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Modulation of Apoptosis by Human Papillomavirus (HPV) Oncoproteins

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Summary

The regulation of host-mediated apoptosis by the E6 and E7 oncoproteins has garnered attention because it is believed to be an important strategy employed by high-risk (HR)-human papillomaviruses (HPVs) to evade immune surveillance. Additionally, the revelation that E5 can protect cells from tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-mediated apoptosis suggests that it may also play a role in undermining host defense mechanisms. Cellular transformation is an unintended consequence of persistent infection by HR-HPVs, and it is therefore likely that the primary function of E5, E6 and E7 is to regulate cell survival throughout the normal viral life cycle in order to ensure viral replication and promote the spread of progeny. The purpose of this article is to review the literature on the regulation of host-mediated apoptosis by E5, E6 and E7 that describe the mechanisms employed by HR-HPVs to persist in the host and create the conditions necessary for cellular transformation.

Introduction

Human papillomaviruses (HPVs) are small, nonenveloped, double-stranded DNA viruses whose natural cellular hosts are keratinocytes. Infection with HPV occurs below the surface of the epithelium in the basal layer, and the life cycle of the virus is closely connected with the differentiation program of the cells it infects. The most common phenotypical manifestations of HPV infection are warts and papillomas of the skin, and various genital hyperplastic epithelial lesions. Over 100 HPV genotypes have been identified and approximately 33% of these genotypes are associated with lesions of the genital tract. HPVs that infect the genital tract can be subdivided into two categories: low-risk and high-risk. Low-risk (LR)-HPVs such as types 6 and 11 generally cause benign warts which rarely progress to cancer. On the other hand, high-risk (HR)-HPVs such as types 16, 18, 31 and 45 are associated with the development of high-grade lesions (cervical intraepithelial neoplasia (CIN) 2/3) that can progress to cancer.

All HPVs have a common genomic organization and encode 8 proteins: E1, E2, E4, E5, E6, and E7 (early) and L1 and L2 (late). E1 and E2 are replication factors and are also involved in transcription control; E4 and E5 are believed to regulate late viral functions although their role is not clearly understood; E6 and E7 are oncoproteins; and L1 and L2 are structural proteins [48]. The E6 and E7 oncoproteins of the high-risk strains are the main contributors to malignant transformation [26,56]. It is believed that a combination of persistent infection by HR strains along with the inability of the immune system to adequately clear the virus from infected cells are the main factors contributing to the integration of HPV genomes into the DNA of the host-

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a critical step in tumorigenesis. As a result of integration, the E2 open reading frame (ORF) is *frequently* disrupted, leading to a loss of E2 expression and its repressive action on E6 and E7, as well as a concomitant rise in the levels of these oncoproteins.

HR-HPVs, such as HPV 16 and HPV 18, play a pivotal role in the pathophysiology of cervical cancer [48,51] which accounts for one-fifth of all cancerrelated deaths among women, and is the second most common cancer worldwide [62,67]. Merck's recently approved vaccine for HPV 16 and 18, and one under development by GlaxoSmithKline hold great promise for the eradication of this deadly disease [11]. While a considerable amount of research has been done on the role of HR-HPVs in the etiology of cervical cancer, many of the actions of these viruses that promote persistence in the host and cellular transformation have not been fully elucidated. A full understanding of the events that occur during the time HR-HPVs establish a successful infection may reveal targets for novel therapeutic approaches.

Immune defense mechanisms against HPV infection

HPVs are persistent viruses that can remain in their hosts for long periods of time before causing any ill effects. Generally, the host reacts to viral pathogens by generating both humoral and cell-mediated responses. Humoral responses are typically antibody-mediated and involve the secretion of antibodies such as immunoglobulin A (IgA) and immunoglobulin G (IgG) by B lymphocytes. Cell-mediated responses, on the other hand, are carried out by immune effector cells such as dendritic cells (DCs), natural killer (NK) cells, macrophages and T lymphocytes which secrete a number of cytokines including interferons (INF) and tumor necrosis factor (TNF), and up-regulate the expression of Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) on their cell surface.

In the case of HPV infection, the immune response is frequently weak or undetectable, and accompanied by little or no inflammation. Even when an immune response is elicited, it may not be able to clear the virus. Although HR-HPVs are 'clever' at evading immune detection, studies suggest that the immune system can be successful at controlling HPV infection [7]. Indeed, a number of reports indicate that systemic Th1 responses against HPV proteins are associated with health [93,94]. These reports demonstrate that the immune system is successful at preventing many cases of HPV infection from progressing to malignancy and may explain instances of spontaneous regression of warts observed in some patients.

