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Epstein-Barr Virus and Human Cancer: Hokkaido University, July 4–6, 2001

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The Twenty-first International Symposium of the Sapporo Cancer Seminar Foundation on Epstein-Barr Virus and Human Cancer was held in Hokkaido University between July 4 and 6, 2001. The Symposium was striking because of its scholarly exchange within the gracious environment provided by the support of Dr. Hiroshi Kobayashi of the Sapporo Cancer Seminar Foundation and the Ministry of Education, Culture, Sports, Science and Technology of Japan. All generations of Epstein-Barr Virus (EBV) researchers were present, from its founders including Sir Anthony Epstein, Dr. Yorio Hinuma, and Dr. Harald zur Hausen to its most recent initiates. The presentations covered new findings on EBV-associated malignancies, current research on the mechanisms of EBV's oncogenesis, and approaches to develop therapies for EBV-associated malignancies.

EBV is the paradigm for human tumor viruses. Prospective epidemiologic studies have indicated that it is a risk factor in the development of Burkitt's lymphoma and nasopharyngeal carcinoma. Much evidence makes it likely that EBV also contributes causally to other malignancies including post-transplant lymphomas and portions of Hodgkin's disease, gastric carcinoma, and T-cell lymphomas. At this meeting important insights into EBV's causal role for nasopharyngeal carcinoma were provided by Dr. Dolly Huang of the Chinese University of Hong Kong. She detailed evidence that chromosomal deletions at positions 3p and 9p occur commonly in low-grade dysplasia of nasopharyngeal epithelia among people at risk for developing nasopharyngeal carcinoma. These genetic changes are not found in histologically normal tissue and precede detectable EBV. EBV could be detected by *in situ* hybridization for its abundantly expressed EBV-encoded small RNAs, EBERs, only in high-grade dysplasia. Thus, although EBV has been found to be a risk factor in the development of nasopharyngeal carcinoma and is present in effectively 100% of the frank tumors among patients in high-risk regions of the world, it is not detected in their earliest pre-neoplastic lesions. These findings are particularly intriguing given the work described by Dr. Irène Joab of the Hôpital Saint-Louis, Paris, France. She detected EBV DNA in 51 of 100 breast carcinomas examined by PCR. The positive tumor samples were microdissected and pools of isolated tumor cells were again assayed. These pools were dramatically heterogeneous in their content of EBV DNA indicating either that the tissue was recently infected or that daughters of infected cells had lost EBV. The former possibility appears consistent with Dr. Huang's findings in nasopharyngeal carcinoma and provides further motivation for analyzing breast carcinomas for the presence of EBV.

EBV is found in a portion of gastric carcinomas. Dr. Masashi Fukayama of the University of Tokyo, Japan noted that while only 7–10% of all gastric carcinomas are EBV-positive, 100% of the lymphoepithelioma-like variants are EBV-positive. He has successfully propagated a gastric carcinoma biopsy through multiple passages in SCID mice. These passaged tumors maintain their EBV DNA and express EBERs and the Epstein-Barr nuclear antigen 1 (EBNA-1) which is common to all EBV-associated malignancies. Dr. Fukayama has found that a cellular inhibitor of apoptosis, c-IAP2, is expressed particularly efficiently in these transplanted tumors, illustrating how powerful they will be for analyzing the association of EBV with gastric carcinoma.

EBV can be detected via its expressed EBERs in approximately 20% of all cases of Hodgkin's disease. Dr. Richard Ambinder (Johns Hopkins University, USA) reported that older female patients with EBV-positive Hodgkin's disease do not survive as well as similar patients with the EBV-negative forms of the disease. EBV is also often associated with a group of lymphomas characterized by Dr. Katsuyuki Aozasa (Osaka University). He has detected EBV through its expression of EBNA-1 in almost all examined nasal NK-T cell lymphomas and pyothorax-associated lymphomas and about one half of adrenal lymphomas. The nasal NK-T cell lymphomas are found primarily in east Asian countries, with their development probably depending on environmental factors in addition to EBV.

