MEDICAL REVIEW

The Molecular Mechanisms of Classic Hodgkin's Lymphoma

Rachael S. Felberbaum

Yale University, Department of Molecular, Cellular, and Developmental Biology, New Haven, Connecticut

Classic Hodgkin's lymphoma is characterized by the appearance of giant abnormal cells called Hodgkin and Reed-Sternberg (HRS) cells. HRS cells arise from germinal center B lymphocytes and in about 50 percent of patients are infected with Epstein-Barr virus. In addition, HRS cells show constitutive NF-κB activation and are resistant to apoptosis. This paper reviews several recent studies that for the first time implicate specific molecules in the pathogenesis of classic Hodgkin's lymphoma. Targeting these molecules could lead to the development of novel therapies for this disease.

INTRODUCTION

Despite its initial characterization more than three centuries ago, little is known about the molecular mechanisms that give rise to classic Hodgkin's lymphoma (cHL)[†] [1]. cHL is characterized by the appearance of giant cells known as Hodgkin and Reed-Sternberg (HRS) cells, which serve as an important diagnostic marker of the disease [1].[§] Although HRS cells had long been suspected to be the pathogenic cells of cHL, only within the last few years has this been proven to be the case.

In 1999, Cossman et al. demonstrated that HRS cells are derived from germinal center B lymphocytes [1]. Germinal centers are specialized structures in secondary lymphoid tissue that arise from the clonal expansion of antigen-specific B cells during an immune response. During germinal center expansion, B cell immunoglobulin genes hypermutate in order to increase antibody affinity. Normally, the B cells

---

To whom all correspondence should be addressed: Rachael S. Felberbaum, Yale University, Department of Molecular, Cellular, and Developmental Biology, Bass 217, 266 Whitney Avenue, New Haven, CT 06520. Tel.: 203-432-6507; Fax: 203-432-5175; E-mail: rachael.felberbaum@yale.edu.
[†]Abbreviations: ABVD, Adriamycin, Bleomycin, Vinblastine, Dacarbazine; Apaf-1, apoptosis protease-activating factor-1; BCR, B cell receptor; cHL, classic Hodgkin's lymphoma; DISC, death-inducing signaling complex; EBV, Epstein-Barr virus; FADD, Fas-associated death domain; FLIP, FADD-like IL-1β-converting enzyme (FLICE)-like inhibitory protein; HRS, Hodgkin and Reed-Sternberg; IκB, inhibitor of kappa B; LMP2A, latent membrane protein 2A; MS, microsatellite sequence; NF-κB, nuclear factor kappa B; siRNA, short interfering RNA oligonucleotide; Smac, second mitochondria-derived activator of caspases; TNF, tumor necrosis factor; XIAP, X-linked inhibitor of apoptosis.
§This is in contrast to nodular lymphocyte-predominant Hodgkin's lymphoma, which is characterized by lymphocytic and histiocytic cells.

with mutated immunoglobulin genes that give rise to low-affinity, unfavorable B cell receptors (BCRs) are eliminated, while those that give rise to high-affinity, favorable BCRs survive. Somehow, HRS cells acquire an ability to survive despite having deleterious mutations.

In addition to evading cell death, HRS cells exhibit other characteristic features. Similar to many cancers, HRS cells show constitutive activation of the transcription factor nuclear factor kappa B (NF-κB), which promotes cell growth. Moreover, a substantial proportion of HRS cells carry Epstein-Barr virus (EBV), a virus that latently infects more than 95 percent of all adults [2]. EBV infects and activates naive B cells, causing them to proliferate. Normally, this proliferation is short-lived and drives the B cells into the B-cell memory pool, where they are harmlessly maintained. Given that EBV genes can promote cell division, one hypothesis is that EBV deregulation might cause aberrant B-cell proliferation and lead to cHL [2]. However, in light of the large number of people that carry EBV but never develop cHL, the significance of this infection remains uncertain. In the past few years, a significant body of research has been published that sheds light on the more detailed molecular mechanisms that might contribute to cHL. This review highlights some of the current advances in the field and discusses the therapeutic implications of this progress as it relates to the development of new therapies.

