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Development of a swine model of secondary liver tumor from a genetically induced swine fibroblast cell line

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Abstract

Aim. Metastatic disease is the most common liver tumor. Although alternative therapies have been developed for non-surgical candidates, those therapies lacked ideal testing prior to clinical application because of a paucity of large animal models. The purpose of the present study was to develop a model for secondary liver tumor in a large animal. *Material and methods.* Fibroblasts were isolated from swine ear lobules and then transfected with amphotrophic retroviruses encoding human or murine genetic material (hTERT, $p53^{DD}$, cyclinD-1, $CDK4^{R24C}$, Myc ^{T58A}, Ras^{G12V}). Transformed cell lines were finally inoculated subcutaneously (s.c.) into: 1) immunodeficient mice (nude), 2) immunocompetent mice (wild type), 3) immunosuppressed swine (under tacrolimus or corticosteroids), 4) immunocompetent swine, and 5) into the liver and portal circulation of swine under steroid-based immunosuppression. *Results.* In the murine model, tumor growth was evident in 100% of the nude mice ($n=5$), with a peak size of 20 mm (15.22 ± 4.5 mm; mean $\pm$ SD) at the time of sacrifice (3 weeks). Tumor growth was evident in 71% of the wild mice ($n=21$), with a peak size of 7.8 mm (4.19 ± 1.1 mm) by the third week of growth. In the swine model, tumor growth was evident in 75% (3/4 ears; $n=2$) of swine under tacrolimus-based immunosuppression versus 50% of swine under steroids-based immunosuppression ($n=2$). Tumor growth was slow in two animals, while in one animal the tumor was larger with a peak growth of 42 mm at 3 weeks. The tumor pattern in the ear lobules was characterized by slow growth, with a peak size of 6–8 mm in the immunocompetent swine at 3 weeks. All tumors were shown to be malignant by histology. In contrast, inoculums of the transformed fibroblast cell line in swine livers showed no evidence of tumor growth at 3 weeks. *Conclusions.* Development of a transformed swine fibroblast cell line was successful, resulting in an *in vivo* malignant tumor. Cell line inoculums had tumorigenic properties in nude mice, wild-type mice, and immunosuppressed swine, as judged by uncontrolled cell growth, invasion of surrounding tissue, neoangiogenesis, and invasion of normal vasculature, resulting in the formation of tumor nodules. Such properties were not observed in swine upon inoculation into the liver/portal circulation.

Key Words: *Cancer models, liver tumors, secondary liver tumors, swine*

Introduction

The most common etiology of a liver mass is metastatic disease, usually from a colorectal primary tumor. Approximately 75,000 patients per year are diagnosed with metastatic liver disease in the USA [1,2]. Only 20% of these patients can have a curative surgical approach because of the extension of the malignant process or because of a medical condition that prohibits surgery [3–6]. Alternative treatment modalities have been developed. Ablative therapies include radiofrequency ablation (RFA), microwave ablation, and cryoablation. Other therapies for the control of hepatic tumor growth include chemo-embolization (TACE) and radio-embolization [5,7–9]. Nevertheless, all techniques have produced inconsistent results [3,7–11]. The purpose of the present study was to develop a model of secondary tumors of the liver in a large animal. The swine model offers the privilege of anatomical, metabolic, and physiological proximity to the human and, if developed, it may help us understand the efficacy of the various ablative modalities under diverse conditions. Development of a similar animal model by the implantation of human cell lines into swine livers has been unsuccessful [12].

Here, we describe implantation of a genetically defined transformed dermal fibroblast cell line from swine into: (i) nude and wild-type mice, to ensure the

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cell line's tumorigenic potential, and (ii) immunosuppressed and immunocompetent swine, to observe its biological behavior. Inoculation into the nude mice and immunosuppressed swine was consistent with development of tumor growth and neoplastic behavior. Tumors manifested in uncontrolled growth with invasion of surrounding tissues, neoangiogenesis, vascular invasion, and tumor thrombus formation. Inoculation of fibroblast cell line into wild-type mice and immunocompetent swine was characterized by slow growth, limited invasion to the surrounding tissue, poor neoangiogenesis, and a paucity of vascular invasion.

