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Review

New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections

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Abstract

Extensive research over the past 20 years provided strong evidence that persistent infections with high risk type human papillomaviruses (HR-HPVs) cause cervical cancer. However, depending on their age, more than 20% of normal women are infected with these viruses and only very few develop clinically relevant dysplastic lesions or even cancer. During an *acute* HPV infection, expression of viral genes, in particular the viral E6 and E7 oncogenes is restricted to differentiated epithelial cells, which lost the capability to replicate their genomes and are therefore at no further risk for acquiring functionally relevant mutations upon genotoxic damage. High grade cervical dysplasia, however, is initiated by deregulated expression of viral oncogenes in replicating epithelial stem cells. Here, the E6–E7 gene products submerge control of the cell cycle and mitotic spindle pole formation through complex interactions with various cellular protein complexes and induce severe chromosomal instability. The detailed molecular analysis of these interactions allowed to define new biomarkers for dysplastic cervical cells. E7 for example induces increasing expression of the cyclin dependent kinase inhibitor $p16^{ink4a}$ in dysplastic cells. This can be used to identify dysplastic cells in histological slides, cytological smears or samples taken for biochemical analyses with an yet unmet fidelity. Detection of specific viral mRNAs derived from integrated HPV genomes in advanced precancers can be used to identify lesions with a particularly high risk for progression into invasive carcinomas (APOT assay). These new markers will result in a modified classification of cervical precancers and improved screening assays. Here, we review the basic concept and potential clinical applications of these new developments.

Keywords: HPV; Cervical dysplasia; E6; E7; Cervical cancer screening; Molecular markers

1. Introduction

1.1. The current cervical cancer screening strategy

Although population wide screening in most Western countries has led to a remarkable reduction in the incidence and mortality of cervical cancer [1], with approximately 400 000 new cases and 250 000 cancer-related deaths each year, and remains a major global health burden. Since its description by George Papanicolaou in 1941 [2], the technical approach of the screening assay has remained essentially the same. It is still based on the morphological interpretation of cells in smears taken from the surface of the cervix. Scoring criteria include the assessment of cell size/nuclear size, the nuclear shape and nuclear staining intensity, nuclear and chromatin architecture, shape of the nuclear membrane and the ratio between cytoplasm and nuclear volume [3], most of these being signs of acute or persistent papillomavirus infections. Due to the ambiguity of these morphological criteria, screening results are affected by a high degree of inter- and intra-observer variability. In particular, 'low grade' changes are often prone to misinterpretation [3,4]. In addition to the insecurity and distress for the affected women, this also causes significant costs for healthcare providers [5–7]. In view of the tremendous scientific achievements which have

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been made to unravel the molecular pathogenesis of cervical cancer, it is now time to translate these significant improvements into new screening concepts and technologies. The purpose of this review is to summarise the major pathogenic features of persistent oncogenic papillomavirus infections in cervical carcinogenesis. Based on this knowledge, new biomarkers for dysplastic cervical cells can be delineated. The basic concepts and potential clinical applications in modified screening programmes will also be discussed.

2. Prevalence of human papillomavirus infection and the association with cervical cancer

The human papillomaviruses (HPV) infect epithelial cells in both mucosal surfaces and the skin. A subgroup of the mucosa-associated HPV types, the high-risk papillomaviruses (HR-HPVs), infect primarily mucosal epithelia of the anogenital tract and are the major cause of cervical cancer (Tables 1 and 2). Women infected with HR-HPVs have an at least 100-fold increased risk of developing a high grade cervical lesion compared with non-infected individuals; nucleic acids of HR-HPVs can be readily detected in almost all high-grade cervical lesions and cervical cancers [8,9]; two genes, *E6* and *E7*, encoded by HR-HPV types are consistently expressed in high grade cervical dysplasia, cervical cancers as well as the cell lines derived from them (Table 2, Fig. 1). They are potent oncogenes and transform cells into continuously growing cell clones, characterised by a high degree of chromosomal instability [10–12]. Continuous expression of the *E6* and *E7* genes is an essential and indispensable prerequisite to maintain the neoplastic growth of cervical cancer cells [13,14]. Finally, studies on the molecular mechanisms, of how these genes provoke transformation have revealed a complex pattern of interactions with cellular gene products involved in the fine tuning of the cell cycle, the programmed cell death pathway (apoptosis), epithelial differentiation and chromosomal homeostasis and stability (reviewed in Refs. [12,14]).

These observations confirmed a pathogenic role for HR-HPVs in cervical carcinogenesis. However, epidemiological studies have revealed that HR-HPV infections are very common and are readily detectable, particularly in young women [15,16]. In cross-sectional studies, the highest infection rates were observed in women aged 20–35 years. Up to 30% of women in this age group were found to be infected with HR-HPV types [15]. The prevalence of infection decreases with increasing age suggesting that in most women the infection is self limited and resolves spontaneously without leading to relevant pathologies. In longitudinal cohort studies, it was observed that most 'acute' HPV infections last approximately 8–10 months [16,17]. Only a few of them persist and induce dysplastic epithelial lesions. The cumulative incidence among sexually active young women is estimated to be in the range of more than 60–80%, being highly influenced by specific risk factors and, particularly, the number of sex partners.

Due to these epidemiological features, the use of virological tests as a primary screening parameter has been controversially discussed. Due to the high prevalence of infections among women without pathologies, the mere detection of an HR-HPV infection has a *low positive predictive value* for the presence of dysplasia. However, due to the very close association of persistent HR-HPV infections and cervical dysplasia or cancer, a negative HR-HPV test has a very *high negative predictive value* for cervical dysplasia or cancer. Thus, HPV testing is very helpful to stratify patients with abnormal smears [18]. However, its use as a primary screening parameter would generate unnecessarily high secondary costs in the work-up of all infected patients without relevant dysplasia (Table 3a). In addition, the psychological distress that would be experienced by any infected women who ultimately would not need any further treatment is a serious concern (Table 3b). These considerations further underline the need to identify more specific biomarkers with a better *positive predictive value* for the presence of cervical dysplasia or cancer. Such tests are expected to dramatically reduce the costs of the current cervical cancer screening programmes and will result in a higher patient compliance because of their assumed immediate therapeutic implications (Fig. 2). A more detailed understanding of the molecular pathogenesis of cervical cancer and its precursors has now allowed us to delineate respective markers.

