Retrovirus-Induced Ovine Pulmonary Adenocarcinoma, an Animal Model for Lung Cancer

Massimo Palmarini, Hung Fan

Studies on the molecular mechanisms of transformation of retrovirus-induced neoplasms in domestic and laboratory animal species have provided insights into the genetic basis of cancer. Ovine pulmonary adenocarcinoma (OPA) is a retrovirus-induced spontaneous lung tumor of sheep that has striking analogies to some forms of human adenocarcinoma. The etiologic agent of OPA, jaagsiekte sheep retrovirus (JSRV), is unique among retroviruses for having a specific tropism for the differentiated epithelial cells of the lung, and it is the only virus known to cause a naturally occurring lung adenocarcinoma. Expression of the JSRV envelope protein is sufficient to induce cell transformation in vitro, possibly via the activation of the phosphatidylinositol 3-kinase/Akt-signaling pathway mediated by the cytoplasmic tail of the transmembrane protein. The aim of this review is to draw the attention of basic and clinical scientists engaged in lung cancer research to this unique animal model, to explore the possible use of OPA as a tool to investigate the mechanisms of pulmonary carcinogenesis, and to underline the similarities between OPA and some forms of human lung adenocarcinoma. The possibility of a viral etiology for the latter will be evaluated in this review. [J Natl Cancer Inst 2001; 93:1603–14]

Lung cancer is the leading cause of human cancer death, accounting for the lives of more than a million people yearly worldwide (1–3). The majority of lung cancers are associated with cigarette smoking; however, about 10% of lung cancers in men and 20%–25% in women are not associated with smoking (4,5). Adenocarcinoma is increasing in frequency and accounts for almost half of lung cancers in some countries, and it is associated less strongly with cigarette smoking (6–9).

Ovine pulmonary adenocarcinoma (OPA) is a naturally occurring adenocarcinoma of sheep. It is interesting that, in a guest editorial that appeared in this Journal almost 20 years ago, it is the only virus known to cause a naturally occurring lung adenocarcinoma. Expression of the JSRV envelope protein is sufficient to induce cell transformation in vitro, possibly via the activation of the phosphatidylinositol 3-kinase/Akt-signaling pathway mediated by the cytoplasmic tail of the transmembrane protein.

OPA is transmitted as an infectious disease. The incidence of the disease is usually 2%–5% (15,18,30,31), although in the U.K. it can be as high as 30%. In an affected flock, the disease may be responsible for more than 50% of the mortality (32). OPA generally affects young adult sheep. The diseased animals develop symptoms of progressive respiratory distress that worsen with increasing size of the lesions and resulting loss of alveolar function. Affected animals usually die within a few weeks of the onset of the clinical signs. A pathognomonic symptom of OPA is the accumulation of fluid within the respiratory tract of the affected sheep. Up to 300 mL of lung fluid can be collected by lifting the rear limbs (and thus the chest) above the head of the affected sheep (“wheel-barrow test”).

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Ovine Pulmonary Adenocarcinoma

OPA (also known as jaagsiekte, sheep pulmonary adenomatosis, or ovine pulmonary carcinoma) is a naturally occurring adenocarcinoma of sheep (11–13). The disease was first recognized in South Africa in the 19th century as a cause of respiratory distress in sheep when they were herded. The Afrikaans name “jaagsiekte” was coined from “driving” (jaag) and “sickness” (ziekte) (14), reflecting the tendency of diseased sheep to lag behind the flock during herding. Since then, OPA has been identified worldwide with the exception of Australia and New Zealand (15–24). OPA acquired a singular place in the study of infectious diseases in the 1930s when it was introduced (along with scrapie, maedi-visna, and paratuberculosis) into Iceland by a small number of sheep imported from Germany (25). Geographical barriers in Iceland allowed the establishment of the relationship between the imported animals and the emerging disease, even if they appeared several months after the introduction of the sheep from Germany. In seminal studies, an Icelandic veterinarian (B. Sigurdsson), coined the term “slow” (25) to describe this group of diseases because they did not fit either the acute or the chronic pattern of a classical infectious disease. Since then, the “slow diseases” of sheep have played important roles in the study of infectious diseases, including the original systems of prion diseases (scrapie) (26,27) and lentiviral infections (maedi-visna) (28,29).

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See “Notes” following “References.”
cranio-ventral portion of the organ (Fig. 1) the cut surface is generally moist with frothy fluid exuding from the bronchioles of the affected areas. In rare cases, an “atypical” pattern (33–35) is observed, with solitary or multiple, hard and dry nodules, usually located in the diaphragmatic lobe. Ultrastructural studies (13,36,37) have shown that the tumor originates from the type II pneumocytes and the nonciliated bronchiolar (Clara) cells. OPA tumor cells maintain the appearance of the cells from which they originated: microvilli, desmosomes, and intracellular lamellar bodies for the type II pneumocytes and cytoplasmic dense bodies for the Clara cells. Both type II pneumocytes and Clara cells are secretory bodies. Type II pneumocytes are the sites of synthesis, storage, and secretion of the alveolar surfactant (38,39), whereas Clara cells in the bronchioles are thought to synthesize, store, and secrete protein components of the extracellular lining layer of bronchioles (38,40).

