ABSTRACT

Although certain neoplasms are unique to man, others occur across species. One such neoplasm is bronchioloalveolar lung carcinoma (BAC), a neoplasm of the Type II pneumocyte that affects humans, sheep, and small animals (dogs and cats). Human BAC occurs largely in nonsmokers. Sheep BAC is caused by the jaagsiekte retrovirus and is endemic and contagious. Feline BAC is neither endemic nor contagious and occurs sporadically and spontaneously in older purebred cats. In these respects, feline BAC is more closely similar to human BAC than sheep BAC (jaagsiekte) is. To study feline BAC further, we established the first immortal cell line (SPARKY) and transplantable scid mouse xenograft (Sparky-X) from a malignant pleural effusion of a 12-year-old Persian male with autopsy-confirmed BAC. SPARKY exhibited a Type II pneumocyte phenotype characterized by surfactant and thyroid-transcription factor-1 immunoreactivities and lamellar bodies. SPARKY’s karyotype was aneuploid (66 chromosomes: 38, normal cat) and showed evidence of genomic instability analogous to human lung cancers. p53 showed a homozygous G to T transversion at codon 167, the feline equivalent of human codon 175, one of the many hot spots mutated in the lungs of smokers. H-ras and K-ras were not altered. By reverse transcription-PCR, SPARKY lacked expression of retroviral JSRV gag transcripts that were present in the lungs of sheep BAC (jaagsiekte). Unlike human BAC xenografts, SPARKY-X retained its unique lepidic BAC growth pattern even though it was grown in murine s.c. tissues. This property may be related to the ability of SPARKY-X to up-regulate its surfactant genes (SP-A, SP-B, and SP-D). These studies of feline BAC may allow insights into the human disease that are not possible by studying human BAC directly.

INTRODUCTION

BAC3, unlike most other histological types of lung cancer, occurs naturally in two nonhuman species: sheep and cats (1, 2). In sheep, it manifests as an enzootic contagious disease called SPA or jaagsiekte (3–5). In cats, it occurs as a sporadic spontaneous disease of geriatric purebred species such as Persians and Himalayans (2). Because of these features, feline BAC is much more analogous to its human counterpart than is sheep BAC. The cause of SPA is an exogenous type B/D retrovirus called the JSRV, a slowly transforming retrovirus lacking oncogenes (6). The relationship of human BAC to smoking, either mainstream or side-stream smoke, has been weak (7). Because domestic cats are not known to be susceptible to JSRV nor often exposed to mainstream or side-stream smoke, a comparative study of feline BAC might be particularly enlightening. To take advantage of this intriguing, although little studied disease, we established the first immortal cell line (SPARKY) and transplantable scid mouse xenograft (SPARKY-X) from a malignant pleural effusion of a 12-year-old Persian male with autopsy-confirmed BAC. The comparative molecular and biological properties of feline BAC, its derived cell line and xenograft, are the subject of this study.

MATERIALS AND METHODS

Case of Feline BAC. A case of feline BAC was confirmed at autopsy of a 12-year-old male Persian cat that presented with shortness of breath and, antemortem, was found to have bilateral pleural effusions that, on pleuralcentesis, contained malignant cells. These cells were grown in cell culture.

Cell Line and Xenograft Establishment. The pleural fluid obtained by pleuralcentesis was spun at 200 × g to concentrate the cells, and the cells were placed in suspension culture containing MEM with 10% FCS (Life Technologies, Inc., Gaithersburg, MD) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) at 37°C in an air-5% CO2 atmosphere at constant humidity. After several hours, the cells in suspension attached to the tissue culture flask and formed monolayers. When confluent, cells were generally seeded to tissue culture flasks at 1/6 to 1/12 the confluent density or 0.5–1 × 106 cells/cm². In this manner an immortal cell line (SPARKY) was established. A xenograft (SPARKY-X) was generated by implantation of 106 cells/200 µl s.c. into the ventrolateral flanks of female scid mice, 4 weeks of age, and the injection sites were monitored for latency, tumorigenicity, and growth rate. When tumors emerged they were allowed to grow to 1–2 cm. Portions were processed for routine histopathology; other portions were removed and transplanted to new host scid mice with a number 10 trocar.

