replication, transcription, malignant transformation and signal transduction. In 1971, two human polyomaviruses were discovered. BK virus (BKV) was isolated from the urine of a kidney allograft recipient with chronic pyelonephritis and advanced renal failure [20]. JC virus was isolated from the brain of a patient suffering from progressive mutifocal leucoencephalopathy (PML) [21].

JCV is very closely related to SV40 and BKV as evidenced by a similar genome size (~5.2 kb), genome organization and DNA sequence. The circular JCV genome has two regions (early and late), whose transcription is initiated in opposite directions from a common non-transcribed regulatory region that also contains the origin of viral DNA replication. The JCV late region encodes the capsid proteins VP1, VP2 and VP3 that arise by alternative splicing and a small regulatory protein known as agnoprotein. The JCV early region encodes the alternatively spliced transforming proteins large T-antigen and small t-antigen (see Fig. 1). These proteins are important in promoting transformation of cells in culture and oncogenesis *in vivo*.

Infection of cells by JCV is initiated by the binding of the virion to a receptor on the outer cell membrane consisting of α (2–3)- and α (2–6)-linked sialic acid residues [22,23]. Since these oligosaccharides are ubiquitously

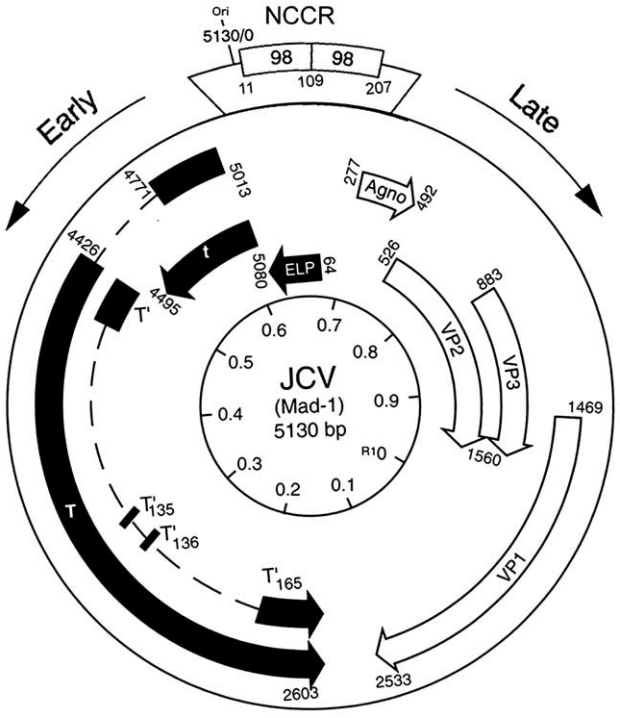

Fig. 1. Organization of the JCV genome (strain Mad-1). A schematic representation showing the JCV genome is shown. Nucleotides are numbered relative to the Mad-1 reference strain (GenBank # NC_001699). T – large-T antigen, t – small-t antigen, T′135, T′136 and T′165 – three additional alternatively spliced forms of T-antigen [118], Agno – the late auxiliary protein, agnoprotein, ELP – putative early leader protein.

present on glycoproteins and glycolipids on the surface of cells, JCV can enter a broad spectrum of mammalian cell types [24]. However, JCV replication is restricted to glial cells and lymphoid cells of the B-cell lineage due to cell-specific intranuclear limitations that dictate JCV tropism. After binding to the cell surface, JCV virions undergo endocytosis and are transported to the nucleus where the viral DNA is uncoated and transcription of the early region begins. After JCV enters the nucleus of a permissive cell, the early region primary transcript is expressed and is alternatively spliced to give rise to large T-antigen and small t-antigen. JCV T-antigen is a large nuclear phosphoprotein that is an essential factor for viral DNA replication. JCV relies on cellular enzymes and cofactors for DNA replication that are only expressed during S-phase. For this reason, JCV T-antigen stimulates cellular signalling pathways promoting cell cycle progression through its ability to bind to a number of cell cycle control proteins. As viral replication proceeds, the late genes become expressed through T-antigen-stimulated transcription of the late promoter leading to expression of the capsid proteins VP1, VP2 and VP3, which assemble with the replicated viral DNA to form intranuclear virions, which are released upon cell lysis.