Material and methods

Swine cell line

Dermal fibroblasts were isolated from swine, cultured, and then transfected with human and murine proto-oncogenes and mutated tumor suppressor genes, as previously described by Adam et al. [13]. Briefly, fibroblasts in culture were transfected with a replication-deficient retroviral vector encoding for six human or murine genes: hTERT, cyclin D1, $CDK4^{R24C}$, Myc^{T58A}, RAS^{G12V}, and $p53^{DD}$. The resulting transformed fibroblasts were then analyzed for the expression of introduced genes by reverse transcriptase-polymerase chain reaction (RT-PCR) techniques [13]. Cells with the full expression of the genetic material were frozen. Inoculums were thawed in a 37°C water bath, washed in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Cleveland, Oh., USA), and cultured in a 75 cm^2 culture flask (Becton Dickinson, Rockville, Md., USA) containing DMEM, 10% fetal bovine serum (Invitrogen, Cleveland, Oh., USA) and 1% penicillin-streptomycin as media (American Type Culture Collection, Manassas, Va., USA). Confluence was reached at 37°C, gassed with O_2: CO_2, 95: 5% by 4 days in a cell incubator (NAPCO CO2 6000; Cole-Parmer Instrument Company, Vernon Hills, Ill., USA). Trypsin-EDTA 0.05% (Invitrogen, Cleveland, Oh., USA) dissolved in pre-warmed media was added to the culture flask for 10 min to obtain cells in suspension. Cells were then centrifuged at 1500 rpm for 10 min at 4°C. Re-suspended cells in 10 ml of phosphate buffer solution (PBS) (Invitrogen, Cleveland, Oh., USA) were counted in triplicate and recorded as number of cells/ml. An aliquot of the injected inoculums was saved for: (i) confirmation of cell concentration administered and (ii) for cell viability by Trypan Blue exclusion method. Consistently, cell concentration was $(1.03 \pm 0.24) \times 10^8$ cells/ml and cell viability was $>90\%$.

RT-PCR on cell lines

RT-PCR techniques were conducted on the initial cell line (6510-6gene) as well as on the cells isolated from the growing tumor (pig8rt) after inoculation into swine ear to compare for the tumorigenic expression at second pass. RNA was isolated using the RNAazole B reagent (Tel-Test Inc., Friendswood, Tx., USA), then further purified by the addition of chloroform and centrifugation. The RNA pellet was reverse-transcribed using Omniscipt reagents (Qiagen, Valencia, Calif., USA) with oligo dT primers (Invitrogen, Carlsbad, Calif., USA). Reverse-transcription reactions, incubated at 37°C, were set up for each cell line. The resulting cDNA was used to verify the absence or presence of expression of specific transgenes by PCR amplification with one primer specific to the transgenes and another specific to a transcribed region of the pBABE plasmids as noted: 5′-TGGCT GTGCCACCAAGCATT and 5′-TTTCCACACCT GGTTGC (hTERT), 5′-GCTCACTCCAGCTACC TGAA and 5′-ATGCCTTGCAAAATGGCG ($p53^{DD}$), 5′-AACATGGACCCAAGGCC and 5′-TTCTGCC TGCTGGGGAG (cyclin D1), 5′-GGTGGTACCT-GAGATGGA and 5′-TAGCTTGCCAAACCTA-CAGG ($CDK4^{R24C}$), 5′-ACGAGCACAAGCTCACC and 5′-TTTCCACACCTGGTTGC (c-Myc^{T58A}) and 5′-GCACGCACTGTGGAATCT and 5′-TAGCTT GCCAAACCTACAGG (H-Ras^{G12V}). Five microliters of the cDNA was added to the appropriate transgene specific primer and the PCR reaction mixture including ddH_2O, dNTPs, Redman Taq buffer, DMSO, and Redman Taq polymerase (Invitrogen, Carlsbad, Calif., USA). The reaction was allowed through a thermocycler with the following settings: 1 cycle at 94.0°C for 5 min followed by 25 cycles at 94.0°C for 30 s, 55.0°C for 1 min, then 72.0°C for 30 s, and one last cycle at 72.0°C for 10 min. The amplified cDNA was finally held at 4°C awaiting gel electrophoresis. Twenty-five microliters of each PCR sample was directly loaded into the wells of 2% agarose gel and run at 100 volts for optimal band separation. The gel was then checked for band expression.

