Some cancers are associated with viral infections, one of the most important being cervical carcinoma for which human papillomavirus (HPV) is a major risk factor. Most HPV infections do not lead to cancer, but sometimes integration of viral DNA into a cell chromosome occurs, resulting in interruption of the viral regulatory gene E2 and continued production of the oncoproteins E6 and E7. This extends the life span of the host epithelial cell and favours the accumulation of further genetic changes which lead to the loss of differentiated characteristics and progression towards becoming a cancer cell. Our research is aimed at interfering with this process. Here, we will first summarise certain aspects of HPV infection, then consider the role of the virus in cancer.

The HPVs are a family of small DNA viruses which infect epithelial cells of the skin or mucosa (reviewed in {1}). Among the HPVs infecting genital mucosa, a certain number - the so-called high-risk viruses, including notably HPV16 and HPV18 - induce lesions with an increased risk of progressing to cervical cancer. The circular viral genome of approximately 8 kb encodes two classes of proteins (see Fig. 1): the early (E) proteins which establish a permissive cellular environment and take part in viral replication, and the late (L) proteins which form the viral capsid.

HPV replication linked to keratinocyte differentiation

HPVs are thought to infect keratinocytes of the basal layer of an epithelium. In these cells there is a low level of E gene expression and the episomal viral DNA replicates, we found, in step with cellular DNA replication. Subsequent viral production is intimately linked to the differentiation of the infected keratinocyte. As the cell terminally differentiates and moves towards the outer edge of the epithelium, viral DNA is amplified to high copy number, capsid proteins are synthesised and progeny viruses assembled.

Papillomavirus replication linked to keratinocyte differentiation

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In HPV-containing cancer cells this viral growth cycle seems to have been interrupted. For reasons which are not yet clear the normally episomal viral DNA integrates into a host cell chromosome (Fig. 2). Integration is generally such that the viral E6 and E7 genes are intact and expressed, while other early genes, notably E2, are disrupted. This process of E2 gene disruption has been reported to correlate with poor disease prognosis {3}. These observations suggested that the HPV E2 protein behaves
like a natural tumour suppressor whose presence is incompatible with continued cell proliferation. E2 can function as a transcription factor for the HPV promoter region, and so control expression of the E6 and E7 genes. However, E2 has effects on cells which are independent of this \cite{4, 5}. We are therefore testing the effects of E2 on cell division, both in human keratinocytes, the normal hosts of HPV, and in yeast cells where mechanisms of effects on the cell cycle are easier to analyse.

**Papillomavirus E2 protein delays the entry of cells into mitosis**

We found, in collaboration with N. Fournier and V. Simanis of ISREC, that expression of the HPV16 E2 protein in the fission yeast *Schizosaccharomyces pombe* retards the G2-M transition, by delaying activation of Cdc2 kinase. In contrast, S-phase progression, and commitment to cell division in late G1 are not affected. The delay is independent of the transcriptional trans-activation function of E2, and does not result from E2 binding to DNA and mimicking DNA damage. Increased expression of E2 also delays mitotic initiation in mammalian cells as judged by flow cytometric analysis. *S. pombe* may thus provide a simple model for the analysis of E2 function and we are now looking into the mechanisms of E2’s effect on the cell cycle.

What purpose could the anti-proliferative function of E2 play in the HPV life cycle? We mentioned above that the expression of viral genes is tightly linked to the differentiation state of the infected cell as it migrates to the outer layers of the stratified epithelium. Although at early times after infection the viral E6 and E7 genes cause the differentiating cell to re-enter the cell cycle and allow the virus to replicate its DNA, while suppressing p53-mediated apoptosis, the late genes L1 and L2, which encode the viral capsid proteins, can only be expressed as the host cell reaches the final stages of differentiation. It therefore appears that proliferation of the infected cell must be restricted to allow it to continue its differentiation program. This role may be fulfilled by the anti-proliferative function of the E2 protein. This is also consistent with the observation that E2 function is lost in HPV-induced tumours. Treatment based on putting E2 back into cervical cancer cells is an interesting possibility for the future, but has not been tested yet.

