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Sheep retrovirus structural protein induces lung tumours

Sarah K. Wootton^{*}, Christine L. Halbert^{*}, and A. Dusty Miller

Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, Washington 98109, USA

Abstract

Jaagsiekte sheep retrovirus (JSRV) causes a contagious lung cancer in sheep and goats, with significant animal health and economic consequences¹. The host range of JSRV is in part limited by species-specific differences in the virus entry receptor, hyaluronidase 2 (Hyal2), which is not functional as a receptor in mice but is functional in humans². Sheep are immunotolerant of JSRV because of the expression of closely related endogenous retroviruses^{3,4}, which are not present in humans and most other species, and this may facilitate oncogenesis. Here we show that expression of the JSRV envelope (Env) protein alone in lungs of mice, by using a replication-incompetent adeno-associated virus vector, results in tumours with a bronchiolo-alveolar localization like those seen in sheep. Whereas lethal disease was observed in immunodeficient mice, tumour development was almost entirely blocked in immunocompetent mice. Our results provide a rare example of an oncogenic viral structural protein, show that interaction of the viral Env protein with the virus entry receptor Hyal2 is not required for tumorigenesis, and indicate that immune recognition of Env can protect against JSRV tumorigenesis.

Oncogenic retroviruses are known to cause cancer by the acquisition and expression of host-derived oncogenes, by the insertional activation of host cell oncogenes or by the expression of auxiliary viral oncogenes such as the *tax* gene of human T-cell leukaemia virus. JSRV is a simple retrovirus (Fig. 1) that does not express a host-derived or auxiliary oncogene and can induce lung tumours in as little as 10 days⁵, a much shorter latency than typically found for the insertional activation of host oncogenes by other retroviruses. The mechanism of oncogenesis is unknown, but the JSRV Env protein has been found to transform cells in culture^{2,6–8}. One mechanism of transformation involves activation of the phosphoinositide-3-OH kinase (PI3K)/Akt pathway and is dependent on the presence of the cytoplasmic tail of Env^{8–10}, and the other involves Env binding to Hyal2, Hyal2 degradation, and activation of the RON receptor tyrosine kinase, which is normally suppressed by Hyal2 (ref. 11).

Further studies of Env oncogenesis in animals are limited by the difficulty and expense of experimentation with a contagious oncogenic virus in sheep and by the inability of JSRV to infect convenient rodent animal models such as mice. However, we have found that adeno-associated virus (AAV) vectors made with AAV type 6 capsid proteins (AAV6 vectors) can promote long-term gene expression in all epithelial cell types in mouse lung¹². To test whether Env alone would induce lung tumours we administered a mixture of 5×10^{10} vector genomes of an AAV6 vector that expressed Env (ARJenv; Fig. 1) and 5×10^9 vector genomes of an AAV6 vector that expressed human placental alkaline phosphatase (AP) (ARAP4; Fig. 1) (ref. 13) to the noses of lightly anaesthetized mice and monitored the mice for AP expression and tumour development. The ARAP4 vector was included to confirm that vector transduction had

Correspondence to: A. Dusty Miller.

Correspondence and requests for materials should be addressed to A.D.M. (dmiller@fhcrc.org)..

^{*}These authors contributed equally to this work

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occurred. We used 8-week-old immunodeficient (Rag2-knockout) C57BL/6 mice as recipients to avoid an immune response that might eliminate Env-expressing cells and because C57BL/6 mice are resistant to the development of spontaneous lung cancer¹⁴. Individual mice were killed at 2, 2.5, 5 and 6 months after vector exposure and their lungs were fixed and stained for AP expression. Lung tumours were present in all mice and increased in size and number with time (2 months, Fig. 2a, e; 6 months, Fig. 2b, f; Table 1). AP staining was visible in some tumours (dark blue/black stain in Fig. 2e, f) and a few tumours stained uniformly for AP (not shown), showing that occasional tumour progenitor cells were transduced by both vectors. The animal killed at 6 months was severely underweight (21 g versus 35 g each for two age-matched mice that received control AAV6 vectors) and was experiencing breathing difficulties and signs of distress that necessitated euthanasia. No tumour or evidence of disease was seen in animals treated identically apart from receiving an AAV6 vector (ARJenvF) that expressed a transformation-defective JSRV Env instead of the vector encoding the active Env (fewer than two tumours per cm² in histological sections of lungs of individual animals killed at 2, 2.5, 5 and 6 months), revealing a highly significant difference in tumour number between mice receiving Env and those receiving the control vector ($P = 0.01$; two-tailed t -test).