Animal studies

In order to characterize the tumorigenic potential of the transformed fibroblasts, 1×10^6 transformed cells in 0.05 ml of PBS were inoculated s.c. into the abdominal wall of 20–30 g xenograft male outbreed nude mice ($n=5$) (Taconic #NCRNU-M, Germantown, N.Y., USA) and CD57 wild-type male mice ($n=21$) (Jackson Lab, Bar Harbor, Maine, USA). The procedures were performed under sterile conditions. The site of injection was inspected daily for 1 week to check for infection or bleed. The growth of the inoculums was measured weekly in all animals. Five wild-type mice were killed at time-points 3, 6, 8, and 12 weeks after injection. All nude mice were killed at 3 weeks due to rapid growth of the tumor. Mice were maintained in standard conditions of temperature, humidity, day/light cycle, and food (Harlan

Teklad Animal Diets and Bedding, Madison, Wisc., USA); water was given ad libitum. Mice were euthanized by cervical dislocation, and then the abdominal wall was dissected and fixed in 10% formaldehyde (Fischer Scientific SF93-4, Florence, Ky., USA). H&E and Masson's Trichrome staining slides were prepared and submitted for morphometric studies

Studies in swine (Yorkshire Swine, Pine View Farms, Valley City, Oh., USA) were performed by inoculation of the transformed fibroblast cell line into the ear lobules and also the liver/portal circulation. 1×10^8 cells suspended in 1 ml of PBS were inoculated s.c. into three groups of swine: (i) tacrolimus-based immunosuppressed animals ($n = 2$), (ii) steroid-based immunosuppressed animals ($n = 2$), and (iii) immunocompetent animals ($n = 2$). Tacrolimus (Prograf®, Novartis, N.J., USA) was given orally every 12 h at a dose of 0.2 mg/kg/day. Treatment started 1 week prior to cell line inoculation. Trough levels in blood were checked twice per week by ELISA (Chemistry Laboratories, University Hospitals-Case Medical Center) (Figure 1). A second group of swine was provided with a single intravenous dose of 250 mg of methyl-prednisolone (Solu-Medrol 125 mg/2 ml, UPJohn, Kalamazoo, Mich., USA) at the time of cell implantation.

Swine were sedated with Ketamine 20 mg/kg prior to general anesthesia. A mixture of Isofluorane: O_2 1:99% was administered while the animals underwent a 4 cm midline incision under sterile conditions. 1×10^8 cells were needle-injected into three different sites in the right liver and intravenously into the portal circulation. Hepatic sites of injection were tagged by non-resorbable suture (SurgiPro5:0's U.S. Surgical Corp., New Haven, Ct., USA). Tumor growth in swine was monitored by follow-up of ear lobule inoculums and weekly liver ultrasound. At 3 weeks, liver tissue and ear lobules were collected, fixed with 10% formaldehyde and H&E, and Masson's Trichrome stained slides were prepared. The sedated swine were then euthanized by an IV sodium pentobarbital overdose. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) according to Case Western Reserve University guidelines for animal research.

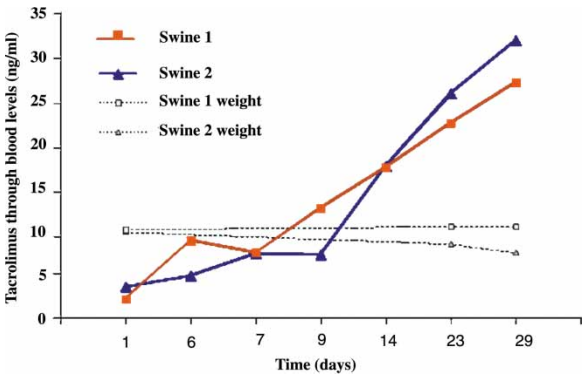

Figure 1. Tacrolimus levels in swine serum: Through levels of tacrolimus in the serum of swine before and after implantation of fibroblast cell line inoculum into ear lobules.