EPSTEIN-BARR VIRUS INFECTION

Nearly half of Hodgkin's patients have EBV DNA in their tumor cells [3]. However, the role EBV infection might play in oncogenesis is only just beginning to be understood. EBV-infected HRS cells express very few viral genes. A notable exception is latent membrane protein 2A (LMP2A), whose gene product localizes to small glycolipid-enriched microdomains in the plasma membrane of these cells [4]. Previous work has indicated that LMP2A might mimic an activated BCR and enable BCR-negative cells that should be eliminated to inappropriately exit the bone marrow and colonize peripheral lymphoid organs [5].

A recent study by Portis et al. explored the transcriptional profile of B cells expressing the LMP2A transgene [4]. Using DNA microarray analysis, the authors compared gene transcription in bone marrow and splenic B cells from LMP2A transgenic mice, LMP2A-expressing human B-cell lines, and LMP2A-positive EBV-infected lymphoblastoid cell lines. Overall, they found similar transcriptional changes in the various cell types, suggesting that LMP2A targets specific cellular pathways. Interestingly, this transcriptional profile showed many alterations in gene expression similar to that found in the HRS cells of Hodgkin's lymphoma patients. For example, many of the same B cell-specific transcription factors and signaling molecules were downregulated, which might provide a mechanism for these cells to avoid immune recognition and apoptosis induction. In addition, various cell-cycle, anti-apoptotic, and anti-immunity genes were overexpressed, which favors cell growth.

Such overexpression is a common feature of HRS cells in general, including those cells that are EBV-negative. It is possible that EBV-negative HRS cells contain other elements that functionally replicate the effects of LMP2A or initially were infected with EBV but lost viral gene expression during later stages of malignancy [4].

CONSTITUTIVE NF-ΚB ACTIVATION

NF-κB is a transcription factor that regulates the expression of many genes involved in cell proliferation, inflammatory response, and apoptosis. Constitutive NF-κB activity is a hallmark of HRS cells, which may help protect these cells from apoptosis and enable them to grow in the absence of

growth signal. NF-κB is comprised of a dimer of different subunits, including p50, p65/RelA, p52, c-Rel or RelB. Normally, dimeric NF-κB is sequestered in the cytoplasm by binding to members of the inhibitor of kappa B (IκB) family, which include IκBα, IκBβ, and IκBε. Upon treatment of cells with certain stimuli, such as the cytokine tumor necrosis factor (TNF) alpha, one or more of the IκB proteins is phosphorylated by the IκB kinase complex. This phosphorylation targets the IκB proteins for polyubiquitination and subsequent degradation by the 26S proteasome, releasing NF-κB and enabling it to translocate to the nucleus, where it binds DNA and activates transcription (for a detailed review of NF-κB regulation, see [6]).

Recent investigations have revealed several mechanisms that appear to lead to constitutive NF-κB activation and expression of NF-κB target genes in HRS cells [7-9]. Previously, it was shown that mutations in the IκBα gene could lead to a nonfunctional inhibitor that was unable to efficiently sequester NF-κB in the cytoplasm [10]. Now, Emmerich et al. have extended their earlier work on IκBα by analyzing IκBε for mutations in patients with cHL as well as in cHL-derived cell lines [7]. PCR and sequence analysis of 228 single HRS cells from the lymph node sections of six patients revealed that one of the patients had a point mutation at position +5 of intron 1 in the IκBε gene, which led to a splicing deficiency. Notably, this patient did not have a mutation in IκBα.

The detected splicing deficiency led to a frame shift followed by a pre-terminal stop codon in exon 3, resulting in a truncated IκBε protein that was unable to interact with NF-κB. In addition, the investigators analyzed the expression of IκBε protein in six cHL-derived cell lines. Western blot analysis revealed loss of IκBε expression in one of these lines compared to the other cHL-derived and control cell lines. Together, these data suggest that defects in the various NF-κB inhibitory molecules

may be more frequently involved in the constitutive activation of NF-κB in HRS cells than previously thought.

