Naturally occurring translational models for development of cancer gene therapy[#]

Review Article

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Summary

Most cancer deaths occur from metastatic spread of cancer cells. Immunotherapy and gene therapy are appealing modalities to treat cancer, not only because tumors that are resistant to conventional treatment such as radiation and chemotherapy can be treated using immunologic and genetic approaches, but also because these modalities can reach distant metastases and tumors that are inaccessible for conventional treatment. One gene therapy-based immunologic approach that has shown preclinical promise in laboratory animals is the use of Fas ligand (FasL) gene transfer. FasL promotes tumor cell killing directly and indirectly, and it induces reliable antitumor immune responses that protect animals against subsequent tumor challenge. Yet, despite the unquestioned benefits to study mechanistic questions, factors such as size, pharmacokinetic distribution, and route of administration preclude precise extrapolation of safety data from laboratory mice to humans. We have used spontaneous cancers of dogs as intermediaries for translational studies because the size and physiology of dogs, as well as the natural history of homologous tumors in this species resemble those of humans more closely than rodent models created in the laboratory. Here, we use appendicular osteosarcoma (OS) as an example to document clinical and biological

similarities between the disease in dogs and humans. Specifically, we underscore the unique properties of this model to develop therapy approaches prior to translation into clinical trials of human cancer patients.

I. Introduction

The utility of preclinical animal models for therapeutic development is dependent on how well they approximate the human disease in question. In the realm of cancer, rodent models are especially powerful to define the impact of single gene abnormalities in disease pathogenesis, and strains that are susceptible to chemical carcinogens are well suited to explore the benefit of interventions for cancer prevention. Conversely, the accelerated growth rate exhibited by many transplantable and inducible tumors in laboratory rodents can make evaluation of therapeutic strategies for pre-existing disease problematic.

Cancer in dogs occurs spontaneously. The relative lifetime cancer risk is similar in dogs and in humans, and the shared environment between people and their pet dogs offers opportunities to examine cancer etiology and response to treatment under more realistic conditions. Nevertheless, canine models for therapy development must be chosen with care, not only to properly frame the hypothesis to be tested, but also to reflect the disease under study. Here, we review the strengths of naturally occurring canine osteosarcoma (OS) as a model for preclinical development of Fas ligand (FasL) gene therapy in the adjuvant setting.

II. Fas ligand gene transfer for cancer therapy

We recently reviewed mechanistic basis and preclinical data supporting the use of adenovirus-based Fas ligand (Ad-FasL) gene transfer for cancer therapy (Modiano et al, 2004). The fundamental rationale to develop this approach is based on its potential as an adjuvant treatment: the gene is delivered into the tumor environment, where it primes immune effector cells that mediate systemic antitumor immunity. This then leads to destruction of metastatic cells, increasing the likelihood of durable remissions with reduced morbidity of cancer patients. It is especially important to note that Ad-FasL can promote antitumor immunity by two distinct mechanisms, depending on whether or not tumors express Fas receptors and are susceptible to FasL-mediated apoptosis. Specifically, in the context of cancer gene therapy, ectopic FasL promotes Fas-dependent apoptosis of susceptible tumors. In the tumor environment, scavenging of apoptotic cells by antigen presenting cells can lead to cross priming that enhances cytokine production and killing by tumor-specific T cells (Bianco et al, 2003). On the other hand, when expressed in tumors that are resistant to Fas-dependent apoptosis, the ectopic FasL (and possibly the response to the adenovirus vector) initiates robust inflammatory responses that result in tumor cell death. Unmitigated inflammation is seen with transduction of Ad-FasL, probably because of the high levels of local expression achieved with this method. In these conditions, there is extensive apoptosis of neutrophils and macrophages (Hohlbaum et al, 2001;