Histopathological evaluation

Blinded slides were assessed by three authors for evidence of lymphocytic infiltration, extent of central necrosis, neoangiogenesis, and tumor invasion. Three digital records from representative areas were saved from each animal in each group using a Digi3 Digital Binocular Microscope (International Falls, Minn., USA). Digital images were printed and then blind graded for lymphocytic infiltration (grade 0 = <10 lymphocytes per field, grade 1 = 10–30 lymphocytes per field, grade 2 = >30 lymphocytes per field; ×40 magnification), extent of central necrosis (grade 0 = no tumor necrosis, grade 1 = <25% tumor necrosis, grade 2 = 25–50% tumor necrosis, grade 3 = >50% tumor necrosis; ×20 magnification), neoangiogenesis (grade 0 = no evidence of new vessel formation within the tumor, grade 1 = ≤5 vessels per field within the tumor, grade 2 = >5 vessels per field within the tumor; ×40 magnification), and tumor invasion (grade 0 = no tumor invasion, grade 1 = tumor invasion to surrounded tissues, and grade 2 = tumor invasion to native vessels with tumor thrombus formation; ×40 magnification) (Figure 2). For purposes of lymphocyte infiltration and neoangiogenesis evaluation, slides were submitted for immunohistochemical staining for CD3 and CD34 cell surface proteins; CD3 is a receptor specific to all T lymphocytes, while CD34 is a hematopoeitic stem cell marker indicative of neoangiogenesis. All immunohistochemical stains where conducted in the clinical laboratories at the University Hospitals Case Medical Center (Cleveland, Oh., USA) with the use of a horseradish peroxidase labeled system, Envision+ (Dako, Carpinteria, Calif., USA). This method uses an unlabeled primary polyclonal antibody to CD3 (ref. #A0452, Dakocytomation, Carpinteria, Calif., USA) and a monoclonal antibody to CD34 (ref. # M7165, Dakocytomation, Carpinteria, Calif., USA), coupled to a secondary horseradish peroxidase labeled polymer conjugated to antimouse immunoglobulins. Positive and negative controls were performed simultaneously.

Statistical analysis

Mean values are presented with standard deviations (mean ± SD). Student's *t*-test (two-tailed) was used to calculate the significance of difference in the mean values (http://www.physics.csbsju.edu) and Fisher's exact test to calculate the significance of categorical percentage data differences (http://www.exactoid.com/fisher). A p-value < 0.05 was considered statistically significant.