Langerhans cells (LC), a type of DC that monitors squamous mucosal surfaces, are an integral part of the immune system's arsenal. These cells are likely the first immune cells to encounter HPV in low-grade squamous intraepithelial lesions (CIN 1). Work by several groups suggests that function of LCs may be impaired because of their low density and stunted dendritic processes within CIN 1 [1,89]. However, using an organotypic culture model to approximate the microenvironment of CIN 1, Hurbert *et al.* demonstrated that LCs were fully capable of inducing apoptosis in HPV positive keratinocytes and that the interaction of LCs with HPV positive cells did not result in their demise [37]. Therefore, the ability of HPV to establish chronic infection is likely not the result of an inadequate host-immune response, but to the ability of the virus to evade host-immune surveillance mechanisms.

Host-mediated apoptosis

Apoptosis, a type of programmed cell death, plays an important role in organism development, cellular homeostasis and the pathophysiology of many diseases [70]. In the immune system, apoptosis is often triggered by cell surface receptors. Many of these receptors belong to the TNF receptor family including tumor necrosis factor receptor 1 (TNF R1), Fas, death receptor 4 (DR4), and death receptor 5 (DR5). These receptors bind to their cognate ligands, TNF, FasL, and TRAIL. The receptors and ligands that make up the TNF receptor and ligand families are

also referred to as death receptors and death ligands, receptively, because their interaction triggers cell death [30]. Typically, cell death receptor-mediated apoptosis is induced by the binding of the death ligands (usually in the form of a homotrimer) to their cognate receptors, which then recruit adaptor molecules and initiator caspases such as FADD and procaspase-8 to their death domains (DD) to form the death inducing signaling complex (DISC). Initiator caspases are then activated by proximity induced cleavage at the DISC and in turn cleave and activate executioner caspases such as procaspase-3. Once activated, caspase-3 cleaves target substrates such as poly(ADP-ribose) polymerase (PARP) and Lamin B, leading to the demise of the cell. In Fas- and TRAIL-mediated apoptosis, this is commonly called the type I model and its hallmark is strong caspase-8 activation which results in robust DISC formation. In contrast, DISC formation in type II cells is weak and amplification of the death signal via the mitochondrial pathway is necessary for apoptosis. In this context, caspase-8 cleaves Bid, triggering mitochondrial depolarization and the release of cytochrome C. Following its release, cytochrome C forms a complex with (apoptosis protease activating factor 1) Apaf-1 and pro-caspase 9 called the apoptosome. The apoptosome, which is analogous in function to the DISC, mediates the activation of caspase-9 which in turn activates caspase-3. The intracellular events of apoptosis give way to the external characteristics of this form of cell death, which include chromatin condensation, phosphatidlyserine exposure, cytoplasmic shrinkage and membrane blebbing.

Not surprisingly, many viruses, including HPV, have developed numerous strategies to block host-mediated apoptosis. The ability of HPV to persist in the host for long periods of time without being eliminated attests to the sophistication of its evasion mechanisms. A growing body of evidence suggests that the oncoproteins of HR-HPVs (i.e., E6 and E7), as well as E5, can inhibit death receptor signaling at key points in the pathway. In so doing, HPV is able to regulate the survival of infected cells in order to facilitate its replication cycle and thus ensure the production and spread of progeny.

Modulation of apoptosis by E5

The role of E5 in protecting HPV from elimination by the host has not received much attention because of its weak transforming properties and the fact that the E5 open reading frame (ORF) is often deleted after the HPV genome has integrated into the DNA of the host [13,53]. Furthermore, direct immunoblot analysis of E5 protein expression has been hindered by its low solubility and many studies have utilized tagged versions of the protein for easier analysis. HPV E5 is a hydrophobic protein and is expressed in cellular membrane structures such as the Golgi apparatus, endoplasmic reticulum and the nuclear membrane [12]. The exact function of E5 is not known but a number of its actions have been described. For example, HPV 16 E5 has been reported to interfere with the actin cytoskeleton and block endocytic trafficking [84]; modulate epidermal growth factor receptor signaling [14,16,65,77]; induce c-jun expression via a ras- and PKC-dependent pathway [10]; trigger the up-regulation of diacylglycerol (DAG) and inositol phosphates in fibroblast cells [15]; and down-regulate the expression of surface MHC class I molecules [3].