Shimizu et al, 2001), which in turn perpetuates the inflammatory response by recruiting additional leukocytes. The adenovirus-mediated expression of FasL is extinguished in <2 weeks because transduced cells are killed as a consequence of the inflammatory response (Regardsoe et al, 2004). Therefore, both mechanisms (Fas-mediated apoptosis and inflammation leading to tumor cell death) minimize persistence of FasL in the system, but they also promote specific, protective antitumor immune responses (Modiano et al, 2004). We have thus proposed that FasL gene transfer can be used as a "tumor vaccine" without the need to identify or enrich specific tumor antigens, and have reached a feasibility stage where it is essential to determine the risk-benefit relationships of FasL gene transfer used in an adjuvant therapy setting to treat solid tumors. Among other targets, we propose that adjuvant Ad-FasL therapy has vast potential to improve outcomes of pediatric patients with OS. Dogs with the same disease offer a clinically and biologically relevant model for development.

III. Comparative aspects of human and canine osteosarcoma

OS is an exceptional model for novel therapeutic development, as it meets the following criteria. It is a highly metastatic tumor and patients would benefit from improved treatment options; the disease is relatively common, the tumors can be visualized externally or using imaging, the tumor is responsive to immunotherapy, and both Fas-sensitive and Fas-resistant forms of the tumor occur spontaneously. According to the American Cancer Society, about 2,570 new cases of cancer of the bones and joints will be diagnosed in 2005, and about 1,200 deaths from these cancers are expected (Jemal et al, 2005). OS is the most common among these tumors; it also is the most common type of primary bone cancer in dogs, accounting for up to 85% of skeletal tumors (Dernell et al, 2001) with an annual incidence of 6,000 - >8,000 new cases per year (Withrow et al, 1991; Hansen and Khanna, 2004). Except for the age of clinical onset, the natural history of the disease is similar in people and in dogs (**Table 1**). The standard-of-care for appendicular OS includes amputation or limb-sparing surgery, followed by adjuvant chemotherapy. In children, this treatment produces an overall survival rate of ~80%, but event-free survival (EFS) is lower, with only ~60% reaching five years and barely 50% reaching 10 years (Bielack et al, 2002). Despite these encouraging facts, 20% of children diagnosed with OS will not survive five years, as many as 50% may not see the tenth anniversary of their diagnosis, and most will have significant morbidity associated with the disease. Clearly, there is need for options that will improve the outcomes of patients with this disease. The timeframe bracketed by these hallmarks in children represents ~10% of an average adult lifetime, which provides a reasonable basis on which to compare clinical outcome with dogs that have bone tumors, where the

Table 1. Comparative aspects of human and canine OS[a]
Common recurrent abnormalities are shown in **bold,** those with prognostic or predictive value shown in red.