JCV has a unique tropism for replication in glial cells that is largely determined at the level of viral early gene transcription which is responsible for the production of T-antigen [25,26]. Detailed analyses of the JCV early promoter have shown that transcription is regulated in a complex fashion. Multiple transcription factors, both general and cell-type specific, regulate the JCV early promoter including Jun, NF-1, GF-1, Sp1, Sμbp-2, Purα, and YB-1 [26,27].

The transmission of JCV is not fully understood. JCV is widespread throughout the human population with greater than 80% of adults exhibiting JCV-specific antibodies [28]. Infection occurs during childhood and is usually subclinical. In the human body, it is thought that JCV can infect cells in the tonsils and spread from there by replication in lymphoid cells [29,30]. JCV may be found in the urine and the kidney is thought to be the major organ of JCV persistence during latency [31]. JCV emergence from latency is associated with PML which is a fatal demyelinating disease of the CNS which is characterized by multiple foci of demyelination caused by lytic infection of oligodendrocytes, the myelin-producing cells of the CNS [32]. Only individuals with severely impaired immunity, mainly AIDS patients, develop PML.

4. JCV and cancer

There is both experimental and clinical evidence for a role of JCV in cancer. JCV is able to transform cells in culture, particularly cells of glial origin including human fetal glial cells and primary hamster brain cells. JCV-transformed cells exhibit the phenotypic properties associated with transformation including growth in soft agar, serum-independence, changes in morphology, plasminogen activator production, etc. The transforming ability of JCV appears to be limited to cells of neural origin and cell-type specific transcriptional regulation of the viral promoter is thought to be responsible for this property, as discussed above. Many studies have established the highly oncogenic potential of JCV in laboratory animals, *e.g.*, JCV induced multiple types of brain tumours when injected into the brains of newborn Golden Syrian hamsters. JCV is the only human virus that induces solid tumours in non-human primates. JCV caused the development of astrocytomas, glioblastomas and neuroblastomas in owl and squirrel monkeys which occurred 16–24 months after inoculation of JCV intracerebrally, subcutaneously or intravenously [33,34]. Transgenic mice expressing JCV T-antigen can develop adrenal neuroblastomas, tumours of primitive neuroectodermal origin, tumours arising from the pituitary gland and malignant peripheral nerve sheath tumours, rare neoplasms that occur in individuals with neurofibromatosis.

The possible association of JCV with human brain tumours was first indicated by the detection of JC virus DNA sequence and expression of viral oncoprotein T-antigen in the brain of an immunocompetent patient with oligoastrocytoma. The identity of PCR-amplified product from this tumour was confirmed as JCV by DNA sequencing. JCV RNA and T-antigen protein were detectable by primer extension analysis and Western blotting, respectively, in the tumour tissue indicating that JCV gene expression occurred in the tumour cells [35]. In other studies, JCV DNA and T-antigen expression were found in 57–83% of tumours of glial origin [36] and in ~50% of pediatric medulloblastomas [37,38]. Many other studies that have employed PCR-mediated DNA amplification and/or immunohistochemistry of neural-origin tumour samples provide support for an association of JCV with a wide variety of tumours of the central nervous system (CNS) and in other tumours such as colon cancer [39] and CNS lymphoma [40].

Thus the association of JCV with human tumours and the effects of JCV on cultured cells and in experimental animal suggest JCV-mediated cellular transformation may be involved in neoplasia. However it remains unknown whether JCV has a causal role in human neoplasia. More extensive reviews of the role of JCV in neoplasia have been published elsewhere [41–45].

5. Deregulation of cellular processes by JCV regulatory proteins

JCV is a very small virus with a genome size of only 5130 bp and encoding only 6 viral proteins: 3 capsid structural proteins (VP1, VP2 and VP3) and 3 regulatory proteins (T-antigen, t-antigen and agnoprotein). Thus it is not surprising that the regulatory proteins of JCV are highly multifunctional. The virus needs to drive the host cell into S-phase in order for viral DNA replication to occur and hence the viral regulatory proteins target a variety of cellular proteins involved in signalling and cell cycle control. A corollary of this is that their expression in a restrictive cell setting can lead to transformation. We will now consider the mechanisms that are involved for each of the three JCV regulatory proteins.

5.1. Large T-antigen

Large T-antigen is an amazing protein. Firstly, it is an essential factor for viral DNA replication. In the case of SV40, T-antigen binds specifically to the origin of replication as a dodecamer where it serves as an ATP-dependent replicative DNA helicase and orchestrates the assembly and function of cellular proteins such as replication protein A and DNA polymerase-α that constitute the viral replisome [16]. High-resolution structural analysis of SV40 T-antigen hexamers in distinct nucleotide binding states has revealed that conformational changes are triggered by ATP binding/hydrolysis. These create an ''iris''-like motion in the hexamer with β-hairpins on the channel surface moving longitudinally along the central channel containing the viral DNA, possibly serving as a motor for pulling DNA into the T-antigen double hexamer for unwinding [46].

