Retroviral Oncogenesis in an Ovine Model of Lung Cancer  
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Ovine pulmonary adenocarcinoma (OPA, sheep pulmonary adenomatosis, jaagsiekte) is a naturally occurring pulmonary neoplasm of sheep caused by jaagsiekte sheep retrovirus (JSRV) [1-5]. Several features of the OPA model underscore its relevance for pulmonary biology and retroviral oncogenesis [6-9]. First, as a virus-induced neoplasm involving two secretory epithelial cells of the lung, alveolar type II (ATII) cells and non-ciliated bronchiolar epithelial (Clara) cells [10], OPA is a unique model for investigating gene regulation and mechanisms of transformation in these important pneumocytes [11,12]. Second, OPA morphologically resembles human bronchioloalveolar carcinoma (BAC) and a JSRV-related protein has been found in some BAC cases [13,14]. Third, OPA is one of only a few known epithelial cancers associated with a retrovirus etiology. Fourth, biologically interesting endogenous proviruses related to JSRV are found in sheep and in humans [15,16]. Further advantages of the OPA carcinogenesis model include a relatively short incubation period, the use of an outbred animal species with a respiratory system widely considered to be the best animal model for the human lung, and the availability of large quantities of tumor material at different stages of disease. Even if oncogenic viruses are not directly involved in human lung cancer, an understanding of the mechanisms of carcinogenesis in the sheep model could lead to improved diagnosis, treatment, or prevention of lung cancer in humans.

The pace of research in the OPA system has markedly increased beginning with the determination of the nucleotide sequence of JSRV [5]. The availability of infectious, pathogenic molecular clones of the virus enables analysis of the pathogenesis of JSRV-induced disease and immune responses, virus-target cell interactions, and molecular mechanisms of carcinogenesis. Some important questions include: What are the early target cells for JSRV infection and transformation? Are there differences in pathogenesis between naturally occurring and experimentally induced OPA? What limits JSRV carcinogenesis to cells of the ATII/Clara cell lineage? What influence does the JSRV envelope have in the disease process and what is the molecular basis for its effect? Does JSRV-induced insertional mutagenesis play a role in carcinogenesis and, if so, what host genes are involved? Do JSRV-related endogenous viruses play a role in OPA pathogenesis or the immune response to JSRV? Finally, do JSRV-like viruses play a role in human lung cancer, or if not, are similar mechanisms in oncogenesis involved?

The Biology of Ovine Pulmonary Carcinoma

Studies of naturally occurring OPA -
OPA is rarely diagnosed in the US, where only 11 cases have been reported, but occurs more frequently other countries such as Scotland and in Peru, where prevalence in affected flocks can be as high as 20% [17,18]. These differences in apparent rates of occurrence of OPA among continents may reflect variations in viral strains, host susceptibility, or simply lack of exposure to the causative agent. The clinical presentation and lesions of OPA in sheep from Scotland, Peru and the US are quite similar [2,17,18]. Most affected sheep produce 15 - 50 ml/day of watery nasal fluid that originates in the lungs and is presumably a product of the pulmonary tumor cells. Recent work indicates that this fluid contains large amounts of surfactant protein A. This fluid, which also contains JSRV capsid antigen detected by immunoblotting and competition radioimmunoassay, can be collected from living animals and is a rich source of JSRV for experimental transmission of OPA or for JSRV molecular characterization [19-22].

Pathologically, OPA is characterized by grey, firm multifocal pulmonary nodules or anteroventral masses which often exude clear fluid from the cut surface. Histologically, there is papillary to acinar proliferation of well-differentiated epithelial cells
which exhibit a bronchioloalveolar pattern. Metastasis occurs in approximately 10% of cases most often as small lesions in pulmonary lymph nodes. The tumor is classified as an adenocarcinoma of papillary, acinar, mixed or bronchioloalveolar subtypes [23]. Ultrastructural and immunohistochemical data indicate that most tumor cells exhibit features of ATII cells and a smaller proportion have features of Clara cells [10]. Tumor cells of ATII cell origin are usually well-differentiated with microvilli, desmosomes, and intracellular lamellar bodies. Tumor cells of Clara cell origin contain cytoplasmic dense bodies instead of lamellar bodies. Both cell types were frequently found in the same tumor. Overall, surfactant protein A and surfactant protein C were expressed in about 75% of tumor cells, whereas Clara cell 10 kDa protein was expressed in 17% of tumor cells. JSRV capsid protein was detected in 71% of tumor cells, and less frequently in macrophages or non-neoplastic alveolar and bronchiolar cells.