Table 1
List of mucosal HPV types

Most prevalent low-risk (LR-HPV) types: HPV 6b, 11,13, 41, 42, 44, 53, 54, 58, 61, 62, 66, 69, and others
Most prevalent high-risk (HR) types: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56

Table 2
Evidence supporting the causal role of HR-HPV in cervical carcinogenesis

• HR-HPV infection is the major risk factor for cervical cancer
• Regular presence of HR-HPV DNA in tumour cells
• Consistent expression of viral oncogenes (*E6* and *E7*) in tumour cells
• Cervical cancer cells cannot grow without the expression of the viral oncogenes
• *E6* and *E7* oncogene products interact with cellular growth regulatory proteins

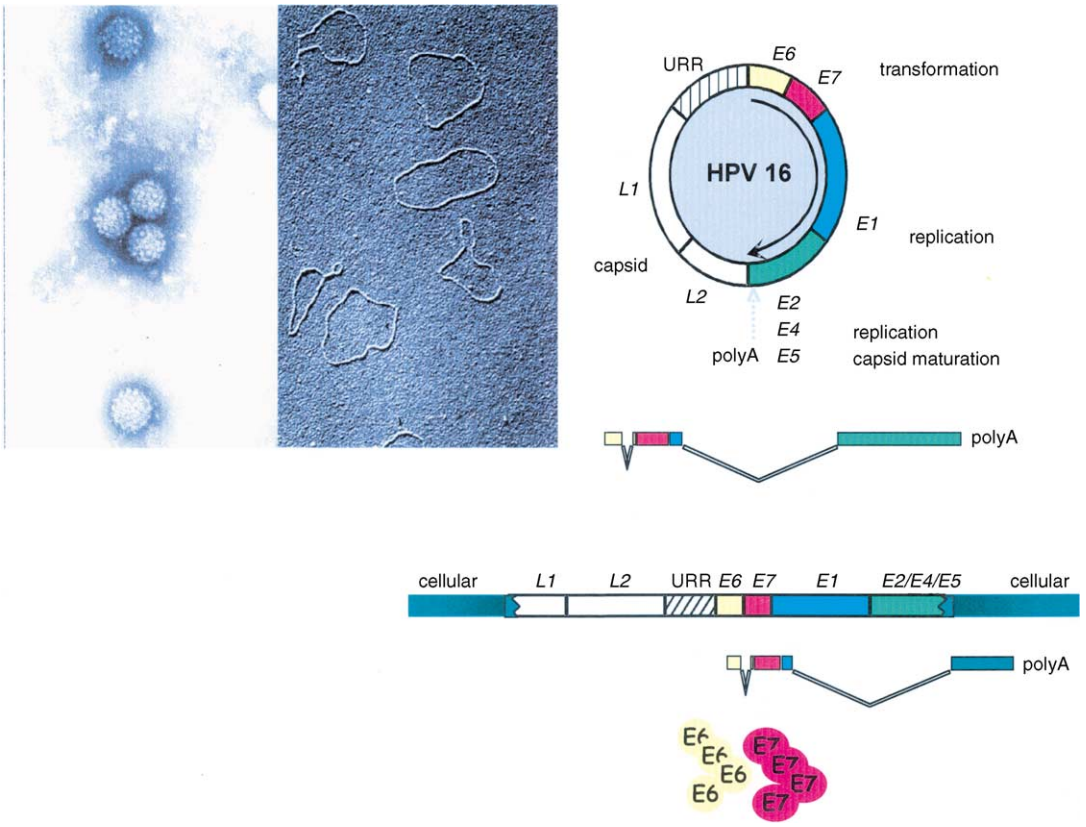

Fig. 1. Human papillomaviruses are small viral particles with a circular DNA genome which encodes several early (E) and late (L) genes. During the normal viral life cycle, episomal molecules are transcribed resulting in an major early gene transcript, which also encodes the *E6* and *E7* oncogenes. In cervical cancers, the viral genome is frequently integrated into the host cell genome and the *E6* and *E7* genes are transcribed as fusion transcripts together with cellular sequences.

3. The natural history of HR-HPV infections

Most HPV-related dysplastic lesions develop within the anogenital tract of females. Therefore, most epidemiological studies on the incidence and prevalence of HPV infections have been performed in female cohorts. Penile lesions are very rare, although a similar percentage of males are likely to be infected and seem to represent the major reservoir for transmission of the oncogenic viruses [19]. This suggests that besides the simple infection, a particular interaction with specific host cells is required for the initiation and progression of HPV-related precancers. In order to initiate a productive, 'acute' HPV-infection, the virus needs to infect an epithelial stem cell, usually at sites of minor wounding or where the anatomical architecture provides easy access (Fig. 3a). Given the above, it is unsurprising that the squamous columnar junction in the transformation zone of the uterine cervix is a major target for HR-HPV-infections. Upon infection, the virus utilises the host cell DNA replication machinery to multiply a few of its episomal viral genomes. These persist in the nucleus of the infected stem cells and are distributed during mitosis on to the daughter cells. These infected stem cells act as a reservoir for cells, which might gain the capacity to multiply the virus to very high copy numbers (Fig. 3b). However, this productive stage is restricted to postmitotic, differentiated cells in the suprabasal layers of the epithelium, which were withdrawn from the cell cycle. Detailed *in situ* hybridisation [20,21] and immunohistochemical studies [22–24] have revealed that high level expression of viral genes, multiplication of the viral genome, synthesis of early (*E6*, *E7*, *E2*, *E4*) and late gene products (*L1* and *L2*), encapsidation of the HPV genome, and release of virion particles along with the exfoliation of upper epithelial layers is strictly linked to terminal differentiation of infected epithelia. Papillomaviruses possess a mechanism to overcome the block in DNA synthesis that occurs along with the differentiation of the epithelial cells, although this does not lead to full replication of the genome of the differentiated host cells. Various lines of evidence suggest that the HPV E6- and E7-oncoproteins play a major part in the deblockade of DNA synthesis in differentiated epithelia. Cells with high copy number multiplication of the viral DNA and expression of viral genes are arrested

Table 3
a Frequently asked questions associated with HPV tests in primary screening programs

- Do all HPV-positive women require clinical work-up?
- Which test should be done on HPV-positive women?
- Colposcopy
- Pap smear
- Biopsy
- Others?

b Frequently asked questions by infected women

- Am I infected with a 'cancer virus'?
- Where did I get this virus from?
- Did my partner infect me with a 'cancer virus'?
- What can I do to prevent cancer? Is there nothing better than repeated tests?
- The second test was negative. Is this diagnosis reliable?

in the postmitotic stage and are determined to die. They are not susceptible to mutagenic effects and damage to the host cells genome associated with multiplication of the virus or its genome, simply because they do not pass their genomes on to the surviving daughter cells.

The virus replicating cells display typical cytopathic changes characteristic for low grade squamous intra-epithelial lesions (LSIL). These include most prominently koilocytosis or koilocytotic atypia, i.e. the squamous cells with a vacuolated appearance and also some minor changes of the nuclear morphology (Fig. 4) [25,26]. This stage of the infection must be clearly differentiated from effects which occur later in persistent HR-HPV infections. These are characterised by more severe morphological changes including marked changes of the staining intensity of the nuclei indicating severe changes of the overall DNA content (aneuploidy), altered chromatin texture, changes of the nuclear membrane, and changes of the cell size and the relationship of the cytoplasm and nuclear volume. Histologically, the more advanced dysplastic lesions have increased numbers of mitotic figures and a significant extension of actively replicating cells into the upper parts of the epithelium. Apparently these latter alterations are due to the expression of viral genes in epithelial stem cells, which subsequently lose in a stepwise manner the capacity to differentiate in a coordinated way. Epithelial stem cells, which express the viral oncogenes replicate their cellular genomes and therefore experience the dramatic mutagenic consequences [12].

