4. Molecular Mechanisms of HPV-induced Carcinogenesis

4.1 Experimental data that support the carcinogenicity of specific HPV genotypes and analyse the mechanism of HPV-linked carcinogenesis

In this section, the molecular mechanisms that contribute to HPV-induced carcinogenesis are described. Numerous studies have ascribed many biochemical activities, e.g. the ability of the viral proteins E6 or E7 to bind cellular factors, and biological properties, e.g. the immortalization of cells, to other HPV proteins. Section 4.1.1 provides a general introduction to molecular studies of cells that established the importance of HPV in cervical and skin cancers. Section 4.1.2 describes the numerous biochemical activities ascribed to HPV proteins. Section 4.1.3 describes the biological properties of HPV gene products in tissue culture, and the role of individual biochemical activities in mediating these properties. Subsequent sections describe other mechanistic aspects of HPV-associated cancers, including the contribution of viral DNA integration, cellular chromosomal alterations and co-factors.

4.1.1 *Transforming capacity of HPV*

HPVs are small DNA viruses that infect various epithelial tissues including the epidermis (cutaneous types) and the epithelial linings of the upper respiratory system and anogenital tract (mucosotropic types). The difference in their ability to promote malignant

transformation is the basis for the classification of HPVs into low- and high-risk types. This concept emanates from observations made more than 20 years ago that some HPV types were more frequently found in cancers than in benign lesions, and was followed by a large number of studies that demonstrated that these risk categories reflect the inherent and differential abilities of the viruses to interfere with proliferation and stability of the genome of the infected cell. A number of assays have been used to evaluate the ability of the E6 and E7 proteins to transform cells *in vitro*. Three types of cell were mainly used: (*a*) established rodent cell lines, e.g. NIH 3T3, (*b*) primary rodent cells (rat embryo fibroblasts, neonatal mouse or rat kidney cells) and (*c*) primary human keratinocytes, the natural host of the virus. The evidence for transformation included immortalization, formation of foci and growth in soft agar, cell proliferation and differentiation.

(*a*) *Mucosal HPV*

A subset of mucosotropic HPVs that belong to the alpha genus, including the high-risk HPV types 16 and 18, are associated with more than 99% of cervical carcinomas (Walboomers *et al.*, 1999). In these cancers, the papillomaviral DNA genome is often found integrated into the host chromosome (Boshart *et al.*, 1984; Schwarz *et al.*, 1985; Yee *et al.*, 1985). Cervical epithelial cells that harbour integrated HPV 16 DNA have a selective growth advantage over cells that harbour normal extrachromosomal viral genomes; this growth advantage correlates with the increased expression of two viral genes in particular, *E6* and *E7* (Jeon *et al.*, 1995). The early proteins, E6 and E7, bind and inactivate the tumour-suppressor gene product, p53, and the retinoblastoma tumour-suppressor protein (pRb), respectively (Dyson *et al.*, 1989; Münger *et al.*, 1989a; Werness *et al.*, 1990). In cell lines derived from HPV-positive cervical cancers, these genes are not inactivated mutationally, whereas they are mutated in cell lines derived from HPV-negative cervical cancers (Scheffner *et al.*, 1991). The expression of the *E6* and *E7* viral genes is required for the continued growth of cell lines derived from cervical cancers (Hwang *et al.*, 1993; Francis *et al.*, 2000; Goodwin & DiMaio, 2000; Goodwin *et al.*, 2000; Nishimura, A. *et al.*, 2000; Wells *et al.*, 2000). These facts support the hypothesis that E6 and E7 are causally related to the onset and maintenance of human cervical cancers. In addition, continuous expression of these early proteins can lead to the accumulation of mutations in the cellular genome that are required for malignant conversion (reviewed in zur Hausen, 1999). Both E6 and E7 co-operate to induce transformation of epithelial cells (Münger *et al.*, 1989b); however, a fully malignant phenotype is only observed after prolonged cultivation of the transformed cells (Hurlin *et al.*, 1991; Dürst *et al.*, 1995), which supports the multistep nature of HPV-induced transformation. In a transgenic mouse model, the expression of HPV 16 E6 alone has been shown to be sufficient to induce carcinomas (Song *et al.*, 1999). In contrast, E6 and E7 of the low-risk mucosal types have very low transforming activities *in vitro* (Farr *et al.*, 1991; Sang & Barbosa, 1992). Exceptions include immortalization by the low-risk type HPV 6 of human mammary epithelial cells which are not the natural host cells of these viruses (Band *et al.*, 1993). A recent report demonstrated the presence of integrated genomes of the low-risk type HPV 11, but not those of HPV 6, in cancers that deve-

loped in patients with early-onset recurrent respiratory papillomatosis (Reidy *et al.*, 2004). This points to the relevance of integration of the viral genome in malignant transformation, as discussed in Section 4.1.4.

(*b*) *Cutaneous HPVs*

Epidemiological studies have clearly demonstrated that a subset of cutaneous HPV types classified into the beta genus (approximately 25 types, also called epidermodysplasia verruciformis (EV)-HPV types, have been sequenced so far) are commonly and consistently found in non-melanoma skin cancers. These skin tumours arise predominantly at sites exposed to the sun and, contrary to mucosal types, the EV-HPV DNA copy number appears to be much lower than one copy/cell (de Villiers, 1998; Iftner *et al.*, 2003). Cutaneous HPVs have been studied less extensively than mucosal types and their capacity for cell transformation and molecular mechanisms are still largely unknown. Most cutaneous HPV types express E6 and E7 gene products that are structurally similar to those of the mucosal types but their genome does not harbour an identifiable E5 open-reading frame (ORF) (Pfister, 2003).

Schmitt *et al.* (1994) performed a comparative analysis of various properties of the E6 and E7 proteins of EV-associated type 8 and non-EV-associated type 1 cutaneous HPVs by transfecting the genes into different cell lines. HPV 8 E6, HPV 16 E6 and E7 and HPV 1 E7 but not cottontail rabbit papillomavirus (CRPV) long E6 or HPV 8 E7 were able to transform immortalized mouse fibroblasts (C127 cell line) while cells that expressed HPV 1 E6 or CRPV short E6 exhibited a weak transformed phenotype. The in-vitro retinoblastoma protein (Rb)-binding affinity (relative to that of HPV 16 E7) was 66% for HPV 1 E7, 34% for HPV 8 E7 and 11% for CRPV E7. None of the E6 or E7 proteins of the cutaneous HPV types 1 or 8 or CRPV revealed true immortalizing activities in primary human keratinocytes. In these cells, only a weak induction of proliferation was observed with HPV 8 E7, and only HPV 8 E7 transformed primary rodent cells co-transfected with the EJ-*Ras* oncogene.

HPV 5 and 8 E7s were shown to form complexes with the Rb protein, but with lower affinities than that of HPV 16 E7 (Yamashita *et al.*, 1993). Ciccolini *et al.* (1994) found that HPV 1 E7 binds to pRb with an affinity similar to that of high-risk E7 proteins but has no transforming activity in primary rodent cells. HPV 8 E6 protein expressed *in vitro* was shown not to bind murine p53 (Steger & Pfister, 1992). Similarly, HPV 1 and 8 E6 proteins bound to neither human p53 nor E6-associated protein (E6-AP) (Elbel *et al.*, 1997). Furthermore, HPV 8 E2, E4 and E6 were shown to interact with the TATA box-binding protein (TBP) and a number of TBP-associated factors (Enzenauer *et al.*, 1998).

Caldeira *et al.* (2003) analysed the in-vitro properties of E7 proteins of cutaneous EV- (HPV 20 and 38) and non-EV- (HPV 10) HPV types that are frequently detected in the skin. It was shown that HPV 38 E7 binds to and inactivates the tumour suppressor pRb and induces loss of G1/S transition control. In contrast, HPV 10 and HPV 20 E7 proteins do not display in-vitro transforming activities. Moreover, E6 and E7 of HPV 38 were shown to immortalize primary human keratinocytes, which suggests a role of HPV 38 infection in skin carcinogenesis.

4.1.2 *Biochemical properties of HPV proteins*

In this section, the biochemical properties of the HPV proteins E5, E6 and E7 are reviewed. Much of this information has been acquired for mucosal high-risk HPV (mainly HPV 16 and 18) proteins. Description of differences in the biochemical and biological properties of proteins of high-risk versus low-risk HPVs is largely provided in Section 4.1.3.

(*a*) *E5*

The study of the HPV E5 protein and recognition of its tumorigenic potential arose from the analysis of the transforming potential of BPV 1 in mouse C127 cells (see Section 3.3) (Yang *et al.*, 1985; DiMaio *et al.*, 1986; Schiller *et al.*, 1986; Schlegel *et al.*, 1986; Settleman *et al.*, 1989). The E5 ORF and the hydrophobic nature of its gene product are conserved in many papillomaviruses, although the degree of conservation of the primary amino acid sequence is variable (Bubb *et al.*, 1988). High-risk HPV E5 is considered to be tumorigenic because it transforms murine fibroblasts and keratinocytes in tissue culture (Leechanachai *et al.*, 1992; Pim *et al.*, 1992; Straight *et al.*, 1993), enhances the immortalization potential of E6 and E7 (Stöppler *et al.*, 1996) and, in cooperation with E7, stimulates the proliferation of human and mouse primary cells (Bouvard *et al.*, 1994b; Valle & Banks, 1995). Two major biochemical activities have been attributed to HPV 16 E5 *in vitro*: the ability to enhance the activity of the epidermal growth factor receptor (EGFR) in the presence of ligand (Leechanachai *et al.*, 1992; Pim *et al.*, 1992; Straight *et al.*, 1993) and the ability to bind and inactivate the 16-kDa pore-forming membrane component of the vacuolar H^+ adenosine triphosphatase (v-ATPase) (Conrad *et al.*, 1993; Adam *et al.*, 2000; Briggs *et al.*, 2001). Multiple studies have suggested that EGFR, a 170-kDa tyrosine kinase receptor, mediates the biological activities of HPV 16 E5 protein, and purport that E5 activates EGFR and induces mitogenic signalling and transformation of cells via this receptor (Straight *et al.*, 1993; Crusius *et al.*, 1998; Tomakidi *et al.*, 2000). Co-immunoprecipitation studies have indicated that HPV 16 E5 can form a complex with growth factor receptors when both proteins are overexpressed (Hwang *et al.*, 1995), but this binding has not always been observed (Conrad *et al.*, 1994). The binding of HPV 16 E5 to the 16-kDa subunit of the v-ATPase (Conrad *et al.*, 1993) is thought to delay endosomal acidification in human keratinocytes (Straight *et al.*, 1995), which has been implicated in the enhancement of EGFR phosphorylation in keratinocytes, since failure to acidify endosomes may result in decreased receptor degradation and increased receptor recycling to the cell surface (Straight *et al.*, 1993, 1995).

(*b*) *E6*

Mucosal high-risk E6 proteins are best known for their ability to associate with the cellular tumour suppressor p53 (Werness *et al.*, 1990). Association of E6 with p53 leads to degradation of p53 via recruitment of an ubiquitin ligase, E6-AP (Scheffner *et al.*, 1990; Huibregtse *et al.*, 1991; Scheffner *et al.*, 1993), and results in the inhibition of the transcriptional regulatory activities of the p53 protein in tissue culture cells (Lechner

et al., 1992; Mietz *et al.*, 1992). E6 proteins from multiple human and animal papillomaviruses bind to cellular proteins other than p53 and E6-AP. These include (*a*) transcription factors such as p300 (Patel *et al.*, 1999; Zimmermann *et al.*, 1999), myc (Gross-Mesilaty *et al.*, 1998), interferon regulatory factor 3 (IRF3) (Ronco *et al.*, 1998) and autocrine motility factor 1 (AMF-1/Gps2) (Degenhardt & Silverstein, 2001); (*b*) factors that determine adhesion, cytoskeleton and polarity, such as paxillin (Tong & Howley, 1997; Tong *et al.*, 1997; Vande Pol *et al.*, 1998), the mammalian homologue of *Drosophila* disk–large tumour-suppressor gene product (DLG) (Kiyono *et al.*, 1997; Lee *et al.*, 1997), Scribble (Nakagawa & Huibregtse, 2000), membrane-associated guanylate inverted-1 (MAGI-1) (Glaunsinger *et al.*, 2000) and multiple PDZ protein 1 (MUPP1) (Lee *et al.*, 2000); (*c*) apoptosis factors such as the pro-apoptic Bcl2 protein, Bak (Thomas & Banks, 1998, 1999); (*d*) replication factors and DNA repair factors such as mcm7 (Kühne & Banks, 1998; Kukimoto *et al.*, 1998) and XRCC1 (Iftner *et al.*, 2002); and (*e*) other proteins such as E6 target protein 1 (E6TP1) (Gao *et al.*, 1999), E6 binding protein 1 (E6BP1) (Chen *et al.*, 1995) and protein kinase PKN (Gao *et al.*, 2000). In addition, E6 can induce telomerase activity by inducing the expression of human telomerase reverse transcriptase (hTERT) (Klingelhutz *et al.*, 1996; Gewin & Galloway, 2001; Oh *et al.*, 2001; Veldman *et al.*, 2001). A more complete compendium of factors that interact with E6 is available (Mantovani & Banks, 2001).

