·Review·

Cell survival and death program modulated by LMP1: implication in antitumor immunity

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[Abstract] The genome of Epstein-Barr virus (EBV) encodes proteins essential for malignant transformation, for example, latent membrane protein 1(LMP1). Whereas, LMP1 up-regulates anti-apoptotic proteins to support viral replication, it also potentiates apoptosis, suggesting that a viral protein contributes to the survival of the virus, and it also elicit host defense leading to the destruction of the infected cells. The antitumor immunity is exerted by infiltrated $CD8^+$ T cells elaborating cytotoxic effectors, like Fas ligand (FasL, CD95L or CD178). As a nuclear factor-κB (NF-κB)-dependent molecule, Fas is induced by LMP1, and LMP1 enhances Fas-mediated apoptosis, according to our finding of stimulus-dependent apoptosis regulation by LMP1. Data has shown that FasL-mediated cytotoxicity has significant therapeutic effect on EBV-associated nasopharyngeal carcinoma (NPC). Recent reports suggest that mutations affecting the Fas-mediated apoptotic pathway reduce individuals' susceptibility to cancers, but cytokine-targeting therapy which precisely regulates the Fas level on tumor cells could still contribute to enhancement of antitumor immunity in cancer patients.

Key words: Epstein-Barr virus, latent membrane protein 1, Fas/Apo-1/CD95, FasL/CD178, antitumor immunity, cytotoxic T lymphocyte, NF-κB

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1 The role of proliferation-promoting potential of Epstein-Barr virus (EBV) in malignant transformation

Epstein-Barr virus (EBV) is a lymphotropic human gammaherpesvirus which infects more than 90% individuals in the human population. Its efficiency to convert B lymphocytes into lymphoblastoid cell lines is attributed to its genomic products which play major role in the malignant transformation. During latency III infection which exists in immunocompromised host *in vivo* and lymphoblastoid cell lines *in vitro*, characterized by a wide spectrum EBV antigen expression, EBV nuclear antigen 2 (EBNA2) and several other nuclear proteins associate with the cellular protein RBP-Jκ/CBF1 and regulate the transcription of downstream promoters of Notch receptor signaling and a membrane integral protein, latent membrane protein 1 (LMP1), expressed during latent Ⅱ and Ⅲ infection, contributes to the malignant phenotype through association with tumor necrosis factor receptor (TNFR) co-factors, and engages in the signaling pathways of necrosis factor-κB (NF-κB), interferon regulatory factor 7 (IRF7), and JNK. Generally, LMP1 is considered as a viral oncogene.

LMP1 associates with tumor necrosis factor receptor-associated factors (TRAFs), tumor necrosis factor receptor-associated death domain protein (TRADD), and receptor-interacting protein (RIP), to activate NF-κB and stress-activated

kinase pathways.[1] It has recently been reported that CTAR2, the membrane distal transformation effector domain of LMP1, but not CTAR1, the proximal domain, triggers JNK activation. Activation of the JNK pathway by LMP1 does not involve TRADD, suggesting that the CTAR2-triggered NF-κB and JNK pathways bifurcate upstream of TRADD.[2]

As a key mediator of type I interferon (IFN) (IFN-α/β) responses, IRF7 plays an important role in immune defenses. LMP1 recruits RIP to fully activate and stimulate RIP-dependent K63-linked ubiquitination of IRF7. It was suggested that RIP may serve as a general activator of IRF7, responding to and transmitting the signals from various stimuli, and that ubiquitination may be a general mechanism for enhancing the activity of IRF7.[3]

NF-κB belongs to the Rel family of transcription factors, which contains five mammalian proteins: RelA (P65), c-Rel, RelB, NF-κB1 (P50/P105) and NF-κB2 (P52/P100).[4] RelA, c-Rel and RelB are synthesized as mature proteins while P50 and P52 are first expressed as precursors (P105 and P100), which are processed by proteasome. To date, two principal pathways for NF-κB activation have been characterized: a canonical and an alternative pathways.[5] The canonical pathway involves the P65 (RelA)/P50 dimer. Activation of this pathway in response to a variety of stimuli [TNF-α, interleukin-1(IL-1), CD95L] relies on the I-κB kinase (IKK) signalosome, which consists of two catalytic subunits (IKK1 or IKK-α and IKK2 or IKK-β) and a regulatory subunit (IKK-γ or NEMO). The IKK complex phosphorylates the inhibitor proteins of NF-κB (I-κBs) causing their subsequent ubiquitination and degradation by the proteasome, resulting in NF-κB translocation to the nucleus and transcriptional activation of specific target genes. An alternative pathway exists which involves the phosphorylation of P100 by the IKK complex and then the processing and nuclear translocation of RelB/P52 dimers. During quiescent phase, subunits with transcription factor activity form a complex with the inhibitor I-κB. The ligation of the surface molecules of TNFRs and some cytokine receptors activates kinase cascades and phosphorylates I-κB. Upon phosphorylation, I-κB is ubiquitinated and degraded by proteasome. NF-κB is activated by canonical and non-canonical pathways as classified by the activated subunits involved.