Secondly, T-antigen interacts with a plethora of cellular proteins that are responsible for driving cells into S-phase and mediates cellular transformation. These interactions will be discussed in this section. The T-antigen proteins of SV40, JC and BK are 708, 688, and 695 amino acid residues in length, respectively, and share 70–80% sequence identity. T-Antigen is mainly localized to the nucleus with a small amount being found at the plasma membrane. Indeed SV40 T-antigen was the first protein for which a nuclear localization signal was characterized [47,48]. T-antigen has multiple post-translational modifications including phosphorylation, O-glycosylation, acylation, adenylation, poly-ADP-ribosylation and N-terminal acetylation [16]. Some investigations of T-antigen have focused on SV40 while others have focused on JCV. This will be made clear in each case and comparative data where available will be discussed.

5.1.1. pRb

One key regulatory protein that binds T-antigen is the retinoblastoma gene product, pRb. This is a central coordinator of cell cycle progression. The Rb family of so-called pocket proteins negatively regulates progression from G_0 through G_1 to S-phase. pRb, p107 and p130 possess a binding pocket for members of the E2F family of transcription factors that control expression of S-phase-related genes, *e.g.*, cyclin A, cyclin E, DNA polymerase-α, ribonucleotide reductase, thymidine kinase, c-*fos*, c-*myc*, etc. [51,52]. During the cell cycle, CDK4 and CDK6 cause phosphorylation of pRb in late G_1 allowing release of the E2F transcription factors that facilitate S phase progression [49,50]. The T-antigens of all three primate polyomaviruses (JCV, BKV and SV40) bind to pRb and displace E2F thereby promoting cell cycle progression. This is a major mechanism whereby T-antigen promotes the inappropriate cell proliferation characteristic of oncogenically transformed cells [53–57]. The Rb-binding domain of T-antigen is highly conserved between JCV and BKV but not between JCV/BKV and SV40 perhaps explaining the lower affinity for pRb exhibited by JCV and BKV T-antigens compared to SV40 T-antigen 56,58]. Inactivation of pRB family members by T-antigen binding leads to increased E2F activity and bypass of pocket protein-mediated G_1 arrest. Binding of T-antigen to pRb family members involves not only the T-antigen pRb-binding domain but

also the T-antigen N-terminal J-domain (see below). This leads to hypophosphorylation of the pocket protein [52]. The pRb-binding domain of T-antigen is required for transforming activity [52].

5.1.2. p53

Another key target of T-antigen is p53. p53 protein is a tumour suppressor encoded by a gene whose disruption is associated with ~50–55% of all human cancers. p53 is the main mediator of the checkpoint in the cell cycle and is able to halt cell cycle progression and in some cases initiates programmed cell death. This occurs when DNA damage has been inflicted on a cell or when cell proliferation is activated inappropriately, *e.g.*, by an oncogene. This serves to eliminate damaged or transformed cells and is an important defense against cancer. DNA damage activates p53 through the protein kinases ATM and Chk2 while oncogenic transformation acts through a different protein $p14^{ARF}$ [59,10]. The release of E2F from pRb by T-antigen activates transcription of the cell cycle regulatory protein $p14^{ARF}$. $p14^{ARF}$ exerts its function by interfering with MDM2 (a negative regulator of p53) that functions by stabilising p53. Thus $p14^{ARF}$ would slow the cell cycle and viral replication through activation of p53 [59,10]. However, JCV T-antigen binds to and inactivates p53 and thus prevents inhibition of the cell cycle or apoptosis [60,57]. Thus T-antigen prevents $p14^{ARF}$ inhibition of the cell cycle. This is important for providing an optimal cell environment for viral replication and packaging during polyomavirus lytic infection and also facilitates transformation in non-permissive cells [59,10]. Interaction of T-antigen with p53 has been demonstrated for SV40 T-antigen [61] and for JCV and BKV T-antigens [57,60]. p53 is a transcription factor that upregulates proteins that are inhibitory for cell cycle progression, *e.g.*, p21Waf1/Cip1 which inhibits the kinase activities if cyclin E/cdk2 and cyclin D/cdk4–6 [62]. T-antigen binding to p53 blocks the ability of p53 to bind to DNA [63] (see Fig. 2).