Comparative Cells and Tissues. Comparative cell lines and xenografts included a human inflammatory breast carcinoma xenograft, MARY-X, established by us (8, 9) and HMS-1/HMS-X, a human myoepithelial cell line and xenograft also established by us (10). Control tissues included normal feline lung tissue obtained from Sparky, sheep lung tissue with jaagsiekte (a gift of Massimo Palmarini, University of California Irvine, Irvine, CA), and murine scid lung tissue.

Ultrastructural and Immunocytochemical Studies. Feline lung tumoral tissues obtained at necropsy, in tissue culture, and portions of the xenografts were prepared for electron microscopy according to standard protocols (11). Immunocytochemical studies were undertaken on fresh-frozen sections of the feline BAC lung tumor, the derived xenograft (SPARKY-X) and a cell pellet of the derived cell line (SPARKY). Immunocytochemical studies used mouse monoclonal antibodies to human surfactant apoprotein A (IgG2b, clone PE-10; DAKO Corporation, Carpenteria, CA), thyroid transcription factor (IgG1, clone 8G7G3/1; DAKO Corporation), and p53 (IgG1, clone Pab1801; Oncogene Research Products, Boston, MA). The dilution of each of these antibodies was according to the manufacturer’s specifications. The second antibody was an affinity-purified peroxidase-conjugated sheep anti-mouse IgG (1/20 dilution). The peroxidase-conjugated secondary antibody was detected with diamobenzidine (Sigma Chemical Co., St. Louis, MO).

Cytogenetics. Metaphase spreads of SPARKY were prepared with a standard technique (12). To confirm feline origin, FISH with total feline genomic DNA as a probe was performed according to established methods (13, 14). Chromosomal abnormalities were characterized using a novel method of computer enhanced fluorescent R-banding (15). In this procedure, metaphase preparations of SPARKY were banded with 4′,6-diamino-2-phenylindole (DAPI), which binds A-T rich regions of DNA and with chromomycin A3, which binds preferentially G-C rich regions, and the DAPI image was displayed.
by the chromomycin image. This technique is similar to that used in comparative genomic hybridization (16). This process gave a high-resolution R-band pattern unique to each feline chromosome.

**RT-PCR and Sequencing Studies.** RT-PCR was performed to examine the presence and the mutational status of several genes implicated in both human and sheep lung cancer. These included *p53*, *H-ras*, *K-ras*, and JSRV gag.

For the *p53*, *H-ras*, and *K-ras* studies, total RNA extracted from SPARKY and normal feline lung as control was reverse transcribed using an oligo(dT) primer (Superscript, Life Technologies, Inc.) and then PCR amplified using primer pairs for the feline *p53* (17), *H-ras* (18) and *K-ras* (18) genes as follows: *p53* [P1] sense: 5’-GGCGCCTATGGTTTCCATTTAG-3’; [P2] antisense: 5’-CATCCAGTTGGCTTCTCTTTTT-3’; *K-ras* [P1] sense: 5’-GACGTAATATAACTTGG-3’; [P2] antisense: 5’-CTATAATGTTGGAATATC-3’. PCR reactions were performed in a Perkin-Elmer Gen Amp 2400 thermal cycler in 100 μl containing 1 x buffer [20 mM Tris-HCl (pH 8.4), and 50 mM KCl], 1.5 mM MgCl₂, 200 μM dNTPs, 200 nM primers and 5 Units of Taq polymerase (Boehringer-Mannheim, Indianapolis, IN). Cycling for the *p53* gene was 30 cycles of denaturation at 94°C (1 min), annealing at 60°C (1.5 min) and extension at 72°C (2 min). The *ras* genes were similarly amplified but used 35 cycles and a 50°C annealing temperature. DNA sequence analysis was performed on an ABI DNA Sequencer Model 377 using 0.1 μg of gel purified DNA as template and 3.3 pmol of sense or antisense primer. Amplified *p53* gene segments were also cloned into a Bluescript plasmid (Stratagene, La Jolla, CA) before sequencing studies. The feline *p53* primers amplified a 664-bp fragment of coding sequence (codons 97–318, exons 4–9) that contained the mutational hot spot codons (17, 19 and 21). The *H-ras* and *K-ras* primers amplified 285 and 289 bp, respectively, of exons I and II, which contained the mutational hot spot codons 12, 13, 59 and 61 (18).