LMP1 activates NF-κB independent of any extracellular ligands, and it presents as the conformation of an activated receptor on cell surface. While a number of proliferation-promoting factors have been identified as NF-κB and/or LMP1 inducible in several studies, some pro-apoptotic factors were noted as well.[6,7] Among these, a death receptor, Fas/ApoI/CD95, was identified by high throughput screening.[8] We have reported the stimulus-dependent apoptotic regulation of LMP1. LMP1 expression in HeLa cells protects against apoptosis induced by TNF-α but not apoptosis induced by Fas or etoposide.[9] LMP1 is generally known as an apoptotic regulator, but some reports suggest that it also acts as an inducer of apoptosis,[10] and apoptosis induced by LMP1 requires the Fas receptor.[11]

The opposing effects of LMP1 on host apoptotic regulation may reflect that a viral protein uses such strategy as apoptosis inhibition to support viral replication, but meanwhile, the genetic products of viral genome are immunogenic and can elicit immune response. The second arm of reactions is intriguing because it may be of assistance in design of antitumor therapy against virus-associated human tumors.

2 Inhibition of apoptosis as a viral strategy to support EBV parasitism

Apoptosis is a host mechanism to eliminate unwanted cell clones, including those infected by viruses. Herpesviruses, most of which establish a lifelong persistence in the infected host, have developed different strategies to interfere with cell-autonomous apoptosis and to block cytotoxic T lymphocyte (CTL)-induced apoptosis mediated by death receptors such as Fas and TRAIL.[12] Herpesviruses can be found principally in two different conditions: episomal persistence with a limited number of genes expressed and lytic replication with expression of almost all genes. To meet the need of the virus to enhance survival of infected cells, herpesviruses have evolved different strategies that function during both episomal persistence and lytic replication. They encode 70 to more than 200 genes, including cell homologous anti-apoptotic genes. The mammalian bcl-2, for example, is synthesized by two tumorigenic human herpesviruses, EBV and human herpesvirus type 8 (HHV8); they code for multifunctional genes that can also regulate apoptosis, and, finally, they modulate the expression of cellular apoptosis-regulating genes to favor survival of infected cells.

NF-κB activation mainly contributes to pro-surviving, anti-apoptotic profile of gene expression; cIAP and cFLIP are NF-κB-dependent factors exerting antagonism to programmed cell death. LMP1 confers a survival advantage on EBV-infected B cells by activation of NF-κB-up-regulated anti-apoptotic genes, such as those encoding A20, Bfl-1, and Bcl-2.[13,14] For the latency Ⅲ in B lymphocytes, the event is important in death and surviving programme. LMP1, expressed during the wide gene expression spectrum of latencies Ⅱ and

Ⅲ, confers surviving and growth advantage in a cell type-dependent manner. In B cells, the earliest known host range of EBV infection, LMP1 transcriptionally activates anti-apoptotic genes, like mitochondrial targeting Bcl-2, and zinc finger protein A20, potentially inhibits apoptosis. Epithelial cells are another host range of EBV infection, and the infection plays an important role in the malignant clonal expansion during the early steps of pathogenesis of nasopharyngeal carcinoma (NPC) and gastric cancer. While LMP1 was been shown to induce Fas in B cells, we have shown that LMP1 modulates apoptosis in epithelial cells in a stimulus-dependent manner; LMP1 inhibits TNF-induced apoptosis, but potentiates Fas/ApoI/CD95 and chemotherapeutic agent-induced apoptosis.[9,15]