Histologically, the lesions in “classic” OPA are characterized by the formation of nodular lesions in which the alveoli are lined with cuboidal or columnar cells, often forming papillary growths. In the majority of cases, the presence of large, swollen macrophages in the alveoli contiguous to the focal neoplasia can be detected. In the “atypical” cases, the histopathology is essentially the same, but the stroma of the tumor usually appears to be heavily infiltrated with mononuclear cells and connective tissue (33–35). Classically, OPA has been classified as a bronchioloalveolar carcinoma resembling human bronchioloalveolar carcinoma (41). However, the new classification by the World Health Organization (WHO) of human lung tumors (9) gives a more stringent definition of bronchioloalveolar carcinoma (see below), and in this respect, OPA would be defined as a mixed adenocarcinoma showing acinar, papillary, and bronchioloalveolar growth patterns.

Metastases in OPA cases, in both thoracic and extrathoracic tissues, can be observed in some instances (15,42–44). In the Awassi sheep of Israel, metastases have been observed in 50% of cases (42). The ability of OPA tumors to metastasize and the capacity to transplant OPA tumor cells and derived cell lines in nude mice (45,46) indicate that the nature of the OPA lesions is neoplastic rather than proliferative, although the majority of OPA lesions show a hyperplastic phenotype.

**Fig. 1.** Sheep lungs with typical ovine pulmonary adenocarcinoma lesions. The neoplastic tissues are localized in the cranio-ventral portion of the lungs (arrows). Note the frothy fluid exuding from the end of the trachea (“lung fluid”). Reprinted from (12), with permission from Elsevier Science.

**SIMILARITIES BETWEEN OPA AND HUMAN PERIPHERAL ADENOCARCINOMAS**

As mentioned above, OPA has been classically compared with human bronchioloalveolar carcinoma (10,47). The existence of bronchioloalveolar carcinoma as a distinct clinical and pathologic type of adenocarcinoma has been somewhat controversial, mainly because of the difficulty in differentiating bronchioloalveolar carcinoma from other adenocarcinomas (48). Indeed, in the new classification of human lung tumors, the definition of bronchioloalveolar carcinoma has been in part modified (9) from its original definition (49–51). Bronchioloalveolar carcinoma is now defined as a type of adenocarcinoma with a pure bronchioloalveolar carcinoma growth pattern and no evidence of stromal, vascular, or pleural invasion. The neoplastic cells grow as a single layer along the walls of the terminal airways and alveoli (49,50). Bronchioloalveolar carcinoma can be divided into nonmucinous and mucinous adenocarcinomas. In the nonmucinous adenocarcinoma, the Clara cells and/or type II pneumocytes grow along the alveolar walls and there is no stromal invasion. The mucinous adenocarcinoma is composed of tall columnar cells with cytoplasmic mucin, which displace the nucleus to the base of the cell, growing along alveolar walls and without stromal invasion. Thus, the current classification of lung cancer considers bronchioloalveolar carcinoma to be the noninvasive form of adenocarcinoma rather than a distinct entity. True bronchioloalveolar lesions appear to precede adenocarcinoma lesions; in the latter, the tumors still show a growth pattern involving replacement of the alveolar lining, but it is possible to observe an active fibroblastic proliferation (52). Bronchioloalveolar carcinoma tends to progress to a multifocal pattern, which is traditionally explained as resulting from intrapulmonary metastasis (53), although in some cases the different foci have been shown to be multiclonal (54).

There are as yet no epidemiologic studies in the literature on bronchioloalveolar carcinoma that take into account the new histologic classification of lung tumors. Various reports are present in the literature based on the old WHO classification (51). Even with the old criteria, the incidence of bronchioloalveolar carcinoma has been debated, and the variability of reports in the literature is probably the result of the above-mentioned difficulty of differentiating bronchioloalveolar carcinoma from other forms of adenocarcinoma (48). A high degree of disagreement around the diagnosis of bronchioloalveolar carcinoma indeed has been reported (55,56). However, the incidence of peripheral adenocarcinomas of all types, including bronchioloalveolar carcinoma, is increasing in the United States and in Japan (57–61) and can account for up to a quarter of lung cancers (62). The majority of studies show the incidence of bronchioloalveolar carcinoma to constitute between 1% and 10% of lung cancers (63–67), which might be an overestimate if the new classification of lung cancers is taken into account. Bronchioloalveolar carcinoma has some features that might differentiate it from other human lung cancers. Compared with other forms of lung cancer, the incidence of bronchioloalveolar carcinoma is higher in females, in nonsmokers, and in a comparatively young age group (49,50,54,68–70). Adenocarcinoma, like any form of lung cancer, is associated with cigarette smoking but less strongly than other lung cancers (6–9).