Tumour histology revealed papillary adenomas (Fig. 2c) progressing to adenocarcinoma (Fig. 2d) at later time points. The mouse tumour histology resembled human peripheral adenocarcinoma (Fig. 2g) and that of tumours arising in sheep after experimental infection with JSRV (Fig. 2h). All mouse tumours expressed surfactant protein C (SP-C) (Fig. 2i), a marker for alveolar type II pneumocytes. With the exception of occasional small areas of staining, tumours did not express Clara cell 10-kDa (CC10) antigen, a marker for bronchiolar non-ciliated Clara cells (Fig. 2j). This pattern resembles that seen for JSRV-induced tumours in sheep¹⁵ and indicates that the primary target for Env oncogenesis might be the type II pneumocyte, the progenitor of type I pneumocytes that line the alveoli. Tumours were not seen in large airways or in the noses of these mice, even though the ARAP4 vector was able to transduce these cells as measured by the expression of AP (ref. 12 and data not shown), and by the expression of spliced Env RNA of the expected size in these tissues (Fig. 3).

We next tested whether immune function might influence Env tumorigenesis in mice. We exposed normal 8-week-old C57BL/6 mice to a mixture of 5×10^{10} vector genomes of the ARJenv vector and 5×10^9 vector genomes of the ARAP4 vector, and killed two mice each at 2 and 4 months after exposure. None of these animals showed signs of disease. A few small tumours were found in one animal killed at 2 months, whereas the rest of the animals were tumour-free (Table 1). Staining of the lungs for AP revealed similar transduction by the ARAP4 vector to that in the identically treated immunodeficient mice described above. We tested the mice for serum antibodies against JSRV Env by measuring the ability of the serum to neutralize the infectivity of a retroviral vector bearing JSRV Env [LAPSN(PJ4)] (ref. 16). Serum harvested at 2.5 and 4 months from two mice receiving the ARJenv AAV6 vector reduced the titre of the JSRV retroviral vector by 50% at a 1:3,000 dilution and completely neutralized the JSRV vector at a 1:100 dilution, whereas a 1:20 dilution of serum harvested at 2.5 months from a mouse that received only the ARAP4 vector (5×10^{10} vector genomes) had no effect on the JSRV vector titre. These results show that normal C57BL/6 mice can mount a strong serum antibody response against JSRV Env expressed from the AAV6 vector. Taken together, our results indicate that Env tumorigenesis can be controlled by the normal immune system of these mice.

The availability of antiserum against JSRV Env allowed us to examine JSRV Env expression in tumours from the immunodeficient mice. Serum antibodies bound to tumours (Fig. 2k) and showed no binding to surrounding lung tissue (Fig. 2k) or to lungs of normal mice (not shown), indicating that the serum was specific for Env. Env staining was primarily localized to the cell membrane at the apical (airway) side of tumour cells (Fig. 2l), an appropriate position for

retrovirus budding into the airway. This staining pattern was similar to that for SP-C, which is secreted into the airway, although SP-C was more diffusely localized to the cytoplasm near the apical membrane (Fig. 2i and data not shown). JSRV-infected sheep show increased secretion of virus-containing nasal fluid, a process facilitated by oncogenic transformation and expansion of the secretory alveolar type II cell population, and this is likely to be the main route for virus transmission. The level of Env staining was also correlated with tumour size (not shown), suggesting that higher levels of Env expression result in faster tumour growth. We were unable to detect Env staining of individual cells in the lung outside the tumours, whereas AP staining was clearly visible in such cells. This may indicate that Env expression was limited to alveolar type II cells or simply that our detection method was not sensitive enough to detect low-level Env expression in cells outside tumours.