3 Antitumor immunity-against tumor antigens including those synthesized by tumorigenic viruses

As discussed above, EBV, like most human herpesviruses, establishes lifetime infection in the host. The majority of healthy individuals will not suffer from life-threatening disorders induced by EBV, because EBV-specific T lymphocytes control and inhibit the growth of EBV-infected or EBV-transformed cells throughout life. Because MHC class I-restricted CTLs are considered as the main effectors for providing protection against virus-associated malignancies, most researchers have focused on characterizing CTL responses to EBV-derived latent and lytic cycle antigens. CTL-based immunotherapy has been recognized as an attractive way to treat EBV-associated malignancies by eradicating EBV-transformed cells. Most results have been obtained using adoptive T-cell immunotherapy with EBV-specific CTLs against Hodgkin's lymphoma (HL)[16] and post-transplantation lymphoproliferative disorder (PTLD).[17] In most instances, the EBV-reactive CTLs raised for adoptive immunotherapy are directly against some immunodominant EBNA antigens, which are not expressed in HL, NPC, and natural killer (NK)/T-cell lymphomas. Thus, to extend adoptive immunotherapy (or active immunization) against HL, NPC, and NK/T-cell lymphomas, it would be necessary to generate T-cell responses against other antigens, such as LMP1. A naturally processed epitope derived from LMP1, peptide LMP1159-175 was found effective in inducing helper T lymphocytes (HTL) responses that were restricted by HLA-DR9, HLA-DR53, or HLA-DR15, indicating that this peptide behaves as a promiscuous T-cell epitope. Moreover, LMP1159-175-reactive HTL clones directly recognize EBV lymphoblastoid B cells and EBV-infected NK/T-lymphoma cells, and naturally process antigens in the form of LMP1-positive tumor cell lysates presented by autologous dendritic cells. Because the newly identified epitope LMP1159-175 overlaps with an HLA-A2-restricted CTL epitope (LMP1159-167), this peptide might have the ability to induce simultaneous CTL and HTL responses against LMP1. The data should be relevant for the design and optimization of T-cell epitope-based immunotherapy against various EBV-associated malignancies, including NK/T-cell lymphomas.[18]

4 Antitumor immunity exerted by cytotoxic effectors elaborated by T-cell lymphoma and NK cells

Tumor-associated antigens (TAA) re-expressed or overexpressed by malignant cells, or viral antigens expressed by infected cells elicit T lymphocyte and NK cell response characterized by the release of cell death-inducing serine proteases, granzymes from cytotoxic granules during granule exocytosis of the killer cells. The entry of granzymes to target cells is mediated by a cofactor, perforin.[19] Besides a well characterized mechanism, that is, extracellular Ca^{2+}-dependent degranulation of cytotoxic molecules perforin and granzymes, the antitumor CTLs which are differentiated CD8+ T cells also express Fas ligand (FasL or CD178), to induce apoptosis of Fas-expressing target cells. Current models suggest that FasL is stored in cytolytic granules and that cell surface expression of FasL would be subjected to the same controls as degranulation. Signaling for FasL expression appears to be finely tuned as a weak signal preferentially induces surface translocation of the stored FasL, whereas a strong signal preferentially triggers the expression of de novo synthesized FasL. The early FasL is differentially regulated from degranulation, as there are multiple circumstances whereby rapid FasL cell surface expression and FasL-dependent killing occurrs in the absence of detectable degranulation. Furthermore, stored FasL resides in vesicles distinct from cytolytic granules. The findings clearly show that CTL degranulation and FasL lytic mechanisms are fully independent with respect to stored component localization and regulation.[20] It is believed that the apoptosis mediated by granzyme B/perforin and by CTL-triggered Fas/FasL is signaled in nearly identical pathway, notably the activation of caspases. But it has been reported that granzyme B directly cleaves the pro-apoptotic molecule Bid, bypassing the need for caspase-8 activation of Bid. These results provide evidence for a two-pronged strategy for mediating target cell destruction and provide evidence of a direct link between granzyme B activity, Bid cleavage, and caspase-3 activation.[21]

5 Fas-mediated apoptosis and counterattack of FasL to antitumor T lymphocytes

Fas/APO-1 or CD95 was identified independently by the laboratories of Shin Yonehara in Tokyo, Japan[22] and of Peter Krammer in Heidelberg, Germany.[23] It is mainly expressed on activated or malignant T lymphocytes. Fas contains an intracellular domain of 80 amino acids called the death domain (DD). Upon stimulation by FasL, Fas becomes trimerized, and DD recruits the adapter molecule FADD (Fas-associated death domain, also called Mort-1), caspase-8 and -10 and a caspase-8-like molecule that lacks proteolytic activity termed c-FLIP (cellular FADD-like IL-1β-converting enzyme inhibitory protein) to form the death-inducing signaling complex (DISC).[24] DISC acts as a molecular scaffold to favor a high local concentration of initiator caspases and their activation by autoproteolysis. The pro-caspase-8 recruited in DISC is activated after removing prodomain through self hydrolysis. Activated caspase-8 either cleaved Bid molecule to permeabilize mitochondrion to activate downstream caspases or directly activates caspase-3, to execute apoptosis.