For the JSRV *gag* studies, total RNA extracted from SPARKY was reverse transcribed using either random hexamers, 0.5 μg oligo(dT), or the antisense primer. Sheep lung tissues from jaagsiekte lungs served as positive control. For studies, total RNA extracted from SPARKY was reverse transcribed using an oligo[dT] primer and after PCR amplifications, the following primer pair amplified a 229-bp fragment. The H- and K-primers amplified 285 and 289 bp, respectively, of exons I and II, which contained the mutational hot spot codons 12, 13, 59 and 61 (18).

**RESULTS**

**Feline BAC.** Sparky (Fig. 1A), a purebred male Persian cat never exposed to either mainstream or second-hand smoke, exhibited, on postmortem examination, a left lower lobe consolidation (Fig. 1B) and smaller multicentric tumor nodules bilaterally. Pathological examination of the involved lobe revealed a primary lung BAC growing along alveolar septa, preserving alveolar spaces in the so-called lepidermal growth pattern of BAC (Fig. 1C). In some areas, the histological pattern was more glandular and papillary, which suggested dedifferentiation toward adenocarcinoma. Using the most recent WHO classification (22), BAC tumors are noninvasive. It appears that this feline equivalent may have started out as a true BAC but later developed an invasive component (as manifested by pleural invasion). In these latter areas, the tumor cells exhibited clear cytoplasmic vacuoles suggestive of surfactant accumulations (Fig. 1D). The primary feline lung carcinoma gave evidence of evolving from a transformed Type II pneumocyte. The tumor exhibited cytoplasmic and plasma membrane surfactant apoprotein A immunoreactivity (Fig. 2A), lamellar bodies ultrastructurally (Fig. 2B), nuclear p53 (Fig. 2C), and nuclear TTF-1 (Fig. 2D) immunoreactivities. No immunoreactivities were observed when the primary antibodies were omitted.

**Feline BAC Cell Line.** The carcinoma cells that were cultured from the pleural effusion almost immediately attached to the plastic culture dish and formed monolayers. These monolayers grew to a high cell density of 1 x 10⁵ cells/cm² and exhibited a doubling time of 16 h during log-phase growth. About 10% of the cells exhibited large cytoplasmic vacuoles (Fig. 3) that became more prominent when the line reached confluent density. These vacuoles probably represented surfactant stores. The established feline BAC cell line was named SPARKY. It has exhibited high plating efficiency and phenotypic stability over >100 passages. SPARKY is immortal.

FISH performed with labeled feline genomic DNA confirmed the feline identity of SPARKY. SPARKY was aneuploid by metaphase spread and detailed karyotype analysis (Fig. 4A). SPARKY exhibited 66 chromosomes (38, normal feline) with the feline male Y chromosome being identified (Fig. 4A). The fluorescent R-banding technique used to characterize cytogenetic aberrations resulted in high-resolution bands. This facilitated chromosomal counting and analysis. SPARKY exhibited genomic instability analogous to human lung cancers. Most abnormalities observed were numerical (Fig. 4A). There were some structural aberrations including an addition of a dark band on the p-arm of an A1 chromosome, an inversion and deletion in one copy of a C1 chromosome (Fig. 4A). SPARKY also exhibited the same positive TTF-1, surfactant apoprotein A and p53 immunocytochemical profile as that exhibited by its primary feline lung BAC.