In addition to mutations in the IκB genes, genomic changes in the loci of the NF-κB subunits have been implicated in the pathogenesis of cHL. Structural aberrations of the short arm of chromosome 2, most often resulting in gains of 2p13~16, have been described as highly recurrent in HRS cells [11]. This gain is correlated with aberrant fluorescence in situ hybridization signal patterns of the NF-κB subunit REL locus.

Recently, Barth et al. investigated the role of c-Rel in cHL [8]. Performing interphase cytogenetics on 26 biopsies from 25 cHL patients, they found that 12 demonstrated gains of chromosomal material of 2p13~16, which contains the REL locus. Furthermore, immunohistochemical analysis of the c-Rel protein revealed that all 12 of these biopsies displayed nuclear staining for c-Rel compared to control tissue, which displayed predominant cytoplasmic staining. Moreover, many of the samples without gains of 2p also displayed nuclear staining of c-Rel, although to a lesser extent than the samples with gains of 2p.

Given that NF-κB is active in the nucleus, it is possible that enhanced nuclear staining of c-Rel is indicative of enhanced NF-κB activity. In all, this study demonstrates a close correlation between chromosomal aberrations in 2p13~16 and nuclear accumulation of c-Rel and suggests that genomic changes in the REL locus might be a genetic mechanism that leads to constitutive NF-κB activity in HRS cells.

Another hallmark of HRS cells is the overexpression of CD30, a member of the TNF receptor superfamily involved in cytokine signaling. Overexpression of CD30 results in ligand-independent constitutive signaling that activates NF-κB, however, the mechanism leading to CD30 overexpression in these cells is unclear.

Watanabe et al. have recently published a study reporting that aberrant

CD30 expression in HRS cells is due to dysregulation of the CD30 promoter region [9]. The CD30 promoter region is comprised of three domains: a core promoter with polymerase binding sites, a downstream promoter element essential for start-site selection, and an upstream microsatellite sequence (MS) that represses core promoter activity. In their study, Watanabe et al. conducted a sequence analysis of the CD30 MS region and found a novel AP-1 binding site. AP-1, like NF-κB, is a dimeric transcription factor comprised of a variety of different subunits, in this case from the Jun and Fos families. Using a gel-shift assay, the authors identified JunB as the nuclear factor that binds this AP-1 site. Furthermore, a reporter assay indicated that this binding blocks the normal suppression of the CD30 MS region and corresponds to an increase in CD30 promoter activity. Finally, the investigators demonstrated that both HRS cell lines and HRS cells from the lymph nodes of cHL patients constitutively expressed JunB. Therefore, they suggest that in HRS cells, elevated JunB levels may lead to inappropriate binding of the AP-1 site in the CD30 MS region, resulting in a loss of normal CD30 promoter suppression and consequent CD30 overexpression.

RESISTANCE TO APOPTOSIS

Apoptosis, or programmed cell death, is an important regulatory mechanism whereby unwanted cells are eliminated. This process is mediated by two distinct signaling pathways, one that involves the triggering of death domain-containing cell surface receptors and the other that involves the release of proapoptotic factors from the mitochondria. One receptor that mediates the former apoptotic pathway is the TNF receptor superfamily member CD95/Fas. Normally, activation of Fas leads to the recruitment of the death-inducing signaling complex (DISC) consisting of Fas, the adaptor molecule

Fas-associated death domain (FADD), and the cysteine protease caspase-8. Oligomerization of procaspase-8 results in its autoproteolytic cleavage, which leads to its activation and the onset of a cascade of effector caspases that ultimately results in cell death. Resistance to Fas-mediated apoptosis is thought to play an important role in the survival of the unfavorable germinal center B cells that give rise to HRS cells.