Fas/FasL system plays an important role in the development and maturation of immune system, and also in antitumor immunity via elimination of tumor cells expressing Fas molecule. Decreased Fas function but no Fas gene mutation was identified in patients with ALPS-like clinical pattern (named autoimmune lymphoproliferative disease, ALD).[25] They also displayed decreased PCD response to ceramide, triggering a death pathway partially overlapping that used by Fas, which suggests that ALD is caused by downstream alterations of the Fas signaling pathway. Decreased Fas function is also involved in tumor development because somatic mutations hitting the Fas system may protect neoplastic cells from immune surveillance.

A study using memory CD8 + T cells from wild type FasL mutant mice showed that memory CD8 + T cells in FasL mutant mice expressed reduced levels of IFN-γ and displayed poor homeostatic and Ag-induced proliferation. 26 Impairment in CD8 + T cell memory in FasL mutant hosts was not due to defective programming or the expression of mutant FasL on CD8 + T cells, but was caused by perturbed cytokine environment in FasL mutant mice. Although adoptively transferred wild type memory CD8 + T cells mediated protection against *L. monocytogenes* in either the wild type or FasL mutant hosts, FasL mutant memory CD8 + T cells failed to mediate protection even in wild type hosts. Thus, in individuals with mutation in Fas pathway, impairment in the function of the memory CD8 + T cells may increase their susceptibility to recurrence or latent infections.[26] NPC is well known of its tight association with EBV infection; all cases of undifferentiated NPC (endemic in southern China) is positive for the EBV-determined nuclear antigen 1 (EBNA1), and a considerable amount of the individuals are also positive for LMP1. It has been reported that an adenoviral FasL recombinant induced more significant cytotoxicity in EBV-positive, NPC-derived C666-1 cell line, than EBV-negative CNE-2Z cells, and C666-1 cells transfected with the adenoviral FasL vector were unable to form tumor when inoculated to severe combined immune deficient (SCID) mice.[27]

Many tumors express FasL and may thus kill tumor-infiltrating lymphocytes, a phenomenon called tumor counterattack. The effect of FasL expression of tumor cells on tumor-specific T cells was studied using an in vitro system with TCR tg T cells and a model tumor antigen.[28] Pre-activated antitumor T cells were able to kill FasL(−) and FasL (+) tumor cells. FasL(+) tumor cells killed activated T cells in vitro and inhibited the expansion of cytotoxic antitumor T cells in mixed lymphocyte tumor reactions. In NPC, Fas/FasL-induced apoptosis in tumor-infiltrating CTL is among immunoescape mechanisms exploited by tumor cells.[29] A study in mice suggests that, however, in vivo FasL expression led to delayed tumor growth or complete tumor rejection.[28] Incidences and growth rates of FasL (+) tumors in mice injected with a neutrophil-depleting or an isotype control antibody were the same. In Fas-deficient *lpr* mice, tumor growth was not altered as compared with that in wild-type mice. Taken together, FasL mediates tumor counterattack *in vitro*, but leads to neutrophil-independent tumor rejection *in vivo*.

6 Mutations in Fas-mediated apoptosis as related to host susceptibility to tumors

Since many different tumor cells express Fas on their membrane, and Fas contributes to mount an antitumor immunity by killing tumor cells, targeting Fas-mediated apoptosis by anti-Fas antibodies may be a promising antitumor therapy. Unfortunately, not all Fas-expressing cells are sensitive to Fas-mediated apoptosis. This has resulted in the discovery of many different inhibition mechanisms of Fas-mediated apoptosis. In addition, mutations in Fas or p53 gene can also influence the sensitivity for Fas-mediated apoptosis.[30]