OPA has some similarities with bronchioloalveolar carcinoma, or more correctly with peripheral adenocarcinoma, that
have been noticed for more than half a century (10,41,47,71). In OPA, like bronchioloalveolar carcinoma and its progressive forms of peripheral adenocarcinoma, the lesions tend to be multifocal and are localized at the periphery of the lungs, and the neoplastic cells are derived from type II pneumocytes and Clara cells. However, from the histopathologic point of view, the somewhat strict current definition of bronchioloalveolar carcinoma does not apply to the histologic appearance of OPA. OPA is rather a mixed adenocarcinoma that grows at the periphery of the lung and shows acinar, papillary, and bronchioloalveolar growth patterns. In addition, the majority of the tumor cells forming OPA lesions do not show malignant characteristics.

From a clinical point of view, it is difficult to compare the disease in sheep and in humans, mainly because OPA in sheep is diagnosed only when the lesions are at an advanced stage resulting in respiratory distress. In humans, bronchioloalveolar carcinoma has been described as having two clinical entities with identical histologic appearance: 1) a focal form with a relatively good prognosis following resection and 2) a multifocal progressive and diffuse form with a worse prognosis regardless of intervention (72–75). In sheep, for practical reasons, only the diffuse progressive form is seen because the diagnosis is made only when the affected animal shows evident respiratory distress and, consequently, the tumor is at a late stage.

In conclusion, although there is no perfect animal model for any given human disease, there are enough similarities between OPA and some forms of adenocarcinomas in humans to support the idea that understanding the molecular basis of OPA could provide an intellectual framework to understand some aspects of pulmonary carcinogenesis (see below) (76).

**JAAGSIEKT SHEEP RETROVIRUS**

Retroviruses have been invaluable in elucidating the multistep processes leading to oncogenesis (77). The capacities of retroviruses to integrate into the host cell genome and to subvert the cellular regulatory machinery make these viruses a great tool for investigating the mechanisms of carcinogenesis. Indeed, the identification of viral oncogenes and their cellular counterparts, the proto-oncogenes involved in positive stimulation of cell proliferation into which OPA-affected sheep had exhaled, resulted from studies of animal retroviruses (78–81).

OPA is caused by a retrovirus known as jaagsiekte sheep retrovirus (JSRV). A viral etiology for OPA was suspected beginning in the late 1940s. At that time, OPA was experimentally reproduced by inoculating lambs with a cell-free glycerin solution into which OPA-affected sheep had exhaled (82). In the 1970s and 1980s, the visualization in tumor cells of retrovirus-like particles by electron microscopy studies and the detection in the same materials of reverse transcriptase activity and of polypeptides related immunologically to Mason-Pfizer monkey virus, a simian betaretrovirus, and to mouse mammary tumor virus, a murine betaretrovirus, suggested that a retrovirus was present in OPA (83–89). In 1992, the complete genomic sequence of JSRV was determined from a complementary DNA λ phage library of viral particles RNA purified from lung secretions of an OPA-affected sheep (90,91). The JSRV genome has a simple genetic organization, characteristic of the replication-competent betaretroviruses (92) containing the canonical structural retroviral genes gag, pro, pol, and env (Fig. 2). An additional open reading frame (orf-x) overlaps pol and has some unusual features, including a codon usage different from that of other genes within JSRV, and a very hydrophobic predicted amino-acid sequence that shows no similarities with any other known protein, with the exception of a low homology to a G-protein-coupled receptor (93,94). The role of this open reading frame is unknown. Although orf-x is conserved among different JSRV isolates (93,94), it does not seem to be required for JSRV replication in vitro (Palmarini M, Fan H: unpublished results) or for cell transformation in vitro (see below) (95).