With regard to its role in tumorigenesis, E5 has been shown to induce fibroblasts to form colonies in soft agar [65,77], increase the efficiency of immortalization of keratinocytes by E6 and E7 [76], and impair cell-cell communication at gap junctions [59]. While these activities may not be necessary for maintaining the malignant status of infected cells, the findings of these studies and others suggest that E5 may play an important role in the initial phase of tumorigenesis. Indeed, prior to integration, when the HPV genome is episomal, the E5 mRNA is the most abundant viral transcript [74]. E5 has also been reported to inhibit the expression of the p21 tumor suppressor [88], which suggest that it may co-operate with E6 and E7 to transform keratinocytes. Interestingly, the E5 protein has been shown to protect HaCat

keratinocytes from both Fas- and TRAIL-mediated apoptosis, albeit by different mechanisms [40]. In their study, Kabsch and Alonso showed that E5 inhibits Fas-induced apoptosis, in part, by decreasing the cell surface expression of the Fas receptor. While E5 did not down-regulate TRAIL receptor expression, it was found to inhibit TRAIL signaling by interfering with the formation of the TRAIL DISC and thereby inhibiting the cleavage of procaspases-8 and -3, as well as of PARP [40]. Therefore, it is possible that E5 interferes with the ability of the immune system to eliminate infected cells by impairing death receptor signaling. Together, the results of these studies provide strong evidence that the E5 contributes to the evasion of immune surveillance during the early stages of HPV infection.

Modulation of apoptosis by E6

The E6 oncoprotein has been widely studied because of its potent tumorigenic properties. E6 is a relatively small protein (150 amino acids) and is produced in two forms: a full-length version of ~16 kDa and a smaller version of about half that size corresponding to the N-terminal half of the full-length protein [51]. One of the primary targets of E6 is the tumor suppressor p53 [71,95]. In the early stages of HR-HPV infection, E7 induces a significant increase in cell proliferation as a result of its interaction with retinoblastoma (RB), which triggers the expression of p53 [48]. Under normal physiological conditions, this rise in p53 levels would lead to cell cycle arrest and/or apoptosis, depending on the intensity of the damage or stimulus. However, in this situation, E6 binds to p53 with the aid of E6-associated protein ligase (E6AP) and prevents p53 from inducing growth arrest and apoptosis by targeting it for degradation through the ubiquitin-proteasome pathway [38]. E6 also precludes the growth-suppressive activities of p53 by cytoplasmic sequestration and by transcriptional suppression of its target genes [51].

A number of proteins other than p53 are targeted by E6. These include proteins involved in the regulation of transcription and DNA replication such as p300/CBP [63,98], IRF-3 [69], hMcm7 [43,44], E6TP1 [28] and ADA3 [28,97]; proteins involved in apoptosis and immune evasion such as Bak [79], c-Myc [34], TNF receptor 1 (TNF-R1) [23] and FADD [24]; proteins involved with epithelial organization and differentiation such as paxillin [87], E6BP/ERC-55 [9], zyxin [18], and fibulin-1 [20]; proteins involved in cell-cell adhesion, polarity, and proliferation control that contain a PDZ-binding motif such as hDLG [42,45], hScrib [57], MAGI-1 [31,81], MAGI-2, MAGI-3 [82], and MUPPI [46]; and proteins involved in DNA repair such as XRCCI and 6-*0*-methylguanine-DNA methyl transferase [73].

Two of these proteins, Bak and myc, were the first apoptosis-related targets of E6 to be identified. Thomas & Banks found that E6 inhibits Bak-mediated apoptosis by direct binding to Bak, an interaction that is conserved from HR-to LR-HPVs [79,80]. In laryngeal cells, E6 was found to inhibit TNF-mediated apoptosis by reducing the expression of Bak without significantly affecting the expression of caspase-3 and -8 [19]. Like p53, both Bak and myc are ubiquitinated by E6AP and degraded in the ubiquitin-proteasome pathway [34,79].