Clinical Features	Human	Dog
Age at diagnosis	Adolescent (peak at ~15 yr) (Gurney et al, 1999)	Adult (peak at ~8 yr) (Gorlick et al, 2003; Hansen and Khanna 2004)[b]
Gender-based prevalence	Male ~1.2X	Male ~1.5X
Site	Long bones of limb (78%)	Long bones of limb (85%)
Phenotype	Aggressive, metastatic (lungs most common)	Aggressive, metastatic (lungs most common)
Standard-of-care	Surgery + adjuvant chemotherapy	Surgery + adjuvant chemotherapy
Median event-free survival	~5 yr (<10% of a lifetime)	~9.6 months (<10% of a lifetime)
Pathogenetic Features		
Cytogenetics		
Karyotype	• Aneuploid • Complex to chaotic (Ozisik et al, 1994; Batanian et al, 2002; Bayani et al, 2003; Gorlick et al, 2003; Lopez-Guerrero et al, 2004)	• Aneuploid (Fox et al,; Setoguchi et al,) • Complex to chaotic (Thomas et al, 2005)
Numerical abnormalities	• Gains and losses identified in all autosomes and X chromosome • Chromosome gains outnumber losses by 20-30% • Gain of HSA 19 or loss of HSA 9 predict poor therapy response (Sztan et al, 1997; Friedmann et al, 2002; Gisselsson et al, 2002; Ozaki et al, 2002; Overholtzer et al, 2003; Squire et al, 2003; Lau et al, 2004; Lopez-Guerrero et al, 2004; Man et al, 2004; van Dartel and Hulsebos, 2004; van Dartel et al, 2004; Zielenska et al, 2004)	• Gains and losses identified in many autosomes (Thomas et al, 2005)
Structural abnormalities	• Many chromosomes involved, but disproportionately more frequent with HSA 20 • Many centromeric rearrangements (Ozisik et al, 1994; Miller et al, 1996; Lonardo et al, 1997; Pellin et al, 1997; Kanoe et al, 1998; Yokoyama et al, 1998; Gisselsson et al, 2002; Bayani et al, 2003; Overholtzer et al,; Lau et al,)	• Various rearrangements and centromeric translocations (Thomas et al, 2005)
Oncogenes		
MYC, RAS, HDM2/MDM2, CDK4, MDR-1	• Amplified, mutated, or overexpressed in small number of OS cases • Unknown predictive value or prognostic significance (Nardeux et al, 1987; Ikeda et al, 1989; Barrios et al, 1993; Ladanyi et al, 1993; Antillon-Klussmann et al, 1995; Gamberi et al, 1998; Kanoe et al, 1998; Yokoyama et al, 1998; Ferrari et al, 2004)	• Amplified, mutated, or overexpressed in small number of OS cases • Unknown predictive value or prognostic significance (Kochevar et al, 1990; Mealey et al, 1998; Mendoza et al, 1998)
SIS/PDGFR	• Expression of PDGF AA (*c-sis*) and PDGFR associated with progression and decreased DFI (Sulzbacher et al, 2003)	• PDGF production, PDGFR expression detected in OS cell lines • Low level amplification of *c-sis* in primary OS cases (Kochevar et al, 1990; Levine, 2002)
MET/HGF	• Met overexpression associated with metastatic phenotype • Allelic imbalance at HSA 7q31 is an independent indicator of poor prognosis (Ferracini et al, 1995; Scotlandi et al, 1996; Naka et al, 1997; Oda et al, 2000; Coltella et al, 2003; Entz-Werle et al, 2003)	• Met amplification and HGF co-expression; greater in a pulmonary metastasis (Ferracini et al, 1995; MacEwen et al, 2003) • MET and HGF BACs involved in structural rearrangements
HER2/Neu (ERB-B2)	• *Conflicting data* • Amplification/overexpression detectable in 100% of cases using laser microdissection • Overexpression associated alternatively with higher metastatic potential and decreased DFI, or with increased DFI in different studies (Akatsuka et al, 2002; Anninga et al, 2004; Fellenberg et al, 2004; Ferrari et al, 2004)	• Overexpressed in 4/10 cases of OS and in 6/7 OS cell lines • Overexpression showed trend to decreased overall survival (371 days vs. 487 days) (Flint et al, 2004)
IGF1/IGF1R	• IGF-1/IGF-1R co-expressed in ~50% of primary	• OncoLAR IGF-1 antagonist reduces