4. The intracellular control of viral gene expression and replication in epithelial stem cells

How expression of viral genes suddenly occurs in the non-senescent, not yet terminally differentiated, epithelial stem cells is largely unknown. Several lines of evidence point to loss of intracellular transcriptional control by specific cellular surveillance factors referred to as cellular interferrin, factor CIF (reviewed in [27]). This permits 'aberrant' expression of the *E6* and *E7* genes in replicating epithelial stem cells (Fig. 3c and d). This concept nicely explains how infections with oncogenic HPV types can be abundant, although the associated cancers occur relatively rarely [28].

Histologically and cytologically, early lesions, in which the postulated CIF function is still active and in which viral gene expression and replication occurs

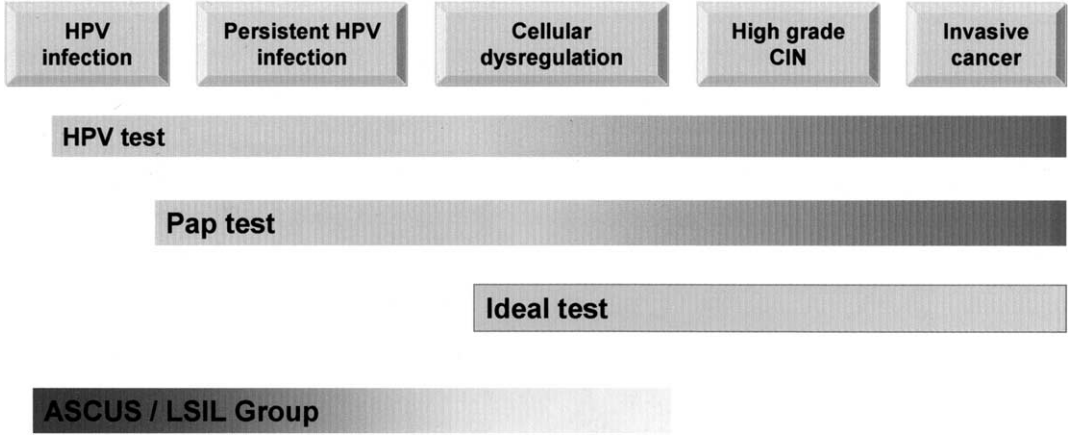

Fig. 2. Diagnostic sensitivity of cervical cancer screening tests. The HPV test is highly sensitive, but due to the many infected women without relevant pathology, its positive predictive value is rather low. The Papanicolaou (Pap)-test identifies rather unspecific cellular changes. It is sensitive, but the specificity is not optimal. The 'optimal test' would only identify true dysplasias (high positive and negative predictive values). The major advantage of such a test would be the lower number of false-positive test results, as the Pap smear currently gives rise to a high number of diagnoses as atypical squamous cells of undetermined significance (ASCUS) or low grade squamous intraepithelial lesion (LSIL).

selectively in terminally differentiated cells are hard to differentiate from early lesions in which the CIF mechanism has already been inactivated and which have already passed the selection process to establish a cell clone with aberrant expression of the viral oncogenes in proliferating basal cells. The latter lesions have a higher grade of dysplasia, compared with lesions which still retain the complete control machinery of viral gene expression.

Simple molecular markers which allow these two cell types to be precisely differentiated might have a substantial clinical impact. It is anticipated that they will help to reduce the subjective interpretation and risk assignment of individual observers (pathologists), particularly in the interpretation of early dysplastic lesions [29]. Since aberrant high level expression of the viral oncogenes *E6* and *E7* in epithelial stem cells is the major