DNA sequence analysis of PCR-derived DNA and plasmid-cloned DNA of the 664-bp fragment of the *p53* gene repeatedly identified a G:C to T:A transversion (Arg → Leu) at codon 167, the feline codon equivalent of human codon 175, one of the many hot spots mutated in lung cancers of smokers (17, 23). A silent mutation was also seen at codon 155 (C:G to T:A), but sequence analysis of feline RNA derived from normal SPARKY tissues revealed the same change from the published sequence (17). Hence, we concluded that this latter silent mutation was a polymorphism. Additional studies confirmed that both alleles showed the identical p53 mutation. The data from direct sequencing of the PCR-amplified cDNA showed no combination of nucleotides at the positions in question. Furthermore the cloned sequences of the plasmids all showed the same mutations (three were sequenced). We also cut the PCR-amplified band from both the Sparky normal and BAC cell line (SPARKY) templates with a restriction endonuclease that specifically cuts the normal p53 site but not the mutated site. This produced a large single band in SPARKY.
because of the mutated sequence and a smaller band in the normal. Hence we were dealing with a homozygous \( p53 \) mutation. Sequencing studies identified no missense mutations in either the \( K-ras \) or \( H-ras \) genes when compared with the normal sequence (18). For all these comparative studies, we used the published sequences of the feline \( p53 \) (17), \( H-ras \) (18) and \( K-ras \) (18) genes. The feline \( p53 \) sequence is registered under the accession number D26608 and is contained in the CSDB, DDBJ, EMBL, and National Center for Biotechnology Information (NCBI) nucleotide sequence databases. The feline \( H-ras \) and \( K-ras \) sequences are registered under the GenBank Accession nos. U62088 and U62089, respectively.

The RT-PCR for the 229-bp region internal to the JSRV\( gag \) gene (position 1598 to 1826) revealed, as expected, evidence of JSRV\( gag \) in SPA (jaagsiekte) lung tissues but absent JSRV\( gag \) in SPARKY (Fig. 4B). The sequence of this 229-bp region was identical to the \( gag \) sequence of the exogenous JSRV retrovirus, containing a \( ScaI \) site at position 129 of the 229-bp amplicon, which distinguishes the exogenous retrovirus from endogenous retroviral sequences (data not shown; Ref. 3).

**Feline BAC Xenograft.** SPARKY cells when injected s.c. into scid mice were 100% tumorigenic. When the cells were initially injected, the latency period was 12 weeks. When xenograft fragments

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Fig. 1. *A,* Sparky, in healthier times, was a purebred Persian cat; *B,* autopsy of Sparky revealed involvement of both lungs but with a complete lobar replacement in the left lower lobe characteristic of the “pneumonic” form of BAC; *C,* photomicrograph of Sparky’s lung shows the so-called lepidic pattern of BAC with transformed Type II pneumocytes growing along alveolar septa. Other areas were papillary; *D,* Other areas of Sparky’s lung show a solid pattern with clear cells. *A* and *B,* gross photographs; *C* and *D,* photomicrographs, H&E, ×250.
were subsequently transplanted, the latency period was only 1–2 weeks. SPARKY-X exhibited a robust growth rate reaching 1 cm in diameter by 3 weeks. SPARKY-X also exhibited the same immuno-cytochemical profile as SPARKY and the primary feline lung BAC. SPARKY-X has exhibited a stable phenotype for >15 transplant generations.

SPARKY-X showed increased surfactant gene expression of SP-A, SP-B and SP-D compared with SPARKY (Fig. 5A, B, and C). In control murine scid lung (because lung contains Type II pneumocytes), there were three strong bands of SP-A, the two characteristic SP-A monomeric bands (M₄ 32,000 and 38,000) and a M₆ 60,000 band. One major M₄ 32,000 band was detected in both SPARKY-X and SPARKY. However, there was an additional major band of M₄ 43,000–45,000 present only in SPARKY-X (Fig. 5A). Overall SP-A expression was increased in the feline xenograft compared with the feline cell line. With respect to SP-B, the results showed that there were two strong bands detected in both

Fig. 2. The feline tumor gave evidence of being of a transformed Type II pneumocyte origin, exhibiting strong surfactant apoprotein A immunoreactivity (A); ultrastructural evidence of numerous lamellar bodies within a single cell of SPARKY. Although not all cells exhibited multiple lamellar bodies, many did (B); p53 overexpression (C) and strong TTF-1 expression (D). A, anti-human surfactant apoprotein A, immunoperoxidase; ×150. B, lead citrate, uranyl acetate; ×54,715. C, anti-p53, immunoperoxidase; ×250. D, anti-TTF-1, immunoperoxidase; ×450.