Over the past several years, our laboratory has discovered novel E6 binding partners that have helped to elucidate the role of E6 in immune evasion. We have shown that E6 can inhibit TNF-mediated apoptosis in mouse fibroblasts, human monocytes/histocytes and osteosarcoma cells by binding to the death domain of the TNF-R1 and preventing it from interacting with TRADD [23]. Paradoxically, stable transfection of E6 in human cells does not always result in protection from TNF-mediated apoptosis. We found that clones with a high level of E6 expression were more sensitive to apoptosis induced by TNF than clones with a low level of E6 expression [25]. This finding is consistent with literature reports which describe both the ‘sensitization’ [47,90] and ‘protection’ properties of E6 [21]. With regard to Fas-mediated apoptosis, we have shown that E6 binds to FADD and mediates its degradation via the ubiquitin

proteosome pathway [24]. Recently, we demonstrated that E6 can also protect human cells (HCT116) from TRAIL-induced apoptosis by accelerating the degradation of FADD and caspase-8 [29]. In these studies, the targeting of TNF-R1, FADD and caspase-8 by E6 resulted in the suppression of caspase activation and protection from apoptosis (Figure 1). However, our unpublished observations that E6 does not interact with TRADD or Fas suggests that the binding of E6 to TNF-R1, FADD and caspase-8 is specific.

Interestingly, we also found that E6 does not interfere with the mitochondrial apoptotic pathway in our cellular models. We arrived at this conclusion based on the results of experiments in which the mitochondrial pathway in E6-expressing HCT116 and U2OS cells was activated by mitomycin C or ceramide. While both agents are known to trigger the mitochondrial apoptotic pathway, their ability to induce cell death in HCT116 and U2OS cells was not hindered in the presence of E6 [24,25,29]. However, others have found that the induction of apoptosis via Bak- and Bax-dependent pathways is impaired in the presence of E6 [79]. This implies that in some cases E6 may inhibit the mitochondrial pathway because it is well-known that both Bak and Bax can trigger cytochrome c release [33]. In the Thomas and Banks study cited above, p53-null mouse 10(1) cells and human Saos-2 cells lacking both p53 and Rb were transfected with Bak alone or Bak plus HPV 18 E6. Survival assays revealed that Bak-mediated apoptosis was markedly inhibited upon co-transfection of Bak with E6 [79]. Recently, Vogt et al. reported that the suppression of Bax activity by E6 was necessary for its (E6's) antiapoptotic function [91]. The results of the study demonstrated that inhibition of E6 expression with RNAi in HPV-positive HeLa cells lead to the transactivation of the PUMA gene by p53, the activation of Bax, and its translocation to the mitochondria. These events were followed by the release of cytochrome c into the cytosol and the activation of caspase-3. The apparent discrepancy between our results and these two studies can be reconciled by noting that the ceramide- and mitomycin C signaling pathways may not require Bak or Bax for apoptosis induction via the mitochondria. Whether this is also true of death receptor-induced apoptosis may not be important because our results suggest that E6 inhibits death receptor-mediated apoptosis upstream of the mitochondrial apoptotic pathway.

The link between the association of E6 with certain cellular proteins and its oncogenic potential is not always obvious. A case in point is a group of proteins containing PDZ (postsynaptic density protein, discs large tumor suppressor, and the epithelial tight junction protein, Z0-1) domains. A number of these proteins are targeted by E6 and are necessary for both its transforming potential [92] and its ability to induce epithelial hyperplasia in mice [58]. A recent study by researchers at the University of Iowa provides convincing evidence that the PDZ-binding domain of E6 is important in mediating resistance to TNF-induced apoptosis [39]. According to the report, the expression of E6 in primary human airway epithelial cells (AECs) leads to an upregulation in the activity of p52-containing NF-κB complexes, as well as the transactivation of nuclear factor kappa B (NF-κB) responsive genes such as the inhibitor of apoptosis protein, cIAP-2. As a result of this cascade of events, AECs acquire partial resistance to TNF and experience protection from apoptosis. The authors also demonstrated that the protection provided by E6 could be abrogated by a mutant form of the protein that lacks the ability to bind the PDZ motif and to activate NF-κB. Intriguingly, this mutant protein retains the ability to bind and direct the degradation of p53. Taken together, the studies discussed above suggest that through the activities of E6, HR-HPVs can interfere with cellular apoptotic pathways by both p53-dependent and -independent mechanisms.