In sheep in the US and in Peru, but not in Scotland, OPC often coexists with ovine lentivirus (OvLV) infection, as indicated by the presence of OvLV-specific serum antibodies and the isolation of OvLV from OPC-affected sheep [17-19]. In many cases of chronic lymphoid interstitial pneumonia induced by OvLV, there is marked epithelialization of alveoli, reflecting ATII hyperplasia and a possible expanded target cell population for JSRV.

Studies of experimentally induced OPA -
Transmission of OPA was achieved in North America using lung fluid or tumor homogenate preparations from naturally occurring or experimentally induced cases of OPA [19,22]. OPA was experimentally-induced in 24 of 35 lambs (69%) injected with tumor homogenate or lung fluid. All 35 lambs also developed lymphoid interstitial pneumonia and were infected with OvLV based on serum antibodies. In serial transmission experiments employing lung tumor homogenate or lung fluid of OPA cases, the agent of OPA was passaged in vivo 3 times to 7 lambs, all of which developed lesions of both OPA and lymphoid interstitial pneumonia.

Comparison of the biological behavior and pathologic features of naturally occurring and experimentally induced OPA has implications for the mechanism of oncogenesis. In experimentally induced OPA, small, disseminated tumor nodules are found within weeks or a few months after inoculation, suggestive of an acutely transforming retrovirus. In contrast, the tumors in naturally occurring cases usually consist of large monocentric coalescing masses in adult sheep after a long incubation period, suggesting a slow transformation mechanism such as insertional mutagenesis. Thus, JSRV displays attributes of both acute and non-acute retroviral oncogenesis. These observations suggest that tumor development in adult animals infected by JSRV may be the result of a multistep process involving viral protein-induced lung cell proliferation and provirus-induced insertional mutagenesis.

The Biology of JSRV
Endogenous viruses related to JSRV -
The sheep genome contains a family of endogenous retroviral sequences (enJSRV) closely related to JSRV [24]. Interestingly, the integration pattern, as assayed by Southern blot hybridization, does not vary much among a wide range of domestic sheep breeds, and even among wild sheep including bighorn sheep and mouflon sheep, the likely ancestor of domestic breeds. Thus, it appears that these viruses became fixed in the genome early in the development of the genus Ovis, and have not shown much recent movement. Closely related endogenous viruses are also found in the genus Capra, which includes wild and domestic goats. Indeed related viruses were found in several other genera of the subfamily Caprinae of the family Bovidae [24].

Much of the interest in this family of viruses has been directed at developing ways to distinguish exogenous JSRV from enJSRV, and to this end restriction maps and partial sequences were derived for several of the enJSRV loci [3,25,26]. This led to the identification of diagnostic restriction endonuclease cleavage sites and hybridization probes that allowed the isolation of two infectious clones of JSRV [4,27]. Recently, three complete enJSRV proviral loci have been sequenced [28]. These were defective in producing virus particles and differed from infectious JSRV by numerous point mutations and deletions. Sequence differences between the LTRs of JSRV and enJSRV seem to be particularly important in determining the differences in expression in different tissues. While JSRV LTRs appear to be most active in lung cells, the enJSRV LTRs seem to be most active in the uterus [29,30]. Further identification and characterization of the enJSRV loci will be necessary in determining their functional significance.