5.1.3. p300/CBP/p400

The SV40 T-antigen/p53 dimer binds to members of the p300/CBP/p400 family of transcriptional co-activators to form a ternary complex [64]. These co-activator proteins are histone acetylases [65] and interact with a variety of cellular regulatory proteins including p53, MDM2 and NFκ B p65 subunit [63]. Knockout mice lacking a CBP allele develop hematological malignancies indicating a tumour suppressor function [66]. The roles of these proteins in the regulation of cell growth and transformation have recently been reviewed [67]. The binding of T-antigen to p300 and p400 is mediated by the C-terminus of T-antigen and mutations in this region that abrogate p53 binding also abrogate p300 and p400 binding [64]. p300 is a co-activator of transcription from p53-dependent promoters such as p21Waf1/Cip1 and Bax

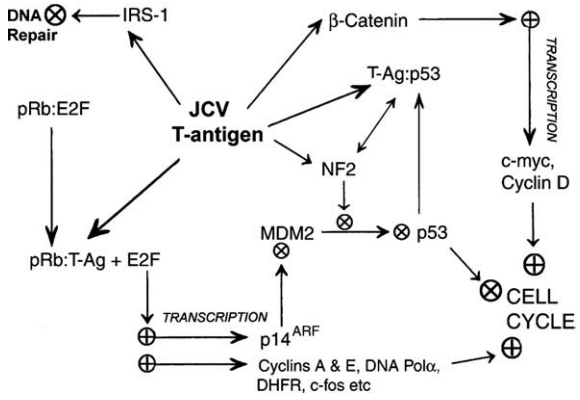

Fig. 2. Interactions of JCV large T-antigen. The interactions of JCV large T-antigen with cellular proteins are shown. T-antigen binds to pRb causing the release of the E2F transcription factors that induce the expression of proteins such as Cyclins A and E, that promote cell cycle progression into S-phase. However, it also promotes transcription of the protein $p14^{ARF}$ that acts to stabilise p53 through the protein MDM2 that controls p53 turnover. p53 acts as a brake on the cell cycle that is inactivated upon T-antigen binding. NF2 is another tumour suppressor protein that neutralizes the inhibitory effect of MDM2 on p53. NF2 forms a ternary complex with p53 and T-antigen. T-antigen also binds to β-catenin and IRS-1 promoting the transcription of c-myc and cyclin D and inhibition of DNA repair, respectively (see text).

and this is disrupted by T-antigen [68]. Interestingly, SV40 T-antigen is acetylated *in vivo* by CBP in a p53-dependent manner [69]. The site of acetylation is lysine 697 near the C-terminus and this residue is conserved in JCV and BKV [69]. All studies on T-antigen interaction with p300 family members have been done with SV40 and not JCV. It would thus be of interest to investigate the interaction of JCV T-antigen with members of the p300/CBP/p400 protein family.

5.1.4. β-catenin

Recently, it has been found that deregulation of the β-catenin/Wnt pathway by JCV T-antigen is another mechanism whereby T-antigen alters cellular transcription and promotes transformation. This is effected by a direct interaction between JCV T-antigen and β-catenin [39,70]. This interaction involves the central domain of T-antigen (residues 82–628), and the C-terminus of β-catenin (residues 695– 781). The binding of JCV T-antigen to β-catenin increases the stability of β-catenin leading to an increase in the level of β-catenin in the cell [70]. The interaction of JCV T-antigen with β-catenin causes β-catenin to translocate to the nucleus where it enhances expression of genes such as c-myc and cyclin D1 that promote progression of the cell cycle. These observations ascribe a new mechanism for the deregulation of the Wnt pathway through stabilization of β-catenin. These signalling events may be important in certain human cancers that are associated with JCV T-antigen expression such as medulloblastoma, colon and esophageal cancer [71,39,72].

5.1.5. NF2

In mice transgenic for the JCV early region, JCV T-antigen associated with the NF2 protein (also known as merlin) in tumours that developed which histologically resembled malignant peripheral nerve sheath tumours [73]. In these tumours, NF2 was present in a ternary complex with T-antigen and p53. Although the function of NF2 is not known, it belongs to the ezrin-radixin-moesin (ERM) subgroup of the Protein 4.1 family, which links cell surface glycoproteins to the actin cytoskeleton [74] and it is a tumour suppressor that is mutated in the autosomal dominant genetic disorder neurofibromatosis type 2 [75].