Modulation of apoptosis by E7

Like E6, the E7 oncoprotein of HR-HPVs is necessary for both cellular transformation and viral pathogenesis. E7's primary target is retinoblastoma (Rb) and its related proteins including p107 and p130 [5,22]. Under normal conditions, Rb forms a complex with histone deacetylase

(HDAC) and binds to the E2F transcription factor in the G1 phase of the cell cycle. This prevents E2F from transactivating genes that are necessary for proliferation until the cell enters the S phase. However, when E7 is expressed in cells, it binds to Rb and HDAC and relieves their repression of E2F, resulting in the constitutive activation of E2F-responsive genes. The actions of E7 cause the cell to reenter the S phase where cellular replication factors that are necessary of viral replication are activated. While much is known about the interaction between E7 and Rb family members and the biological consequences of this association, a number of unanswered questions remain regarding the interaction of E7 and HDAC. One of these questions is why a mutation in the HDAC binding domain of the E7 oncoprotein of HPV 31 results in defective maintenance of viral episomes [49]. Several possibilities have been proposed [48]. One is that the binding of HDAC to E7 prevents it from deacetylating the E2F transcription factors, which causes these proteins to relocate outside the nucleus. Another more attractive possibility is that the interaction of these proteins uncovers an important, but as yet unrecognized, role of E7.

E7 binds to other proteins besides Rb and HDAC. They include proteins involved in cell cycle control such as cyclin-dependent kinase cdk2 and cycline A [86]; proteins regulating transcription such as the TATA box-binding protein and the AP1 transcription factors [2,52, 64]; and proteins with other cellular functions such as TAF-110 and TBP [55], the S4 ATPase of the 26S proteosome [4], Mi2 [8], Interferon Regulatory Factor-1 [61], IGFBP-3 [50], M2-PK [54], Skip [68], and PP2A [66].

Besides its role in cell proliferation and viral replication, E7 also regulates apoptosis. However, its effect on cellular apoptotic pathways is pleiomorphic. For example, in some studies the actions of E7 appear to be anti-apoptotic. A case in point is a recent study by Yuan *et al.* which suggests that E7 can inhibit TNF-mediated apoptosis in keratinocytes by up-regulating the expression of the inhibitor of apoptosis protein, c-IAP2 [96]. In another study, it was reported that the expression of E7 in fibroblasts delayed Fas-mediated apoptosis and prevented TNF-mediated apoptosis by a mechanism involving the suppression of caspase-8 activation [83]. However, the majority of studies suggest that E7 serves in pro-apoptotic role. For instance, E7 has been shown to sensitize mouse lymphoma cells (JD3) to IFN-alpha-induced apoptosis [85]; co-expression of E7 and p21 induced apoptosis in U2OS osteosarcoma cells [41]; and overexpression of E7 in genital keratinocytes induced spontaneous cell death and sensitized the cells to TNF-mediated apoptosis [75]. The pleiotrophic effect of both E6 and E7 on apoptosis is indicative of their important role in immune evasion and underscores the complexity of HPV-host interactions.

Combined effects of E2, E6 and E7 on cell survival

Studies in which the activities of E6 and E7 have been examined independently (ie, by overexpression) have uncovered many of the molecular mechanisms employed by these proteins to modulate cell survival. However, in an attempt to mirror physiological conditions, other studies have examined the effect of E6/E7 on cell survival within the context of the whole HPV genome. One such study took advantage of the fact that E6 and E7 are expressed bicistronically [78] and investigated the consequences of siRNA-mediated transcriptional repression of the genes encoding these proteins. The authors found that inhibition of E6 and E7 with siRNA resulted in a significant reduction in DNA replication as measured by [^{3}H] thymidine incorporation in HeLa cells [35]. Furthermore, they found that the reduction in cell proliferation was not due to apoptosis, but to senescence [35]. Thus, within the context of the whole HPV genome post-transcriptional silencing of E6 and E7 alone is sufficient to inhibit the growth of cervical carcinoma cells. It is noteworthy that similar observations have been achieved by exogenous expression of E2 in cervical carcinoma cells [32]. Not only does the overexpression of E2 result in senescence, but studies by the DiMaio, Gaston and Theirry

laboratories have demonstrated that E2 can induce apoptotic cell death [6,17,60]. This finding and the fact that E2 can regulate the activities of E6 and E7 via transcriptional control or by direct interaction [27], suggests that HPV genome integration may result from a strong selective pressure on the virus to avoid E2-induced apoptosis while modulating the survival of infected cells through the activities of E6 and E7.