Several E6 targets (paxillin, IRF3, E6BP1, E6TP1 and E6-AP) share a common α-helical structural motif that is known or suspected to mediate their binding to E6 (Tong *et al.*, 1997; Chen *et al.*, 1998; Vande Pol *et al.*, 1998; Be *et al.*, 2001). Mutations in E6 that disrupt its ability to bind α-helical partners also cause defects in multiple biological properties (Liu *et al.*, 1999). One of these partners, E6-AP, has attracted considerable attention because it is believed to be responsible for mediating high-risk HPV E6-dependent destabilization of multiple targets including p53 (Scheffner *et al.*, 1993), myc (Gross-Mesilaty *et al.*, 1998) and the PDZ domain protein, Scribble (Nakagawa & Huibregtse, 2000). This ubiquitin ligase, however, probably does not account completely for the ability of E6 to target cellular factors for proteasome-mediated degradation. Among the cellular partners of high-risk HPV E6 that are destabilized independently of E6-AP are human DLG and members of the MAGI family (Grm & Banks, 2004). It is also important to note that cutaneous HPV E6s, including the EV-associated HPV 8 E6, do not bind E6-AP (Elbel *et al.*, 1997). Another putative α-helical partner, E6TP1, a Rap guanosine triphosphatase-activating protein, has been implicated in the capacity of E6 to immortalize mammary epithelial cells (Gao *et al.*, 2001). It has been demonstrated that, for bovine papillomavirus (BPV) 1 E6, the selective binding of E6, but not of E6-AP, to paxillin mediates its transforming potential (Vande Pol *et al.*, 1998); however, it remains unclear whether an interaction with paxillin is of biological importance for the transforming potential of HPV E6 protein.

The interaction of high-risk E6 proteins with proteins that contain the PDZ domain, such as DLG, Scribble, MAGI1–3 and MUPP-1, has drawn a lot of interest. E6 can target PDZ-domain proteins for degradation (Gardiol *et al.*, 1999; Nakagawa & Huibregtse,

2000; Massimi *et al.*, 2004). The intracellular location and levels of DLG are altered in cervical cancers and their precursor lesions (Watson *et al.*, 2002; Cavatorta *et al.*, 2004; Lin *et al.*, 2004). Disruption of the C-terminal domain in E6 that mediates its interaction with PDZ-domain proteins leads to defects in the transforming potential of E6 in certain tissue culture-based assays (Kiyono *et al.*, 1997) and its tumorigenic potential in mice (Nguyen *et al.*, 2003), but not its capacity for immortalization.

(*c*) *E7*

High-risk HPV E7 proteins are best known for their ability to associate with the cellular tumour suppressor, pRb (Dyson *et al.*, 1989; Münger *et al.*, 1989a; Gage *et al.*, 1990). Association of high-risk E7 with pRb also promotes the degradation of pRb (Boyer *et al.*, 1996; Jones *et al.*, 1997a) through a proteasome-mediated pathway (Berezutskaya & Bagchi, 1997; Gonzalez *et al.*, 2001) and disrupts the capacity of pRb to bind and inactivate functionally cellular E2F transcription factors (Phelps *et al.*, 1991; Chellappan *et al.*, 1992). In addition to binding pRb, high-risk E7 proteins can bind to other pocket proteins (p107 and p130) that are related to pRb (Dyson *et al.*, 1992; Davies *et al.*, 1993) and also interact with different members of the E2F family of transcription factors (Dyson *et al.*, 1993; Classon *et al.*, 2000). The inactivation of pocket proteins by E7 is necessary but not sufficient to elicit the transforming potential of E7 (Heck *et al.*, 1992; Phelps *et al.*, 1992; Kiyono *et al.*, 1998). High-risk E7 is also purported to complex with cyclins (Dyson *et al.*, 1992; Arroyo *et al.*, 1993; Tommasino *et al.*, 1993; McIntyre *et al.*, 1996) and to inactivate the cyclin-associated kinase inhibitors p21 and p27 (Funk *et al.*, 1997; Jones *et al.*, 1997b). Thus, E7 can associate with and/or alter the activities of multiple cellular factors that normally contribute to the regulation of the cell cycle. Other interactions have been identified between high-risk E7 and cellular factors including the S4 subunit of the 26 S proteasome (Berezutskaya & Bagchi, 1997), Mi2beta, a component of the nuclease remodelling and deacetylase (NURD) histone complex (Brehm *et al.*, 1999), the fork head domain transcription factor MPP2 (Lüscher-Firzlaff *et al.*, 1999), the transcription factor activator protein-1 (AP-1) (Antinore *et al.*, 1996), insulin-like growth factor binding protein 3 (Mannhardt *et al.*, 2000), TBP (Massimi *et al.*, 1996, 1997; Phillips & Vousden, 1997), TBP-associated factor-110 (Mazzarelli *et al.*, 1995) and a novel human DnaJ protein, hTid-1 (Schilling *et al.*, 1998).

4.1.3 *Biological properties of HPV proteins*

(*a*) *Immortalization*

The E6 and E7 proteins of mucosal high-risk HPVs have transforming activity in tissue culture. They act independently or synergistically to immortalize multiple cell types including human foreskin keratinocytes, cervical epithelial or mammary epithelial cells (Dürst *et al.*, 1987a; Pirisi *et al.*, 1987, 1988; Hawley-Nelson *et al.*, 1989; Kaur *et al.*, 1989; Band *et al.*, 1990; Hudson *et al.*, 1990; Halbert *et al.*, 1991; Wazer *et al.*, 1995). To date, the E6 and E7 proteins of only one cutaneous EV-associated HPV type, HPV 38, have shown to

immortalize human primary keratinocytes (Caldeira *et al.*, 2003). Mucosal high-risk (HPV 16, 18, 31) E7s but not low-risk (HPV 6, 11) E7s cooperate with an activated *ras* to transform neonatal rat kidney or human cervical epithelial cells (Matlashewski *et al.*, 1987; Crook *et al.*, 1988; Phelps *et al.*, 1988; Storey *et al.*, 1988). The contribution of HPV 16 E7 to immortalization correlates with its disruption of the p16/pRb pathway (Kiyono *et al.*, 1998; Jarrard *et al.*, 1999). While E7 appears to be critical for efficient immortalization of multiple human epithelial cell types (Hawley-Nelson *et al.*, 1989; Halbert *et al.*, 1991), it may be dispensible depending on the method by which the cells are cultured (Ramirez *et al.*, 2001). Low-risk mucosal HPV 6 E7 and cutaneous EV-HPV 8 E7 demonstrate weak immortalizing activity compared with that of high-risk HPV 16 E7 (Halbert *et al.*, 1992; Schmitt *et al.*, 1994). This difference potentially correlates with the relative capacity of these E7 proteins to induce the degradation of pRb rather than their affinity for pRb (Giarrè *et al.*, 2001). The E5 protein can enhance the immortalization of keratinocytes effected by the combination of E6 and E7 (Stöppler *et al.*, 1996). The mechanism by which E6 contributes to immortalization is controversial. Some studies have correlated its potential for immortalization with its ability to induce the expression of telomerase (Kiyono *et al.*, 1998), since telomerase activity is clearly induced by E6 (Klingelhutz *et al.*, 1996). Whereas E6 modulates the transcription of hTERT through a direct stimulation of myc-mediated transactivation of the hTERT promoter (Veldman *et al.*, 2003), another study argued that induction of myc was not found (Gewin & Galloway, 2001). An alternative hypothesis that was recently put forward is that E6 relieves the repression of the telomerase promoter by inducing degradation of the transcriptional repressor, NFX1-91 (Gewin *et al.*, 2004). The level of telomerase activity in E6-positive cells increases further when they become immortalized although levels of E6 expression do not change (Fu *et al.*, 2003), which indicates that other events contribute to telomerase activation. Other studies have linked the immortalization potential of E6 in mammary epithelial cells and keratinocytes to its inactivation of p53 (Dalal *et al.*, 1996; McMurray & McCance, 2004).

(*b*) *Genomic instability*

Another hallmark of cells that express E6 and E7 is genomic instability, which has been observed in multiple epithelial cell types (Smith *et al.*, 1989; Hashida & Yasumoto, 1991; Reznikoff *et al.*, 1994; White *et al.*, 1994; Coursen *et al.*, 1997; Steenbergen *et al.*, 1998; Duensing & Münger, 2002; Shen *et al.*, 2002b). Abnormalities included monosomies and trisomies, chromatid gaps and breaks, double minutes and aberrant chromosomes. Structural changes are more commonly detected in chromosomes 1, 3 and 5 and less frequently in chromosomes 7, 8, 10, 12, 13, 16 and 22. Some of these allelic losses have been associated with particular genes that could be involved in malignant conversion and/or progression. Among these, losses in 3p and 10p have been associated with telomerase activation (Steenbergen *et al.*, 1998), which is a crucial step for cell immortalization mediated by high-risk HPVs (Klingelhutz *et al.*, 1996; Coursen *et al.*, 1997). Mitotic abnormalities can be induced by high-risk HPV 16 (but not low-risk HPV 6) E6 and E7 proteins by direct subversion of the mitotic spindle checkpoint (Thomas & Laimins, 1998; Duensing *et al.*, 2000).

The ability of E6 to induce genomic instability probably reflects its ability to inhibit the function of p53 (Havre *et al.*, 1995), which leads to the disruption of normal DNA repair processes and a consequent accumulation of genetic change. The genomic instability induced by E7 may reflect its effect on centrosome biogenesis and the consequent defects in segregation of daughter chromosomes during cell division (Duensing *et al.*, 2000; Duensing & Münger, 2001; Duensing *et al.*, 2001a,b). However, the manner in which E7 induces genomic instability remains unclear. While studies in mice have indicated that inactivation of pRb is sufficient to induce centrosome abnormalities (Balsitis *et al.*, 2003), other studies have demonstrated that E7 can induce centrosome abnormalities through a pRb-independent mechanism (Duensing & Münger, 2003).

Using an HPV 16-positive cell line (W12) derived from a low-grade squamous intraepithelial lesion (LSIL), Pett *et al.* (2004) recently suggested that acquisition of chromosomal instability is correlated with integration of the viral genome. The contrary has been shown in raft cultures of human keratinocytes that contain episomal HPV 16 in which genomic instability was observed in the absence of viral integration (Duensing *et al.*, 2001b). Progress in this area has been hampered by the lack of experimental models and methods to determine HPV integration that are suitable for use on large series in human biological specimens.

(*c*) *DNA damage responses*

Both E6 and E7 can abrogate normal DNA damage responses (Kessis *et al.*, 1993; Slebos *et al.*, 1994; Demers *et al.*, 1996). This is thought to reflect the activity of both E6 and E7 in inhibiting p53-mediated cell-cycle arrest. This correlates at least in part with the ability of E6 to bind and inactivate p53 (Song *et al.*, 1998); for E7, this correlates not only with its ability to disrupt the function of the cell-cycle regulator pRb, but also with its ability to inactivate p21 (Funk *et al.*, 1997; Jones *et al.*, 1997b), the cyclin-dependent kinase inhibitor that is induced when p53 is activated in response to DNA damage (Helt *et al.*, 2002). Abrogation of the DNA-damage responses is hypothesized to contribute to the accumulation of genetic alterations in HPV-positive cells, which include those that might contribute to tumorigenicity.

(*d*) *Cell proliferation and differentiation*

Whereas suprabasal cells are withdrawn from the cell cycle in normal stratified epithelia, HPV can re-programme suprabasal cells to sustain DNA synthesis which may contribute to the production of progeny virus through amplification of the viral genome. E7 is necessary (Flores *et al.*, 2000) and sufficient (Cheng *et al.*, 1995; Herber *et al.*, 1996) for this phenotype. It remains uncertain whether the ability of E7 to induce DNA synthesis in differentiated cells reflects the failure of cells that express E7 to withdraw from the cell cycle (Sacco *et al.*, 2003) or an ability of E7 to re-programme the differentiated cell to re-enter the cell cycle (Cheng *et al.*, 1995; Chien *et al.*, 2002). Inactivation of pRb alone can cause suprabasal cells to sustain DNA synthesis (Balsitis *et al.*, 2003); however, other properties of E7 may also contribute to this phenotype, notably its recognition by casein

kinase as a substrate for phosphorylation (Chien *et al.*, 2000). E6 has also been shown to induce suprabasal DNA synthesis (Song *et al.*, 1999), a p53-independent activity that correlates with the ability of E6 to bind PDZ-domain proteins (Nguyen *et al.*, 2003). A potentially related activity of E6 and E7 is their ability to inhibit keratinocyte differentiation (Schlegel *et al.*, 1988; Barbosa & Schlegel, 1989; Pan & Griep, 1994; Herber *et al.*, 1996; Gulliver *et al.*, 1997; Sherman *et al.*, 1997; Pei *et al.*, 1998; Song *et al.*, 1999). For E7, this probably reflects its inactivation of pRb (Gulliver *et al.*, 1997; Balsitis *et al.*, 2003), but inactivation of p53 by E6 is not sufficient for the inhibition of keratinocyte differentiation (Sherman *et al.*, 1997). It remains unclear whether the re-programming of suprabasal cells to sustain DNA synthesis by E6 and E7 or their inhibition of differentiation contribute to the tumorigenic potential of HPV; however, recent studies indicate that the ability of E6 to bind PDZ-domain proteins, which is required for the induction of both of these acute effects on stratified squamous epithelia, correlates with its contribution to the promotion phase of carcinogenesis (Simonson *et al.*, 2005).