Despite all of the available data pointing strongly to JSRV as the etiologic agent of OPA, it was unclear for many years whether JSRV alone was sufficient to induce lung cancer. The lack of a suitable cell culture system to propagate JSRV in vitro has hampered efforts to establish with certainty the etiology of the tumor. To conclusively establish the role of JSRV in causing OPA, we recently obtained an infectious molecular clone of the integrated DNA form of JSRV (termed “JSRV21”) from a spontaneous OPA case (96). The lack of a suitable cell culture system was bypassed by using a derivative of pJSRV21, in which the cytomegalovirus immediate early promoter was used to drive high-level expression of viral proteins (pCMV2JS21) in transiently transfected human 293T cells. This system resulted in the production of substantial amounts of virus particles. Intratracheal inoculation into four newborn lambs of concentrated virus produced from transfected 293T cells resulted in the development of clinical OPA in two of them by 4 months. Moreover, immunohistochemistry indicated that the lesions were positive for viral protein, and molecular markers of JSRV were found by polymerase chain reaction in the tumor lesions (96). This evidence provided the first conclusive demonstration that JSRV is necessary and sufficient to induce OPA, and it opened the way to exploring the mechanisms of oncogenesis as described below. Recently, another JSRV molecular clone (JSRVJS7) has been obtained by DeMartini et al. (97).

**MOLECULAR BASIS FOR THE LUNG TROPISM OF JSRV**

JSRV appears to be unique among retroviruses in inducing transformation of the differentiated epithelial cells of the lungs (77). Moreover, the only sites where JSRV is highly expressed in vivo are the transformed epithelial cells of the lung (Fig. 3) (98), although viral DNA and RNA are detected in vivo in various lymphoid organs (98–100). In lymphoid cells, JSRV can be detected in naturally infected sheep before the onset of clinical disease and even before the development of discernible neoplastic lesions (101).

The envelope gene (env) and the long terminal repeat (LTR) are the major determinants of retroviral tropism. The env gene encodes the viral glycoprotein that specifically interacts with the cellular receptor(s) necessary for viral entry (102). Retroviruses are able to infect only cells expressing their specific receptor. On
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highest transcriptional activity in cell lines derived from type II pneumocytes and Clara cells. In addition, the JSRV LTR inter-
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Gee-Estrada K, Palmarini M, Fan H: unpublished results) (112). Thus, the restriction for JSRV expression in type II pneumocytes and Clara cells is due to the preferential activation of the JSRV LTR in these cells.
In the future, the preferential expression of the JSRV LTR for
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designing of viral vectors for gene therapy of lung diseases. Efficient transduction and expression in lung cells have proven
to be a difficult task with other vectors (113–115).
It is interesting that the lung tropism of JSRV was apparently acquired relatively recently in evolutionary terms. The sheep
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(91,116,117), and the estimated time of integration of some of
these elements is between 500,000 and 2 million years ago (117,118). enJSRVs are transcriptionally active at a low level in many tissues (119) but are highly expressed in the epithelium of the lumen and the glands of the uterus (118,120). Thus, the ancestor(s) of JSRV were preferentially expressed in the sheep genital tract rather than in the lungs. Indeed, most of the major differences between the exogenous JSRV and its endogenous counterparts are in the U3 region of the LTR, where the viral enhancers are located (118,119,121). In reporter assays, the enJSRV LTRs do not show the high transcriptional specificity for lung epithelial cells that the JSRV LTR does (118). In ad-
dition, the enJSRV LTRs do not interact with HNF-3β but do respond to hormonal stimuli such as progesterone (Palmarini M, Murgia C, Spencer T, Fan H: unpublished data).
In one sense, enJSRVs may be indirectly involved in the pathogenesis of OPA. OPA-infected sheep do not show an antibody response toward JSRV (87,122,123). The likely reason is the expression of endogenous elements during fetal develop-
ment, leading to the tolerance toward the exogenous JSRV. In-
deed, in recent studies, we have detected high levels of expres-

**Retroviruses and Cancer**

The importance of retroviruses in understanding carcinogenesis is due to the fact that common mechanisms are involved in retrovirus-induced neoplasms and in tumors that lack retroviral etiology. For example many proto-oncogenes transduced by retro-
viruses (e.g., ras, myc, abl, and erbB) are now known to be
activated in certain human cancers (77,124–128). With regard to oncogenesis, retroviruses can be divided into two classes: acutely transforming retroviruses and nonacute retroviruses (77,104).

The acutely transforming retroviruses are probably among the
most powerful carcinogens present in nature. They have captured a cellular proto-oncogene into their genome, altering its structure (deletions, point mutations, etc.) and expression in the process (78–80,129,130). Cellular proto-oncogenes are usually conserved over long evolutionary distances, suggesting that they are indispensible for fundamental cellular functions (131). Viral oncogenes work through the synthesis of proteins (oncoproteins) that have the potential to transform a normal cell into a neoplastic one. Most retroviral oncogenes have homology with cellular genes (proto-oncogenes), a hallmark of an oncogene. For example, the avian erythroblastosis virus strain H carries the erbB oncogene that has close homology to the chicken epidermal growth factor receptor (132). As a result of the transduction of the oncogene, the acutely transforming retroviruses typically lack portions of their structural genes and are replication defective. Acutely transforming retroviruses are thus propagated in conjunction with a replication-competent virus called “helper” that encodes all of the viral structural and enzymatic proteins (133–135). Viruses containing oncogenes are relatively rare in naturally occurring retrovirus-induced tumors for several reasons: 1) Oncogene capture is an unlikely event, 2) the rapid and lethal course of the induced disease could limit the spread of the infection, and 3) there is probably no evolutionary-positive se-
lection of replication-defective oncogene-containing retrovi-