Here we have shown that the Env protein of JSRV is sufficient to induce lethal adenocarcinoma in mice, with tumour appearance, location and alveolar type II pneumocyte marker staining similar to those found in sheep exposed to JSRV¹⁵. We found no significant homology of the JSRV Env protein to cellular proteins by database searches, arguing against an oncogenic mechanism involving the viral acquisition of part or all of a cellular oncogene. Because of the role of Hyal2 in human epithelial cell transformation¹¹ we were concerned that mice might not provide a suitable model for Env oncogenesis because mouse Hyal2 does not bind JSRV Env¹⁷. However, our more recent experiments showing that Madin–Darby canine kidney (MDCK) epithelial cell transformation is dependent on the PI3K/Akt pathway and not the Hyal2 pathway⁸, and our inability to detect Hyal2 regulation of RON/Stk activity in fibroblasts¹⁸, encouraged us to test for Env tumorigenesis in mice. The fact that JSRV Env can induce tumours in mice shows that Env interaction with Hyal2 is not required for oncogenesis, although it may facilitate oncogenesis in other species.

AAV6 vectors promote efficient gene transfer and long-term gene expression in the lungs of young and adult animals. Their use provides some advantages over the use of transgenic mice engineered to study oncogenes, such as the ability to test new genes or combinations of genes rapidly, and the ability to introduce genes into a controllable number of cells at specific times without the possibility of leaky gene expression at earlier times during development. Avian retrovirus vectors have also been used in studies of lung cancer, but these require the use of transgenic mice that express the avian retrovirus receptor and, because the vectors infect only dividing cells, require infection of the lung in utero¹⁹.

The finding that JSRV Env can mediate the infection of human cells¹⁶ raised the possibility that JSRV or a related virus might cause cancer in humans. Furthermore, antibodies against the JSRV capsid proteins were found to react with about 30% of human bronchioloalveolar carcinoma and pulmonary adenocarcinoma samples but not with adenocarcinomas from other organs or with normal tissues²⁰. Attempts to identify such viruses by polymerase chain reaction (PCR) have been unsuccessful so far^{21,22}. Results presented here show that immune responses against JSRV Env can limit oncogenesis in species other than sheep, and such immune responses may provide one level of protection against JSRV oncogenesis in humans. □

Methods

AAV6 vectors

AAV6 vectors expressing either the native (ARJenv) or a carboxy-terminal Flag-tagged (ARJenvF) JSRV Env were constructed by inserting the respective cDNAs and upstream splicing signals from pSX2-Jenv¹⁶ and pSX2neo-JenvFlag^{10,17} into the ARAP4 AAV vector¹³ in place of the AP cDNA (Fig. 1). ARJenv and ARJenvF are identical with the exception of the Flag sequence, which is present only in ARJenvF. ARJenvF had very poor

transforming activity in cultured cells and was used as a negative control for tumorigenesis by the native Env protein. Virus was generated from the vector plasmids as described²³.

Vector administration to mice

Animal experiments were performed under guidelines approved by the Institutional Review Office of the Fred Hutchinson Cancer Research Center. Lightly anaesthetized mice received AAV vectors by the administration of drops to the nose, which were spontaneously inhaled. Mice killed at various times after vector administration were perfused by way of the heart with phosphate-buffered saline to remove blood from the lungs. Lungs were fixed in 2% paraformaldehyde in phosphate-buffered saline for 2.5 h at 22°C and were washed with phosphate-buffered saline. Endogenous AP was inactivated by incubation at 68°C for 1 h and lungs were stained for vector-encoded heat-stable AP as described²⁴.

Safety precautions

Vector production, vector administration to mice and harvesting of mouse tissues were performed in laminar-flow biological safety cabinets under BL2 containment using BL3 practices with careful attention to prevent aerosol generation. Workers were further protected by the use of particulate respirators. Agents determined to inactivate AAV vectors effectively (for example 1% bleach) were used for disinfection. Standard detergents and 70% ethanol were found to be relatively ineffective in the inactivation of AAV vectors.