High-risk E6 and E7 proteins modulate apoptosis. E7 can induce apoptosis in mouse lens and retina (Howes *et al.*, 1994; Pan & Griep, 1994; Nakamura, T. *et al.*, 1997). E7 can also sensitize human keratinocytes, mammary epithelial cells and uroepithelial cells to agents that induce apoptosis such as tumour necrosis factor (TNF) (Stöppler *et al.*, 1998; Basile *et al.*, 2001), ionizing radiation (Puthenveettil *et al.*, 1996) and ultraviolet (UV) radiation (Carlson & Ethier, 2000) but it has the opposite effect on TNF-induced apoptosis in human fibroblasts (Thompson *et al.*, 2001) or hydrogen peroxide-induced apoptosis in astrocytes (Lee, W.T. *et al.*, 2001). In mouse lens fibre cells and mouse fibroblasts, apoptosis is mediated through the inactivation of pRb by E7 (Alunni-Fabbroni *et al.*, 2000) and the consequent up-regulation of E2F activity (McCaffrey *et al.*, 1999). In hepatocytes, however, it is hypothesized that E7 mediates apoptosis through inactivation of pRb and induction of p21 (Park *et al.*, 2000a). The apoptosis induced by E7 occurs through p53-dependent as well as p53-independent pathways (Pan & Griep, 1995). E7 induces expression of p53 (Song *et al.*, 1998; Seavey *et al.*, 1999), but this p53 is not fully transcriptionally active (Eichten *et al.*, 2002). Whereas E7 can inhibit p53-induced cell-cycle arrest, it does not inhibit p53-induced apoptosis (Wang, Y. *et al.*, 1996) which indicates that these two pathways are separable, and that E7-induced p53 is capable of triggering apoptosis.

4.1.4 *Experimental evidence for a role of mucosal high-risk HPV in malignant conversion and in human cervical cancer*

(*a*) *Requirement of HPV gene expression for cell growth and invasion*

Expression of HPV E6 and E7 proteins is essential for cellular immortalization, but other factors are required for the acquisition of a fully transformed phenotype. HPV-immortalized cells have been shown to become tumorigenic either spontaneously (Pecoraro *et al.*, 1991) or after treatment with chemical carcinogens (Garrett *et al.*, 1993) or exposure to γ-irradiation (Dürst *et al.*, 1995). The necessity of expression of viral proteins for

transformation to the malignant phenotype has been demonstrated using various cell lines in these experimental models. Using an inducible promoter, it has been shown that continued expression of HPV 16 E7 in the presence of activated *Ras* is necessary for the maintenance of a transformed phenotype in primary rodent cells (Crook *et al.*, 1989). Non-malignant revertants were obtained in a model in which transcription of E6 and E7 was impaired (von Knebel Doeberitz *et al.*, 1992, 1994). In another study, stimulation of non-tumorigenic HeLa (HPV 18)-fibroblast hybrids to invasive growth was shown to involve loss of TNFα-mediated repression of viral transcription and participation of AP-1 (Soto *et al.*, 1999). Moreover, a key role for the constitutive expression of c-fos in the transformation of cervical cancer cells has been demonstrated (van Riggelen *et al.*, 2005).

Interference with the expression of HPV 16 E6 and E7 was studied in the HPV 16-positive cervical cancer cell line SiHa using E6 short-interfering RNA (siRNA). Yoshinouchi *et al.* (2003) showed that E6siRNA decreased the levels of mRNA that encode E6 and E7 and induce nuclear accumulation of p53. Moreover, E6siRNA suppressed monolayer and anchorage-independent growth of SiHa cells, which was associated with induction of p21 and hypophosphorylation of pRb.

The contribution of HPV genes to the development of malignancy has also been studied *in vivo* through the generation and characterization of HPV transgenic mice. These studies are described in Section 4.1.6.

(*b*) *Integration of HPV sequences*

In most invasive cancers, high-risk HPV genomes are integrated into the host genome. Integration of HPV can also be found in premalignant lesions, particularly in grade 2/3 cervical intraepithelial neoplasia (CIN2/3). In contrast, HPV DNA is commonly found extrachromosomally in benign and low-grade lesions. Low-risk HPV types are very rarely found integrated in tumours. However, Reidy *et al.* (2004) studied tissue specimens from patients with a history of benign early-onset recurrent respiratory papillomatosis who developed laryngeal cancer. Integrated HPV 11 was found in these specimens as judged by the absence of full-length E2 transcripts measured by real time-polymerase chain reaction (PCR) in a manner similar to that of high-risk HPVs in cervical cancers. An increased ability of high-risk HPV types to integrate into host DNA compared with low-risk types also has been suggested *in vitro* (Kessis *et al.*, 1996).

Integration is considered to be an important molecular event in HPV-induced carcinogenesis. Integrated sequences of DNA have been consistently identified in cervical cancers by southern blot hybridization (Dürst *et al.*, 1985; Cullen *et al.*, 1991). A systematic analysis of large series of HPV-infected cells and tissues has been hampered by a lack of less time-consuming and labour-intensive methods to determine integration of HPV. Since integration of HPV often disrupts the E2 gene (Schwarz *et al.*, 1985; Romanczuk & Howley, 1992), determination of a lack of amplification of E2 sequences by PCR-based protocols has been considered; however, results obtained with these methods are ambiguous because of the concomitant presence of non-integrated and integrated molecules in the biological specimens and to technical failures due to presence of inhibitors or other

factors. However, detection of early gene transcripts by reverse-transcription PCR is more sensitive both in cancers (Park *et al.*, 1997) and in benign or dysplastic cervical swabs, in which the presence of integrated genomes has been shown to correlate with severity of disease, particularly for HPV 18 (Hudelist *et al.*, 2004). Recently, alternative methods for the accurate determination of the physical status of HPV genomes have been proposed (Klaes *et al.*, 1999; Luft *et al.*, 2001; see Section 1.3.3). In the study by Klaes *et al.* (1999), transcripts derived from integrated HPV were more frequently detected in high-grade lesions and cervical cancer than in normal or low-grade dysplastic tissues. Integration of HPV 16 and 18 in high-grade lesions is often accompanied by chromosomal abnormalities (Hopman *et al.*, 2004). This supports the potential use of measurements of HPV integration as markers of progression in cervical cancer. In tonsillar cancers, the presence of extrachromosomal HPV 16 genomes and high viral loads were correlated with better prognosis (Mellin *et al.*, 2002).

Integration of HPV genomes affects both viral and host gene expression. HPV gene expression is regulated by viral and cellular transcriptional activators and repressors (see Section 1.1.6). Normal regulation is altered by viral integration and leads to the continuous expression of E6 and E7 proteins and, consequently, selective growth advantage, as shown by Jeon *et al.* (1995), who used clonal populations of the W12 cell line that harbour non-integrated or integrated HPV 16 DNA. Integration correlated with increased E7 protein synthesis; cells with integrated viral DNA had growth advantages and phenotypic changes compatible with those of high-grade neoplasia compared with cells that harboured extrachromosomal viral DNA.

This consistent pattern of disruption seen in the viral genome does not seem to occur in the host genome. The occurrence of integrated HPV DNA sequences at preferential sites of human chromosomes has been reported and suggests a non-random pattern of integration. In cervical carcinomas, Ferber *et al.* (2003a) observed HPV integration into and around the *hTERT* gene, which resulted in an increase in hTERT expression. Furthermore, HPV 18 DNA was found integrated in the proximity of *c-myc* in several cervical cancers (Ferber *et al.*, 2003b) but no up-regulation of endogenous proto-oncogene expression was observed. Cytogenetic and molecular studies have shown that HPV 16 and 18 DNA sequences can be found integrated in particular chromosomal loci known as common fragile sites in cervical cancers (Ferber *et al.*, 2003b; Thorland *et al.*, 2003), in HPV 16-immortalized keratinocytes (Popescu & diPaolo, 1990) and in an HPV 16-positive cell line derived from a cancer of the tongue (Ragin *et al.*, 2004). One of these regions, the *FRA*3B common fragile site that encompasses the fragile histidine tetrads (*FHIT*) tumour-suppressor gene is mapped on chromosome 3p. Butler *et al.* (2002) showed a clear association between the loss of *FHIT* expression and progression of HPV 16-positive CIN. Invasive cervical cancers that express high-risk HPV E6 and E7 transcripts were shown to contain normal *FHIT* transcription, while fewer viral transcripts were detected when *FHIT* was abnormally expressed, which suggests that E6 and E7 could be repressed in the presence of *FHIT* aberrations (Segawa *et al.*, 1999).

The studies described above favour the concept that HPV genomes may interfere with critical cellular functions by insertional mutagenesis. However, this has not been confirmed in recent studies that used HPV transcript and genome-based amplification techniques (see Section 1.3.3). A comprehensive analysis of integration sites of HPV 16 and 18 in 21 anogenital cancerous and pre-cancerous lesions revealed only single integration events in which E6 and E7 transcripts could be detected (Ziegert *et al.*, 2003). This could be an indication that the major function of HPV integration is the conservation and stabilization of HPV gene expression. A thorough review of integration sites of HPV in cervical dysplasia and cancer (Wentzensen *et al.*, 2004) concluded that these are randomly distributed over the whole genome with a clear predilection for genomic fragile sites. The relative impact of physical and functional disturbance of viral and cellular genes in HPV-mediated carcinogenesis needs further study. Many observations have demonstrated that the malignant phenotype cannot be attributed exclusively to the expression of HPV genes. It has been hypothesized that modification of host cell genes that interfere with the expression or function of viral genes will eventually contribute to immune evasion, and tumour progression and invasion (zur Hausen, 1999).

(*c*) *Chromosomal abnormalities in HPV-associated cancers*

Numerical chromosomal changes have been described in several HPV-associated cancers including cervical (see below), vulvar (Pinto *et al.*, 1999; Rosenthal *et al.*, 2001) and head and neck tumours (Braakhuis *et al.*, 2004).

Aneuploidy has been observed in cervical cancers and their precursor lesions (reviewed in Lazo, 1999). Loss of heterozygosity (LOH), which most frequently involves chromosomes 1, 3, 6, 11, 17 and 18 has been reported. Losses in the short (p) arm of chromosome 3 and gain on the long (q) arm are among the most frequent events associated with progression from high-grade lesions to cervical cancer (Heselmeyer *et al.*, 1996; Larson *et al.*, 1997; Wistuba *et al.*, 1997; Lin, W.M. *et al.*, 2000; Nishimura, M. *et al.*, 2000). An association between the severity of anal intraepithelial neoplasia (AIN) and chromosomal changes detected by comparative genomic hybridization has been described (Haga *et al.*, 2001). The most common alteration involved 3q, similar to the commonest alteration seen in cervical cancer, which suggests that a common molecular pathway for these HPV-associated malignancies exists. Moreover, LOH at 3p was more frequent in HPV 16- and 18-positive cervical tumours, whereas LOH at the 5p regions was more frequent in HPV-negative tumours (Mitra, 1999). Kersemaekers *et al.* (1999) analysed the CIN component, invasive carcinoma and lymph node metastases from 10 patients with primary squamous-cell carcinoma of the cervix for LOH. In CIN lesions, LOH was frequently found at 3p, 6p and 11q. During progression to an invasive tumour, losses of genes were observed on 6q, 17p and 18q. It was suggested that loss of an additional locus on the X chromosome and activation of the *erb*B2 oncogene are important in progression to metastases. Evidence that integration of high-risk HPV is associated with genomic alterations measured by comparative genomic hybridization was provided in a study that examined different degrees of cervical squamous intraepithelial lesions (SIL): more numerical chromosomal aberrations

were found in high-grade lesions with integrated HPV DNA than in low-grade lesions (Alazawi *et al.*, 2004). ELhamidi *et al.* (2004) analysed 164 CIN for LOH at 12 microsatellite loci and found that LOH at D3S1300, D3S1260, D11S35 and D11S528 was associated with CIN, which showed a tendency to persist and/or progress. An indication of geographical distribution of genetic alterations in oesophageal carcinomas has been reported by Si *et al.* (2004): HPV-positive tumours from Hong Kong, but not from Sichuan, had a higher frequency of LOH at D5S82, D6S397 and D13S260 than HPV-negative tumours.