Two approaches were used to investigate the distribution of proviral loci in the sheep genome [31]. Fluorescence in situ hybridization (FISH) to metaphase chromosome spreads using viral DNA probes was used to detect loci on chromosomes. Hybridization signals were reproducibly detected on seven sheep chromosomes and eight goat chromosomes in seven several
cell lines. In addition, a panel of 30 sheep/hamster hybrid cell lines, that each carry one or more sheep chromosomes and collectively contain the whole sheep genome, was examined for enJSRV sequences. DNA from each of the lines was used as a template for PCR with JSRV-gag specific primers. A PCR product was amplified from 27 of the hybrid lines indicating that JSRV-gag sequences are found on at least 15 of the 28 sheep chromosomes including those identified by FISH. Thus enJSRV proviruses are essentially randomly distributed among the chromosomes of sheep and goats. FISH and/or Southern blot hybridization on DNA from several of the sheep/hamster hybrid cell lines suggests that loci containing multiple copies of enJSRV are present on chromosomes 6 and 9. The origin and functional significance of these arrays is not known.

Exogenous JSRV -
With the identification of JSRV-specific sequences within the TM and LTR-U3 regions of the virus, efforts focused on the isolation of full-length proviral clones. Genomic DNA libraries prepared from the tumor cell line JS7 and from whole tumor tissue from an OPA cases in Scotland both yielded infectious molecular clones and in vivo experiments confirmed that JSRV is the etiology of OPA [4,27]. The full-length clones were sequenced and the JSRVJS7 provirus was determined to be 7840 bp in length and they contained the typical retroviral genes of gag, pro, pol, and env as well as a novel open reading frame, orf X. Comparison of deduced env amino acid sequences of 6 JSRV strains from 3 continents identified residues that defined two distinct genotypes of JSRV; African (Type 1) and North American/European (Type 2) [26]. At the nucleotide level, the greatest differences between Type I and II isolates were seen in the LTR (87% identical) and to a lesser degree (91%) in the gag and env genes. The closely related enzootic nasal tumor virus (ENTV), which causes nasal adenocarcinoma in goats and sheep, is only 60% identical to the JSRV LTRs and 83% and 88% identical to env and gag [32].

The isolation of infectious, pathogenic proviral clones has greatly facilitated investigation of the role that JSRV plays in OPA. For example, the isolation of the JSRVJS7 env gene allowed the determination of the host range of the virus as well as the isolation of the cell surface receptor [33]. Studies on the role of the JSRV-21 LTR in the expression and replication of the virus in various cell types has increased our understanding of the mechanism by which JSRV gene expression may be controlled, thus possibly limiting the cells affected in OPA [34]. Furthermore, as described below, the cellular mechanism leading to carcinogenesis is now an active area of research in several laboratories.

Development of a replication system for JSRV -
Progress in elucidating the mechanism by which JSRV induces OPA has been hampered by the lack of a permissive cell culture system for propagating wild-type virus. Traditional methods, including co-culturing JSRV transformed cells with cell lines known to be permissive for other type D viruses, have not been successful. Two tumor cell lines established from OPA cases in Scotland, JS7 and JS8, have been maintained for over 140 passages in vitro [35]. Although the cell lines are clearly of ATII cell origin and are transformed, neither expresses JSRV. Recently, a permissive cell culture system for propagating wild-type virus has been developed by transfecting cells with full-length proviral clones driven by the human cytomegalovirus immediate-early promoter [27,36]. The availability of infectious, pathogenic molecular clones of the virus enables investigation of JSRV-target cell interactions, and the molecular mechanisms of carcinogenesis.

Since the ATII cell is the principal host cell type affected by JSRV, efforts have been directed at the isolation of these cells from sheep using approaches employed for the isolation of rat ATII cells. Typical preparations of cells from lambs 1 - 10 days old yield approximately 3 x 10^8 cells with a viability of 80% and a ATII cell purity of 79% (J.Platt and D. Voelker, unpublished). The cells are cultured on millicells coated with a collagen matrix using a sheep lung fibroblast monolayer on the underside of the millicell filter. In conjunction with the retention of differentiated appearance, these ATII cells synthesize and secrete pulmonary surfactant. Furthermore, they can be transfected using JSRV pseudotype vectors and infected with JSRV (T. Allen, unpublished). This system can be used to observe and analyze the effects of JSRV on the cell type it transforms.