**Papillomavirus-like particles and vaccination**

There is good reason to believe that the cellular immune system helps control HPV infection and disease. Patients under- going immunosuppressive treatments, or infected with HIV, or with an inborn defect in immunity are all more susceptible to the development of HPV-induced lesions. It would be very desirable to have HPV viral particles for use in vaccines. However, HPVVs cannot be easily propagated in cell culture because, as pointed out already, viral replication depends on the differentiation of epithelial cells. Therefore an important goal of several research groups, including our own, has been to produce artificial virus-like particles (VLPs) by synthesising the viral capsid proteins L1 and L2 in recombinant baculovirus or vaccinia virus-based expression systems \cite{6, 7}. To test whether DNA could be incorporated into such VLPs, we used recombinant vaccinia viruses expressing the HPV18 L1 and L2 proteins to infect human 293-T cells carrying an episomal plasmid DNA with an SV40 origin of replication and the β-galactosidase gene as marker (Fig. 3). The results showed that this non-papillomavirus DNA can be packaged into HPV VLPs. These particles are infectious and may provide a much needed reagent for infectivity and vaccination studies with HPV18. Clinical trials on the effectiveness of VLPs as vaccines are already under way in Europe and the USA \cite{8}.
It is not clear that vaccination would be useful against existing lesions so other approaches are being sought. One of these involves retinoids. Retinoic acid reduces expression of the HPV oncoproteins E6 and E7, and in addition has marked effects on epithelial cell differentiation. We are studying the mechanisms of retinoic acid’s effects on the expression of E6 and E7 in cervical tumour cells and the accompanying changes in cell cycle progression. We showed that transcription of the integrated HPV18 E6 and E7 oncogenes in C4-1 cervical tumour cells is reduced by retinoic acid, and in vivo footprinting suggested that this may be due to reduced occupation of AP1 sites in the promoter. The possibility of using retinoids, in the presence or absence of other drugs, against HPV-induced lesions is being explored [9].

Anti-proliferative effects of the adeno-associated virus Rep protein

A very different approach makes use of a virus which would counteract HPV. In contrast to HPV, the adeno-associated virus (AAV) inhibits tumorigenesis in experimental systems, and infection by AAV was reported to be less frequent than average in women with cervical carcinoma [10]. We are therefore very interested in the interactions between AAV and HPV in keratinocytes and the effects of the AAV regulatory protein, Rep, on the cell division cycle.

The anti-oncogenic effects of AAV are very likely mediated, at least in part, by the viral regulatory protein Rep. Rep is a multi-functional protein with properties including site-specific DNA binding and cleavage, helicase and ATPase activity (see [11] and references therein). We are investigating the possible oncosuppressive activity of the Rep protein by introducing a recombinant retroviral vector expressing Rep into different cell types. Rep was found to have a strong anti-proliferative effect. Flow cytometric analysis showed that primary rat fibroblasts expressing Rep accumulate in G1 and G2 with fewer cells in the S-phase of the cell cycle.

To test whether the block imposed by Rep can be overcome by proliferative signals we used cell populations over-expressing oncogenes including adenovirus E1a, v-H-ras, c-myc and HPV E6 and E7, alone or in combinations. None of these oncogenic stimuli could antagonise Rep’s anti-proliferative activity. On the contrary, some of these cells were even more sensitive to Rep, possibly due to induction of apoptosis.

Papillomavirus genes contribute helper functions for adeno-associated virus replication

AAV is a defective virus and needs a helper for its replication. There is evidence that HPV and AAV can co-exist in the cervical epithelium [12]. If HPV could function as helper it would lead to the production of AAV proteins including Rep. To test if this is the case, we transfected into human keratinocytes an infectious clone of AAV DNA along with plasmids encoding various HPV16 proteins and then tested whether AAV was produced.

In the absence of helper effect, only very little or no AAV was detected. Co-transfection of the E2 gene gave an increase of more than 100 fold. The HPV16 E1 gene was also able to increase AAV production, whereas E4 had no effect. We conclude that HPV16 gene products including notably E2 and E1 are able to stimulate AAV replication in these cells. This finding suggests that HPV belongs to the group of viruses which provide helper functions allowing AAV replication. In view of the dramatic effects which the AAV Rep protein has on cells, it clarifies how AAV may interfere with HPV infection and so hinder cervical cancer development.

Knowledge of how AAV-coded proteins achieve their effects on cells should enhance our understanding of HPV-mediated cervical disease and is a potential key to developing therapeutic strategies.

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