One key player in Fas-mediated apoptosis is c-FLIP, the cellular homolog of the viral protein v-FLIP (FADD-like IL-1β-converting enzyme (FLICE)-like inhibitory protein). c-FLIP can also be recruited to the DISC, however, its recruitment inhibits the recruitment of caspase-8. Consequently, high levels of c-FLIP can block Fas-mediated apoptosis by preventing the activation of the caspase cascade.

A significant amount of work has been published in the last few years implicating the overexpression of c-FLIP as a major mechanism responsible for Fas-resistance in cHL [12-15]. The first piece of evidence came from Thomas et al. who demonstrated that c-FLIP was overexpressed in both Fas-resistant HRS cell lines as well as in 18 of 19 cases of primary Hodgkin's lymphoma, as revealed by immunohistochemical analysis [12]. A subsequent study by Maggio et al. explored whether Fas-resistance is likely due to this overexpression of c-FLIP or if instead is due to mutations in Fas [13]. They analyzed FAS gene mutations and c-FLIP expression in 20 Hodgkin's lymphoma tissue samples and four HRS-derived cell lines and found a causative FAS mutation in only one of the 20 primary cases and in one of the four cell lines. Conversely, they detected high c-FLIP expression by RT-PCR or immunohistochemical analysis in all of the cell lines and primary cases. They suggest that Fas mutations are rare and c-FLIP overexpression is a more likely cause of Fas-resistance in HRS cells.

In April 2004, two studies were published providing strong evidence that c-FLIP indeed mediates Fas-resistance and protects HRS cells from cell death [14, 15]. Using small interfering RNA oligonucleotides (siRNAs) specific for c-FLIP or Fas ligand, Dutton et al. showed that the downregulation of c-FLIP in two cHL-derived cell lines resulted in increased cell death, whereas the concomitant downregulation of Fas ligand in both of these cell lines prevented this cell death [14].

Mathas et al. also demonstrated that the specific downregulation of c-FLIP by siRNA transfection in two cHL-derived cell lines sensitized these cells to Fas-mediated apoptosis [15]. In addition, Mathas' group performed immunohistochemical analysis of the DISC members in 59 cases of Hodgkin's lymphoma and found that Fas and c-FLIP were overexpressed in 55 of these cases, and FADD was overexpressed in several compared to surrounding normal cells. These data indicate that the Fas-mediated apoptotic pathway is upregulated in HRS cells and that c-FLIP is a key regulator of resistance in these cells.

In addition to death domain-mediated apoptotic signaling, apoptosis can be signaled through the mitochondria. In this case, proapoptotic factors such as cytochrome c and second mitochondria-derived activator of caspases (Smac) are released from the mitochondria and initiate a caspase cascade via caspase-9. More specifically, cytosolic cytochrome c, procaspase-9, and apoptosis protease-activating factor-1 (Apaf-1) form a complex called the apoptosome, which induces the activation of caspase-9 by dimerization. Similar to caspase-8, activated caspase-9 initiates a cascade of effector caspases, which ultimately leads to cell death.

A family of proteins, known as the inhibitors of apoptosis, modulates the activity of the caspases and prevents unwanted cell death. Recently, Kashkar et al. have implicated one of these molecules, X-linked inhibitor of apoptosis (XIAP), in the pathogenesis of cHL [16]. XIAP can bind the effector caspase, caspase-3, and block its activation, thereby inhibiting apoptosis. In their study, Kashkar et al. showed via coimmunoprecipitation that XIAP, Apaf-1, and caspase-3 were bound in cHL-derived B cells and that stimulation by exogenous cytochrome c could not block this interaction. Furthermore, they demonstrated that caspase-3 activity could be restored in these cells in the presence of the XIAP inhibitor Smac or if the cells were immunodepleted of XIAP. Finally, the investigators showed that both cHL-derived B cells and HRS cells from primary cases of Hodgkin's lymphoma constitutively and abundantly expressed XIAP. Together, these data suggest that XIAP overexpression may confer apoptosis resistance to HRS cells.