Fig. 3. SPARKY, a cell line, of feline BAC origin was derived from the pleural effusion. Prominent vacuoles are in evidence in this cell line. Phase contrast, ×200.
SPARKY-X and SPARKY, a Mr 18,000 and a Mr 27,000–28,000 band of approximate equal intensity in the feline xenograft compared with the feline cell line. However there was an additional strong band at Mr 25,000 detected only in SPARKY-X (Fig. 5B). Therefore, overall SP-B expression was increased in the feline xenograft compared with the feline cell line. SP-C expression was not detected in either SPARKY-X or SPARKY. With respect to SP-D, there were two prominent bands detected in SPARKY-X, one representing the Mr 43,000 band characteristic of SP-D and a Mr 32,000 band (Fig. 5C). These two bands, however, were essentially absent in SPARKY. Overall SP-D expression was markedly increased in the feline xenograft compared with the feline cell line. Other non-BAC control tissues (MARY-X, HMS-1, and HMS-X) did not show evidence of surfactant gene expression. Dexamethasone had no effect on the expression of SP-A, SP-C (which was not expressed constitutively), or SP-D in SPARKY. Increasing dexamethasone concentrations decreased the levels of the Mr, 18,000 band of SP-B by ~50% in SPARKY (Fig. 5D).

Interestingly SPARKY-X also exhibited a lepidic (BAC) growth pattern even though it was grown s.c. in the absence of a lung environment (Fig. 5E). Obviously this meant that the lepidic growth pattern exhibited by SPARKY-X, and which is characteristic of BAC, is an inherent property of its transformed Type II pneumocytes and has nothing to do with the presence of preexisting alveolar spaces within lung parenchyma.

DISCUSSION

Feline BAC has been an understudied disease compared with its human and sheep counterparts. Because cats do not routinely undergo Chest X-ray or computed tomography screening, most feline BAC presents as late stage with multinodular and diffuse pulmonic involvement. The cause of feline BAC is unknown, and there is no known or suspected etiological agent. Until now, however, there has not been an established cell line and xenograft of feline BAC that has maintained its BAC autochthonous phenotype allowing further study. Our initial comparative oncological studies of feline BAC, its derived cell line, SPARKY, and xenograft, SPARKY-X, have allowed additional insights into this disease of transformed pneumocytes. By comparing SPARKY and SPARKY-X with normal feline lung tissues obtained from Sparky at autopsy, we were able to distinguish acquired tumoral mutations from constitutional polymorphisms. This normal feline tissue comparison will be invaluable for future allelotyping. Unfortunately, this normal feline DNA source is not continuing nor self-renewing as SPARKY and SPARKY-X are. Even if we had cultured normal feline fibroblasts from Sparky at autopsy, they would not have been immortal.

Although the etiologies of the BACs among different species may be different, the mechanisms of oncogenesis may be similar, or at least have overlapping molecular pathways, which make a comparative study worthwhile. Human BAC is a form of lung cancer the etiology and pathogenesis of which are controversial and whose link
to either mainstream tobacco smoking or secondhand smoking unproven, but both BAC and PAC have been increasing in frequency (7) in both smokers and nonsmokers (24). Some of the distinguishing pathological, biological, epidemiological, and perhaps etiological features of PAC/BAC include its peripheral location, its association with desmoplasia (scarring), its significant occurrence in nonsmokers, its comparatively high female:male ratio, and its high incidence of multifocality, which reflects its multiclonal origins (25). Because of the rising incidence of BAC and PAC in humans, any insights into the mechanisms of oncogenesis of the Type II pneumocyte in any species would be welcome.