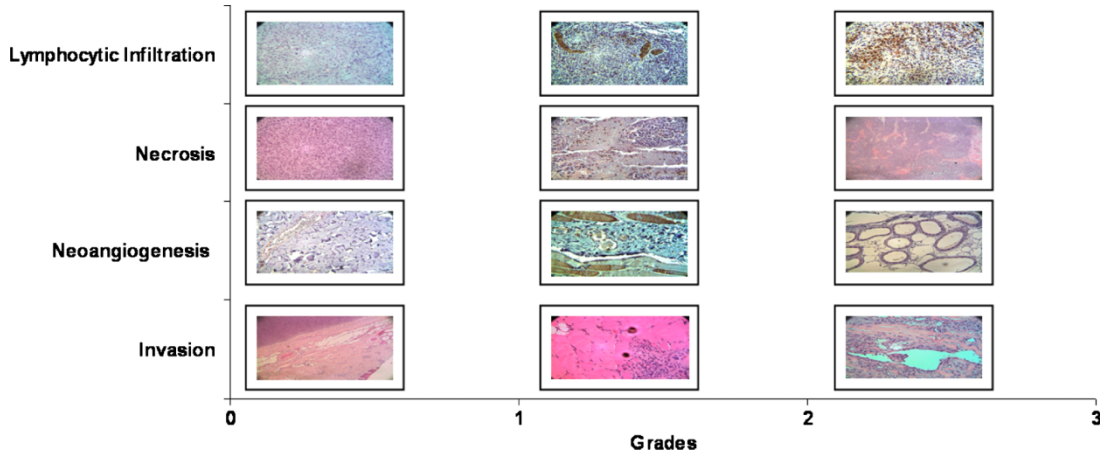

Figure 2. Histological grading system for fibroblast cell line growth: Histologic findings of s.c. inoculums into the abdominal wall of nude and wild mice, and into the ear lobules of immunosuppressed and immune intact swine. Lymphocyte infiltration assessed by anti-CD3 immunostaining is graded into grade 0 = <10 lymphocytes per field, grade 1 =10–30 lymphocytes per field, grade 2 = >30 lymphocytes at high power field; ×40 magnification. Extent of central necrosis is rated by grade 0 =no tumor necrosis, grade 1 = <25% tumor necrosis, grade 2 =25–50% tumor necrosis; ×20 magnification. Neoangiogenesis confirmed by anti-CD34 stained slides is graded 0 if there is no evidence of new vessel formation within the tumor, grade 1 = ≤5 vessels, grade 2 = ≥5 vessels per high-power field; ×40 magnification. Tumor invasion is defined by grade 0 if the tumor is confined to its capsule, grade 1 =tumor invasion to surrounded tissues, and grade 2 =tumor invasion to native vessels with tumor thrombus formation at high-power field; ×40 magnification.

Results

RT-PCR of the cell lines

Transformed fibroblasts isolated from the ear inoculums expressed genes including $p53^{DD}$, cyclin D1, $CDK4^{R24C}$, c-Myc^{T58A}, and H-Ras^{G12V}, but not hTERT when compared to the mother cell line. The hTERT RNA was slightly degraded in the transformed fibroblast 6510-6 gene line, and does not appear to be expressed in the cells extracted from the pig8rt tumor growth (Figure 3).

Characterization of the tumor in the murine model: Nude versus wild

Tumor growth was documented in 100% (5/5) of the nude mice versus 71% (15/21) of the wild CD57 mice at 3 weeks ($p < 0.02$; Student's *t*-test). Peak tumor growth in nude mice was evident at 3 weeks, when most of the mice were showing signs of sickness, reaching a mean size of 15.22 ± 4.5 mm, at the time of sacrifice (Figure 4). The biological behavior of the tumor was characterized by uncontrolled proliferation, dense angiogenesis, and local invasion. Tumors in the nude mice had a pseudo-capsule formed by connective tissue; some extended beyond the s.c. layer, invading and adhering into the adjacent peritoneal layer and reaching the bowel. In a 3-week span, tumors progressed to outgrow their vascular supply, resulting in a central necrotic zone. In contrast, wild-type mice showed slow growth of the inoculums. Tumor growth was 4.19 ± 1.1 mm at 3 weeks after implantation. After 3 weeks, most tumors became non-palpable, though they remained histologically detectable in 60% of the immunocompetent rodents (Figure 4).

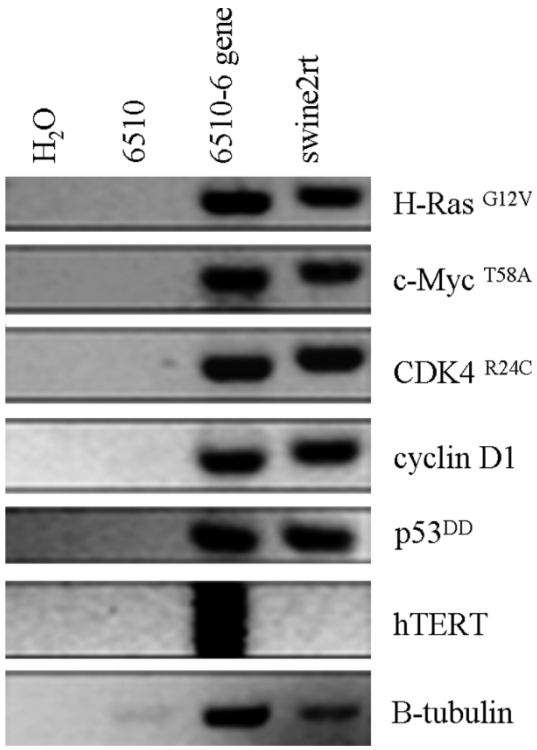

Figure 3. The expression of mutations of genetically induced fibroblast cell line before and after inoculation by RT-PCR: the expression of H-Ras^{G12V}, c-myc^{T58A}, $CDK4^{R24C}$, cyclin D1, $p53^{DD}$, and hTERT in the primary pig fibroblast cell line (6510) is compared to the initial transformed pig fibroblast cell line (6510-6 gene), and to the cell line derived from the tumor removed from the ear of the injected swine (swine2rt). The water lane served as a control to ensure that there was no reagent contamination giving us false-positive bands. The beta-tubulin served as a loading control ensuring that the RNA extract from the cells was not degraded and was isolated properly. The tumorigenic expression appears similar in the initial fibroblast cell line and the post-inoculation cell line except for the expression of hTERT, which did not express in the tumor isolate.