EBV is the paradigm for human tumor viruses not only because it is a risk factor in the development of multiple human tumors, but also because it has been successfully analyzed in cell culture. This analysis began with the detection of EBV in biopsies of Burkitt's lymphoma by Sir Anthony Epstein (Oxford University, UK) and was facilitated by Dr. George Miller's (Yale University, USA) isolation of the B95-8 cell line which efficiently releases infectious EBV. Dr. Miller described his recent dissection of the cellular pathways that need to be triggered to induce the productive phase of EBV's life-cycle. EBV cannot be propagated in cell culture by direct lytic infection of sus-

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ceptible cells as can herpes simplex virus type I. Rather it can infect primary B-lymphocytes, induce them to proliferate, and only in some proliferating progeny cells undergo its productive cycle. Dr. Miller described his findings that, although agonists of protein kinase C can induce EBV's lytic cycle as shown by Dr. Harald zur Hausen (German Cancer Research Center, Heidelberg, Germany), inhibitors of histone deacetylases can induce its lytic cycle independently of protein kinase C.

EBV DNA is maintained extrachromosomally in the cells it infects and induces to proliferate. Only a handful of viral genes are expressed in these latently infected cells. Among these viral genes, EBNA-1 is central in that it both positively regulates transcription of other latently expressed viral genes and supports extrachromosomal replication of the viral genome. Dr. Wolfgang Hammerschmidt (GSF-National Research Center for Environment and Health, Munich, Germany) reported his finding that EBNA-1 binds components of the cell's origin recognition complex (ORC) to position ORC at *ori*P, EBV's origin of plasmid replication. This finding has far reaching consequences because it identifies *ori*P as the only defined mammalian origin of DNA synthesis which is compact and tractably studied. Dr. Elliott Kieff (Harvard University, USA) described his work with fusions of the DNA-binding domain of EBNA-1 to the cellular HMG-1 and histone H1 proteins that can functionally substitute for EBNA-1 in its support of replication. This surprising observation may indicate that the cellular moieties of the fusions also associate with ORC.

Additional latently expressed proteins that contribute to EBV-induced cell proliferation and presumably thereby to tumor formation were also discussed. Dr. Paul Farrell (Ludwig Institute for Cancer Research, London, UK) described studies with EBNA-2, which has been found to subvert the Notch signaling pathway in infected cells by binding suppressor of Hairless or CBF-1. He has found the EBNA-2 induces IL-16 which potentially could inhibit the CD4 immune response. He also noted that not all genes induced by EBNA-2 have CBF-1-binding sites, indicating that additional stimulatory pathways are regulated by this oncogene of EBV. Dr. Georg Bornkamm (GSF-Research Center for Environment and Health, Munich, Germany) has found that EBNA-2 also inhibits induction of EBV's lytic cycle. Dr. Nancy Raab-Traub (University of North Carolina, USA) has developed mice transgenic for EBV's latent membrane protein-1 (LMP-1), a viral oncoprotein which mimics much of CD40's signaling. These transgenic mice express LMP-1 from an IgH chain promoter and enhancer and develop B-cell lymphomas. When crossed with p16 null animals, the LMP1-positive, p16-negative progeny develop lymphomas more rapidly than do p16-positive animals. Dr. Huang's finding that deletions of chromosome 9p in nasopharyngeal carcinomas would delete the *p16* gene and Dr. Raab-Traub's transgenic studies underscore the importance of this tumor suppressor in EBV's oncogenesis. Dr. Lawrence Young (University of Birmingham, UK) discussed his charting of the route by which LMP-1 induces jun N-terminal kinase (JNK) to promote its signaling. He has found that the tpl-2 MAPK kinase kinase mediates LMP-1's induction of JNK. Dr. Bill Sugden (McArdle Laboratory for Cancer Research, University of Wisconsin, USA) noted that although LMP-1 and CD40 signal through common pathways, and that both molecules move to lipid rafts in association with cellular TRAF molecules to signal, their requirements for assembling a signaling complex differ. LMP-1 initiates signaling apparently in a ligand-independent manner and does so particularly efficiently.

The EBERs in addition to EBNA-1 have been found to be generally expressed in EBV-associated malignancies. Dr. Kenzo Takada (Hokkaido University) described his elucidation of the role the EBERs perform in the transformed phenotype of EBV-positive Burkitt's lymphoma tumor cells. He made this study possible by isolating variants of the EBV-positive Akata tumor cell line which he established to have lost their EBV. He could then introduce individual viral genes into the EBV-negative Akata derivative to determine which viral genes recapitulate EBV-positive Akata's phenotypes. Dr. Takada has determined that the EBERs induce IL10, which acts as an autocrine growth factor for EBV-positive Akata cells.