The majority of oncogenic retroviruses do not carry an on-

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**Fig. 3.** Immunohistochemistry of a lung section of a sheep with ovine pulmonary adenocarcinoma. Brown staining represents positive reaction of a rabbit anti-
serum toward the major capsid protein of jaagsiekte sheep retrovirus. Positive reaction is confined to the cytoplasm of the transformed cells. No brown staining is observed in the untransformed alveolar cells. Original magnification ×250.
cogene in their genome (77,104,131,136,137). Such viruses are called “nonacute retroviruses” because the incubation period of the resulting neoplasia is usually several months. Nonacute retroviruses are replication competent. Tumor induction appears to occur through multiple steps, many with similarities to non-virus-induced cancer. The best understood mechanism of nonacute retroviral carcinogenesis is LTR activation of proto-oncogenes, also known as insertional activation, cis-activation, or insertional mutagenesis. In these tumors, a provirus is integrated in the vicinity of a cellular proto-oncogene, resulting in overexpression of the proto-oncogene by one of several mechanisms. Retroviruses that act by insertional activation generally induce tumors with a long incubation period because retroviral integration is essentially a random event with respect to the host DNA (138–141). Thus, multiple rounds of infection are typically necessary before integration near (e.g., few to a hundred kilobases) a proto-oncogene occurs in one cell. Under the influence of the viral LTR, the transcription of the proto-oncogene is elevated in that cell and triggers transformation (142–146).

Insertional inactivation of tumor suppressor genes in retrovirus-induced tumors is very rare because, unlike proto-oncogenes, the loss of function of a tumor suppressor gene requires that both copies of the gene be damaged or lost. However, the insertional inactivation of one allele could potentiate spontaneous mutations occurring in the other allele, leading to transformation. For instance, the insertional inactivation of p53 has been observed in a proportion of non-B-, non-T-cell lymphomas induced by the Cas-Br-E virus (147).

Other mechanisms involved in retroviral carcinogenesis have been proposed for human T-cell-leukemia virus (HTLV-I) in adult T-cell leukemia (148–151) and for bovine leukemia virus (BLV) (152–154). Both viruses lack oncogenes and do not show common sites of viral integration in the induced tumors. The tumors are monoclonal or oligoclonal, and viral expression in the tumor cells is minimal (148). The tumors of both humans and cattle appear after a very long incubation period (many years) (155). Besides activating viral transcription, the viral Tax trans-activator protein also activates the expression of cellular genes involved in T-cell growth and differentiation, such as interleukin (IL)-2, IL-2 receptor, IL-3, and granulocyte–macrophage colony-stimulating factor (156). Many properties of Tax, such as the ability to transform rodent fibroblasts (157) or to be oncogenic when expressed in mice as a transgene (150,158), indicate the oncogenic potential of this gene and of its product. Analogies have been found with the Tax protein of BLV (152). One explanation for HTLV-I leukemogenesis is that the Tax protein leads to hyperproliferation of T lymphocytes, and these cells subsequently progress to end-stage leukemia cells by other spontaneous “hits.” This process could explain the very long latency for HTLV-I-induced leukemia as well as the low incidence of leukemia among infected individuals.

MECHANISMS OF ONCOGENESIS IN OPA

One of the most intriguing questions regarding the biology of JSRV concerns the mechanism of virus-induced cell transformation. Key features of the OPA model are that the experimental induction of OPA in newborn lambs with field isolates of the virus is quite rapid, typically in 4–6 weeks but in as little as 2 weeks after inoculation (123,159). At postmortem examination, the affected lungs show multifocal lesions. Moreover, the only cells in the animal that display detectable JSRV antigen are the tumor cells themselves (98). The apparent lack of viral expression in normal cells in the infected animal distinguishes JSRV from all other oncogenic retroviruses. For other retroviruses, tumorigenesis typically occurs in the context of high levels of viral infection and expression in normal cells in both the target tissue and other tissues (77). Thus, it has been unclear if JSRV induces tumors by insertional activation (LTR activation of proto-oncogenes) or if it carries a viral oncogene. An argument in favor of LTR activation of proto-oncogenes is that analysis of the JSRV genome does not reveal any gene that resembles known cellular oncogenes and the incubation period in the natural disease appears to be several months (25). On the other hand, the low levels of viral expression in the infected animals would make it difficult to achieve the multiple, independent infections necessary for high-efficiency insertional activation of a proto-oncogene. The possibility that the JSRV genome carries an oncogene would be consistent with the rapid development of the disease in the absence of a high viral load, as well as the multifocal pattern of the lesions.