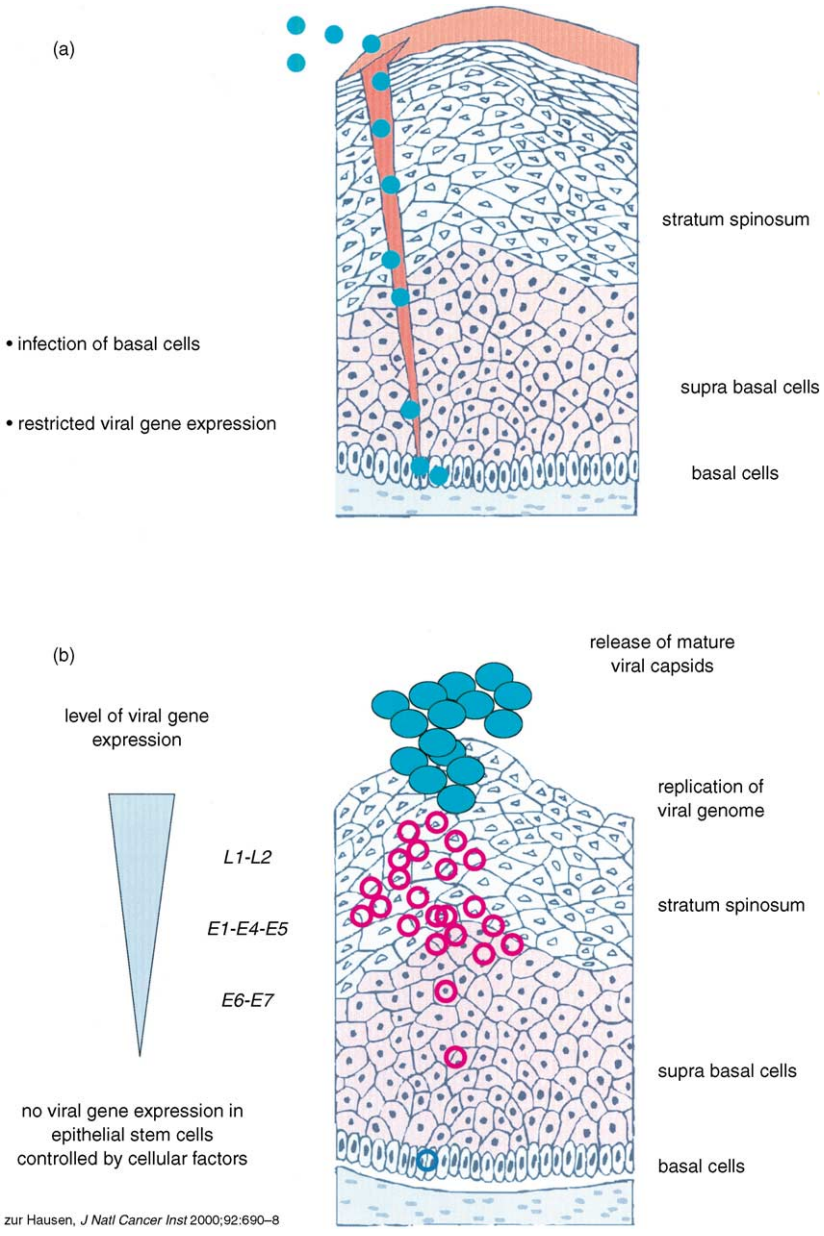

Fig. 3. (a) HPV Life Cycle I: latent HPV infection. Schematic representation of the initial steps of an HPV infection: The virus infects epithelial stem cells after getting access to this cell compartment through little scars or wounds. Initially, it remains as a silent passenger in the epithelial stem cells. (b) HPV Life Cycle II: replication of HPV particles. A few of the infected stem cells give rise to daughter cells capable of replicating the viral genome in the terminally differentiated cell layers in the stratum spinosum or above. Finally, mature viral particles are released along with the squamous debris which is exfoliated at the surface of the epithelium. In the basal cells, viral gene expression is effectively prevented by a tight intracellular control machinery. (a) and (b) reflect the situation of an acute HPV infection, which usually lasts a couple of months.

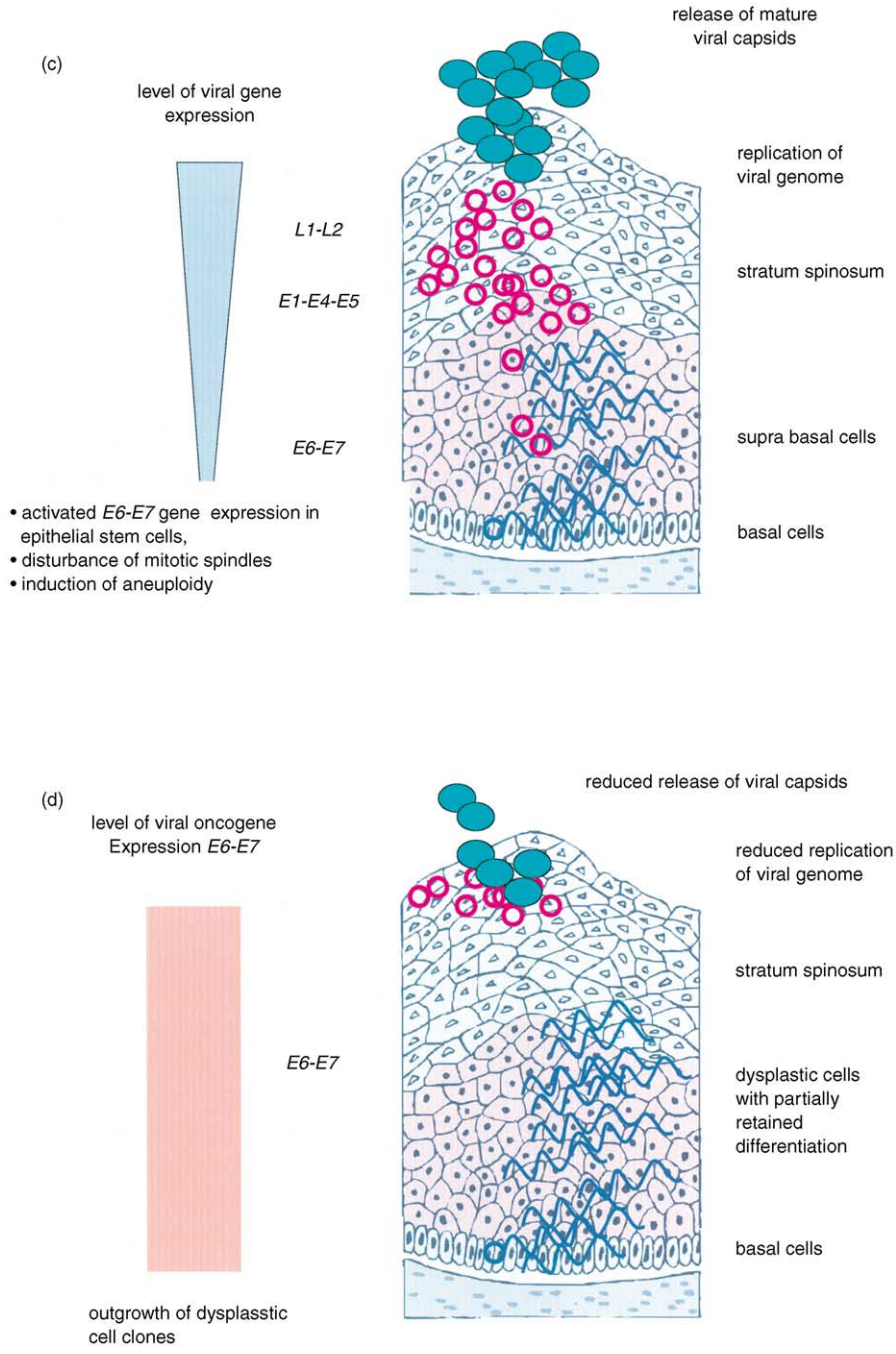

Fig. 3. (c) HPV Life Cycle III: deregulated HPV gene expression. Due to loss of function of the intracellular surveillance mechanism, viral oncogenes are expressed in the epithelial stem cells and initiate chromosomal instability. This is the rate-limiting 'gate keeper' event in the pathogenesis of cervical dysplasia. Expression of the viral genes *E6* and *E7* permits selection of dysplastic cells displaying chromosomal instability. (d) HPV Life Cycle IV: deregulated HPV-gene expression and selection of cells which express *E6–E7* genes. Due to selective growth advantages achieved by some of the stem cells with deregulated viral gene expression, the 'fittest' cell clones are selected and grow out to dysplastic lesions.

difference between them, it was tempting to monitor the expression level of these genes either at the RNA or protein levels. However, quantification of the transcripts is difficult, since it requires sophisticated RNA or cDNA-amplification techniques. Moreover, *E6* and *E7* transcripts are also expressed in the differentiated cells of lesions in which the CIF mechanism is functionally preserved in the basal cells. Earlier reports suggested that in clinical lesions with advanced cervical dysplasia, more *E6–E7* transcripts can be detected [30]. However, quantification of the *E6–E7* transcripts or gene products is technically extremely challenging and the standardisation and reproducibility of the respective assays is likely to be a major problem. Immunohistochemical

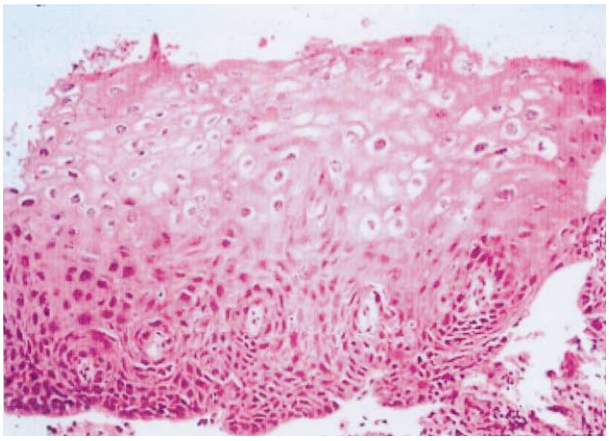

Fig. 4. Typical histological picture of koilocytes. Active replication of the papillomavirus result in the marked halo around the nucleus and the atypical changes of the nuclei. Koilocytes are densely packed with papillomavirus particles.