	OS	IGF-1 levels but provides no clinical benefit (Khanna et al, 2002)
	• Inhibition of IGF-1R pathway ineffective to slow growth or induce apoptosis, probably due to other autocrine growth loops	
	• OncoLAR IGF-1 antagonist reduces IGF-1 levels but provides no clinical benefit (Burrow et al, 1998; Benini et al, 1999; Mansky et al, 2002)	
CTNNB1 (ß-catenin)	• Accumulation of -catenin in cytoplasm of 33/47 primary OS (Haydon et al,)	• Unknown, but osteocalcin is frequently undetectable in our samples of primary OS and OS cell lines
	• ß-catenin-induced activation of LEF-1 inhibits Runx2-mediated osteocalcin expression (Kahler and Westendorf, 2003)	
Ezrin	• High Ezrin expression associated with metastatic phenotype and poor prognosis (shorter DFI)(Leonard et al, 2003; Khanna et al, 2004)	• High Ezrin expression associated with metastatic phenotype and poor prognosis (shorter DFI) (Khanna et al, 2004)
Tumor suppressor genes		
RB1	• Associated with heritable OS	• Variable results
	• In sporadic OS, LOH, allelic imbalance, or mutations in 20-70% of cases	• Inactivation of Rb, p107, p130 in 1/4 OS lines (Levine and Fleischli, 2000)
	• Abnormal RB or loss of HSA 13q14 are indicators of poor prognosis (Araki et al, 1991; Scholz et al, 1992; Entz-Werle et al, 2003; Lopez-Guerrero et al, 2004)	• Expressed in 21/21 primary OS with no detectable structural abnormalities (Mendoza et al, 1998)
		• Undetectable in 12/14 OS lines tested by our group
TP53	Associated with heritable risk (Li Fraumeni syndrome)	• Inactivating mutations in 5/5 OS cell lines and in 8/21 primary OS cases (Mendoza et al, 1998; Levine and Fleischli, 2000)
	• In sporadic OS, LOH, allelic imbalance, or mutations in 10-80% of cases	
	• Tumors with mutant TP53 have higher level of genomic instability	
	• Abnormal TP53 or loss of HSA17p13 are indicators of poor prognosis (Scholz et al, 1992; Al-Romaih et al, 2003; Entz-Werle et al, 2003; Overholtzer et al, 2003; Squire et al, 2003; Ferrari et al, 2004; Lopez-Guerrero et al, 2004)	
CDKN2A (p16, INK4A), PTEN	• Inactivated in 30-100% of OS lines or cases	• Inactivated in 5-100% of OS lines or cases
	• Unknown predictive value or prognostic significance (Nielsen et al, 1998; Ozaki et al, 2002; Park et al, 2002; Entz-Werle et al, 2003; Nielsen-Preiss et al, 2003)	• Unknown predictive value or prognostic significance (Levine and Fleischli 2000; Levine et al, 2002; Thomas et al, 2005)
Death receptors		
FAS	• Loss of expression associated with aggressive metastatic phenotype in xenogeneic transplant model (Worth et al, 2002)	• Loss of expression in ~50% of cases with acquired insensitivity to FasL-mediated apoptosis

[a]Only some representative aspects or genes where canine counterparts are known to be affected are shown

[b]In an ongoing study including 65 dogs with primary appendicular osteosarcoma, the peak age at diagnosis was 8-9 years (34/65 cases with known age), the 95% confidence interval of the mean was 7.25 – 8.75 years, and the range was 2-14 years. This is similar to previously reported values (Dernell et al, 2001)

median overall survival in different studies ranged from ~six to ~11 months, with <30% of dogs surviving two years and <10% of dogs surviving three years (Dernell et al, 2001). Since extraneous factors independent of disease can influence overall survival in dogs with OS, a better indicator may be EFS. A recently completed study from one of our research groups (S. Lana et al, unpublished) showed the mean (median) EFS in dogs treated with standard-of-care was 287 (169) days (about 10% of a lifetime).

The efficiency of naturally occurring OS in dogs as a model platform for controlled preclinical study is not only due to higher incidence of the disease in dogs, but also to more rapid progression and apparent similarities in molecular pathogenesis (**Table 1**). Most OS cases in dogs are stage 2b (they present outside the periosteum, have high grade histologic appearance and no detectable metastases). Metastatic disease occurs in >50% of treated animals within one year and in >90% within three years, and it is greater in the lungs than bone. Predictive factors are similar in dogs and people, including age at diagnosis, anatomic location and size of the tumor, histologic grade, serum alkaline phosphatase concentrations, and initial response to therapy (Ehrhart et al, 1998; Dernell et al,