Recently, we have carried out experiments that support the notion that JSRV can directly cause tumor formation (95). By performing a standard DNA transfection and cell transformation assay in murine NIH-3T3 cells, we tested the hypothesis that the JSRV genome carries a transforming gene (160). The pCMV2JS21 expression plasmid reproducibly induced foci of transformed cells, whereas control plasmids did not (Fig. 4). When the transformed cells were recovered, they all expressed JSRV RNA. It should be noted that the transformation did not result from spread of infectious virus within the transfected cultures, since murine cells do not have a functional JSRV receptor

Fig. 4. Transformation of NIH-3T3 cells by Jaagsiekte sheep retrovirus DNA. A typical focus of transformation is shown in panel B. Foci are evident 3–4 weeks after transfection. In panel A, compare the appearance of NIH-3T3 cells transfected with vector plasmid DNA only.
Phosphorylation of the pro-apoptotic protein BAD which is mediated by the phosphorylation and subsequent inactivation has been shown to have several antiapoptotic properties, one of which is mediated by the phosphorylation and subsequent inactivation has been shown to have several antiapoptotic properties, one of which is the activation of the PI-3K/Akt pathway. The PI-3K initiates a cell-signaling pathway that involves the phosphorylation of Akt (protein kinase B). Akt regulates the production of several downstream targets, including the expression of the JSRV envelope. By western blotting analysis, we have detected phosphorylated Akt in MP1 cells but not in the parental NIH-3T3 cells (166). These data strongly point to the cytoplasmic tail of the JSRV transmembrane protein for being important for cell transformation and suggest a new mechanism of retroviral transformation (Fig. 5). Both PI3-K and Akt have been described as oncogenes transduced by retroviruses. In other words, they have been transduced by acutely transforming retroviruses. The catalytic subunit of PI-3K has been transduced by the avian sarcoma virus 16 (170), whereas Akt has been transduced by an ectropic murine leukemia virus (171,172). In addition, the PI-3K/Akt pathway has been shown to be involved in the induction of erythropoietin independence of erythroid cells after infection with Friend SFFV (173), further demonstrating that the activation of the PI-3K pathway can lead to cell transformation.

The concept of a retroviral envelope protein functioning as an oncogene has precedents in other oncoviral retroviruses. The spleen focus-forming virus (SFFV) component of the Friend erythroleukemia complex directly transforms erythroid cells via a truncated recombinant envelope glycoprotein, gp55. This protein binds to the erythropoietin receptor at the cell surface of erythroid cells, leading to constitutive growth stimulation through that receptor (161). More recently, the envelope protein of avian hemangiosarcoma virus has been shown to directly transform NIH-3T3 cells (162).

There are several possible mechanisms by which JSRV envelope protein could transform NIH-3T3 cells. The retroviral envelope is formed by two components: 1) the surface (SU) protein that interacts with the cellular receptor(s) and 2) the transmembrane (TM) protein that fixes the SU into the lipid bilayer. First, it is possible that the SU of the JSRV envelope binds to murine HYAL2 protein (the JSRV receptor) at the cell surface, leading to inhibition of a negative growth-regulatory function of HYAL2. This possibility is exciting because some human lung cancers show loss of heterozygosity (LOH) on the region on human chromosome 3 that contains the HYAL2 gene (3p21.3) (163) (see also below). LOH in tumors is often a signature for loss of a growth-inhibitory (tumor suppressor) gene (164,165). A second possibility is that the SU protein binds to HYAL2 protein at the cell surface, resulting in positive stimulation of cell growth through HYAL2. Even though murine HYAL2 does not function as an efficient receptor for JSRV, it is still possible that envelope protein can bind to murine HYAL2 and affect its function. A third possibility is that the SU protein binds to some other cell surface protein and, in doing so, activates a growth-stimulatory signal. Finally, it is possible that the TM of the JSRV envelope binds to an intracellular protein through its cytoplasmic tail, resulting in activation of a signal cascade. Our latest results favor this last possibility (166). By mutation of a single tyrosine in the cytoplasmic tail of the JSRV TM, we abolished the capacity of the JSRV envelope to transform NIH-3T3 cells. The mutated tyrosine is part of a Y-X-M motif that is recognized by the phosphatidylinositol 3-kinase (PI-3K). The PI-3K initiates a cell-signaling pathway that appears to inhibit apoptosis and to be required for a number of mitogens during the G1- to S-phase transition of the cell cycle (167). PI-3K activates Akt (also known as protein kinase B). Akt has been shown to have several antiapoptotic properties, one of which is mediated by the phosphorylation and subsequent inactivation of the pro-apoptotic protein BAD (168,169).