5.2. Transcription factors

In the case of SV40, T-antigen is also able to directly influence transcription by interacting with TATA-binding protein-associated factors (TAFs). In this way, T-antigen promiscuously activates simple promoters containing a TATA box or initiator element and at least one upstream transcription factor-binding site. Ribosomal RNA synthesis by RNA polymerase I (Pol I) is tightly associated with cell growth and proliferation. T-antigen of SV40 upregulate the rate of Pol I transcription by directly binding to the Pol I transcription factor SL1 [76]. The 538 amino-terminal domain of T-antigen is necessary for SL1 binding. Human rRNA synthesis by Pol I requires at least two auxiliary factors, upstream binding factor (UBF) and SL1 (TATA-binding protein/TAF(I) complex). UBF is a DNA binding protein that binds to the ribosomal DNA promoter. The carboxy-terminal region of UBF is necessary for transcription activation and is extensively phosphorylated. SL1 is recruited to the promoter and is mediated by specific protein interactions with UBF. UBF phosphorylation plays a critical role in the regulation of the recruitment of SL1 [77]. UBF is phosphorylated by a kinase activity that is strongly associated with T-antigen and the carboxy-terminal activation domain of UBF is required for the phosphorylation to occur. T-antigen-induced UBF phosphorylation promotes the formation of a stable UBF-SL1 complex, *i.e.*, the large T antigen-associated kinase appears to mediate the stimulation of Pol I transcription [78].

SV40 T-antigen can also stimulate Pol II promoters by associating with the transcription factor TFIID [79], and Pol III promoters through interaction with the TBP-containing Pol III transcription factor human TFIIB-related factor [80].

JCV T-antigen binds to c-Jun, a member of the AP-1 family of transcription factors [81]. Results from band shift assays showed that the binding efficiency of c-Jun to the AP-1 site was reduced in the presence of T-antigen. Similarly, SV40 T-antigen inhibits AP-2-dependent transcriptional activation and sedimentation studies suggest that protein–protein interactions between AP-2 and T-antigen block AP-2 binding to DNA [82].

5.2.1. p185/p193/Cul7

Kohrman and Imperiale [83] reported an unknown protein with an apparent molecular weight of 185 kDa that was specifically co-immunoprecipitated with SV40 T-antigen. Binding to this protein mapped to the N-terminal 121 amino acid residues of T-antigen. Tsai and colleagues cloned a protein with an apparent molecular weight of 193 kDa [84] and it co-immunoprecipitated with T-antigen and bound to its N-terminus. Cloning and sequencing of p193 revealed that it had a bcl-2 homology type 3 (BH3) domain suggesting a role in the regulation of apoptosis. Expression of p193 in NIH-3T3 cells promoted apoptosis whereas mutants of p193 lacking the BH3 domain did not. p193 localized to the cytoplasm of transfected cells. Apoptosis induced by p193 was antagonized by co-expression of SV40 T-antigen providing evidence for a p53-independent mechanism of apoptosis suppression by T-antigen [84]. Recently, p185/p193 was identified as Cul7, a member of the cullin family of proteins [85]. Cullins form the scaffold for the multi-subunit complexes that promote the ubiquitination of targeted substrates [86]. Cul7-binding mutants of T-antigen that retain pRb and p53 binding are transformation defective [85]. The specific E3 ubiquitin ligase activity of Cul7 and the identity of the putative ubiquitination substrate are unknown at present.

5.2.2. T-antigen as a chaperone

There is sequence similarity between the N-terminus of T-antigen and the J-class of chaperonins [87]. Domain-swapping experiments have shown that the N-terminus of T-antigen can functionally substitute for the J-domain of *E. coli* DnaJ in a bacteriophage λ growth assay [88]. J-proteins are co-chaperonins for the DnaK (Hsp70) family of proteins that undergo a global conformational change upon J-protein binding. This stimulates hsp70 ATP-ase activity and is involved in binding of protein substrates [87]. The N-terminus of SV40 T-antigen functions as a J-protein in assays *in vitro*, *e.g.* stimulation of bovine Hsp70 ATP-ase activity [89]. The J-domain is essential for many of the functions of T-antigen including DNA replication. In addition to the Rb-binding domain (amino acid residues 101–118), the J-domain of T-antigen is required in cis for binding to the Rb family of proteins described above. It is thought that the J-domain may recruit Hsp70 to the Rb-E2F complex where the action of the Hsp70-mediated ATP hydrolysis liberates E2F. The role of the J-domain in other aspects of transformation is complex and has been reviewed recently [87]. It is possible that the chaperonin functionality of T-antigen is important in conferring the ability of T-antigen to bind to its plethora of cellular targets.