2001; Malawer et al, 2001; Gorlick et al, 2003). Finally, accrual of dogs into clinical studies is rapid, and autopsy compliance is high. For example, a protocol to examine the role of limb-sparing surgery, chemotherapy, and radiation included eligibility criteria of "localized" disease and <50% bone length involvement (Withrow et al, 1993). Forty-nine dogs were accrued, allowing rapid confirmation that, with appropriate candidate selection, this was a suitable treatment option. Interestingly, dogs with infected limb repairs lived twice as long as dogs without infection, suggesting that inflammation at the tumor site with the consequent activation of the immune system has therapeutic benefit. Another randomized study using the insulin growth factor-I (IGF-I) inhibitor, OncoLar, accrued 64 dogs in just eight months (Khanna et al, 2002). In this case, lack of therapeutic benefit of this compound could be confirmed in less than two years. For comparison, 21 people aged between 16 and 35 years old with advanced OS were recruited into a Phase-I multi-institutional study in three years (Mansky et al, 2002). This dose escalation study was stopped due to lack of drug availability when the manufacturer decided toxicities seemed to outweigh clinical benefit. When one considers the accrual rate and the course of disease progression, it might have taken >10 years to show similar negative results in a clinical trial of newly diagnosed (virgin) OS patients.

IV. Molecular features of canine OS and suitability for FasL gene therapy

As noted above, laboratory animal models have a number of limitations that can make translation to humans difficult. Specifically, transplantable tumors or tumors induced by genetic modifications in mice are not always representative of natural tumors. In addition, the homogeneous genetic background in mice strains that can accept tumors (or that develop tumors when exposed to chemicals or when genetic modifications are introduced) do not account for the heterogeneous genetic backgrounds of humans, which can significantly influence tumor progression and response to therapy. Development of better translational models could improve decision-making algorithms to move new therapeutic agents along the development process into clinical trials for human patients. Among all the models available, naturally occurring tumors of dogs present an unparalleled opportunity for use as intermediate steps in the drug development process. Dogs are extensively used in the laboratory setting to define compound safety. However, these controlled conditions still do not approximate the effects that a compound (or a gene) might have in patients that may be debilitated and who may respond differently than healthy individuals. More importantly, cancers of dogs recapitulate the clinical progression of homologous diseases of people, and these animals benefit from participation in clinical studies that can improve their outcome. For studies using canine cancer patients for drug development, safety of the animal "patient" is a major consideration, as the intent is to help these animals in the process of defining a safe (and effective) dose range for the gene therapy that can be translated to humans. Since

studies are also designed to identify dose-limiting toxicity or untoward side effects, dog owners recognize there are risks involved (as is true for any clinical study). If toxic events were to occur, this process makes translation more honest and cost-effective because it allows those to be addressed before a Phase-I clinical trial is instituted in human patients (for example, see the case of OncoLar described above).

We have completed preliminary assessments of the suitability to use FasL gene therapy in dogs *in vitro* and *in vivo*. We have established >60 cell lines derived from primary canine OS. These cells grow autonomously in tissue culture, and morphologically they resemble other established canine OS cells lines (Levine and Fleischli, 2000; Liao et al, 2005). The primary tumors and OS cell lines show similar molecular profiles to those seen in humans. For example, they tend to be genetically unstable and have "chaotic" karyotypes (**Figure 1**). We have confirmed the presence of a variety of numerical and structural cytogenetic abnormalities in these tumors, many of which localize to regions that harbor potential oncogenes and tumor suppressor genes (Thomas et al, 2005). In addition, similar gene families seem to be targeted for aberrant expression (activation or silencing) in OS of humans and dogs (**Table 1**).