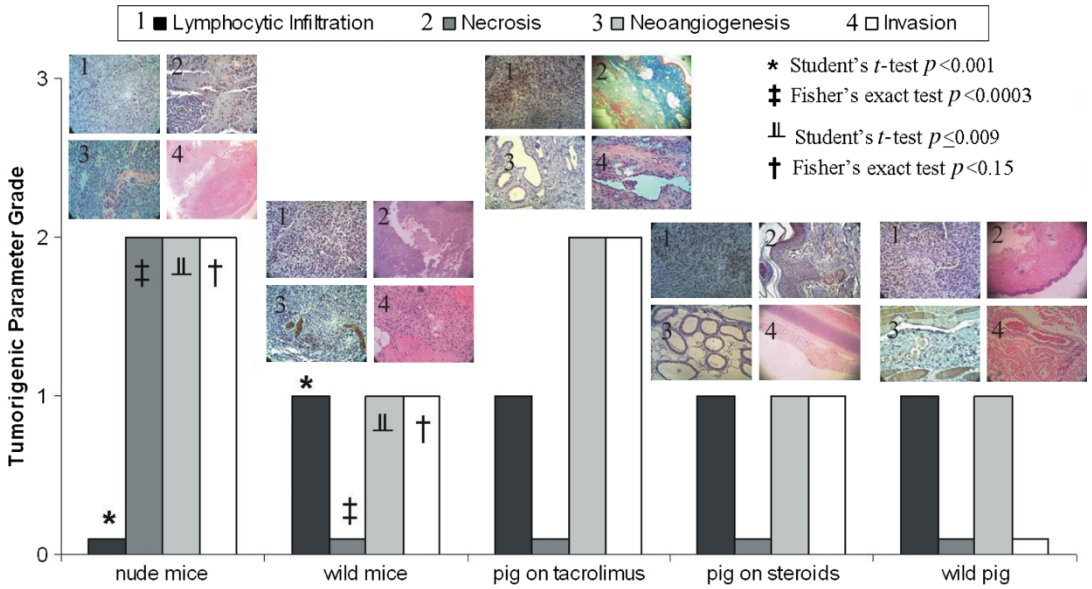

Figure 4. Histologic grading of the genetically defined tumorigenic swine cell line. Cell lines were inoculated into the abdominal wall of nude and immunocompetent mice as well as into ear lobules of swine immunosuppressed with tacrolimus, with corticosteroids, or that were immunocompetent. Inoculums were evaluated at 3 weeks in all animals.

Cell line behavior in the swine model: immunosupressed (tacrolimus and steroids based) versus immunocompetent

In swine, the transformed fibroblast cell line had a different pattern of growth depending on the degree of immunosuppression and site of the inoculum. Tumor growth was evident in 75% of ears lobules in the tacrolimus-based immunosuppressed swine, in 50% of ear lobules in the steroid-based immunosuppressed swine, and in 25% of ear lobules in the immunocompetent animals. Tumor growth was evident in the tacrolimus-exposed ear lobules, with a peak growth of 42 mm at 3 weeks. In contrast, tumor growth was slower in the ear lobules of steroid-based immunosuppressed swine (6.17 ± 3.04 mm) and in ear lobules of immunocompetent swine (8 mm). The biological behavior of the cell line growth was similar in both immunosuppressed groups of swine; we observed increased cellular proliferation, with regional invasion into the deeper cartilaginous and muscle layers, neoangiogenesis, invasion of native vessels with tumor thrombus formation (Figure 4). The tumor growth pattern in the immunocompetent group was characterized as poor, with a lack of neoangiogenesis and formation of a pseudo-capsule of dense connective tissue with various degrees of lymphocyte infiltration. Upon inoculation of the tumor cell line into the liver and portal circulation, no tumor growth could be detected. The development of a fibrotic process with various degrees of inflammation was noted without any sign of malignant growth.