detection of the *E6* and *E7* gene products is hampered by the lack of sufficiently specific and sensitive monoclonal antibodies and by the very short half-life time and high turnover rates of the *E6* and *E7* gene products, which are degraded within several minutes after translation [31,32]. Thus, it is more tempting to look for surrogate markers, which indirectly indicate the expression and activity level of the viral oncogenes in replicating, non-senescent epithelial stem cells. Besides extensive RNA expression profiling [33,34], 'evidence based' approaches were helpful to identify surrogate markers, which reflect the expression level and functional activity of the transforming papillomavirus oncogenes in epithelial stem cells (Sano *et al*. 1998c [35,36]). To consider these concepts in greater detail, it is worthwhile to review some major molecular effects of the viral oncogene products.

5. Molecular functions of the *E6* and *E7* oncogene products

As described above, replication of the HR-HPV genomes is restricted to terminally differentiated cells, which have left the cell cycle and switched off their cellular DNA replication machinery (Fig. 3a–d). The viral genome therefore contributes to the cell's genetic functions, compensating for the missing cellular functions required for the replication of viral DNA. One major player here is the *E7* gene product, which has been shown to mediate the initiation of DNA synthesis as a consequence of cell cycle activation at the G1/S phase transitions (see below) (for review see (Munger *et al*., 2001a)). However, inappropriate activation of the cell cycle and cell proliferation initiates the programmed cell death machinery in normal cells and induces apoptosis [37]. This would prevent survival of respective host cells and result in less effective multiplication of the virus. Presumably for these reasons, the virus developed a molecular machinery, to prevent untimely premature apoptotic cell death, that is mediated by the *E6* gene product (reviewed in [38]). Thus, both genes seem to encode complementary functions, *E7* being the promoter of DNA replication and cell growth and *E6* counteracting apoptosis and mediating the prolonged survival of even severely damaged and mutated cells. The precise physiological functions of these genes in terminally differentiated cells, in which they are normally expressed during the viral life cycle, is largely unknown due to the lack of appropriate experimental systems.

The E6-protein consists of approximately 150 amino acid residues (AA) and carries two zinc-finger binding motifs [39]. It acts as a potent oncogene in various experimental settings. One of its first characterised activities was its complementary transforming function of primary rodent cells along with an activated *ras*-oncogene [40]. *E6* genes of the HR-HPV types, but not of the LR-HPV types readily transform primary human keratinocytes if they are coexpressed together with HR-HPV *E7* genes [41]. Keratinocytes transformed by HR-HPV *E6* and *E7* genes, however, are not fully malignant cell clones. Prolonged culture and/or additional mutagenic agents are required before they establish their fully malignant growth potential [42,43]. This clearly underlines the multistep nature of HPV-mediated transformation once the viral oncogenes are expressed. E6 expressed in the stem cells of the skin in transgenic mice under control of a keratin 14 promoter induces various squamous cell neoplasias, even in the absence of a cooperating *ras* or *E7* gene [44,45]. The transforming functions of E6 are apparently mediated through its interaction with a variety of cellular gene products. The first identified target of E6 was p53, which is directed to the ubiquitin-proteasome pathway (reviewed in [46]). Therefore, cells which express E6 display reduced levels of functionally active p53 and do not respond to DNA damage with an appropriate induction of the p53 levels. Under physiological conditions, p53 degradation is mediated via the ubiquitin–proteasome pathway through Mdm2, which is abolished under cellular stress conditions. In E6-expressing cells, Mdm2-directed degradation of p53 is abolished and p53 degradation is mediated via the binding of E6 to the E6-associated protein E6-AP [47], which is not susceptible to signals upon DNA damage, but continuously degrades p53. This leads to an inappropriate activation of p53 upon genomic stress and permits the survival of cells with damaged genomes. In agreement with these findings, expression of E6 in cells grown in cell culture induces gross genomic damage including translocations and aneuploidy [48]. However, a couple of studies clearly

demonstrate, that p53 activities are not completely abolished in E6-expressing cells and that cervical cancer cells still retain the capacity to induce p53 despite the expression of E6 [49]. This might be due at least in part, to the negative interference of the E6I* splice variant derived gene product, which apparently inhibits the p53 degrading activity of the E6–E6 AP–p53 complex [50,51] and presumably allows a fine tuning of the p53 activities in HR-HPV E6-expressing cells.

Beside its well documented interaction with p53, numerous additional interactions with a variety of further cellular targets have been described (Mantovani and Banks 2001a). These can be subdivided into interactions with factors involved in (i) transcriptional regulation and DNA replication, (ii) further factors involved in the regulation of apoptosis, (iii) factors regulating epithelial organisation and differentiation, and (iv) factors involved in cell polarity and cell-cell interactions. It is also likely that many further interactions have not yet been identified. Thus, E6 is a modulator of many important physiological functions, which of these are most important for cancer initiation and progression is not clear. The multifunctional role of E6 fits in with its short half-life time and the very low amounts that are expressed in cells. Taken together, E6 seems to contribute, through highly complex interactions with many cellular targets, a new regulatory feature to HPV-infected cells. It is able to modulate, through slight alterations of the activity levels of these various interaction partners, important functions such as the transcription of genes involved in chromosomal homeostasis, regulation and fine tuning of apoptosis and cellular differentiation and cell adhesion and organisation of cellular polarity. With regard to the potential diagnostic exploitation of these results, so far only the detection of the telomerase subunit, hTERT, in cervical smears or biopsy has been extensively studied [Keating *et al*., 2001a, 52,53]. Although the results reported so far in the literature suggest an increasing level of *hTERT* transcripts in cervical lesions with increasing grade of dysplasia, many false-positive as well as false negative test results have limited the clinical use of this marker in cervical cancer screening [54].

The second major player in HPV-mediated carcinogenesis is the E7 protein. With its 100 AA residues it is even smaller than the *E6* gene product, but characterised by a comparable complex pattern of interactions with cellular proteins. It cooperates with the HR-HPV E6 oncogene in the transformation of rodent and human cells (reviewed in (Munger *et al*., 2001b). Like E6, E7 is characterised by two copies of a Cys-XX-Cys motif which acts as a dimerisation/multimerisation domain. E7 shares homologies with other oncogenes of DNA-tumour viruses such as E1A of the adenoviruses and the T-Antigens of the polyomaviruses [55,56]. E7 seems to be a predominantly nuclear protein and its intracellular levels are low due to an effective turnover via ubiquitin-mediated lysis [57].