It is important to remember that a suitable model for FasL gene therapy must provide samples that are susceptible to FasL-mediated apoptosis, as well as samples that are resistant to FasL-mediated apoptosis in order to provide the means to determine if both mechanisms of FasL-induced immune activation provide equivalent clinical benefit, or if case selection would be necessary *a priori* (Bianco et al, 2003). For this reason, we first examined Fas expression in canine OS cells. As was true for melanoma (Bianco et al, 2003), approximately 50% of canine OS expressed Fas (for example see **Figure 2**), and in some tumors, we detected anomalous transcripts. Intriguingly, spontaneous metastases of some of the dogs showed loss of Fas expression, suggesting that, as is true in humans with OS (Worth et al, 2002), loss of this pathway might participate in tumor progression and metastasis.

Previously, we showed that Fas expression by canine melanoma cells correlated with susceptibility to death mediated by transduction with Ad-FasL (Bianco et al, 2003). To verify if this was also true for canine OS, we examined cell viability in culture after transduction with Ad-FasL or Ad-GFP. **Figure 3** shows representative Fas receptor-positive cells (OSCA.36.1) that were susceptible to FasL-mediated cell death, and Fas receptor-negative cells (OSCA2) that were not. The distribution of FasL-sensitive and FasL-resistant OS cells from the lines tested so far is almost exactly 50:50. It is therefore crucial to reiterate the importance of this observation, as it establishes canine OS as a suitable model to confirm the findings in mouse models where tumors that are resistant to FasL-mediated apoptosis *in vitro* are still killed (indirectly) by the inflammation induced by FasL *in vivo*. Also significant is that canine endothelial cells are highly susceptible to adenovirus infection and are killed by Ad-FasL. This minimizes concerns of systemic distribution by
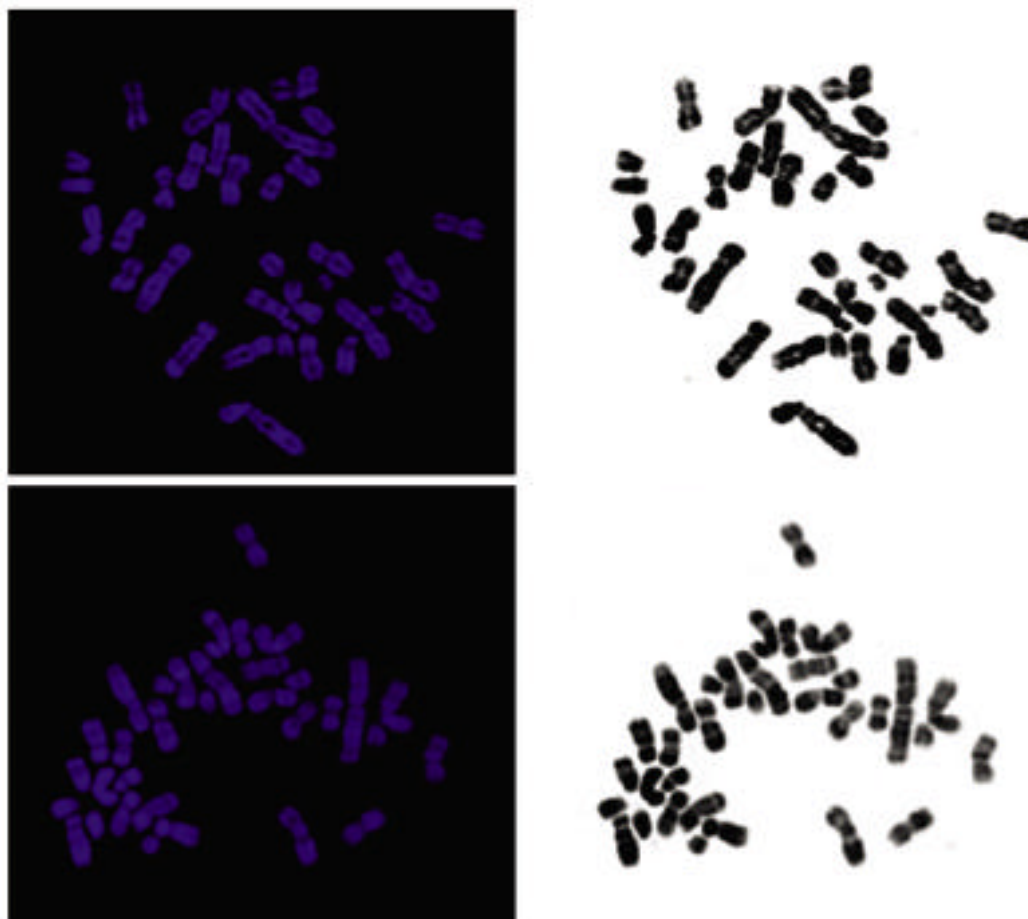
Figure 1. "Chaotic" Karyotypes in Canine OS Cells. The images on the left show DAPI stained metaphase spreads and the images on the right show the corresponding inverted DAPI banded preparations. The modal chromosome number in these cells is significantly reduced (2n=34) compared to normal dog cells (2n=78), and most chromosomes are metacentric, compared to the usual acrocentric morphology of normal canine chromosomes. This 'chaotic' cytogenetic appearance is typical for the canine OS samples we have analyzed (Thomas et al, 2005).
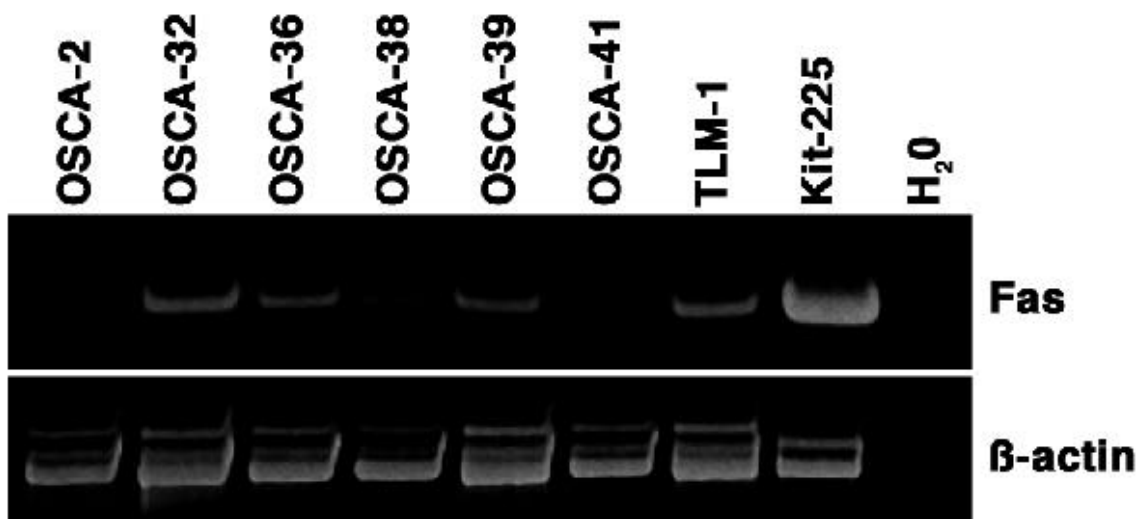