JSRV-Cell Interactions and Their Role in Neoplasia
Multistep Carcinogenesis, Retroviral Oncogenesis, and Lung Cancer -
Strong evidence indicates that carcinogenesis is a multistep process involving genetic damage that takes a variety of forms and results in unregulated cell growth [20,21]. Research on the molecular pathogenesis of human lung cancer has revealed that two mechanisms operate in various histologic forms of lung cancer [37,38]. These are:

1. Activation of dominant cellular proto-oncogenes, such as those of the ras and myc families through high level expression, amplification, point mutation, rearrangements, transactivation, or lack of transcriptional attenuation, and
2. Inactivation or deletion of tumor suppressor genes (anti-oncogenes), especially in small cell lung cancers, most notably the retinoblastoma gene \((rb)\) and p53 [39,40]. In addition to these genetic changes, lung cancer is induced by autocrine growth factors (such as gastrin_releasing peptide, insulin_like peptide, and transferrin_like peptide) as well as \(jun\) gene family activation, which also may activate transcription and mediate tumor promotion. Moreover, suppression of apoptosis pathways also has been recognized to have a considerable impact on cancer cell survival and tumor progression [41].

Infection of animals by retroviruses can induce tumorigenesis by at least six mechanisms [42-44]:

1. Transduction of oncogenes,
2. Proviral cis-activation of host proto-oncogenes by promoter or enhancer insertion,
3. Proviral trans-activation of host proto-oncogenes,
4. Inactivation of host genes by insertional mutagenesis,
5. Mitogenic effects of viral products such as envelope glycoproteins, and
6. Indirect depression of immune responses.

Studies of acutely transforming avian, murine (MuLV) and feline (FeLV) leukemia viruses, which have captured cellular genes after integration, led to the identification of more than 40 oncogenes and proto-oncogenes as well as elucidation of their role in regulating cell growth and inducing neoplasia. In slowly transforming viruses, integration of the provirus disrupts the normal regulation of gene expression. The majority of naturally occurring animal retroviruses, and even some endogenous viruses, act by insertional activation [7]. Recombination between exogenous retroviruses and endogenous retroviral elements may lead to enhanced viral pathogenicity (\(env\), FeLV), or oncogenicity (LTR, AKR mice) [45,46]. Lymphomas induced by MuLV, bovine leukemia virus, and human T-lymphotropic virus, do not appear to arise from proviral insertion near known proto-oncogenes, but rather by a trans-activating mechanism [42]. The initiating trans-activation event is transient and not essential to maintain the transformed state, leading to conjecture that these viruses transactivate a cellular gene whose product is involved in a positive feedback loop. In cats persistently infected with FeLV, de novo generation of acutely transforming FeLV/\(myc\) recombinant viruses appears to be an additional mechanism by which viruses without oncogenes can induce tumors [47]. Of relevance to the multistep nature of cancer, certain strains of the Friend MuLV induce multistage erythroleukemias characterized by polyclonal proliferation of erythroid precursor cells followed by clonal expansion of malignant cells involving insertional activation of \(ets\) oncogenes and inactivation of the p53 tumor suppressor gene [48].

**Mechanisms of JSRV Oncogenesis**

JSRV is unique among replication-competent retroviruses in its ability to transform cells \(in\) \(vitro\) via the expression of its viral envelope (Env), which functions essentially as an oncoprotein. Transfection of chicken [49] or rodent [50] fibroblasts with expression plasmids or vectors for JSRV Env results in the appearance of classic foci of transformed cells in days or weeks after transfection. Several studies have investigated the mechanisms of JSRV Env-induced cell transformation \(in\) \(vitro\) [49,51-56]. Hyaluronidase-2, which is encoded by a putative tumor suppressor gene, is the cellular receptor for JSRV, [56] and both receptor-dependent [52] and–independent [53] mechanisms of transformation have been suggested. Activation of the phosphatidylinositol 3-kinase/Akt pathway mediated by the JSRV Env transmembrane domain helps but is not essential for transformation of chicken or rodent fibroblasts [49,54,55,57].