Alterations in the *pRb* and *p53* genes have also been studied. Kim *et al.* (1997) reported infrequent LOH at *pRb* (14%) and *p53* (5.5%) loci in 55 primary cervical carcinomas. The genes *p53* and *pRb* are less frequently mutated in HPV-positive than in HPV-negative cervical cancer cell lines and tumours (reviewed in Tommasino *et al.*, 2003). Similarly, expression of HPV 16 E6 in head and neck cancers correlated with the absence of mutations within the *p53* gene (Braakhuis *et al.*, 2004; Dai *et al.*, 2004).

Continuous expression of papillomavirus early genes may directly promote genetic abnormalities that often result in impaired function of genes that are critical for cell homeostasis. Furthermore, expression of high-risk HPV *E6* and *E7* genes in the basal cell layer induces chromosomal instability and aneuploidy. It has recently been suggested that this event precedes integration of the viral genomes which in turn triggers the continuous expression of early genes (Melsheimer *et al.*, 2004). It could therefore be speculated that the physical status of the HPV genome and testing for aneuploidy could be used as prognostic tools (Kashyap & Das, 1988).

(*d*) *Alterations of specific proto-oncogenes*

Several investigations have addressed the structural or functional alteration of different proto-oncogenes. Most of these were descriptive in nature and few were designed to correlate the observed alteration with progression of disease. Therefore, a direct role for these genetic alterations in HPV-associated carcinogenesis is difficult to establish.

(i) RAS

Mutations in the *RAS* family of oncogenes have been described in both premalignant and malignant cervical lesions. Levels of H- and N-*RAS* mRNA were significantly higher in cervical carcinomas than in normal tissue or CIN; however, no correlation was found between levels of expression of each *RAS* gene and the presence of HPV (Mammas *et al.*, 2004). This contrasts with the results of Golijow *et al.* (1999) who found higher rates of mutation in K-*RAS* codon 12 in non-cancerous cervical smears that contained high-risk HPV types than in samples infected with low-risk HPV. One study *in vitro* showed that activation of Ha-*RAS* genes and inhibition of protein phosphatases by okadaïc acid stimulated HPV 18 p105 promoter activity (Medina-Martínez *et al.*, 1997). This activation depended on the presence of an intact AP-1 binding site. Leis *et al.* (1998) reported a missense mutation within c-*RAS*^{Ha} codon 61 in the metastasis of an HPV 18-positive penile squamous-cell carcinoma that was absent in the primary tumour and previous metastases. This suggests that alterations in *ras* may be associated with late-stage disease.

(ii) MYC

HPVs have been shown to integrate in the proximity of *c-MYC*, which justifies the search for alterations of this proto-oncogene in HPV-associated lesions. However, the results have not been consistent. Recently, Abba *et al.* (2004) described *c-MYC* amplification in a high proportion of cervical cancers compared with benign and premalignant cervical lesions. Moreover, a significant association between *c-MYC* amplification and HPV 16 infection was observed. Elevated levels of *c-MYC* have been found in several HPV-positive cervical carcinoma cell lines (Dürst *et al.*, 1987b). More recently, Hukku *et al.* (2000) described genetic changes associated with progression to a malignant phenotype of a non-tumorigenic HPV 18-immortalized human prostate cancer cell line, which included amplification of *c-myc* that was considered to be central to this process. However, the significance of these events in HPV-mediated transformation is not clear. The involvement of the Myc protein in HPV-induced immortalization was recently addressed (Veldman *et al.*, 2003). High-risk HPV E6 was shown to associate with Myc complexes (Myc/Max) and activate the hTERT promoter. The specific Myc antagonist, Mad, represses E6-transactivation of hTERT.

(iii) ERB

HER-2/*NEU* mRNA and protein were detected in a large proportion of cervical adenocarcinomas. Preferential expression of this proto-oncogene was more strongly associated with lesions that contained HPV 16 than with those that contained HPV 18 (Roland *et al.*, 1997). Similar patterns of expression were previously observed in different mammary and cervical tumours compared with normal tissues: increased levels of *c-ERB*B-2 mRNA expression were found in two advanced and poorly differentiated endometrial adenocarcinomas, two ductal mammary carcinomas and three carcinomas *in situ* of the cervix (Brumm *et al.*, 1990). An increase in ERBB2 expression was also correlated with progression of disease and has been considered to be a late event in cervical carcinogenesis (Kersemaekers *et al.*, 1999).

(iv) *Other*

Epigenetic events that involve the methylation of viral genes or the long control region (LCR) of high-risk HPVs have been described. The LCR of HPV 16 (Badal *et al.*, 2003) and 18 (Badal *et al.*, 2004) is hypermethylated in normal and low-grade cervical smears and is gradually methylated to a lesser degree in high-grade smears. This correlates with an increased transcriptional activity of the early region and consequently greater availability of the E6 and E7 proteins.

Hypermethylation of cellular gene promoters has also been observed in cervical carcinomas (Dong *et al.*, 2001; Virmani *et al.*, 2001; Steenbergen *et al.*, 2004), and down-regulation of the expression of tumour-suppressor genes could therefore be considered of importance in tumour progression.

4.1.5 *Interactions between HPV and environmental agents*

(*a*) *Effects of other infectious agents*

The proposed mechanisms through which infectious agents might act as co-factors in HPV-associated tumorigenesis include direct biological interactions, such as modification of HPV replication and transcription, and indirect effects, such as inflammation and damage to the epithelial barrier that protects against HPV infection. Herpes simplex virus-2 (HSV-2) is one of the infectious agents that has been most frequently studied as a potential co-factor for cervical cancer. However, epidemiological studies have provided conflicting results (see Section 2.7.2) for an association of HSV-2 with cervical cancer.

Because of the lytic behaviour of many herpesviruses, including HSV-2, abortive infections would need to be involved for HSV-2 to have a direct effect on HPV-associated tumorigenesis. Accordingly, UV-inactivated HSV-2 can transform rodent cells *in vitro* (Duff & Rapp, 1971a,b, 1973). Several studies have demonstrated an interaction between HSV-2 and HPV in transformation *in vitro* (Dhanwada *et al.*, 1993), whereas others have found that HSV-2 can suppress HPV gene expression (Fang *et al.*, 2003). Thus, laboratory data similarly to the epidemiological data are not consistent with regard to a possible interaction of HSV-2 in HPV-associated tumorigenesis.

Laboratory studies conducted during the 1970s demonstrated the ability of UV-inactivated HSV-2 and HSV-1 to transform hamster cells, and showed that these transformed cell lines caused tumours in newborn rodents (Duff & Rapp, 1971a,b, 1973). The continued presence of the HSV genome was observed in some tumorigenic cell lines, but the transformed phenotype was also found to persist in the absence of detectable HSV viral sequences (Davis & Kingsbury, 1976). These findings and the inconsistent detection of HSV DNA in specimens of human cervical cancer gave rise to hypotheses of a possible 'hit and run' mechanism (Davis & Kingsbury, 1976; Skinner, 1976; Galloway & McDougall, 1983), i.e. the concept that a virus may be involved in the initiation or promotion of cancer without being required for the maintenance of the transformed phenotype.

At least two separate genomic regions of HSV have been shown to transform rodent cells *in vitro*: the morphological transforming region II in the *Bgl*II N fragment and the morphological transforming region III in the *Bgl*II C fragment (Jones, 1995). In contrast to rodent cells, the *Bgl*II N fragment in human keratinocytes was found to induce tumorigenic clones in cells that had been immortalized by HPV but not in normal cells (DiPaolo *et al.*, 1990), a finding that is consistent with the hypothesis that HSV is a co-factor in HPV-associated cervical tumorigenesis, but is not itself an important etiological agent. Moreover, although *Bgl*II N sequences were not detected in tumour-derived cell lines (DiPaolo *et al.*, 1990, 1998), when only the Xho2 segment of the *Bgl*II N fragment was used, HPV-immortalized cells could still be transformed and the Xho2 segment was found to be maintained stably in an integrated form in the host cell genome (DiPaolo *et al.*, 1998). These data were interpreted as evidence that the Xho2 fragment contains the transforming sequences of the *Bgl*II N fragment, and that the remaining sequences have an inhibitory effect on stable integration. However, a study of 200 specimens of human

cervical cancer failed to detect any HSV-2 sequences using sensitive PCR methods (Tran-Thanh *et al.*, 2003). In-vivo studies of the detection of HSV and other infectious agents in cervical specimens are mainly reviewed in Section 2.7.2.

Other herpesviruses that are reported to infect the cervix have also been shown to transform epithelial cells in tissue culture, including cytomegalovirus (CMV) (Galloway *et al.*, 1984; Doniger *et al.*, 1999), human herpesvirus (HHV) 6 (Razzaque, 1990; Kashanchi *et al.*, 1997) and Epstein-Barr virus (EBV) (Lopes *et al.*, 2003; Busson *et al.*, 2004; Thompson & Kurzrock, 2004), but there is no strong evidence that these viruses are involved in cervical cancer.

Chlamydia trachomatis is a microbial agent that has most consistently been shown to be associated with cervical cancer in epidemiological studies that controlled statistically for HPV infection (see Section 2.7.2). Although it is not believed to have a direct effect on host DNA or on the transcription of HPV genes, several biological mechanisms by which *C. trachomatis* may increase the risk for cervical cancer have been described. First, it may have anti-apoptotic effects (Fan *et al.*, 1998): resistance of infected cells to apoptosis ensures the persistence of *C. trachomatis* infection, while cell death at the end of the infection cycle triggers release and initiates a new infection cycle (Fan *et al.*, 1998, Perfettini *et al.*, 2003a,b). These anti-apoptotic effects could result in increased persistence of epithelial cells that are co-infected with HPV and/or reduce the probability of cell death following the development of chromosomal abnormalities, which increase in frequency with increasing grade of cervical neoplasia (Lorenzato *et al.*, 2001, Melsheimer *et al.*, 2001). Second, infection by *C. trachomatis* is associated with squamous metaplasia and hypertrophic ectopy, which have been shown to be a risk factor for cervical neoplasia (Moscicki *et al.*, 1999). Third, *C. trachomatis* may cause human cervical epithelial cells to separate from each other due to the breakdown of the cadherin–catenin junctions in the epithelium (the N-cadherin/β-catenin complex) and thereby increase the exposure of basal cells to HPV (Prozialeck *et al.*, 2002). Fourth, it may increase the risk for HPV infection and its persistence through modulation of immune factors: *C. trachomatis* is reported to inhibit the expression of interferon (IFN) γ-inducible major histocompatibility complex (MHC) class II (Zhong *et al.*, 1999), as well as the expression of MHC class I (Zhong *et al.*, 2000; Hook *et al.*, 2004). If this is true, it could impair the adaptive immune response to HPV. Quantitative or qualitative alterations in expression of MHC class I (e.g. due to the presence of viral antigens) can result in stimulation of natural killer (NK) cells (core effector cells of the innate immune system that can kill a broad range of intracellular microbially infected cells without prior sensitization). *C. trachomatis* may, however, inhibit NK cell function and result in a decrease in the lytic capability of NK cells, reduced NK cell production of TNFα and IFNγ and a decrease in antibody-dependent cellular cytotoxicity (Mavoungou *et al.*, 1999). Last, chronic infection with *C. trachomatis* is associated with a predominantly T-helper (Th)2 (humoral immune) cytokine pattern, whereas Th1 (cellular immune) cytokines are important in the control of intracellular microbes such as *C. trachomatis* and HPV (Stephens, 2003).

Overall, the immune response to microbial infection (i.e. cervical inflammation) may play a role in HPV-associated tumorigenesis and help explain the possible associations of cervical cancer with a range of pathogens, including herpesviruses, *C. trachomatis*, *Trichomonas vaginalis, Neisseria gonorrhoeae, Candida albicans* and others (Castle & Giuliano, 2003). The mechanisms by which inflammation might cause an increased risk for cervical cancer have been best described for *C. trachomatis*. Many of the cytokines that are secreted during *C. trachomatis* infection, including TNFα and IFNγ, could cause tissue damage by inducing apoptosis of uninfected cells (Perfettini *et al.*, 2000), and infiltrating macrophages may cause further tissue damage through release of reactive oxygen species (Castle & Giuliano, 2003). Together, these effects probably result in partial disruption of the tissue barrier and exposure of basal cells to HPV infection. Furthermore, it has been hypothesized that the reactive oxygen species released by infiltrating macrophages could cause host cell DNA damage that leads to increased risk for cervical cancer in cells that are protected against apoptosis by HPV (Gravitt & Castle, 2001; Smith *et al.*, 2004). Support for this proposed mechanism has come from laboratory studies that showed an association between inflammatory host responses and oxidative DNA damage (Zhuang *et al.*, 2002; Touati *et al.*, 2003).