5.2.3. T-antigen as a mutator

In addition to deregulating pathways that control cell proliferation and gene transcription, infection of cells by JCV (or other polyomaviruses) also causes damage to cellular DNA which may be important in the pathogenesis of PML and JCV-associated tumours. Infection with JCV increases spontaneous mutation frequencies up to 100-fold in cultured lymphoid cells or human peripheral blood lymphocytes [90] and also in human colonic cells [91]. In the case of the closely related virus, SV40, large T-antigen rapidly induced numerical and structural chromosome aberrations in human fibroblasts [93,92]. Thus T-antigen has a mutagenic effect on cellular DNA and induces karyotypic instability. This endows the primate polyomaviruses with a ''mutator'' phenotype (as described above in the section on genetic changes associated with cancer). Since p53 is inactivated by T-antigen, the elimination of mutated cells by p53-mediated apoptosis would be expected to be impaired. It seems likely that secondary mutations induced by T-antigen contribute to the tumourigenic progression.

The mechanism of T-antigen-induced genetic instability is due, at least in part, to an interference with cellular DNA repair processes. SV40 T-antigen disturbs the formation of the nuclear DNA-repair foci containing MRE11/Rad50/Nbs1 that are involved in both homologous recombination-directed (HR) and non-homologous end joining (NHEJ) DNA double-strand break repair [94]. Nbs1 also functions to inhibit re-initiation of DNA replication during S-phase and interaction of Nbs1 with SV40 T-antigen inhibits Nbs1 DNA replication control. In cells expressing T-antigen, this prevents Nbs1 inhibition of re-initiation of DNA synthesis and leads to DNA re-replication (also known as chromosomal hyperreplication) in a manner dependent on T-antigen/Nbs1 complex formation [95]. Expression of the JCV large T-antigen inhibits HR-DNA repair in cultured cells [96]. JCV T-antigen has been shown to directly bind to insulin receptor substrate 1 (IRS-1) and cause it to be translocated to the nucleus [97]. A dominant negative mutant of IRS-1 inhibited growth and survival of JCV T-antigen-transformed cells. IRS-1 nuclear translocation regulates Rad51 trafficking and hence HR-DNA repair [98,96]. There is also evidence that interaction with IRS-1 is involved in transformation by SV40 T-antigen [99,100].

In the case of SV40 T-antigen, another mechanism that is involved in the induction of genetic instability is the disruption of the mitotic checkpoint of the cell cycle [101]. This may be mediated through the binding of T-antigen to the mitotic checkpoint protein Bub-1 [102].

5.3. Small t-antigen

The second of the two proteins encoded by the early region of primate polyomaviruses is small t-antigen. t-antigen is 174, 172 and 172 amino acid residues long for SV40, JCV and BKV, respectively. The N-terminal 82 amino acids of t-antigen are the same as the N-terminus of T-antigen but the C-terminus is a unique domain that is incorporated by alternative splicing of the early region primary transcript. The N-terminus of t-antigen, which contains the J-domain described above, is highly conserved between the three primate polyomaviruses (82–89% amino acid sequence identity) while the C-terminus is less conserved (56–69%). Published research on primate polyomavirus small-t has concentrated almost exclusively on SV40. t-Antigen is found in both the nucleus and the cytoplasm [103]. Deletion mutants of SV40 t-antigen are able to replicate (at least in some cells) indicating that it is not essential for viral lytic infection [104]. However, t-antigen augments viral DNA replication [105] and enhances transformation [106]. t-Antigen has a mitogenic role in the transformation of cells by SV40 [107,108]. Cellular proteins of 36 and 63 kDa that associate with SV40 small t antigen were shown to be the catalytic (C) and structural (A) subunits of protein phosphatase 2A (PP2A) [109]. Phosphatases are the negative regulators of the growth-promoting protein kinase signal transduction pathways. PP2A is the major serine/threonine-specific protein phosphatase of eukaryotic cells [110]. By inactivating the major negative regulator, PP2A, t-antigen is able to simultaneously activate several pathways that promote cell proliferation. These include the mitogen-activated protein kinase (MAPK) pathway [111], stress-activated protein kinase (SAPK) pathway [112] and PKC-ζ/NF-κ B [113]. PP2A is a key regulator with multiple functions in cellular signalling that are due to the presence of an assortment of holoenzymes. Each holoenzeyme contains a conserved AC dimer with an array of different regulatory (B) subunits. The specificity of PP2A is conferred by regulation of its endogenous subunit composition. Distinct classes of B subunits bind to AC to give a wide variety of ABC heterotrimers with differing substrate specificities [114]. Small t-antigen is also able to bind to the AC dimer and this interaction occurs via the t-antigen unique C-terminal domain that possesses two cysteine cluster motifs that are conserved in all polyomaviruses [115]. The association of t-antigen with AC displaces the cellular regulatory B subunit and results in the inhibition of phosphatase activity [116]. Thus small t-antigen is an oncoprotein that stimulates multiple growth promoting pathways.