Immunohistochemistry

Immunohistochemistry was performed on 4-µm sections of 2% paraformaldehyde-fixed paraffin-embedded tissues by following a standard avidin–biotin–peroxidase method. Cells with alveolar type II cell markers were detected with the use of two SP-C antibodies, Pro-C (provided by J. Whitsett) or anti-SP-C (Santa Cruz Biotechnology), and Clara cells were detected by using anti-CC10 antibodies (provided by J. Whitsett). After incubation with the primary antibodies and washing, the sections were sequentially incubated with biotinylated anti-rabbit IgG and the ABC-Elite reagent (Vector Labs). For immunohistochemical staining of tissues with anti-JSRV Env serum from ARJenv-transduced C57BL/6 mice, samples were incubated with unconjugated anti-mouse IgG and were then incubated with anti-JSRV Env serum or with control serum from an ARAP4-transduced mouse. After being washed, the sections were incubated sequentially with biotinylated horse anti-mouse IgG and the ABC-Elite reagent. In all cases 3,3'-diaminobenzidine (with nickel chloride enhancement for SP-C and CC10 staining) was used as the chromagen and sections were counterstained with methyl green.

Analysis of Env RNA expression in lung and airways

Total RNA was isolated from lung, from trachea and from epithelial tissue scraped from the inside of the nose, by using a Polytron tissue homogenizer (Brinkmann) and Trizol RNA isolation reagent (Invitrogen). Samples were treated with DNase and with reverse transcriptase in the presence of a 3' Env primer; they were then subjected to 30 cycles of PCR amplification with primers flanking the intron in ARJenv (Fig. 1). Products were subjected to electrophoresis in agarose gels and were stained with ethidium bromide.

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Figure 1.

Scale drawings of the JSRV genome and the AAV vectors encoding JSRV Env (ARJenv) and AP (ARAP4). The JSRV-coding regions are staggered vertically to indicate the three different reading frames that encode the proteins. Gag, core polyprotein; kb, kilobase; LTR, retroviral long terminal repeat; Orf-X, open reading frame of unknown function; Pol, polymerase; Poly (A) signal, polyadenylation signal; Pro, protease and dUTPase; RSV, Rous sarcoma virus; TR, AAV terminal repeat.

Figure 2.

Characteristics of lung tumours induced by JSRV Env in mice. **a**, Lung tumours (arrows) 2 months after vector exposure. **b**, Lung tumours at 6 months. **c**, Papillary adenoma surrounded by normal peripheral lung tissue (compressed near tumour) at 2.5 months. **d**, Adenocarcinoma at 6 months. **e**, Surface tumour at 2 months. **f**, Multiple tumours at 6 months. **g**, Human lung peripheral adenocarcinoma. **h**, Lung tumour in a lamb 2 months after JSRV infection. **i**, SP-C immunostaining. Tumour and type II cells in alveoli are stained but nearby airway is not. **j**, CC10 immunostaining of the tumour shown in **i**. Tumour is not stained but Clara cells lining the nearby airway are. **k**, JSRV Env immunostaining. **l**, Higher-magnification view of apical membrane Env staining. Arrows point to Env staining of apical membranes that face away from lung tissue and towards airways. Staining in **c–h** was with haematoxylin and eosin.

Figure 3.

Env RNA expression in mouse lung and airways 4 months after the administration of ARJenv vector. RNA samples were subjected to reverse transcription and PCR amplification with primers flanking the intron in ARJenv (Fig. 1). The predicted product for spliced RNA is 597 base pairs (bp), whereas that for vector DNA is 1,065 bp. The 597-bp product was detected for all airway tissues of mice exposed to ARJenv vector and for Env-transformed NIH 3T3 mouse cells but was not detected for normal mouse lung. Amplification of residual vector DNA in RNA prepared from the cell line that was not treated with DNase or reverse transcriptase (—RT lane) showed the expected 1,065-bp vector DNA band.

Table 1

Tumour number and area in lungs of mice receiving the ARJenv vector

Mouse strain	Time (months)	Tumours/cm ²	Tumour area (%)
C57BL/6 Rag2	2.0	44	3
	2.5	48	12
	5.0	92	25
	6.0	81	30
C57BL/6	2.0	5	<2
	2.0	<2	n.a.
	4.0	<2	n.a.
	4.0	<2	n.a.

Tumour number and area were determined in haematoxylin and eosin-stained histological sections of lungs (the total area examined for each mouse was 0.5-1.0 cm²) at the indicated times after vector exposure. Tumour areas were calculated from digitized images by using imaging software and are expressed as a percentage of the total lung area examined. Ignoring differences in the time of killing, tumour numbers in C57BL/6 Rag2 mice were significantly different ($P = 0.01$; two-tailed t -test) from those in C57BL/6 mice. n.a., not applicable.