The incidence of cervical cancer is significantly increased in women who have human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) (Mbulaiteye *et al.*, 2003), and biomarkers of host immune status in HIV-positive women, including HIV RNA level and $CD4^+$ T-cell count, are associated with risk for HPV infection and cervical neoplasia (see Section 2.8.3). It is uncertain, however, whether there might also be a direct biological interaction between HIV and HPV. In-vitro studies have shown that HIV TAT protein can co-activate HPV (Tornesello *et al.*, 1993; Vernon *et al.*, 1993; Buonaguro *et al.*, 1994). Whereas some studies have reported that epithelial cells can be infected by HIV *in vitro* (Moore *et al.*, 2003; Yeaman *et al.*, 2003), there is little evidence of this *in vivo* (Spira *et al.*, 1996; Greenhead *et al.*, 2000; Miller & Shattock, 2003; Wu, Z. *et al.*, 2003). Taken together, it seems improbable that HPV-infected cervical epithelial cells could be co-infected with HIV, which limits the opportunity for the two viruses to interact directly at the molecular level.

Adeno-associated virus (AAV) may have a protective effect against HPV-associated cervical tumorigenesis. AAV is a helper-dependent parvovirus that requires co-infection with other DNA viruses, such as adenovirus, for its replication (Leonard & Berns, 1994). In tissue culture, AAV inhibits the tumorigenic effects of HPV (Hermonat, 1994a) and BPV (Hermonat, 1989) and furthermore, HPV can support replication of AAV (Walz *et al.*, 1997; Meyers *et al.*, 2001), a finding that is consistent with possible HPV/AAV co-infection in nature. Initial laboratory studies found that AAV suppressed papillomavirus replication (Hermonat, 1992) and attributed this effect to Rep 78, the major non-structural regulatory protein of AAV. Rep 78 can interfere with transcription factors and HPV promoter activity (Zhan *et al.*, 1999; Su *et al.*, 2000; Prasad *et al.*, 2003). However, recent studies have found that the effects of AAV on HPV replication are complex (Agrawal *et al.*, 2002). High levels of AAV decreased but low levels increased HPV replication

(Meyers *et al.*, 2001) and, under certain culture conditions, AAV actually increased the tumorigenicity of papillomavirus (Hermonat *et al.*, 1998). The significance of these recent findings is not yet clear, but AAV also has anti-neoplastic effects that are independent of its proposed biological interaction with HPV. Through the direct interaction of AAV proteins with cellular genes, AAV has been shown to induce differentiation of tumour cell lines (Bantel-Schaal, 1995), down-regulate *c-FOS* and *c-MYC* (Hermonat, 1994b), inhibit cell proliferation (Walz & Schlehofer, 1992) and reduce carcinogen-induced mutagenicity (Schlehofer & Heilbronn, 1990).

(*b*) *Hormones and anti-estrogens*

Epidemiological data suggest an association between hormonal status, use of hormonal contraception and parity and the risk for preneoplastic lesions of the cervix and cervical cancer (see Section 2.7). The experimental evidence for such an association derives primarily from the fact that the endogenous level of the steroidal hormone, progesterone — the major ingredient of oral contraceptives and injectable hormonal contraceptives — increases during pregnancy (Pater *et al.*, 1994), and experimental studies indicate the presence of hormonal recognition elements in the LCR of high-risk mucosal HPV and increased production of the E6 protein in response to exogenous hormonal stimulation *in vitro* (reviewed by Pater *et al.*, 1994; Moodley *et al.*, 2003; de Villiers, 2003). There is also experimental evidence that hormones may mediate changes in the immune status of the cervical mucosa (Roche & Crum, 1991).

In a review of the literature, Pater *et al.* (1994) summarized the evidence that supports an association between hormones and HPV-mediated tumorigenesis. Several reports before 1994 observed hormone-enhanced transformation of primary rodent cells, immortalization of genital keratinocytes and enhanced expression of HPV in cervical cells. In addition, inhibition of transformation and expression by anti-hormones (e.g. RU-486) had been observed in several reports. Since that time, the evidence to support a role of hormones, including estrogen and progesterone, in the increase in HPV expression has accumulated (Chen *et al.*, 1996; Khare *et al.*, 1997; Webster *et al.*, 2001).

In addition to the above-mentioned effects of hormones on the expression of HPV and the ensuing carcinogenesis, there is evidence for other mechanisms of action of hormones in the development of HPV-related cervical cancer. Auborn *et al.* (1991) observed enhanced 16α-hydroxylation of estradiol activity in both cervical and foreskin cells immortalized with HPV 16. As 16α-hydroxyestron is known to be a risk factor for other estrogen-sensitive cancers, increased concentrations of this metabolite in target cells in the cervix may enhance cervical carcinogenesis by increasing cell proliferation in the presence of HPV 16. Monsonego *et al.* (1991) examined estrogen and progesterone receptor profiles in CIN and invasive cervical cancers and found high levels of expression of progesterone receptors in the underlying stromal cells of preneoplastic lesions of the cervix. These data suggest that, *in vivo*, sex steroid hormones, particularly progesterone, may act indirectly on HPV-infected epithelial cells and be implicated as co-factors in HPV-related cervical neoplasia. These

results could also explain the relative predisposition to malignant transformation of the cervical mucosa compared with vulvar and penile mucosa.

More recently, evidence of a co-carcinogenic role for hormones in cervical cancer has accumulated from studies conducted in HPV-infected transgenic mice. Arbeit *et al.* (1996) demonstrated that exposure of K14-HPV 16 transgenic mice to 17β-estradiol increased the incidence of proliferating cells in the cervical and vaginal squamous epithelium and resulted in a concomitant up-regulation of *E6*/*E7* gene expression through all stages of carcinogenesis. In addition, exposure of these K14-HPV 16 transgenic mice to estrogen induced hyperplasia in the lower uterine gland, and continuous exposure to estrogen resulted in the development of squamous metaplasia and neoplastic progression (Elson *et al.*, 2000). In transgenic mice that express HPV 16 E6 or E7 alone, Riley *et al.* (2003) showed that E7 and estrogen combined are sufficient to induce cervical cancer, and that E6 contributes to increased tumour growth. In a more recent study, estrogen was found to contribute not only to the genesis but also to the maintenance and malignant progression of cervical cancers in HPV 16 transgenic mice (Brake & Lambert, 2005). Michelin *et al.* (1997) observed significant activation of the viral upstream regulatory region in response to exogenous estrogen and progesterone and pregnancy in HPV 18 transgenic mice.

(*c*) *Nutrients*

A large number of different food constituents and nutrients have been associated with a reduction in the persistence of HPV and the development of preneoplastic lesions and invasive cancer of the cervix. The mechanisms by which these food-derived compounds confer protection are not entirely clear. Three main types of nutrient and/or nutrient metabolite have been identified: those that are involved in oxidation reactions (e.g. carotenoids, vitamins C and E), those that are involved in methylation or one-carbon transfer reactions (e.g. folic acid, vitamin B12, vitamin B6, cysteine and the biological marker, serum homocysteine) and nutrient metabolites that have hormone-like activity (e.g. retinoic acid and its isomers).

Epidemiological research over the past few decades has indicated that anti-oxidant nutrients such as carotenoids that are found in fruit and vegetables as well as vitamins C and E may confer protection against the persistence of HPV and the development of preneoplastic lesions and invasive cervical cancer (see Section 2.7.1). The mechanism by which these nutrients might prevent cervical cancer remains unclear. Reactive oxygen species appear to play a central role in cell signalling by activating transcription factors, AP-1 and nuclear factor (NF)-κB, cell proliferation and apoptosis (Palmer & Paulson, 1997). In animal and in-vitro models, reactive oxygen species increased viral titres (Peterhans, 1997) and the infectivity of influenza virus (Hennet *et al.*, 1992). As anti-oxidants, carotenoids and vitamins C and E have a multitude of effects that may be chemopreventive. These compounds have been shown to quench reactive oxygen species that can lead to cellular damage and dysregulation of cell signalling (Palmer & Paulson, 1997). Carotenoids and vitamins C and E may also potentiate host cellular and humoral immunity (Meydani *et al.*, 1995). Studies of HIV and influenza virus also indicate a role for

anti-oxidants (in particular nutrient anti-oxidants) in the down-regulation of viral replication and expression.

Administration of anti-oxidants to animals infected with influenza virus protected against the lethal effects of influenza (Oda *et al.*, 1989). *In vitro*, increases in the cellular oxidant load have been shown to increase the replication of HIV (Pace & Leaf, 1995; Peterhans, 1997). This effect is thought to be due to the fact that reactive oxygen species activate NF-κB, a nuclear transcriptional factor that is obligatory for HIV replication (Pace & Leaf, 1995), and in-vitro studies have consistently demonstrated inhibition of NF-κB activation by anti-oxidants (see review by Epinat & Gilmore, 1999). Further molecular epidemiological studies are required to delineate to what extent anti-oxidant nutrients have a protective effect *in vivo* and the mechanisms by which they act.

Evidence has accumulated to suggest that reactive oxygen species and their down-regulation by anti-oxidants may have a similar effect on HPV infection. Activation of AP-1, a central transcription factor for the expression of E6 and E7 proteins of high-risk HPV types (Cripe *et al.*, 1990; Offord & Beard, 1990), has been shown *in vitro* to be inhibited by anti-oxidants. Rösl *et al.* (1997) demonstrated that the anti-oxidant pyrrolidine-dithiocarbamate selectively suppressed AP-1-induced HPV 16 gene expression in HPV 16-immortalized human keratinocytes, and suggested that manipulation of the redox potential may be a novel therapeutic approach to interfere with the expression of high-risk HPVs.

In addition to its effects on target cells and cell signalling, the oxidant–anti-oxidant balance is an important determinant of immune cell function, and affects the maintenance of immune cell membrane lipids, control of signal transduction and gene expression of immune cells (Meydani *et al.*, 1995; Anderson & Theron, 1990), events that are important for the loss of HPV infection and regression of CIN.

Whitehead *et al.* (1973, 1989) proposed a correlation between folic acid status and the use of oral contraceptives and increased risk for cytological abnormalities among women in the USA. This increased risk was thought to be due to a deficiency of folic acid in local tissues, which could not be detected by measuring folate in serum or in the diet. In support of this hypothesis, several laboratory studies have delineated a role for folic acid in the prevention of cancer at several sites. Folic acid is essential for the synthesis of purine nucleotides and thymidilate, which are essential for the synthesis of DNA during cell replication and repair. In addition, folic acid is necessary for the synthesis of *S*-adenosylmethionine, the main donor of methyl groups in various methylation reactions, such as methylation of the DNA base cytosine (Poirier, 2002). Low tissue levels of folate increase the frequency of fragile sites on DNA (Ames & Wakimoto, 2002), enhance the risk of attack on DNA by carcinogens and viruses (Hsieh *et al.*, 1989), and decrease DNA repair (Ames & Wakimoto, 2002) and DNA methylation (Wainfan *et al.*, 1988). Kim *et al.* (1994) observed significant increases in global DNA methylation in cervical tissue with increasing grade of cervical lesion, which suggested that the change in methylation status may be an early event in cervical carcinogenesis. Extending the work to genital HPV types, DNA methylation within the upstream regulatory region has been shown to regulate expression of high-risk HPV *in vitro* (Rösl *et al.*, 1993; Thain *et al.*, 1996). One

in-vitro study demonstrated that sequence-specific methylation of CpG sites in the constitutive enhancer region of the HPV 18 upstream regulatory region resulted in a down-regulation of transcriptional activity (Rösl *et al.*, 1993). Methylation of a novel transcription factor-binding site decreased the activity of the HPV 16 enhancer and suppressed viral transcription (List *et al.*, 1994). Additional in-vitro studies demonstrated that methylation of specific CpG sites within the HPV 18 E2-binding site abolishes binding (Thain *et al.*, 1996) and leads to a direct effect on E6 and E7 transcription. In summary, changes in the methylation of host and viral DNA may result in an increase in the production of viral proteins and hence in the risk for carcinogenesis. However, it remains unclear whether nutritional status of methyl donors such as folic acid and vitamin B12 influence the methylation patterns of host and viral DNA.