The mechanisms by which JSRV induces type II pneumocytes and Clara cells to become neoplastic have not been studied in detail. Tumorigenesis is a multistep process, and the expression of JSRV Env might not be sufficient to transform these cells \(in\) \(vivo\). The JSRV long terminal repeats (LTRs) are specifically active in the cells that are the target for viral transformation [29]. Abundant viral antigens are present in the tumor cells, and infectious virus is present in the lung secretions of OPA-affected animals [58]. Thus, JSRV replicates in ATII cells and Clara cells, and this could allow the viral LTR to activate nearby genes through the classical mechanism of insertional activation used by most oncogenic retroviruses. Insertional activation may consequently be part of or ultimately be the cause of JSRV-induced carcinogenesis \(in\) \(vivo\).

Only two JSRV integration sites have previously been cloned [4,27], both by screening of genomic DNA libraries. One, from an OPA tumor cell line, JS7, was in the pulmonary surfactant protein A gene. The other, from an OPA tumor, was uncharacterized because of repetitive elements in the clone. The analysis of the JSRV insertion sites was greatly complicated by the presence in the sheep genome of the endogenous retroviruses highly related to JSRV.
To test the hypothesis that JSRV insertional mutagenesis is involved in the oncogenesis of OPA, 70 independent integration sites from 23 cases of OPA were cloned and characterized [59]. Multiple integration sites were identified in most tumors. BLAST analysis of the sequences did not disclose any potential oncogenic motifs or any identical integration sites in different tumors. Thirty-seven of the integration sites were mapped to individual chromosomes by PCR using a panel of sheep/hamster hybrid cell lines. Integration sites were found on 20 of the 28 sheep chromosomes, suggesting a random distribution. However, four integration sites from four different tumors mapped to chromosome 16. By Southern blot hybridization, probes derived from two of these sites mapped to within 5kb of each other on normal sheep DNA. These sites were found within a single sheep bacterial artificial chromosome sheep genome clone and were further mapped to only 2.5 kb apart, within an uncharacterized predicted gene and less than 200kb from a MAP-kinase gene. This common integration site was mapped to chromosome 5q11.2 on the human genome map. This agrees with the somatic cell hybrid mapping of the integration sites, as sheep chromosome 16 is syntenic to HSA5. These findings suggest that there is at least one common integration site for JSRV in OPA, and add weight to the hypothesis that insertional mutagenesis is involved in the development of this tumor.

Conclusion
There are several observations regarding JSRV infection and the development of OPA that may prove relevant to the pathogenesis of the disease. The target cells for the virus (ATII and Clara cells), both function as progenitors or stem cells for vulnerable cell populations following epithelial injury caused by microbial infection or inflammation. A heightened replicative state of ATII cells and Clara cells may be necessary for the replication in genomic integration of JSRV. A second feature common to the target cells for JSRV is their production of surfactant proteins. This may be significant because at least one integration site for JSRV is within the gene for the abundantly expressed SP-A. This raises the possibility that the carcinogenic potential of JSRV may be enhanced by high-level expression in specific host genetic loci. Given the transforming properties of the JSRV ENV protein, there is little doubt that ATII and Clara cell proliferation are a direct consequence of JSRV infection, at least early in the course of the disease. Whether sustained viral ENV protein production is required to maintain the proliferative state of the infected cells is presently unknown, although widespread expression of JSRV-CA is observed in tumor cell populations. Taken together, these data suggest that OPA oncogenesis is consistent with a multistep carcinogenesis model in which tumor development results from early viral envelope protein-induced lung cell proliferation and subsequent provirus-induced insertional mutagenesis involving cis- or trans-activation or inactivation of host genes important in regulation of lung cell growth and replication.

References