The Type II pneumocyte seems to be a cell particularly susceptible to transformation by various mechanisms. Because, in sheep, the Type II pneumocyte is transformed by a slowly transforming retrovirus, JSRV (1, 3–5), and because cats are very susceptible to slowly transforming retroviruses (26) such as the feline leukemia virus, we investigated the presence of JSRV gag in SPARKY and found no evidence for it. Although we did not find JSRV gag sequences in SPARKY, it is possible that feline BAC is caused by other transforming viruses and that these viruses could be transmissible to humans. Although this is an outside chance, if true, it would make SPARKY a valuable resource to study these viruses. Because in humans the Type II pneumocyte is often transformed by alterations in p53 and/or ras (H-ras or K-ras), we investigated their status in SPARKY. p53 exhibited a G:C to T:A homozygous transversion (Arg → Leu) at codon 167, the feline codon equivalent of human codon 175, one of the many hot spots mutated in lung cancers of smokers (17, 23). The p53 mutation at codon 167 was present in both the primary feline BAC and in SPARKY but not in normal Sparky tissues, so it was not acquired as a result of the selection pressure of survival in culture, but was part of the tumorogenic process of feline BAC. Codon 167, the feline codon equivalent of human codon 175, is however a site at which mutations occur in lung cancer at a much lower frequency than many of the other hot spots of the p53 gene (27, 28). Sparky was not exposed to mainstream or sidestream smoke anytime during his lifetime. p53 alterations have been observed in both canine and feline mammary and hematopoietic neoplasms although these mutations are uncommon, occurring in only 10% of the neoplasms examined (17, 29). Sequencing studies identified no missense mutations in either the K-ras or the H-ras genes when compared with the normal sequence (18). K-ras and H-ras alterations are common in human lung adenocarcinomas, especially when these cancers are smoking related. K-ras mutations have also been detected in canine lung cancers (30). The vast majority of human lung cancers exhibit evidence of genomic instability manifested by aneuploidy and chromosomal alterations. These were both observed in SPARKY.

It is important to emphasize that although these molecular and genomic alterations undoubtedly contributed to the tumorigenic phenotype of SPARKY and SPARKY-X, neither the cell line nor the xenograft lost its Type II pneumocyte identity. This was manifested by the presence of lamellar bodies within the cytoplasm of SPARKY and intense nuclear TTF-1 and cytoplasmic and

and HMS-X (a human myoepithelial tumor), exhibit no expression; B, the pattern of SP-B expression was also more pronounced in SPARKY-X than in SPARKY with an additional major band of M, 43,000–45,000 detected in the xenograft. Control murine lung also exhibits strong expression but non-Type II pneumocyte xenografts, MARY-X (a human inflammatory breast cancer),

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Pulmonary surfactant is a mixture of phospholipids and proteins (SP-A, SP-B, SP-C, SP-D) which are thought to function by reducing surface tension at the air/fluid interface that lines the alveolar space (31–36). The secretion of surfactant undoubtedly alters this alveolar/space interface and may be linked to the BAC phenotype of lepidic spread. SPARKY-X manifested a lepidic phenotype, but it may also be related to the actual mechanism of carcinogenesis. SPARKY-SPARKY-X to up-regulate surfactant and grow in a lepidic fashion may be related. In any case, they are features of surfactant-X that are unique. Previous reports of established human and animal cell lines that express BAC features have been limited and most cell lines established from human pulmonary BAC neoplasms have lost their BAC identity. One common human pulmonary adenocarcinoma cell line, the A549, reported to be of Type II pneumocyte origin on the basis of structures resembling lamellar bodies, failed to express surfactant, and its designation as a BAC cell line proved to be erroneous (37). Gazdar et al. (37) developed a group of human cell lines with Type II pneumocyte markers, ultrastructural features and surfactant expression, some of which manifested a tubulopapillary morphology when grown as xenografts, but not a true lepidic growth pattern (defined by single cell spread along spaces). The loss of the ability of human BAC xenografts to grow in a lepidic fashion has been a deterrent to unraveling the BAC phenotype.

Not only is surfactant expression possibly linked to the BAC phenotype, but it may also be related to the actual mechanism of oncogenesis. Although the exact mechanism by which JSRV transforms Type II pneumocytes into sheep BAC (jaagsiekte) is not known, a recent report observed that a lung tumor cell line (JS7) derived from sheep BAC, contained the jaagsiekte provirus integrated into the SP-A gene (38). This finding implies a relationship between JSRV and surfactant in the pathway of oncogenesis. Because surfactant potentially is such a central player in BAC oncogenesis and pathogenesis, the properties of SPARKY/SPARKY-X to up-regulate surfactant and grow in a lepidic fashion makes it unique as a global model of BAC across species.

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We dedicate this study to the memory of Sparky, a Persian cat who brought his owners much joy.

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Comparative Oncological Studies of Feline Bronchioloalveolar Lung Carcinoma, Its Derived Cell Line and Xenograft

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