As described in Section 2.7.1, topical all-*trans*-retinoic acid was found to effect regression of CIN2 lesions in one placebo-controlled clinical trial (Meyskens *et al.*, 1994). Chemoprevention studies that used retinoic acid to prevent preneoplastic lesions or make them regress at other epithelial sites have been successful. A growing body of basic experimental research indicates that retinoic acid and related compounds also have chemopreventive activity in the cervix. Retinoic acid is essential for terminal differentiation of cervical epithelial cells because it decreases cellular proliferation and DNA replication. It differentially inhibits the growth and differentiation of HPV 16-immortalized cervical epithelial cells (Agarwal *et al.*, 1991; Eckert *et al.*, 1995) and low-passage human foreskin keratinocytes (Pirisi *et al.*, 1992; Khan *et al.*, 1993; Creek *et al.*, 1994) compared with normal human keratinocytes in the absence of an HPV infection. In addition to decreasing cellular proliferation in HPV 16-immortalized low-passage human keratinocytes, physiological concentrations of retinoic acid inhibit the expression of HPV 16 E6 and E7 (Pirisi *et al.*, 1992; Khan *et al.*, 1993; Creek *et al.*, 1994). Retinoic acid may indirectly reduce levels of HPV mRNA by influencing the activity of AP-1 (Schüle *et al.*, 1991) or the expression of transforming growth factor β (TGFβ) (Batova *et al.*, 1992). In addition, retinoic acid suppresses cell growth. However, this suppression appears to be lost in late stages of HPV 16-induced transformation of human keratinocytes (Borger *et al.*, 2000) and cervical carcinoma cell lines. In several in-vitro model systems, cells in the late stages of HPV 16-induced transformation acquire resistance to retinoic acid-induced differentiation through several different mechanisms, including loss of growth inhibition, loss of sensitivity to TGF β (Borger *et al.*, 2000), continued growth stimulation (Higo *et al.*, 1997; Sizemore *et al.*, 1998) and loss of expression of the retinoid receptor (Bartsch *et al.*, 1992). This resistance to retinoic acid is consistent with observations that therapy with retinoic acid does not reduce recurrence rates of invasive cervical cancer (Wadler *et al.*, 1997; Look *et al.*, 1998; Weiss *et al.*, 1998) nor does it increase regression of CIN3 (Meyskens *et al.*, 1994). Taken together, these data suggest that retinoic acid may only be effective in the early stages of cervical carcinogenesis by modulating the clearance and persistence of HPV, viral load and the regression of moderate CIN.

(*d*) *Tobacco smoke*

The evidence from epidemiological studies is sufficiently strong to conclude that, in the presence of HPV infection, tobacco smoking is a co-factor in the development of pre-neoplastic lesions of the cervix and invasive cervical cancer (see Section 2.7.1). Currently, there appear to be two different mechanisms by which tobacco smoking can increase the risk for cervical diseases. Cigarette smoke contains mutagens, carcinogens and other components that may act as initiators and/or promoters of uterine cervix carcinogenesis. These components can either affect immune function and allow HPV infection to persist and progress or act directly as co-carcinogens in cervical tissue or both.

Products of cigarette smoke have been found in body fluids outside the lung such as in the breast fluid of lactating smokers, the amniotic fluid of smokers, the urine and saliva of infants of mothers who smoke and secretions of the cervical mucus of smokers (Holly *et al.*, 1986). Both nicotine and cotinine are measurable in the cervical tissue of smokers (Sasson *et al.*, 1985; Schiffman *et al.*, 1987; Hellberg *et al.*, 1988; McCann *et al.*, 1992) and nonsmokers exposed to secondhand tobacco smoke (Jones *et al.*, 1991). On average, concentrations of nicotine were more than 45 times higher in cervical tissue than in serum among women who smoked (Sasson *et al.*, 1985).

Nicotine is metabolized by oxidative *N*-nitrosation to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone that is in turn metabolized by α-hydroxylation to nitrosamine, which is considered to be one of the most potent carcinogens in cigarette smoke (Hellberg *et al.*, 1988). Farin *et al.* (1995) demonstrated that both HPV 16-immortalized oral and cervical cell lines express cytochrome P450 enzymes that are necessary for the activation of nitrosamines and polycyclic aromatic hydrocarbons. Significantly higher concentrations of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Prokopczyk *et al.*, 1997) and benzo[*a*]pyrene metabolites (Melikian *et al.*, 1999a) have been observed in the cervical mucus of smokers compared with that of nonsmokers.

As determined by the Ames *Salmonella* mutagenicity test, smokers are at an increased risk for having mutagenic cervical fluid (Holly *et al.*, 1986). Cervical tissues from smokers have significantly elevated levels of DNA damage as measured by DNA adducts compared with nonsmokers (Simons *et al.*, 1993, 1995; Melikian *et al.*, 1999b). In-vitro experiments (Nakao *et al.*, 1996; Yang *et al.*, 1996) suggest that cigarette-smoke condensate induces faster growth in serum, higher saturation density, anchorage-independent growth and tumorigenicity in cells immortalized by HPV 16 and 18. Melikian *et al.* (1999b) also suggested that HPV 16-infected cells *in vitro* are more susceptible to DNA damage by benzo[*a*]pyrene than non-HPV-infected cells. Finally, tobacco smoke has been associated with aberrant hypermethylation of the tumour-suppressor gene *p16* which significantly correlated with the grade of cervical disease (Lea *et al.*, 2004). Collectively, these data provide biochemical evidence that components of cigarette smoke have a carcinogenic potential on cervical tissue which, in an HPV-infected cervix, would increase the risk for progression to cervical carcinoma.

In addition to its direct role in carcinogenesis, tobacco smoking has been associated with a generalized suppression of the immune system, including a significant decrease in NK cells and NK cell activity, in circulating levels of immunoglobulin (Ig)G and IgA (Ferson *et al.*, 1979) and in Langerhans cells (Barton *et al.*, 1988; Poppe *et al.*, 1996). Langerhans cells are dendritic cells that are localized in the epithelium and present antigen to T lymphocytes. A reduction in the number of Langerhans cells available to detect and present viral antigens may facilitate the establishment and persistence of local viral infection. Giuliano *et al.* (2002c) demonstrated that tobacco smoking was associated with an increased risk for persistence and duration of high-risk HPV infection. The resultant viral persistence may increase the probability of the development of virally induced neoplastic transformation.

(*e*) *Radiation*

(i) *Ionizing radiation*

Carcinomas in EV patients usually show very slow progression, are only destructive locally and show very low invasive and metastatic potential. However, treatment of EV patients with ionizing radiation (γ-rays, X-rays) provokes rapid metastasis, which is probably due to its co-carcinogenic effect (IARC, 2002) and/or the release of large amounts of TNFα (Jablonska & Orth, 1985; Jablonska & Majewski, 1994). These findings have led to strict regulations of the use of ionizing radiation in the therapeutic treatment of EV patients.

Similarly, a 16-fold increase in the risk for malignant transformation has been reported after X-ray radiation therapy for multiple laryngeal papillomas, with a latency period of 5–40 years (summarized in Lindeberg & Elbrond, 1991). A later report showed relatively high frequencies of anaplastic transformation after irradiation of primary laryngeal carcinomas, and recommended a surgical approach rather than radiotherapy for the treatment of these tumours (Hagen *et al*., 1993).

The effect of radiation *in vitro* on human epithelial cells that contain HPV provides a useful model to study the genetic alterations that contribute to transformation. The exposure of HPV 16-immortalized human foreskin cells to X-radiation resulted in malignant conversion after approximately 100 additional tissue culture passages (Dürst *et al*., 1995). HPV 18-immortalized human bronchial epithelial cells exposed to ionizing radiation showed several chromosomal alterations but were not tumorigenic in nude mice despite their ability to grow in soft agar (Willey *et al*., 1993). HPV 18-immortalized bronchial cells irradiated with a single dose of radon-simulated α-particles and maintained in culture for a period of up to 3 months became tumorigenic. No mutation in the K-, H- or N-*RAS* genes was found in four of the tumours (Hei *et al*., 1994).

Since HPV DNA is occasionally found in bronchogenic carcinomas (see Section 2.6.2), it may be of interest to assess the risk of environmental or occupational exposure to radon on the progression of HPV lesions of the respiratory epithelium.

(ii) *Ultraviolet radiation (UV)*

EV has been regarded as a model for the development of non-melanoma skin cancer on sites that are exposed to the sun. Infection with a specific group of HPV types (classified in the beta genus and also termed EV-HPV types) has been associated with the benign and malignant lesions that occur in EV patients. The presence of EV-HPV DNA was also demonstrated in non-melanoma skin cancer in immunocompetent patients (Iftner *et al*., 2003), as well as in up to 90% of non-melanoma skin cancers in organ transplant recipients. EV-associated and other cutaneous HPV types were also demonstrated in normal skin biopsies (35%) and in a small number of melanomas. The frequent presence of more than one HPV type within a lesion was noted in immunosuppressed transplant recipients, and at least one type was EV-associated. The data indicate that primary infection with the majority of HPV types apparently occurs early in life, after which it remains latent. Prolonged UV radiation is needed either to activate viral gene functions and/or to inactivate the cellular genes responsible for controlled cell growth (de Villiers, 1998; Pfister, 2003).

UV activation of the HPV promoter

It is well known that UV radiation stabilizes and activates p53 in the skin. A consensus p53-responsive element was identified in the LCR of the non-EV cutaneous HPV type 77. This virus type is found in warts and skin cancers of renal transplant recipients and has a high degree of sequence homology with other common HPVs that are found in warts in the general population. Consistent with the presence of the p53-responsive element, the HPV 77 LCR was transcriptionally activated by p53. Thus, UV radiation can stimulate expression of HPV 77 genes through activation of p53 (Purdie *et al.*, 1999).

The effect of UV on the LCR from cutaneous HPV types 1, 2, 3, 5, 7, 20, 23, 27, 38, 41 and 77 was evaluated in three cell lines that harbour wild-type or mutant p53 or lack p53. Each of the HPV types reacted differently to the irradiation, and reactions varied from strong inhibition to strong activation of LCR activity (de Villiers *et al.*, 1999b). In another study, a transient transfection assay was conducted in primary human epithelial keratinocytes to determine whether UVB radiation modulates LCR promoter activity of the EV-HPV types 5, 8, 9, 14, 23, 24 and 25. The LCR promoters of HPV types 5 and 8 were activated by UVB in these cells, which suggests a role of this interaction in the development of non-melanoma skin cancer (Akgül *et al.*, 2005).

UV radiation also influences pathways of pro-inflammatory cytokines and mitogen-activated protein kinases. Ruhland and de Villiers (2001) analysed the influence of interleukin (IL)-1 receptor antagonists and inhibitors of the p38 and JUN N-terminal kinase pathways in the presence or absence of UV on the LCRs of EV-HPV types 20 and 27 in RKO, HaCaT and H1299 cell lines that express wild-type or mutated p53 or that lack p53, respectively. The results showed that IL-1α, IL-1β, IL-6, IL-17, TNFα and IFNα, -β and -γ activated the promoter in the HPV 20 LCR but inhibited the HPV 27 LCR promoter. UV radiation induced a prolonged activation of JUN N-terminal kinase in HaCaT and H1299 but not in RKO cells, and the dephosphorylation of this protein was enhanced in the presence of p53 and the HPV LCRs. UV is known to induce expression of cellular genes

that encode cell-cycle inhibitory and apoptotic factors. However, the overall consequence to the cell of the induction of viral genes by UV radiation is not known.

Anti-apoptotic effect of viral protein E6

The E6 proteins of cutaneous HPV types were shown to inhibit apoptosis *in vitro* in response to UV-induced damage. This occurs in both p53-null and wild-type cells and does not require degradation of p53 (Jackson & Storey 2000). A recent study demonstrated that expression of HPV 77 E6 can effectively block UV-induced apoptosis in cells that have UV-activated p53, by selective attenuation of the *trans*-activation of the p53-regulated pro-apoptotic genes *FAS*, *PUMAbeta*, *APAF-1* and *PIG3*. This suggests that HPV 77 E6 may play an important role in specifically deregulating p53-dependent apoptosis upon UVB irradiation (Giampieri *et al.*, 2004).

One of the cellular responses to UVB damage in the skin is the induction of apoptosis, which involves a number of signalling factors that include the pro-apoptotic Bak protein. In a study to investigate the role of HPV proteins in UV-induced apoptosis, Bak was found to be targeted by the E6 proteins of cutaneous HPV type 77 and EV-associated type HPV 5 for degradation *in vitro* and in regenerated epithelium. These data support a link between the virus and UVB radiation in the induction of HPV-associated skin cancer and suggest a survival mechanism for virally infected cells (Jackson *et al.*, 2000). It is conceivable that individuals who are infected by these HPV types are at an increased risk for developing actinic keratoses and squamous-cell carcinomas, possibly through their chronic prevention of UV light-induced apoptosis (Bouwes Bavinck *et al.*, 2001). However, because this activity is shared among E6 proteins of HPV types that are exclusively associated with benign skin lesions (e.g. HPV 10), it is probably not sufficient to confer carcinogenic properties *in vivo*.

UV-induced immunosuppression

It is well known that UV radiation modifies the immune system in the skin (Kripke & Morison, 1985). Local photo-immunosuppression occurs when the skin is exposed to low doses of UVB and may prevent an inflammatory reaction that could damage skin that is exposed to the sun. For immunocompromised patients, the additional role of photo-immunosuppression is particularly important. Renal transplant recipients have an increased risk for squamous-cell carcinomas, the great majority of which are present on skin that has been exposed to the sun. HPV DNA is found in many of these skin lesions, which suggests that UVB affects the local immune response and renders the skin incapable of clearing the infection (Vermeer & Hurks, 1994). This effect may contribute to the postulated interaction between HPV infection and exposure to UV.