Several recent reports described that, however, as related to the counterattack to T lymphocytes, Fas ligation hampers host immunity against tumorigenesis by elimination of tumor-infiltrating T cells via triggering Fas-mediated apoptosis. Corruption of Fas-mediated signaling pathway has been shown to participate in immune escape

and tumorigenesis. Reduced expression of Fas but elevated expression of FasL in many types of human cancers including lung cancer, and an association between functional polymorphisms in Fas (−1377G→A) and FasL (−844T→C) and risk of esophageal cancer have been noted.[31] A report on single nucleotide polymorphism (SNP) of caspase-8 promoter suggests that a 6-nucleotide mutant decreasing caspase-8, the initiator caspase recruited in death receptor-mediated apoptosis, reduced the host susceptibility to different types of cancers.[32] The study on the association between functional variants of Fas (−1377G→A and −670A→G), FasL (−844T→C), and caspase-8 (CASP8) 6-nucleotide deletion polymorphism (−652 6N ins →del) and risk of pancreatic cancer suggests that genetic variations in the death pathway genes FasL and CASP8 are involved in susceptibility to developing pancreatic cancer.[33]

7 Cytokine regulation of the level of LMP1 molecules

LMP1, a transforming protein, is expressed during both latencies Ⅱ and Ⅲ infection of EBV, using different transcriptional regulatory mechanisms. Transcription of LMP1 gene can be initiated from two promoters in the viral genome. pLMP1 (also named ED-L1) is the proximal promoter, while TR-L1 is the distal one located in the terminal repeats.[34] Both viral and cellular factors regulate the activities of the LMP1 promoters. LMP1 mRNA is transcribed by the transactivation of nuclear antigen EBNA2 during latency Ⅲ infection, with the involvement of a set of transcription factors. But during latency Ⅱ infection which is adopted by EBV during the occurrence of NPC and HL, the transcription of LMP1 gene is initiated upon binding of another set of transcription factors to the LMP1 promoters in an EBNA2-independent manner. EBNA2-dependent transactivation involves only pLMP1 and needs cellular transcription factors, alternatively, pLMP1 can be activated in an EBNA2-independent manner by several cellular proteins, including IRF7,[35] STAT3,[34] Notch1,[36] and Sp1.[37] pLMP1 activity can also be inhibited by a Max-Mad1 and histone deacetylase (HDAC) complex.[38] By contrast with pLMP1, TR-L1 activation does not require EBNA2 but could be due to action of cellular transcription factors Sp1/Sp3[39] or STAT3.[34] Under the influence of cytokines, some type I cells may transiently acquire the type II pattern. Kis et al.[40,41] reported that the expression of LMP1 is regulated by several extracellular signals, notably cytokines, for example, IL-4 and IL-10. Occasional cells that did not differentiate correctly in the germinal center are doomed to elimination. EBV-carrying cells could share this fate, although some of them may survival, as indicated by the in vitro experiments.[42,43] Further cellular changes and cytokine-induced LMP1 expression may enhance interaction with the surrounding normal cellular component of the immune system and the development of HL granuloma tissue.

There are many connections between EBV and IL-10. EBV-infected B cells are known to produce homolog of human IL-10, and LMP1 was identified as the gene responsible for its induction of IL-10. Taken together with the finding that IL-10 can induce the expression of LMP1, this suggests the existence of an autocrine regulatory loop between IL-10 and LMP1. A similar regulatory loop was defined in epithelial cells for LMP1 and IL-6. It has been noted that the induced LMP1 expression mimics the latency II LMP1 production triggered by the cytokines that elaborated by locally infiltrating T lymphocytes at the lesion of NPC and HL tumors.

8 Perspectives

Because several different cytotoxic drugs are able to induce Fas membrane expression, combination therapy of antitumor drugs with anti-Fas antibodies or FasL is conceivable as an antitumor strategy. The efficiency of the induction of Fas-mediated apoptosis by anti-Fas antibodies, FasL-expressing cells or recombinant FasL (rFasL) in tumors has been demonstrated in vivo in solid tumors implanted in mice.[30]

The knowledge on regulated LMP1 expression is very helpful in design of biotherapy against EBV-associated human tumors. The engineered cytokines not only exert an proliferation-promoting effects on the antitumor T lymphocytes and NK cells, they may also up-regulate some cytotoxic molecules via acting on stimuli such as LMP1 and CD40, leading to the elimination of the malignant cells on which Fas and/or other death receptors are expressed. Based on the discussion above on induction of death receptor Fas by LMP1, implanting CLT isolated from the tumor patients and stimulated with LMP1 *in vitro* to the same patients could also be a feasible therapeutic approach.

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