5.4. Truncated variants of large T-antigen

In the case of the early regions of SV40 and JCV, splice variants can occur that yield shorter versions of T-antigen containing the N-terminal portion of the protein. SV40 can express a 135 amino acid residue protein,

17kT, which is comprised of the 131 N-terminal amino acids of T-antigen plus 4 C-terminal amino acids that come from a different reading frame [117]. Similarly in the case of JCV, three additional forms of T-antigen (T'_{135}, T'_{136} and T'_{165}) have been identified that are generated by alternative splicing [118]. The T′ proteins interact differentially with proteins of the retinoblastoma family [119], and may contribute to viral DNA replication [120] and cell transformation [121,122].

5.5. Agnoprotein

The late region of primate polyomaviruses encodes the viral capsid proteins VP1, VP2, and VP3 and a small regulatory protein known as agnoprotein that is encoded near the 5′ end of the primary late transcript. Agnoprotein is highly basic and is 62, 71 and 66 amino acid residues in length for SV40, JCV and BKV, respectively [16]. Agnoprotein is produced late in the infectious cycle although it is not incorporated into virions [16,123]. The predominant intracellular localization of agnoprotein is in the cytoplasm and especially the perinuclear region in association with the outer nuclear membrane for all three primate polyomaviruses [124–126]. A small amount agnoprotein is also found in the nucleus in the case of JCV and SV40 [124,125] but not in the case of BKV [126]. Interestingly, BKV agnoprotein is phosphorylated and associates with three cellular proteins of unknown function [126]. Agnoprotein has a role in the lytic cycle since SV40 mutants in which the agnogene is deleted produce virions more slowly than wild-type virus [127]. Agnoprotein has regulatory roles in viral transcription, translation as well as in virion assembly and maturation and this has been reviewed recently [129]. JCV agnoprotein can interact with the large T-antigen and downregulate viral gene expression and DNA replication [128]. It also interacts with YB-1, a cellular transcription factor that contributes to JCV gene expression in glial cells, and negatively regulates YB-1-mediated JCV gene transcription [129,130].

Interestingly, agnoprotein can exert profound effects on cells when it is expressed in the absence of the other viral proteins. NIH-3T3 mouse fibroblasts that constitutively expressed JCV agnoprotein showed deregulation of cell cycle progression and accumulated at the G_2/M phase [131]. Concomitantly, a decline in cyclin A and B-associated kinase activity was observed in these cells. Agnoprotein showed the ability to augment the activity of the p21/WAF-1 promoter and increased the level of p21/WAF-1 protein in cells. In addition, agnoprotein was shown to bind p53. Activation of p21/WAF-1 gene expression in cells expressing agnoprotein may be mediated, at least in part, through cooperation with p53 [131]. Results from a p53 null cell line revealed that agnoprotein can induce p21/WAF-1 transcription but to a much lesser extent than in p53-expressing cells indicating the existence of a p53-independent mechanism for p21/WAF-1 activation by agnoprotein [131].