Psoralen–UV treatment and HPV

Patients who have psoriasis and are treated with psoralen–UV (PUVA) are at an increased risk for skin cancer, but the exact cause of this increased incidence is not well understood. It has been suggested that PUVA may increase expression of HPV in the skin

by directly stimulating virus replication, immune suppression or both, and thereby leads to the development of skin cancer. The prevalence of HPV, as measured by PCR, in the skin (hair follicles) is increased in patients who have psoriasis and a history of exposure to PUVA compared with those who do not (Wolf *et al.*, 2004).

4.1.6 *Transgenic models for HPV-associated cancers*

Numerous HPV transgenic mouse models have been generated and characterized and provide a wealth of information regarding the in-vivo biological properties of HPV genes, in particular *E6* and *E7* of HPV 16. In this section, the tumorigenic properties of viral genes that have been discovered through studies in mice, are discussed, as well as the value that HPV transgenic mice have provided to the assessment of host immune responses to these viral antigens.

(*a*) *Tumorigenic properties of HPV genes in mouse skin*

The most common type of cancer caused by HPVs in humans is squamous carcinoma of the anogenital tracts and oral cavity. The first evidence in HPV transgenic mice that HPV 16 genes could induce squamous carcinoma derived from the analysis of αAcryE6/E7 mice (in which the viral genes were placed under transcriptional control of the αA crystallin promoter) that expressed these genes ectopically in the skin (Lambert *et al.*, 1993). The mice developed squamous carcinomas as adults (Lambert *et al.*, 1993), as well as tumours of the lens (Griep *et al.*, 1993) and retina (Griep *et al.*, 1998). Transgenic mice in which the expression of HPV 16 *E6* (Song *et al.*, 1999), HPV 16 E7 (Herber *et al.*, 1996) and all early HPV 16 genes (Coussens *et al.*, 1996) was directed to the basal compartment of stratified squamous epithelia by the keratin 14 (K14) promoter developed cancers. The severity of the cancers that arose in mice that expressed *E6* or *E7* differed individually. In K14E7 mice, the tumours were primarily benign or low-grade carcinomas, whereas in the K14E6 mice, the majority of tumours were malignant carcinomas of higher grade (Song *et al.*, 2000). These differences were also seen in the synergy between HPV genes and chemical carcinogens in the induction of skin cancer. *E6* was found to contribute to two stages in carcinogenesis — promotion, which is a required step in the formation of benign papillomas, and progression, which is the process that converts a benign tumour to a malignant cancer — whereas *E7* only contributed to promotion (Song *et al.*, 2000). Exclusively benign skin tumours were observed in other strains of mice in which the expression of HPV 16 or 18 *E6* and *E7* was directed by the tyrosinase or human keratin (hK) 1 promoters (Greenhalgh *et al.*, 1994; Kang *et al.*, 2000). Activated forms of the *Ras* proto-oncogene acted synergetically with HPV 16 *E6* and *E7* to produce tumours (Schreiber *et al.*, 2004).

The carcinogenic properties of a cutaneous HPV in mice have now been described (Schaper *et al.*, 2005). The early region of the EV-HPV 8 genome was placed under transcriptional control of the hK14 promoter. While multiple independent lines of HPV 8 transgenic mice did not display acute phenotypes of epithelial hyperplasia as seen in mice that expressed HPV 16 *E6* and *E7* genes separately or together (see above), they deve-

loped both benign and malignant tumours of the skin at frequencies of 91 and 6%, respectively. The frequency of tumours was higher and tumours occurred at an earlier age than those in HPV 16 transgenic mice. This provides the first evidence that a cutaneous HPV can cause tumours *in vivo* in an animal model.

(*b*) *Tumorigenic properties of HPV genes in the reproductive tracts of mice*

The first mouse model for HPV-induced cervical cancer used a recombinant retrovirus to transduce HPV 16 *E6* and *E7* genes into the mouse cervix (Sasagawa *et al.*, 1992). Cervical cancer developed when the mice exposed to the E6/E7 recombinant retrovirus were treated with the tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate or the mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. A more tractable model for HPV-induced carcinogenesis was developed in which K14HPV16 transgenic mice were treated with exogenous estrogen. These mice developed a progressive disease that led to the formation of squamous carcinoma of the cervix over a 6-month period and closely reflected the histopathological characteristics of the progressive disease that leads to cervical cancer in humans (Arbeit *et al.*, 1996; Elson *et al.*, 2000). The individual role of *E6* and *E7* in cervical cancer was found to differ from that observed in the skin; *E7* played a more dominant role in the cervix and *E6* in the skin (Riley *et al.*, 2003).

The validity of these HPV 16 transgenic mice as models for human cervical cancer has been demonstrated at several levels. First, the histopathological progression of disease in mice closely parallels that in human cervical cancer (Riley *et al.*, 2003). Second, the role of estrogen as a co-factor in the development and progression of cervical cancers in mice (Brake & Lambert, 2005) parallels the epidemiological evidence for a role of estrogen in human cancers (see Section 2.7.1). Third, there is a close parallel in the expression pattern of biomarkers for human and murine cervical cancer (Brake *et al.*, 2003). Last, the demonstration that the anti-estrogenic drug, indole-3-carbinol, inhibits cervical cancer in the HPV-transgenic mouse model (Auborn *et al.*, 1991) has led to its successful use in the clinical treatment of CIN2/3 (Bell *et al.*, 2000).

(*c*) *Use of HPV transgenic mice for immunological studies*

Many investigators have used HPV transgenic mice to investigate host immune responses to viral antigens and to develop protocols to induce antigen-specific therapeutic immune responses against viral antigens. Although viral genes that are expressed as transgenes should be considered as self-antigens, many studies have demonstrated that HPV transgenic mice can mount immune responses to E7 when stimulated appropriately. When immunized with E7 protein, HPV transgenic mice (line 19 αAcryE6E7) that develop squamous-cell carcinomas (Griep *et al.*, 1993) produced antibody and showed Th responses to E7 that were indistinguishable from those seen in immunized syngeneic, non-transgenic mice (Frazer *et al.*, 1995; Herd *et al.*, 1997). Non-immunized line 19 mice, when allowed to age and develop skin lesions, showed spontaneous E7-specific immune responses (Frazer *et al.*, 1995). While these antigen-specific responses neither contributed

to nor modulated the skin diseases that occurred in these mice, non-specific local inflammatory responses did contribute positively to skin disease (Hilditch-Maguire *et al.*, 1999). K14E7 mice that express E7 from the K14 promoter display split tolerance to E7, which is characterized by the ability to mount normal Th and B-cell responses to E7, but the inability to mount E7-specific CTL responses on genetic backgrounds (i.e. H-2b) in which E7-specific CTL-restricted epitopes exist (Doan *et al.*, 1998; Frazer *et al.*, 1998; Doan *et al.*, 2000).

The absence of CTL responses in K14E7 mice was associated with a non-specific down-regulation of CD8-positive T cells (Tindle *et al.*, 2001) that was probably attributable to transgene-associated disturbance of the K14E7 thymic architecture (Malcolm *et al.*, 2003). Similar split tolerance was observed in HPV transgenic mice in which the expression of E7 was directed from the K10 promoter (Borchers *et al.*, 1999). The underlying reason for this split tolerance is not understood, but could reflect the fact that keratin promoters are active in thymic epithelial cells (Frazer *et al.*, 1998).

The generation of HPV transgenic mouse models that express viral antigens in the skin offered the opportunity to use graft studies to investigate immune recognition of these antigens, when expressed in clinically relevant amounts in keratinocytes by naïve, immunologically intact animals. E7 did not function as a classical minor transplantation antigen with regard to its expression in mouse epidermis. When grafted onto non-transgenic mice, skin from K14E7 mice was not rejected even when these mice were immunized against E7 and developed E7-specific CTL responses (Dunn *et al.*, 1997; Frazer *et al.*, 2001). Similarly, grafts in which E6 antigen was expressed from the K14 promoter were also accepted (Matsumoto *et al.*, 2004). However, recipient animals were induced to reject the skin grafts in an E7-specific manner after stimulation of systemic pro-inflammatory responses (Frazer *et al.*, 2001) or through passive transfer of E7-specific CTLs in combination with E7-specific immunization (Matsumoto *et al.*, 2004). The reason why the immune system has difficulty in recognizing E7 antigen in the skin remains unclear, but E7 may possibly inhibit host immune responses. In one study in transgenic mice, it was suggested — on the basis of a comparison of expression levels of various IFN-responsive genes using real-time PCR in E7 transgenic versus non-transgenic mice — that E7 can suppress innate immune responses through its interaction with IRF1 (Um *et al.*, 2002). In another study, however, no effect of E7 was noted on the levels of MHC class I protein, a major IFN-response gene product, following induction by IFN when keratinocytes from K14E7 transgenic mice were compared with those from non-transgenic mice (Leggatt *et al.*, 2002). An alternative explanation for the poor recognition of E7 by the host immune response is that, as a non-secreted antigen, it is poorly cross-presented by antigen-presenting cells and/or that the local immune environment of the skin is not conducive to recognition by the host immune system of keratinocyte-expressed antigens. Regardless of the mechanism, the insights gained from these graft studies have clear implications with regard to the design of effective therapeutic vaccines to treat patients with HPV-associated disease.

4.2 Immune mechanisms and HPV-associated neoplasia

4.2.1 *Immunosuppression*

Impaired immunity is a host factor that has been associated with increased numbers of HPV-related lesions, as reported in studies of various populations of immunosuppressed patients. Renal transplant patients, who suffer from cell-mediated immune suppression, have an increased risk for cutaneous and genital HPV lesions (Rüdlinger *et al.*, 1986; Bouwes Bavinck & Berkhout, 1997). Generalized T-cell deficiency has also been associated with an increased incidence of anogenital neoplasia (Tindle & Frazer, 1994), as demonstrated by the increased relative risk of 5.4 (95% confidence interval, 3.9–7.2) for cervical cancer in the AIDS/National Cancer Registry study in the USA (Frisch *et al.*, 2000). Among patients with EV, NK cell-mediated cytotoxicity against keratinocytes that harbour EV-HPV DNA was markedly decreased (Majewski *et al.*, 1990). In healthy individuals, most HPV-associated lesions regress spontaneously (Stern *et al.*, 2000). Occasionally, virus persists and lesions progress, although it is not understood why some immunocompetent individuals fail to clear the infection. Both the innate and adaptive arms of cell-mediated immunity play a critical role in the determination of the outcome of an initial infection and the prevention of recurrences. A better understanding of these areas is critical for the development of therapeutic vaccines against HPVs (for reviews, see Konya & Dillner, 2001; Stern *et al.*, 2000; Melief *et al.*, 2002).

4.2.2 *Histological studies*

As HPV infection is restricted to the epithelium, clearance must be mediated by local immune defences. To address the question which effectors mediate clearance, several studies have performed histochemical analyses of genital lesions and in some cases have compared lesions from patients who responded to IFN therapy with those from patients who did not.

In naturally regressing genital warts, an increase in the infiltration of macrophages, NK cells and both $CD4^+$ and $CD8^+$ T cells into the epithelial layer was observed (Coleman *et al.*, 1994). Warts that responded to treatment with IFNg and IFNa2a showed increases in Th1 inflammatory cells, macrophages and $CD4^+$ T cells (Arany & Tyring, 1996; Hong *et al.*, 1997). Furthermore, endothelial cells that line capillaries in the underlying stroma expressed the up-regulated adhesion molecules VCAM and E-selectin and the cytokine RANTES. In contrast, warts that did not respond had fewer infiltrating T cells and reduced levels of IL-1, granulocyte macrophage colony-stimulating factor and TNF. These results suggest that a Th1 response is important in the generation of a milieu that clears HPV infection.

CIN and cancers show somewhat different features. Notably, there is a decrease in Langerhans cells in low-grade lesions and a more pronounced depletion in high-grade lesions (Viac *et al.*, 1990). Langerhans cells are specialized epithelial dendritic cells that function in the uptake of antigens. Upon migration to draining lymph nodes, they change into potent antigen-presenting cells with enhanced expression of MHC and co-stimulatory

molecules that are necessary to generate a vigorous T-cell response. Recent studies have shown that activation (or maturation) of dendritic cells depends on signals from either innate immunity triggers such as Toll-like receptors or adaptive immunity triggers such as the CD40 ligand on $CD4^+$ T cells (Melief *et al.*, 2002). In the absence of antigen presentation by fully activated dendritic cells, the resulting $CD8^+$ T-cell response is more likely to be tolerogenic than cytolytic. A study that examined Langerhans cells in low- and high-grade cervical lesions with two markers, CD1 antigen and S-100 protein (Connor *et al.*, 1999), showed a significant reduction in S100-positive Langerhans cells in high-grade compared with low-grade lesions and normal tissues. The lack of S100 expression suggested an early failure of activation of Langerhans cells in HPV-infected tissue. Lesions that contain HPV 16 have reduced numbers of Langerhans cells in the infected epidermis (Matthews *et al.*, 2003), which was attributed to reduced levels of E-cadherin on the surface of basal keratinocytes. In culture, the E6 protein mediated the reduction in levels of E-cadherin. Tobacco smoking, which has been shown to be a risk factor for the development of several anogenital cancers (Daling *et al.*, 1996), also results in the depletion of Langerhans cells in the cervical mucosa (Poppe *et al.*, 1996).