Conclusions

HPV, like many of its viral counterparts has developed sophisticated mechanisms to evade host defenses and establish a successful infection. The primary weapons at its disposal are the E5, E6 and E7 oncoproteins. Although the role of these proteins, particularly E6 and E7, in cellular transformation (an unintended consequence of the HPV life cycle) has been well-studied, less attention has been given to their role in HPV persistence in the host during the normal viral life cycle, and to their ability to circumvent host-mediated apoptosis. Recent reports suggest that an important effect of E5 might be to create conditions conducive to the integration of HPV DNA into the host genome, in part by fending off apoptotic stimuli from immune effector cells. As noted, E5 achieves this by modulating signal transduction triggered by apoptotic stimuli from these cells [40] and also by interfering with MHC class I protein expression [3]. Furthermore, E5 has transforming potential and can co-operate with E6 and E7 to immortalize cells [76]. Following intergration, the E5 ORF is usually deleted and the transcriptional repression of E6 and E7 by E2 is relieved, allowing these oncogenes and their protein products to take center stage.

The interaction of E6 and E7 with their primary cellular targets, p53 and Rb, respectively, plays an important role in the initiation of tumorigenesis, and these oncoproteins are also required for retaining the transformed status of infected cells [48].

Intriguingly, E6 and E7 can inhibit apoptosis induced by stimuli such as TNF as well as sensitize cells to TNF and other apoptotic stimuli [25,83,96]. The significance of these observations is still being investigated, but clues to their possible role in the viral life cycle are beginning to unfold. The fact that HPV encodes proteins that perform both functions (protection/sensitization) suggests that (1) during the viral life cycle both functions may be required, and/or that (2) E6 can compensate for E7 or vice versa if the expression of one or the other protein is lost (i.e., through deletion) or silenced (e.g., by antisense or siRNA molecules produced by the host) during the viral life cycle. Indeed, while the expression of both E6 and E7 are necessary to efficiently immortalize cells, they can also induce cell transformation on their own [36,72]. This finding as well as the ability of both proteins to modulate cell survival suggests that there is some redundancy in their functions. If true, this would be highly advantageous to the virus as it tries to persist in the host and avoid elimination. Together, the studies presented here strongly suggest that targeting E5, E6 and E7 is a prudent strategy for therapies aimed at preventing the development of premalignant intraepithelial lesions and their progression to cancer.

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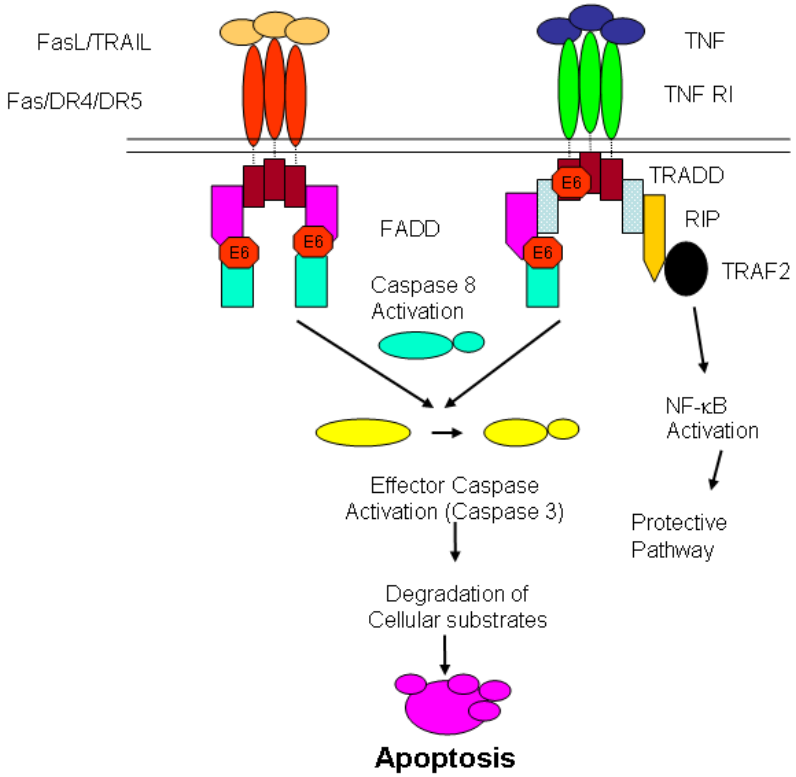

Figure 1.

HPV 16 E6 exploits similarities in the apoptotic signaling pathways of TNF, FasL and TRAIL. E6 inhibits TNF- and Fas-mediated apoptosis by binding to TNF R1 and to FADD. E6 protects cells from TRAIL-mediated apoptosis by accelerating the degradation of FADD and caspase-8.