Central physiological effects of E7 are linked to the virus strategy to use as few genes as possible for its own multiplication. Replication of the viral genome therefore relies on an at least partly functioning DNA synthesis machinery, which in normal differentiating cells of the squamous epithelium is shut down during differentiation (reviewed in [58]). E7 has been shown to uncouple differentiation and switch off DNA replication in HPV-infected host cells, thereby permitting full DNA synthesis in terminally differentiated cells [59]. To do so, expression of E7 results in the activation of a number of cellular genes which all are regulated by the E2F-family of transcription factors (reviewed in [60,61]). These include, for example, DNA-polymerase α, dehydrofolate-reductase, thymidine kinase, cyclin E and many others. In normal cells, the activity of E2F as transcription factor is regulated by the cyclin/cyclin-dependent kinase (CDK) dependent phosphorylation of pRB and other pocket proteins including p107 and p130 (Dyson, 1998b). Phosphorylation of pRB and the other pocket proteins results in the release of free E2F from the pRB/E2F transcriptional repressor complex and activation of E2F as a transcriptional activator of a group of genes required for the promotion of cells in the G1-phase of the cell cycle into S-phase. E2F is associated with another group of proteins referred to as DP. E2F-DP heterodimers act as DNA-binding transcriptional activators. The pocket proteins (pRB, p107, p130) act as regulators of these complexes. After binding, they mask the transcriptional activation domain of E2F/DP complexes and convert them to transcriptional repressor complexes, which actively restricts entry into S-phase. Upon cyclin-dependent kinase-mediated phosphorylation of the pocket proteins, these complexes dissociate and E2F is again converted to a transcriptional activator of the S-phase entry genes. Introduction of E7 into cells disturbs this network and results in an enhanced transcriptional activation activity of E2F-dependent genes (Fig. 5).

Differentiation of normal keratinocytes is linked to the enhanced expression of p21 and p27, two cyclin-dependent kinase inhibitor proteins, which inhibit the activity of CDKs, in particular CDK-2 and thereby prevent further activation of S-phase entry genes. To create an intracellular milieu which allows continuous DNA replication, although the post-mitotic cell has already undergone terminal differentiation and expresses enhanced levels of p21 and p27, E7 inhibits the activity of p21 and p27. Consequently, CDK-2 remains active, mediates ongoing phosphorylation of the pocket proteins and release of E2F-transcriptionally activating factors and activation of genes involved in DNA-synthesis and thus creates an intracellular milieu, in which viral genome replication can take place, although the

cells have already entered the terminal differentiation pathway (reviewed in [Munger *et al.*, 2001c]). Interestingly, this does not directly affect the replication of the host cell genome, since E7 expression in differentiated cells with high levels of cyclin E and p21 does not lead to induction of proliferating cell nuclear antigen (PCNA) and cellular DNA-synthesis [62]. Taken together, the ability of E7 to abrogate the inhibitory effects of CDK-inhibitors p21 and p27 in combination with sustained inactivation of the E2F-pocket protein-repressor complexes results in enhanced and/or sustained synthesis of S-phase entry proteins and thus recruits these cellular factors for the replication of the viral genome. Although the full permissive DNA-replication cycle of the cellular genome is not reinitiated in these terminally differentiated cells, the milieu permits sufficient DNA synthesis for replication of the episomal viral genomes.

Originally, the biochemical concept which was proposed for the E7-mediated inactivation of the pocket proteins pRB, p107, and p130 suggested that the interaction of the pocket proteins with the E2F transcription factor family is disturbed by a direct stoichiometric interaction of E7 with pRB or its homologues, resulting in increased free E2F upon binding of E7 to pRB. However, through a number of more recent studies it has been suggested that it is more likely to be E7-mediated degradation of the pocket proteins through the proteasome–ubiquitin pathway than the mere interaction with the pocket domain which contributes the major E7-mediated activity. These studies further suggest that it is not only the interaction of pRB with E2F-like factors but also other potential E2F-independent activities which contribute to the E7-mediated transformation (reviewed in (Munger *et al.*, 2001d).

E7 is also linked to the abrogation of the cytostatic effects of TGF-β, interferons and other factors on E7-expressing cells. In addition, E7 affects the activity of several enzymes involved in the energy metabolism of the cell, for example, M2 pyruvate kinase, α-glucosidase [63,64], as well as enzymes involved in the regulation of intracellular alkalinisation [65]. How far these mechanisms contribute to transformation is largely unknown. However, the activation of the α-glucosidase in particular nicely explains the very well known observation that dysplastic HPV-transformed epithelial lesions display significantly reduced levels of glycogen, a feature which is broadly used to identify the extent of cervical lesions during clinical investigation using iodine solution (Lugol's test) [66].

One particularly important function of E7 is its remarkable activity to induce polyploidy and aneuploidy, once expressed in actively growing cells. This is linked to multipolar mitotic figures in contrast to the bipolar mitotic figures in normal mitotic cells. Recent studies have shown that HR-HPV *E7* gene products interfere with the ordered synthesis of centrosomes [[67,68]. Centrosomes are the major cellular components which organise the microtubular apparatus and serve as mitotic spindle pole bodies (reviewed in [69]). Within normal cells, only two centrosomes are formed once per cell cycle to ensure, that the condensed chromosomes find only the two poles before they segregate during mitosis. E7 initiates inappropriate synthesis and formation of centrioles, the core elements of centrosomes. Consequently multipolar mitotic figures are formed as a key feature of genetic (chromosomal) instability reviewed in [12]). Recently, E6 was shown to induce marked nuclear atypia and concomitant accumulation of centrosomes [70]. Thus, both viral oncoproteins cooperate in central features of chromosomal instability following expression in replicating epithelial cells.

6. Diagnostic exploitation of molecular pathways induced by the viral oncogenes: E7-mediated overexpression of p16ink4a identifies HPV-transformed cells

In searching for new diagnostic concepts one aspect of the interaction of E7 and the pocket proteins, in particular pRb, seems particularly worthwhile to consider further. As discussed above, the transcriptional inhibitory activity of the complex of pRB and E2F seems to be degraded, particularly due to E7-enhanced ubiquitination and proteasome-mediated decay. pRB and its complex with E2F, as discussed earlier, controls the initiation of the G1/S phase progression at least in terms of activation of S-phase promoting genes like DNA-polymerase α and others (Fig. 5). Inactivation of pRB is a very common feature in human cancers (for review see [71]) . It might occur at the protein level, as for example in cancers where the *RB* gene is mutated or like in cervical cancers, where tumour virus encoded gene products inhibit the pRB function. However, overexpression of cyclins like cyclin D in several tumour entities results in similar effects, since the complex of cyclins and CDKs are responsible for the phosphorylation of pRB thereby regulating its G1/S phase promoting activity. Increased cyclin/CDK activity results in the hyper-phosphorylation of pRB and thus release of E2F-transcription factors and promotion of the cell cycle. The activity of the complex of cyclins and cyclin-dependent kinases, in particular cyclin D and CDK4, is regulated by CDK inhibitors including p16ink4a. p16ink4a consequently was also found to be inactivated by mutation, hypermethylation of its promoter and/or deletion of the gene in a variety of different human cancers and therefore is thought to be a classical tumour suppressor gene. Due to this hierarchy of regulation of the various cyclin-dependent kinase inhibitors, cyclins and CDKs as well as their primary targets, i.e. the pocket proteins (Fig. 5), it was tempting to speculate that loss of pRB function