Agnoprotein expression has effects on the response of cells to DNA damage. Cells expressing ectopic agnoprotein were more sensitive to the cytotoxic effects of cisplatin and exhibited increased chromosome fragmentation and micronuclei formation [132]. Under conditions where moderate chromosome damage was observed in control cells, severe chromosomal pulverization was seen in agnoprotein-expressing cells. Non-homologous end joining is a major mechanism of double strand DNA break repair and is impaired in nuclear extracts from cells that express agnoprotein. This appears to be due to agnoprotein binding to the Ku70 DNA repair protein and sequestering it in the perinuclear space. Indeed, *in vitro* synthesized agnoprotein could directly bind to Ku70 in cell extracts and inhibit non-homologous end joining [132]. Agnoprotein also impaired DNA damage-induced cell cycle arrest. The inhibition of DNA repair that is exerted by JCV agnoprotein is a likely contributor to the genomic instability conferred on cells when they undergo polyomavirus infection. Thus, like T-antigen, agnogene is a "mutator" gene as defined above. Agnogene expression may thus contribute to the progression of cells to a malignant phenotype. JCV is associated with some human cancers and it has recently been found that some of these JCV-associated tumours express agnoprotein. Immunohistochemical analysis of 20 well-characterized medulloblastomas showed expression of agnoprotein in the neoplastic cells in 11 of the samples [133]. A similar fraction of oligodendrogliomas samples exhibited immunoreactivity for expression of agnoprotein [38]. Agnoprotein expression has been detected in colon cancer [39], primary central nervous system lymphoma [40], and esophageal cancer [72]. In tumour tissues, agnoprotein exhibits its characteristic perinuclear localization.

Thus agnoprotein, like T-antigen, has multiple roles both in the polyomavirus life cycle and in interacting with cellular proteins to disturb normal cellular functions leading to tumour progression.

6. Conclusions

The three non-capsid regulatory proteins that are encoded by the primate polyomaviruses are highly multifunctional agents that disrupt cell signalling homeostasis at numerous levels. We have discussed three types of genetic mutation that occur along the road to cancer and the consequent intracellular changes. These are the activation of oncogenes, the inactivation of anti-oncogenes (tumour suppressors) and the acquisition of a mutator (mutagenic) phenotype leading to genetic instability. Interestingly, the viral proteins can do all three. Small t-antigen is an oncogene that stimulates kinase signalling pathways as do the oncogenes v-*src* and v-*raf*, but

t-antigen is able to alter many pathways at once since it inhibits a common phosphatase, PP2A. Large T-antigen incapacitates the tumour suppressor proteins pRb and p53. Inactivation of these proteins occurs in most if not all non-viral human cancers indicating their importance. T-antigen and agnoprotein also act as mutators inducing genomic instability through their abilities to inhibit HR and NHEJ DNA repair, respectively, *i.e.* these proteins are mutagenic. This is expected to lead to the susceptibility of the cell to further deleterious mutational events and tumour progression.

We presume that the genes of the polyomaviruses evolved to allow the viruses to push resting cells into S-phase so that viral DNA can be replicated. A corollary of this deregulation of cell growth control is that the viruses can advance oncogenesis. The salient properties of the multifunctional polyomavirus proteins have been described in this review and reveal how multiple "hits" can be delivered to cells in which they are expressed. These would be expected to propel them down the multi-step road to malignancy.

In humans, JCV is widespread but usually dormant, becoming active only in PML and in certain malignancies. It is possible that the expression of JCV proteins could be an early or a late event in tumourigenesis. On the one hand, it is possible that expression occurs at an early stage in tumour progression and that the genetic instability caused by T-antigen induces further pathological changes [91]. Alternatively other early abnormal cellular changes may occur first that precede T-antigen and agnoprotein expression. For example, conceivably there could be a mutation in the structure of, or a change in the level of expression of, one of the cellular transcription factors that bind to the control region of JCV that regulates the viral genes [27]. This could lead to a transcriptional activation of latent JCV genomes during the course of tumourigenesis leading to the onset of production of viral oncoproteins such as T-antigen and subsequently to a more malignant phenotype.

The relatively high incidence of JCV T-antigen involvement with certain malignancies indicates that molecular strategies for the disruption of the interaction of T-antigen with cellular proteins may represent a fruitful avenue for the development of new types of therapeutic interventions. For example, delivery of antisense transcripts to the early region of SV40 using an adenoviral gene therapy vector achieved significant growth inhibition and apoptosis of a T-antigen-expressing human mesothelioma cell line [134,135]. ELISA-based assays for T-antigen/p53 interaction have been used to screen chemical libraries for agents that inhibit SV40 T-antigen binding to p53 [136]. Such an approach could also be employed for JCV T-antigen. We have recently demonstrated the efficacy of a strategy of RNA interference using JCV T-antigen and agnoprotein siRNA to inhibit JCV protein expression in JCV-infected human fetal astrocytes. The inhibition of T-antigen production by siRNA reduced the rate of JC viral replication [137]. Further investigation of the utility of gene therapy agents that target JCV in the treatment of JCV-associated tumours is warranted.

Conflict of interest statement

None declared.

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