We have developed an NIH-3T3-derived cell line (MP1) from a focus of JSRV-transformed cells. MP1 expresses the JSRV envelope. By western blotting analysis, we have detected phosphorylated Akt in MP1 cells but not in the parental NIH-3T3 cells (166). These data strongly point to the cytoplasmic tail of the JSRV transmembrane protein for being important for cell transformation and suggest a new mechanism of retroviral transformation (Fig. 5). Both PI3-K and Akt have been described as oncogenes transduced by retroviruses. In other words, they have been transduced by acutely transforming retroviruses. The catalytic subunit of PI-3K has been transduced by the avian sarcoma virus 16 (170), whereas Akt has been transduced by an ectropic murine leukemia virus (171,172). In addition, the PI-3K/Akt pathway has been shown to be involved in the induction of erythropoietin independence of erythroid cells after infection with Friend SFFV (173), further demonstrating that the activation of the PI-3K pathway can lead to cell transformation. Indeed, the PI-3K/Akt pathway has been shown to be activated in a number of solid human tumors, including lung cancer (174–177). The JSRV envelope needs to be expressed at high levels to be able to transform NIH-3T3 cells. Indeed, transformation is inefficient when the JSRV envelope is under the control of the JSRV LTR, a relatively weak promoter in NIH-3T3 cells (95). The necessity for high levels of virus expression to induce cell transformation in vitro might reflect what happens in JSRV-infected animals. JSRV infects a wide variety of lymphoid tissues and cells (99–101), but viral antigen is consistently detected only in differentiated lung epithelial cells (98). Thus, it appears that high levels of viral expression are achieved only in the cells targeted for viral transformation, where the JSRV LTR is specifically active (111).

Recent data (178) suggest that, in type II pneumocytes, the surfactant protein A (SP-A) regulates the production of pulmonary surfactant secretion via activation of the PI-3K/Akt pathway. SP-A increases transcription of another surfactant protein, SP-B, by increasing the activity of lung-specific transcription factors like HNF-3 (179). Thus, it might be hypothesized that JSRV expression in the transformed type II pneumocytes involves an autocrine loop, where lung-specific transcription factors activate the JSRV LTR; the resulting ENV expression leads to constitutive activation of the PI-3K/Akt pathway, which in turn activates a growth-stimulatory signal. Finally, it is possible that the TM of the JSRV envelope binds to an intracellular protein through its cytoplasmic tail, resulting in activation of a signal cascade. Our latest results favor this last possibility (166). By mutation of a single tyrosine in the cytoplasmic tail of the JSRV TM, we abolished the capacity of the JSRV envelope to transform NIH-3T3 cells. The mutated tyrosine is part of a Y-X-M motif that is recognized by the phosphatidylinositol 3-kinase (PI-3K). The PI-3K initiates a cell-signaling pathway that appears to inhibit apoptosis and to be required for a number of mitogens during the G1- to S-phase transition of the cell cycle (167). PI-3K activates Akt (also known as protein kinase B). Akt has been shown to have several antiapoptotic properties, one of which is mediated by the phosphorylation and subsequent inactivation of the pro-apoptotic protein BAD (168,169).

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We cannot exclude the possibility that HYAL2 is necessary for JSRV oncogenesis by giving the virus the opportunity to produce more viral particles and, consequently, having more chances to be transmitted from an OPA-affected animal to a healthy sheep.

Obviously, there might be differences between the mechanisms of cell transformation observed in immortalized rodent fibroblasts and those observed in vivo. Additional hits might be required besides the activation of the PI-3K/Akt pathway in natural settings. However, these results strengthen the fact that OPA can be useful in gaining an understanding of carcinogenesis in some forms of human lung cancer (107).

**A Retrovirus in Some Forms of Human Adenocarcinoma?**

Tobacco smoking is associated with the majority of human lung cancers (180). However, as we mentioned above, this association is less strong in adenocarcinomas (6–9), suggesting that other etiologic factors might be present.

At the molecular level, several steps forward have been made in understanding the events governing lung carcinogenesis. A detailed analysis of these events is beyond the scope of this review, and the reader is referred to excellent reviews published in recent years on this topic (181–183). It is, however, pertinent to mention that one of the most common genetic changes occurring in the pathogenesis of lung cancer is the LOH involving the short arm of chromosome 3 (181). Regions of DNA susceptible to LOH are likely to contain tumor suppressor genes. Homozygous deletions have been found in lung cancer cell lines, uncultured lung tumors, and premalignant lesions in the 3p21.3 region (163,184–189). It is interesting that the cellular receptor for JSRV, HYAL2, maps to this region (107); therefore, it is attractive to link, based on this gene, the oncogenesis in OPA with that in some human lung cancers (108). It is difficult, however, to reconcile the putative tumor suppressor activity envisaged for HYAL2 with the activation of the PI-3K/Akt pathway by the cytoplasmic tail of the JSRV ENV. At the moment, we cannot exclude the possibility that HYAL2 is necessary for JSRV oncogenesis, but, if it is, it would appear to favor, rather than to suppress, oncogenesis.