might also have an impact on the regulation of the genes grouped above pRB in the hierarchical order of the regulatory events controlling the G1/S-phase progression in the cell cycle. Indeed, to achieve a fine tuning of the regulatory events it was observed that the complex of pRB-E2F also inhibits the transcription of the *p16ink4a* gene itself [72]. Consequently, loss of pRB function on the level of the *pRB* gene product, either by mutation or protein–protein interaction with a viral oncogene product (e.g. E7), should result in the release of the *p16ink4a* gene from negative transcriptional feedback control in the respective cells (Fig. 5). Enhanced transcription and translation of *p16ink4a* should be the consequence. Due to the downstream block of pRB by the viral E7 oncogene product, enhanced expression of p16ink4a should remain functionally inert. Indeed, in line with this hypothesis, very high levels of p16ink4a were found in HPV-transformed cell lines as well as in cervical pre-cancer or cancerous lesions [35,36,73], although some other reports have suggested that in cervical lesions the *p16ink4a* gene might be down-regulated by hypermethylation or even mutation [74,75]. The latter observation, however, could not be confirmed by subsequent analyses [35,76–78]. Overexpression of p16ink4a has meanwhile been investigated in many primary cervical dysplastic lesions and cancers. These studies confirmed the high overexpression in advanced cervical dysplasia in virtually all cases, whereas no p16 expression was observed in normal squamous cell epithelium of the cervical mucosa. In early cervical intraepithelial neoplasia (CIN) 1 (low grade SIL) lesions, approximately 60% of the cases stained strongly positive for p16ink4a in the proliferating basal cells, although the remaining 40% did not stain in this compartment, although they could be shown to be infected by HR-HPV types and displayed features of HPV replication (i.e. koilocytosis). Although not yet formally shown, this observation fits well with the hypothesis that only a part of the early lesions display deregulated viral oncogene expression in the basal or parabasal cell compartment. In line with this hypothesis, the p16ink4a overexpressing lesions are likely to display the 'aberrant' pattern of HPV *E6–E7* gene expression, whereas the p16ink4-negative lesions presumably do not express the viral oncogenes in the replication competent basal cells and are therefore not at a comparably high risk for progression. Most interestingly, the strong expression of p16ink4a is closely associated with lesions infected by high-risk, but not low-risk HPV types (Fig. 6). This is consistent with the well documented inhibitory effects of

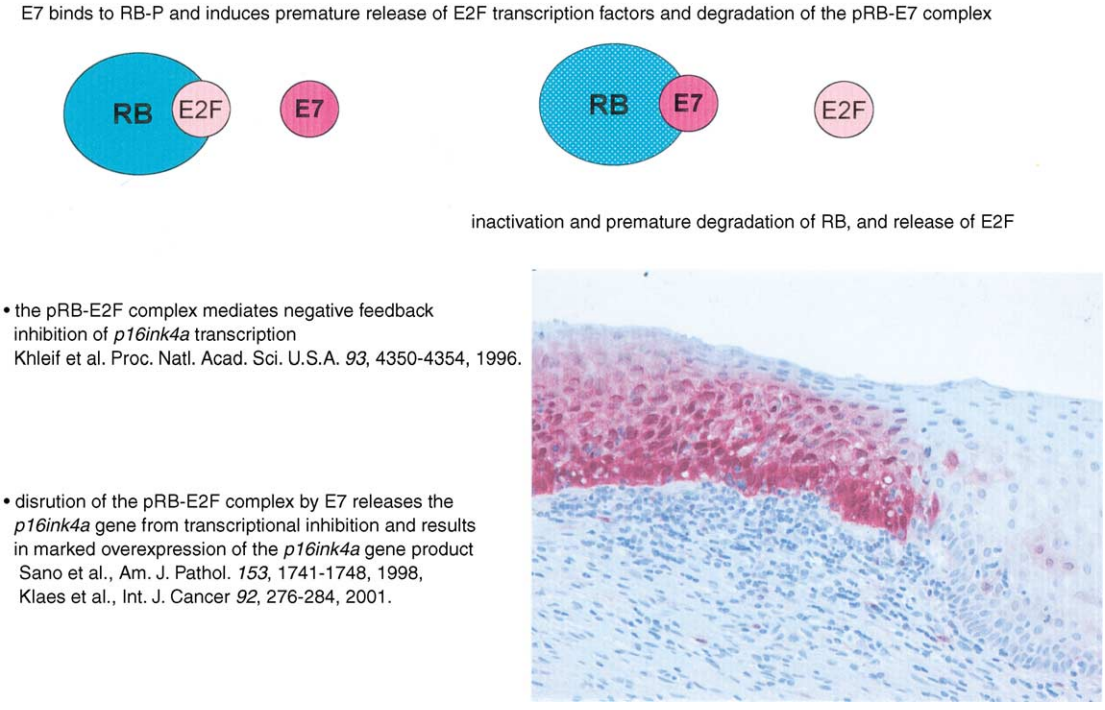

Fig. 5. Schematic representation of the mechanism leading to p16ink4a overexpression in HPV-transformed dysplastic epithelia. Binding of the E7 oncoprotein to pRB results in premature degradation of the pRB-E2F complex and increased release of free E2F. Reduced levels of active pRB result in the release of the *p16ink4a* gene from negative transcriptional feedback control and marked overexpression of the gene in epithelial cells which express the *E7* oncogene product. The high grade cervical lesions was stained with a monoclonal antibody E6H4 directed against p16ink4a epitopes (produced by MTM-Laboratories AG, Heidelberg, Germany).