Grading of the cell line potential for lymphocytic infiltration, necrosis, neoangiogenesis, and tumor invasion

We observed a different lymphocytic response and a superior potential for necrosis, neoangiogenesis, and tumor invasion in the nude mice compared to the wild-type mice (Figure 4). While the nude mice lacked a lymphocytic response (athymic mice), the wild type presented a robust immune reaction (26 ± 6 lymphocytes per high-power field; $\times 40$ magnification) (Student's t-test; p-value < 0.001). Necrosis was also observed in 4/5 of the nude mice but not in any of the wild-type mice. For the neoangiogenic potential, nude mice showed more than 5 vessels per high-power field (grade 2) versus no evidence for new vessel formation in the wild-type mice (Student's t-test; p-value < 0.009). Tumor in 2/5 of the nude mice extended into the peritoneal space, while in only 2/21 of the wild-type mice did the tumor extend beyond the pseudo-capsule and into the stomach (Fisher exact test, 2-tailed; p-value < 0.15).

Tumor growth in the swine model shared a similar lymphocytic infiltration despite the varying degrees of immunosuppression. In addition, tumors in the swine presented no evidence of necrosis. Tacrolimus-treated animals differed in both the observed neoangiogenesis and in the degree of tumor invasion compared to the steroid-immunosuppressed and immunocompetent swine. The tumor in swine on tacrolimus demonstrated neoangiogenesis and vascular invasion. This was not observed in the immunocompetent or steroid-treated animals (Figure 4).

Discussion

The purpose of these studies was to develop a model of secondary liver tumor using a genetically defined allogenic fibroblast cell line from swine. A transformed fibroblast cell line was grown in culture [13]. S.c. inoculums of the cell line showed typical malignant behavior in nude and wild-type mice. A

similar pattern of growth was observed upon inoculation into ear lobules of tacrolimus-immunosuppressed swine, less so when animals were treated with steroids, and to an even lesser extent in ear lobules of immunocompetent swine. No malignant growth resulted from direct inoculation of the transformed cells into the liver parenchyma or through the portal circulation in steroid-treated swine.

A model of secondary liver tumors in a large animal should ideally express specific characteristics. The animal model must resemble the human scenario in terms of size, anatomy, and physiology. The tumor should be easy to develop and reproduce, with a highly predictable pattern of growth. Finally, the process of reproducing a tumor model in a large animal must be financially accessible and cost effective. In our case, we were successful at developing a reliable and reproducible s.c. fibroblast tumor model only when swine were tacrolimus-treated. Tacrolimus (FK506) acts directly on the T-lymphocytes by binding to the FK-binding protein (FKBP) and competitively inhibiting calcineurin, a phosphatase that is active only when bound to calcium and calmodulin [14]. As a result, T cells do not produce IL-2, which is necessary for full T-cell activation [14]. Tacrolimus-based immunosuppression of swine, however, raises logistic and financial challenges. Animals must ingest the required dose every 12 h. Some authors have achieved adequate levels by placement of a percutaneous gastrostomy tube [15]. In addition, the cost of this immunosuppressant regimen lies in the range of 500 US dollars per week with an additional 150 dollars per blood level measurement [16]. To limit the experimental cost with no compromise to the higher aim, tacrolimus was substituted for corticosteroids. Corticosteroids exert their effects on cells involved in immune and inflammatory responses primarily by modulating the transcription of a large number of genes, subsequently affecting the function of both T and B cells, and macrophages [17–19]. In addition, corticosteroids have been implicated in promoting apoptosis of T lymphocytes [20]. Swine were then treated with a single injection of methylprednisolone at the time of inoculation, at a dose of 15 mg/kg and a total treatment cost well below 50 US dollars. However, tumor growth was achieved in only 50% of ear lobule inoculums and in none of the swine liver inoculums.