Beyond direct defects in the apoptotic pathways, apoptosis resistance can be incurred in cells that acquire changes that allow them to bypass the cellular checkpoints that would normally stimulate the apoptotic pathways. One class of genes that regulate such checkpoints is the tumor suppressor genes. Loss of function of both copies of a tumor suppressor gene is characteristic of many cancers and enables inappropriate growth. Epigenetic inactivation of one such tumor suppressor, RASSF1A, has been observed in many solid tumors. Murray et al. have recently published data that this might also be the case in cHL [17]. They found that of six cHL-derived cell lines, four had hypermethylated RASSF1A promoters and did not express the gene. Demethylation of the promoter restored RASSF1A expression in these cells. In addition, hypermethylation of RASSF1A was detected in 34 of 52 (65 percent) primary cases of Hodgkin's lymphoma. Consequently, inactivation of RASSF1A may be yet another mechanism by which HRS progenitors escape apoptosis and become pathogenic.

CONCLUSIONS

One hallmark of cHL is the presence of HRS cells. Until recently, very little was

known about the molecular mechanisms of these pathogenic germinal center B cells, except that they show constitutive NF-κB activity, are resistant to apoptosis, and sometimes carry Epstein-Barr virus. In the past few years, significant advances have been made further defining the mechanisms at play (Table 1). In those patients infected with EBV, the expression of viral LMP2A has been shown to give rise to a transcriptional profile that enables cells to inappropriately survive and proliferate. In other cases, mutations in IκB or REL, or overexpression of JunB have been shown to lead to constitutive NF-κB activity. In addition, the overexpression of c-FLIP or XIAP, or the inactivation of the RASSF1A tumor suppressor gene has been shown to contribute to the resistance of HRS cells to apoptosis. Taken together, these mutations begin to paint a clearer picture of what goes wrong in cHL and represent potential targets for new therapies.

According to the United States National Cancer Institute, an estimated 7,300 new patients will be diagnosed with Hodgkin's lymphoma this year. Notably, 65 percent of Hodgkin's patients are under the age of 40 years. Older treatments for the disease were often associated with serious complications from therapy, including sterility and high incidence rates of secondary cancers. Given the young

population of patients, these toxicities were not acceptable.

Today, the standards of care, such as the chemotherapy combination ABVD (Adriamycin, Bleomycin, Vinblastine, Dacarbazine) followed by the selective use of low-dose radiation, have dramatically reduced treatment side effects and improved cure rates. Over 90 percent of patients with early-stage disease will be cured by this or a similar type of treatment. Moreover, about 40 percent of patients suffering a relapse will be cured by other treatment regimens, such as high-dose chemotherapy and autologous peripheral stem cell transplantation. Despite this improvement, there is still a need to develop new therapies.

By defining the molecular mechanisms of cHL, new therapeutic targets are identified that could give rise to a new generation of rationally designed therapies that may be even more effective and less toxic than current treatments. For instance, in those patients infected with EBV, a drug that could successfully downregulate LMP2A could also potentially alleviate the aberrant transcriptional profile of infected cells. LMP2A is an excellent drug target since it is a viral protein and is not expressed in normal cells. Consequently, a therapy designed against LMP2A would

Table 1. Summary of the molecular mechanisms of classic Hodgkin's lymphoma reviewed in this paper.

Molecular Mechanisms of Classic Hodgkin's Lymphoma

Epstein-Barr Virus-positive cells:
Viral LMP2A expression leads to an aberrant transcriptional profile

Epstein-Barr Virus-positive or negative cells:
Constitutive NF-κB activity leads to inappropriate cell growth:
• Arises from mutations in IκB
• Arises from mutations in REL
• Arises from JunB overexpression leading to CD30 overexpression
Resistance to apoptosis prevents cell death:
• Arises from c-FLIP overexpression
• Arises from XIAP overexpression
• Arises from inactivation of the tumor suppressor gene RASSF1A

be very specific for infected cells and potentially less toxic to patients. In addition, the studies on c-FLIP indicate that HRS cells are already primed for apoptosis. Therefore, the selective downregulation of c-FLIP could also serve as a novel treatment that would specifically kill HRS cells. Recently, the use of proteasome inhibitors has been found to be an effective treatment for some cancers. For example, the drug bortezomib, which is a proteasome inhibitor now approved for the treatment of multiple myeloma, has been shown to not only inhibit NF-κB but also promote cell cycle arrest and apoptosis [18]. Given the elevated activity of NF-κB and the anti-apoptotic character of HRS cells, it would be interesting to determine whether a similar approach would also prove fruitful for cHL, although this remains to be tested. It is important that drug designers incorporate these and future findings into the development of new therapies so that the best possible treatments become available.