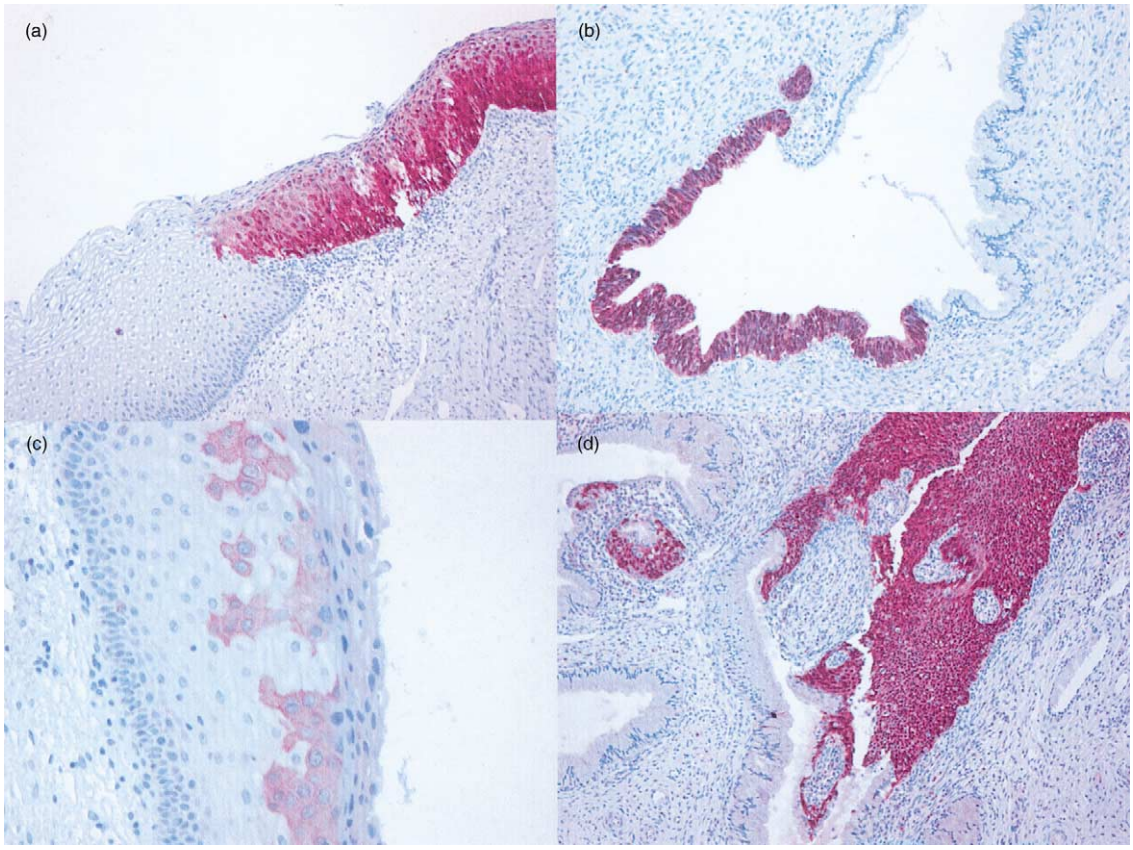

Fig. 6. Several biopsies stained with the monoclonal antibody E6H4 (MTM-Laboratories AG, Heidelberg, Germany). Virtually all high grade cervical dysplasia, either squamous cell (a) or glandular (b) lesions, as well as invasive cancers (d) stain intensely positive for p16ink4a. (c) A regressing lesion still retains some dysplastic, p16ink4-positive cells. From beneath, these cells were replaced by healthy, normal non-dysplastic cells. Note the marked atypia of the nuclei in the p16ink4a-stained cells.

high-risk, but not low-risk, HPV E7 oncoproteins on pRB [79]. These studies indicate, that the restricted expression pattern of p16ink4a in cervical dysplasia and derived cancers can be used to identify HPV-transformed cells. Therefore, p16ink4a fulfills the request for the specific biomarkers indicating the 'aberrant' expression of the viral oncogenes in replication-competent epithelial stem cells and marks those cells which have undergone activation of the mutagenic principle which initiates and promotes the outgrowth of cervical cancer [36]. First results suggest that staining for p16ink4a using specific new protocols and reagents also identifies dysplastic cells in cervical smears [80].

7. Integration of viral sequences into the cellular genome is a progression marker for cervical precancer

Disturbances of the spindle pole apparatus in proliferating cells generates a tremendous potential for conflicts on 'where to go' for individual chromosomes not tied simply to two poles, but to a complex of additional aberrant centromeres. This might be a strong promoter for chromosomal breakage and recombination, a well described hallmark of HPV-related carcinogenesis (reviewed in [81]). Interestingly, recombination of viral and cellular genes also seems to be promoted by this mode of E6- and E7-mediated genetic instability [82]. This is reflected by the fact, that in the vast majority of cervical cancer cells at least some HPV genome copies are integrated into the host cell genome. For many years it has been realised that integration of the viral genome promotes the expression of the *E6* and *E7* oncogenes, which are in most cervical cancer cells translated from multicistronic transcripts which consists of a viral part encompassing the *E6* and *E7* sequences and 3′ adjacent cellular transcripts which are spliced to the viral sequences. These co-transcribed cellular sequences apparently contribute to the stability of the viral transcripts and if tested in *in vitro* transformation assays contribute to their increased transformation potential compared with wild type *E6–E7* transcripts encoded by episomal viral genomes [83,84]. Moreover, integration of viral genomes favours deregulated

expression of the viral oncogenes since the transcriptional control elements of the virus can be overrun by *cis*-acting cellular transcription regulating elements at the site of integration [85]. Since the expression level of the viral oncogenes is directly linked to the number of replication rounds and mitotic activity of cells expressing these genes [13,86], recombination of cellular and viral genes appears to be an independent progression factor favouring further genomic damage in cells which experience integration and expression of the viral oncogenes derived from integrated viral genome copies. Consequently, detection of integrated viral genome copies is clearly associated with advanced cervical dysplasia and might serve as a useful clinical tool for the prognosis of cervical precancer or early cancer [87,88]. Due to the individual highly specific recombination of viral and cellular sequences in each HPV-transformed cell clone, characterisation of the joining region allows the establishment of a highly sensitive, but very specific, detection system to monitor early relapse after primary therapy of HR-HPV induced lesions. Integration of human papillomavirus genomes might further disrupt critical tumour suppressive genes and thus contribute to cervical carcinogenesis through 'insertional mutagenesis' [89]. A systematic survey of a large series of anogenital lesions, and particularly cervical cancer samples, showed that targeted knock-out of tumour-suppressive genes through the integration of viral genome copies contributes only in very rare cases (if at all) to the pathogenesis of cervical cancer [90]. These data suggest that the series of molecular accidents associated with cervical carcinogenesis all favour expression of the *E6* and *E7* oncogenes and, consequently, their strong mutagenic functions. High level E6 and E7 oncogene expression therefore seems to be the major 'darwinistic' selection force in the initiation and progression of cervical cancer.

8. Conclusions

Detailed analysis of the biochemical pathways contributing to the HPV-mediated transformation of epithelial cells has the potential to predict new biomarkers for the detection of dysplastic cells in clinical samples. In particular, marked overexpression of the cyclin dependent kinase inhibitor p16INK4a was regularly observed in HR-HPV induced cervical lesions with deregulated viral oncogene expression in replicating basal or parabasal cells. Implementing staining with p16INK4a-specific antibodies in histological and cytological studies will add significant diagnostic precision in the assessment of CIN lesions. In further advanced lesions, detection of integrated HR-HPV genomes points to considerable chromosomal instability and is a useful parameter to assess the risk of progression risk of cervical precancers.

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