The transformed fibroblast cell line revealed malignant behavior ranging in severity depending on the species, the animal's immune status, and the site of inoculation. The inoculums were more successful in the murine model versus the swine. The tumor not only grew in a higher percentage of either nude or wild-type mice, but also peaked at a higher rate, reaching proportionally larger sizes despite the number of cells in the inoculums relative to the animal size. The tumor reached a peak of 20 mm in the nude mice from an initial inoculum of 10^6 cells, and hardly 8 mm in the steroid-treated swine from an initial inoculum of 10^8 cells at 3 weeks. Furthermore, tumor aggressiveness was markedly stronger in the immunocompromised animals compared to the immunocompetent animals. Tumors grew at a faster rate replacing the abdominal wall of the nude mice with invasion of the abdominal cavity. Tumor biological behavior was altered by the immune regimen; the inoculums grew in proportion to the immune-suppression status of the animal (i.e. tacrolimus 42 mm versus steroids 8.32 mm, versus immunocompetent 6 mm). As to how the immune response differed upon inoculation into the liver versus the ear lobules remains a matter of speculation. Perhaps the high perfusion rate to the liver, as well as the presence of Kupffer cells, provided this organ with more robust defense against the nesting of tested malignant cells relative to the s.c. area of the ear lobule or the abdominal wall [21].

In response to an antigenic encounter, the immune system generally provides both a non-specific and a specific response. When an antigen crosses a defense barrier such as the skin, a non-specific immune response is elicited, resulting in an inflammatory exudate with activation of macrophages, neutrophils, and mast cells. This initial response is clinically manifested by erythema, edema, hyperthermia, and pain. Once an antigen is processed and presented to lymphocytes by class 1 or 2 MHC receptor-mediated mechanisms, it promotes a Th_1 or Th_2 response depending on the antigen type, be it a tumor cell, a non-self cell, or even both. Activation of CD4+ and CD8+ T cells is then expressed by the release of cytokines and activation of natural killer (NK) cells, among other cells, which are involved in tumor destruction [22]. Acute cellular rejection of alloantigenic cells is also characterized by strong lymphocyte infiltration with destruction of the graft. A decrease in the tumor size noted in the immunocompetent models by the second week may be partly explained by the disappearance of local edema as the inflammatory reaction subsided, and partly by the launching of a tumor-specific immune response. At 3 weeks from inoculation, tumor growth persisted in only 60% of the immunocompetent mice, while 100% of the nude mice tumors progressed at constantly increasing rates.

The results of the present study must be seen in light of its limitations. A population of more than two swine undergoing tacrolimus treatment would have empowered the predictability of tumor growth. Liver inoculation of tacrolimus-treated swine should also have been attempted. However, survival of swine under tacrolimus treatment was greatly compromised by the increased incidence of acquired infections; one of the swine on tacrolimus died of diarrhea just prior to the end of the third week. The rise in infection would then be elicited by the absence of immune defenses combined with the animal's habits and behavior in the setting of a surgical skin site. No signs

of infection in the wound or peritoneum were observed in the present studies.

To achieve the ideal model of secondary liver tumor in swine, future directions will include genetic, technical, and immune-related considerations. Further genetic manipulations could enhance the malignant properties of the cell line. Tumor cells may then overcome the immune boundaries of the liver [13,23]. Other techniques for cell line inoculation into the liver could prove fruitful. The VX2 tumor model is an anaplastic squamous-carcinoma cell line that grows in the livers of wild rabbits. Tumor cells are initially inoculated s.c. or intramuscularly, with the resulting solid tumor being sectioned and implanted into the liver [24]. Such a technique may prove successful in our fibroblast cell line. Finally, as the role of immunosuppression has been clearly established in the development of this tumor, a balance in the degree of immune control should be attained to ensure tumor growth while maintaining survival of the animal until study completion and at reasonable cost. In this perspective, we may consider the use of immunosuppressants such as Alemtuzumab (Campath-1), a monoclonal antibody to the CD-52 lymphocyte receptor. It depletes T populations up to a year. To the best of our knowledge, however, this approach has not been explored in swine [25]. Finally, the immune response of allogenic cells could be potentially reduced by the inoculation of transformed cell lines into genetically identical cloned swine; a process that may increase the experimental cost.

In conclusion, the results of these studies demonstrate the development of a secondary tumor model in a large animal by s.c. inoculation of a genetically manipulated fibroblast cell line into immunocompromised swine. The tumor in the immunocompetent animal is characterized by slow growth and poor potential for neoangiogenesis, regional, and vascular invasion. The malignant potential of the fibroblast cell line is greatly enhanced at deeper levels of immunosuppression. Thus, future efforts by further genetic manipulation to the fibroblast cell line, as well as refinement of the inoculum administration techniques, stand to amplify the aggressive potential of the transformed cells, thus reaching a faster and a more secure chance of tumor growth.

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