The unique epidemiologic features of bronchioalveolar carcinoma suggest that the causes of this carcinoma are different from those of other lung malignancies. A possible genetic predisposition to bronchioalveolar carcinoma has been suggested in some studies, including the almost simultaneous onset of the malignancy in identical twins (67,190,191). Familial predisposition, however, could also be explained by common exposure to carcinogens or infectious agents. A viral etiology for bronchioalveolar carcinoma has been postulated from time to time (7.8,192–194). For instance, the multiclonality of at least some multifocal cases of bronchioalveolar carcinoma might suggest an environmental carcinogen or an infectious agent (54). Human papillomavirus DNA has been detected in approximately a third of bronchioalveolar carcinoma cases (n = 22) (193). Of course, the similarities between OPA and bronchioalveolar carcinoma do not suggest per se a viral etiology for bronchioalveolar carcinoma. An intriguing finding, however, has been made recently by De las Heras et al. (195). Analysis of 249 human lung tumors by immunohistochemistry employing a rabbit antiserum toward the major capsid protein of JSRV revealed immunoreactive cells in 23% of the cases (Fig. 7). Among others, tumors included 129 bronchioalveolar carcinomas, 65 adenocarcinomas, and 41 squamous cell carcinomas. Positive samples included 39 (30.2%) of 129 bronchioalveolar carcinomas and 17 (26.2%) of 65 adenocarcinomas. It is interesting that no positive staining was observed in 51 other lung tumors or in 25 nontumor lesions or normal lung tissues. Thus, the possibility of a JSRV-related retrovirus associated with some form of bronchioalveolar carcinoma is attractive, although no other virologic or molecular data obtained thus far support this observation. The antigenic positivity observed might simply reflect cross-reaction with the JSRV antiserum of a protein whose expression is increased in the transformed cells. Alternatively, a human endogenous retrovirus (HERV) related immunologically to JSRV might be overexpressed in the neoplastic cells. The expression of some HERVs in human malignancies has been observed, for example, in testicular tumors, but it is not clear if this expression is etiologically related to the tumor (196–198).

**Fig. 6.** Hypothesized autocrine loop favoring jaagsiekte sheep retrovirus (JSRV) expression in transformed lung cells. Lung-specific transcription factors, such as HNF-3β, activate the JSRV long terminal repeat. The resulting envelope protein (ENV) expression leads to constitutive activation of the PI-3K/Akt pathway, which in turn enhances expression of lung-specific transcription factors. LTR = long terminal repeat; SP-A = surfactant protein A; PI-3K = phosphatidylinositol 3-kinase; HNF-3 = hepatocyte nuclear factor 3β.

**Fig. 7.** Immunohistochemistry of a lung section with human bronchioalveolar carcinoma. Brown staining represents positive reaction and has been obtained with the use of a rabbit antiserum toward the major capsid protein of jaagsiekte sheep retrovirus (the same as in Fig. 3). Original magnification x100. (Courtesy of Marcelo De las Heras (University of Zaragoza, Spain).
CONCLUSIONS

Studies on the oncogenesis of OPA can advance our understanding of the molecular mechanisms governing lung carcinogenesis. Future studies need to further elucidate the mechanisms of JSRV-induced cellular transformation by addressing the role, if any, of HYAL2 and to establish if activation of the PI-3K/Akt pathway and/or additional mechanisms are necessary to achieve transformation in vivo. In addition, the expression of the JSRV envelope as a transgene might be useful in obtaining even more convenient laboratory animal models for lung cancer. Studies on the molecular biology of JSRV should concentrate in further understanding the molecular basis of the viral tropism and on establishing the function of the orf-x gene.

In relation to human carcinogenesis, research should be directed at testing the hypothesis of the possible involvement of a retrovirus in the etiology of at least some forms of human adenocarcinoma. In any case, the mechanisms of tumorigenesis between OPA and some forms of human adenocarcinomas might have common themes even if they have a different etiology.

REFERENCES


Hughes SH, Shank PR, Spector DH, Kung HJ, Bishop JM, Varmus HE, et al. Proviruses of avian sarcoma virus are terminally redundant, co-


NOTES

1In a recent workshop on jaagsiekte sheep retrovirus (JSRV) held in Missillac, France, workers in the field agreed to call the disease induced by JSRV ovine pulmonary adenocarcinoma (OPA) instead of ovine pulmonary carcinoma (OPC) or sheep pulmonary adenomatosis (SPA).

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