A viral gene family, the *Bam*A rightward transcripts, has also been found to be generally expressed as RNAs in EBV-associated tumors. In 80% of nasopharyngeal carcinoma biopsies one member of this family is found to be translated, BARF1. Dr. Tadamasa Ooka (Université Claude Bernard, France) showed that this EBV homologue of the c-fms receptor is also an oncogene and transforms Balb/c 3T3 cells in culture. His work makes testing nasopharyngeal carcinoma biopsies for a possible dependence on CSF1, the ligand for c-fms, an exciting prospect.

EBV encodes at least one latently expressed protein, LMP2A, that limits the escape from latent into productive infection. Dr. Richard Longnecker (Northwestern University, USA) demonstrated that LMP2A inhibits signaling by the B-cell receptor (BCR). LMP2A associates with the src family kinases, *Lyn* and *Syk*, in lipid rafts to carry out this inhibition. Both he and Dr. Ingemar Ernberg (Karolinska Institute, Sweden) have found that LMP2A binds members of the Nedd4 family of ubiquitin ligases which promotes degradation of Lyn and Syk and thereby inhibits BCR signaling. When host cells are successfully induced to support EBV's lytic cycle, a battery of viral genes are expressed which include seven required to synthesize progeny viral DNA. Dr. Tatsuya Tsurumi (Aichi Cancer Center Research Institute, Nagoya) has expressed these viral proteins in insect cells, purified them, and found that

EBV's DNA polymerase, BALF5, associates directly with the viral-encoded helicase-primase complex. This viral machinery can 1000-fold amplify viral DNA, which is then encapsidated and enveloped to yield progeny virions. Dr. Lindsey Hutt-Fletcher (University of Missouri-Kansas City, USA) has found that two viral glycoproteins, gN and gM, form a complex required for progeny virions to be enveloped. gN^-, gM^- virions remain in the nucleus of infected cells.

Once infectious EBV is released from cells *in vivo* it is found in the saliva and in a growing number of instances, in the bloodstream. Preliminary studies by PCR by Dr. Richard Ambinder have detected more high levels of EBV in Hodgkin's patients' blood than in that of controls.

The prevalence of EBV in more than 90% of adults worldwide indicates that therapies for EBV-associated malignancies must deal with a pre-existing immune response that has failed to prevent the disease. Dr. Dennis Moss (University of Queensland, Australia) has pioneered the identification of T-cell recognized epitopes on EBV latent antigens in order to assemble them into a poly-epitope vaccine to stimulate the host's cytotoxic lymphocytes. Epitopes on EBNA-3a, -b, and -c are particularly robustly recognized by these cytotoxic cells. He is developing this vaccine to treat infectious mononucleosis, a common, self-limiting, B-cell lymphoproliferation caused by EBV and hopes to extend its use to patients with nasopharyngeal carcinomas. Dr. Cliona Rooney (Baylor College, Houston, USA) has successfully treated patients with EBV-induced post-transplant lymphoproliferations by culturing their cytoxic T-cells directed against EBV-positive autochthonous B-cells *in vitro* and introducing these specific, cytotoxic cells back into the tumor patients. She is extending this therapeutic approach to relapsed, EBV-positive Hodgkin's patients. She is using LMP2-expressing dendritic cells to reactivate LMP2-specific cytotoxic T-cells *in vitro* so that on introduction into the patient, these specific cytotoxic cells could kill LMP2-expressing Reed-Sternberg cells. The work of Dr. Moss and Dr. Rooney is particularly exciting in its capitalizing on the viral nature of EBV's oncogenesis to develop therapies for these tumors.

EBV is a human virus; its tropism obviously limits its study. Dr. Fred Wang (Harvard University) described his characterization of a Rhesus virus which shares 65% sequence identity with EBV. This rhesus lymphocryptovirus infects its natural host as does EBV and is associated with B-cell lymphomas in simian immunodeficiency virus (SIV)-infected, immunosuppressed macaques. This close relative of EBV may well permit answering questions that cannot be addressed directly with EBV in people.

All of the research presented by the speakers at the Twenty-first International Symposium of the Sapporo Cancer Seminar Foundation was extended and enriched by poster sessions. Thirty-four scientists detailed their ongoing research on EBV and human cancers in these sessions. This international meeting brought more than 100 scientists together to foster their scholarly exchange on the paradigm of human tumor viruses. It both advanced the participants' science and their pleasure in doing it.

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