Several groups have investigated the numbers and types of T cell in cervical dysplasia with varying results (IARC, 1995). Such discrepancies may arise because cross-sectional studies cannot determine whether the lesion is progressing, regressing or static. Taken together, the results indicate that early lesions show little change in the numbers of intraepithelial T cells, whereas high-grade lesions and cancers show an increase in $CD8^+$ T cells. However, although human leukocyte antigen (HLA) class II molecules are up-regulated in late-stage lesions (see Section 4.2.4(*b*)), there is no corresponding increase in $CD4^+$ T cells.

4.2.3 *Cell-mediated immunity*

The evidence that cell-mediated immunity plays a role in the control of HPV infections derives from several lines: (*a*) most HPV-infected tissues show an inflammatory response at the time of regression; (*b*) individuals who have genetic or acquired immune deficiency are at increased risk for persistent HPV infections that progress to cancer; (*c*) efforts at immunomodulation, e.g. the use of IFN and imiquimod (which indirectly enhance cell-mediated immunity), can promote the regression of HPV lesions; and (*d*) numerous animal models have shown that inoculation of viral proteins resulted in a delayed-type hypersensitivity response that required the presence of $CD4^+$ T cells and that clearance of tumours depended on the presence of $CD8^+$ T cells (IARC, 1995; Konya & Dillner, 2001). The host immune system develops a multifactorial response that involves innate immune cells as well as helper and cytolytic T-cell responses.

(*a*) *T-Helper cell responses*

Most priming of $CD4^+$ T cells occurs in the lymph nodes following the transit of Langerhans cells from infected tissues. Most infiltrating T lymphocytes carry HLA-DR

antigens — a late activation marker — but only a few express CD25 — an early activation marker (Coleman *et al.*, 1994), which indicates that they are probably activated at distant sites. T-Helper cells largely function through the up-regulation of CD40 ligand which interacts with CD40 on dendritic cells and leads to their maturation (Melief *et al.*, 2002). CD40L on $CD4^+$ T cells can also directly signal to $CD8^+$ CTL. In addition, $CD4^+$ cells play a crucial role by interacting with B cells to induce antibody production. The interaction of T-helper cells with dendritic cells determines whether they secrete Th1 or Th2 cytokines.

Activation of $CD4^+$ occurs through recognition of MHC class II/peptide complexes present on antigen-presenting cells. Much effort has been made to identify which viral peptides can be recognized by $CD4^+$ T cells and what are the consequences of having viral-specific $CD4^+$ T-cell clones. In theory, all viral antigens can serve as targets that will be presented by class II antigens. Numerous studies have shown that $CD4^+$ T cells proliferate in response to both early and late antigens (Tindle *et al.*, 1991; Kadish *et al.*, 1994; Luxton *et al.*, 1997), and the responses tended to be type-specific. Which T-cell epitopes are presented is governed by the ability of a particular MHC molecule to bind a given peptide.

In addition to their critical function in priming other cells in the cell-mediated immune response, $CD4^+$ T cells deliver cytokines to the infected tissue, thereby influencing the outcome of the infection. The production of Th1-type cytokines is benefical for the reduction of lesions, whereas the elaboration of Th2 cytokines has a deleterious effect. Under the influence of IL-12, a Th1 response yields TNF and IFNg, whereas under the influence of IL-10, the Th2 cytokines IL-4 and IL-5 are generated, which favour B-cell development. The extent to which $CD4^+$ T cells kill keratinocytes that have up-regulated HLA-DR is unclear.

Assays to detect $CD4^+$ T-cell responses have relied on the use of peptides to stimulate T-cell proliferation, as measured by incorporation of tritium. More sensitive assays now measure the release of IL-2 from stimulated cells. Numerous studies have detected $CD4^+$ T-cell responses in sera of patients. In some studies, Th1 responses to either E7 or E2 correlated with the clearance of lesions (Kadish *et al.*, 1997). However, in other studies, the frequency of responses was similar in cases with low-grade lesions and in controls (de Gruijl *et al.*, 1996), even in studies that used virgin women as controls (Nakagawa *et al.*, 1996).

(*b*) *Cytotoxic T-cell responses*

The role of $CD8^+$ T cells in killing HPV-infected cells is extremely important as most therapeutic vaccine strategies are aimed at induction of this type of T cell. It is now recognized that the ability of CTL to kill is not based solely on the interaction of the $CD8^+$ T-cell receptor with antigenic peptides presented by MHC class I. These interactions, in the absence of other signalling cascades, frequently lead to tolerance. In contrast, productive CTL memory and killing requires signalling from highly activated dendritic cells and $CD4^+$ T cells (Melief *et al.*, 2002).

Numerous mouse models have shown that tumours that express HPV 16 or 18 E6 or E7 antigens are killed by generating a CTL response that is antigen- and MHC-restricted (Chen

et al., 1991; Feltkamp *et al.*, 1993; Tindle & Frazer, 1994). In these cases, the epitopes that served as recognition sequences were specified by the mouse MHC class I loci.

CTLs to E7 have been difficult to detect in the peripheral blood of patients known to have been exposed to HPV 16 or 18 (Borysiewicz *et al.*, 1996); however, they can be detected at higher frequency in lesions that contain infiltrates from tumours (Evans *et al.*, 1997). Oligopeptide epitopes or HLA-matched cervical cancer cell lines rarely stimulated peripheral blood CTLs, whereas viral vectors with recombinant E6 and/or E7 stimulated CTLs from 30-60% of HPV 16-positive CIN patients *in vitro* (Nakagawa *et al.*, 1997; Nimako *et al.*, 1997).

In a longitudinal study, more CTL responses to HPV 16 E6 were detected in women who had HPV 16 infection without SIL than in women who had SIL (Nakagawa *et al.*, 2000). Fifty-one women who had HPV 16 infection and three HPV 16-negative control women were enrolled; 22 (55%) of 40 women who cleared HPV 16 infection had an E6 CTL response at least once compared with none of nine women who had persistent HPV 16 ($p = 0.003$). No such difference was demonstrated for E7; 25 (63%) of 40 women who cleared HPV 16 infection responded versus five (56%) of nine women with persistent HPV 16 ($p = 0.720$). It appears that the lack of response to E6 is important in the persistence of HPV 16 infection. More studies on the CTL response to HPV antigens are needed to understand fully the role of CTLs in mediating regression.

4.2.4 *Major histocompatability complex (MHC)*

(*a*) *MHC class I*

MHC class I antigens are expressed ubiquitously on human cells, including cervical keratinocytes. They present peptides that are derived from the processing of endogenous antigens of both host and viral origin to $CD8^+$ T cells. When a vigorous $CD8^+$ T-cell response is generated through stimulation by activated dendritic cells and by CD40 signalling pathways, cytolytic $CD8^+$ T cells can kill cells that present viral peptides on the MHC class I antigen.

Early results on the expression of class I antigens gave conflicting results, probably due to the antibodies that were used for immunostaining. However, more recent studies consistently observed down-regulation of class I expression in cervical cancers (IARC, 1995). HLA class I A and B genes are highly polymorphic and inherited alleles for each gene are expressed. Examination of cervical tumours revealed that down-regulation of MHC class I expression was variable (Keating *et al.*, 1995). Certain alleles were more frequently down-regulated including A2, A3, the A9 group, the B5 group, B7, B8 and B44. Several studies have examined whether certain HLA class I genotypes confer a risk for progression to cancer (see Section 4.2.5). The risk associated with some of these alleles is probably due to their more frequent down-regulation, although it is unclear whether this is an early event that would affect progression. Down-regulation of MHC class I has also been observed in laryngeal papillomas (Vambutas *et al.*, 2000).

Many viruses target various steps in the class I antigen presentation pathway to escape immune detection. The mechanism by which class I down-regulation occurs in HPV infection is not clear and probably involves multiple mechanisms. Cromme *et al.* (1994) showed that directly diminished expression of class I genes was a rare event. The defect in class I expression in some tumours was found to be due to a loss of the peptide transporter 1 associated with antigen processing (TAP1) which occurred through down-regulation of the transcription of TAP1. HPV 16 and 18 E7 have been reported to down-regulate the promoters of both the TAP1 and MHC class I heavy chain (Georgopoulos *et al.*, 2000). Other studies have reported that the HPV 11 E7 binds to the TAP transporter protein and blocks the loading of peptides onto class I antigens (Vambutas *et al.*, 2001). HPV 16 E5 was recently reported to cause the retention of HLA class I complexes in the Golgi apparatus and impede their transport to the cell surface; this was rectified by treatment with IFN. Unlike BPV E5, HPV 16 E5 did not affect the synthesis of HLA class I heavy chains or the expression of the transporter associated with the antigen that processes TAP (Ashrafi *et al.*, 2005).

(*b*) *MHC class II*

MHC class II antigens are generally only expressed on antigen-presenting cells, and present antigens derived from an exogenous antigen-presenting pathway to $CD4^+$ T cells. However, in the majority of cervical premalignant and malignant neoplasias, HLA class II molecules are up-regulated on keratinocytes (Glew *et al.*, 1992; Cromme *et al.*, 1993). Normal ectocervical epithelium does not express detectable levels of class II antigens, nor did expression of HPV genes in cultured epithelium result in class II expression (Coleman & Stanley, 1994), which suggests that the up-regulation of class II was indirect and was mediated by the pro-inflammatory cytokine IFNγ and in part by TNFα (Majewski *et al.*, 1991). Consistent with this explanation is the finding that cultured keratinocytes that expressed HPVs also expressed class II antigens when treated with IFNγ and the fact that class II expression is increased in various inflammatory skin diseases. Also consistent with this hypothesis is the correlation between increased inflammatory response in the stroma and increased infiltrating inflammatory cells and the extent of class II upregulation (Coleman & Stanley, 1994). The DR, DP and DQ class II MHC subloci are differentially expressed on keratinocytes within cervical squamous tumours, which suggests independent regulation (Glew *et al.*, 1992). It has also been reported that the HPV 16 E5 protein can block INF-induced surface expression of MHC class II by preventing degradation of the invariant chain (Zhang *et al.*, 2003).

4.2.5 *Modulation of innate immune responses by HPV*

HPVs display multiple activities that may contribute to down-modulation of both antigen-specific and innate immune responses. Previous sections have described studies that demonstrated an ability of HPV gene products to modulate MHC class I and class II cell surface expression, as well as the expression of TAPs, all of which are components of

the cellular machinery that mediates antigen-specific responses. This section reviews studies that demonstrate that HPV gene products can modulate innate immune responses. For a more in-depth summary of the effects of HPVs on the host immune response the reader is referred to the review by O'Brien and Campo (2002).

A number of laboratories have identified the effects of HPV proteins E6 and E7 on specific cellular factors that modulate the cellular response to IFNs. Ronco *et al.* (1998) discovered that HPV 16 E6 can bind to and destabilize IRF3 and thereby inhibit the induction of IFNβ. Li *et al.* (1999) provided evidence that HPV 18 E6, but not HPV 11 E6, binds tyrosine kinase 2 and impairs activation of the Jak-signal transducer and activator of transcription pathway by IFNα. Thus, E6 proteins of high-risk but not low-risk HPVs studied to date can inhibit IFN responses.

Barnard and McMillan (1999) proposed that a direct interaction of HPV 16 E7 with the p48 component of the IFN-stimulated gene factor 3 transcription complex contributes to the inhibition by E7 of IFNα-inducible genes. In a subsequent study, it was proposed that E7 from both high- and low-risk HPVs can inhibit IFNα-inducible genes, and that this activity required the region in E7 that is involved in Rb-binding (Barnard *et al.*, 2000). Other studies found that HPV 16 E7 can impair the function of IRF1 (Park *et al.*, 2000b; Perea *et al.*, 2000) and that this correlates with the ability of E7 to interact with IRF1 at least in part through its Rb-binding domain (Park *et al.*, 2000b).

Indirect evidence for an effect of HPVs on the innate immune response derives from gene expression profiling studies on human epithelial cell lines that harbour intact high-risk HPV genomes or express high-risk HPV genes, in which a down-regulation of the expression of many IFN-responsive genes was observed (Chang & Laimins, 2000; Nees *et al.*, 2001).