REFERENCES

1. Cossman J, Annunziata C, Barash S, et al. Reed-Sternberg cell genome expression supports a B-cell lineage. Blood 1999;94:411-6.
2. Thorley-Lawson D. EBV the prototypical human tumor virus – just how bad is it? J Allergy Clin Immunol 2005;116:251-61.
3. Hammerschmidt W and Sugden B. Epstein-Barr virus sustains Burkitt's lymphomas and Hodgkin's disease. Trends Mol Med 2004;10:331-6.
4. Portis T, Dyck P, and Longnecker R. Epstein-Barr Virus (EBV) LMP2A induces alterations in gene transcription similar to those observed in Reed-Sternberg cells of Hodgkin lymphoma. Blood 2003;102:4166-78.
5. Caldwell R, Wilson J, Anderson S, and Longnecker R. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. Immunity 1998;9:405-11.
6. Viatour P, Merville M, Bours V, and Chariot A. Phosphorylation of NF-kB and IκB proteins: implications in cancer and inflammation. Trends Biochem Sci 2005;30:43-52.
7. Emmerich F, Theurich S, Hummel M, et al. Inactivating I kappa B epsilon mutations in Hodgkin/Reed-Sternberg cells. J Pathol 2003;201:413-20.
8. Barth T, Martin-Subero J, Joos S, et al. Gains of 2p involving the REL locus correlate with nuclear c-Rel protein accumulation in neoplastic cells of classical Hodgkin lymphoma. Blood 2003;101:3681-6.
9. Watanabe M, Ogawa Y, Ito K, et al. AP-1 mediated relief of repressive activity of the CD30 promoter microsatellite in Hodgkin and Reed-Sternberg cells. Am J Pathol 2003;163:633-41.
10. Emmerich F, Meiser M, Hummel M, et al. Overexpression of I kappa B alpha without inhibition of NF-kB activity and mutations in the I kappa B alpha gene in Reed-Sternberg cells. Blood 1999;94:3129-34.
11. Joos S, Menz C, Wrobel G, et al. Classical Hodgkin lymphoma is characterized by recurrent copy number gains of the short arm of chromosome 2. Blood 2002;99:1381-7.
12. Thomas R, Kallenborn A, Wickenhauser C, et al. Constitutive expression of c-FLIP in Hodgkin and Reed-Sternberg cells. Am J Pathol 2002;160:1521-8.
13. Maggio E, van den Berg A, de Jong D, Diepstra A and Poppema S. Low frequency of FAS mutations in Reed-Sternberg cells of Hodgkin's lymphoma. Am J Pathol 2003;162:29-35.
14. Dutton A, O'Neil J, Milner A, et al. Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. PNAS 2004;101:6611-6.
15. Mathas S, Lietz A, Anagnostopoulos I, et al. c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. J Exp Med 2004;199:1041-52.
16. Kashkar H, Haefs C, Shin H, et al. XIAP-mediated caspase inhibition in Hodgkin's lymphoma-derived B cells. J Exp Med 2003;198:341-7.
17. Murray P, Qiu G, Fu L, et al. Frequent epigenetic inactivation of the RASSF1A tumor suppressor gene in Hodgkin's lymphoma. Oncogene 2004;23:1326-31.
18. Chauhan D, Hideshima T, and Anderson K. Proteasome inhibition in multiple myeloma: therapeutic implication. Annu Rev Pharmacol Toxicol 2005;45:465-76.