Figure 2. Expression of Fas mRNA by Canine OS Cell Lines. Fas expression was examined by RT-PCR in representative canine OS cell lines established from primary tumor explants. Primers were designed to amplify a 146 bp canine Fas mRNA product. Fas-positive TLM-1 canine melanoma cells and Kit-225 human leukemia cells were used as positive controls; dH$_2$O without input RNA (in the PCR reaction) was used as a negative control. Expression of ß-actin was used to verify the integrity of the RNA samples and to control for loading differences. (OSCA = osteosarcoma cell line-AMC).
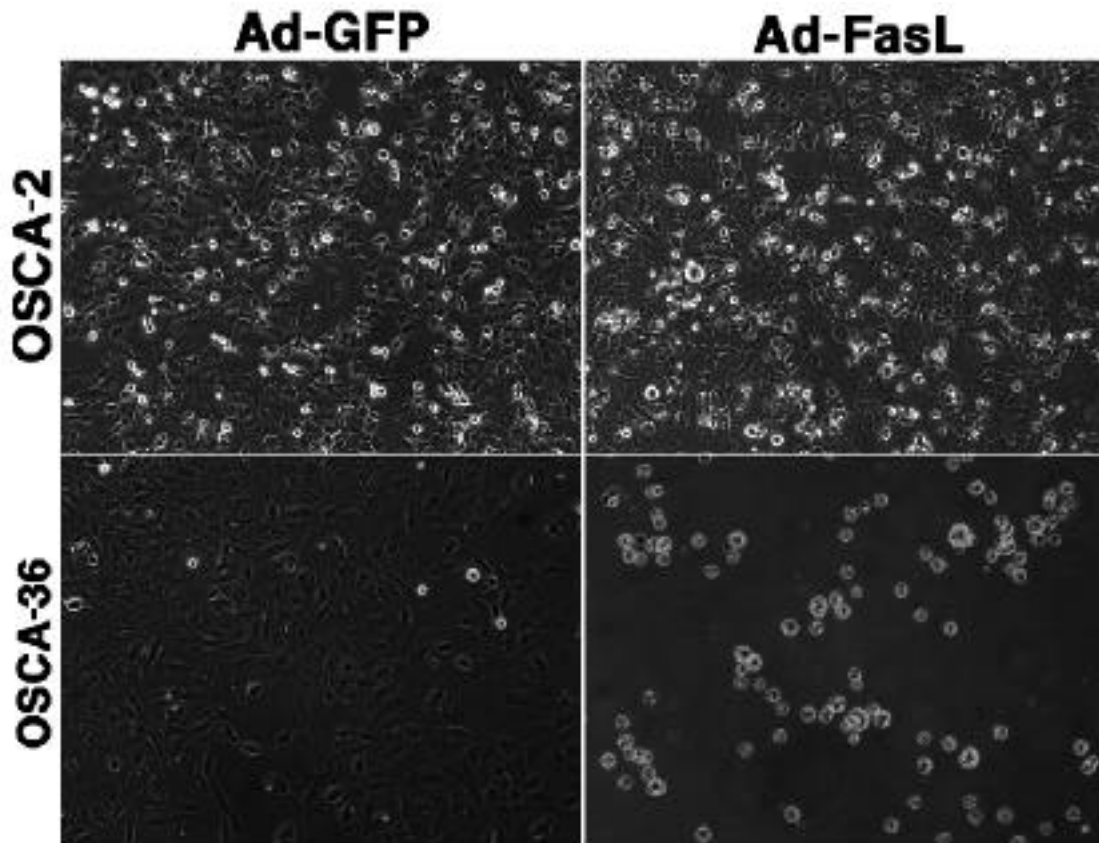
Figure 3. Susceptibility of OS cells to Fas-mediated death. OSCA2 (Fas receptor-negative, top) and OSCA36.1 (Fas receptor-positive, bottom) cell lines are shown to represent, respectively, Fas-resistant cells and Fas-sensitive cells. Subconfluent cultures were transduced using 2,000 pfu of Ad-GFP (left) or Ad-FasL (right). After 6 hr, cultures were photographed under phase-contrast microscopy. While OSCA-2 cells showed virtually no effects of transduction with either adenovirus, more than 90% of OSCA-36 cells showed characteristic apoptotic changes (condensed chromatin, rounded morphology, and detachment from the plastic substrate). OSCA-2 cells continued to grow unhindered in the presence of either adenovirus, as did OSCA-36 cells transduced with Ad-GFP. In contrast, no live cells remained after 24 hr from OSCA-36 cells transduced with Ad-FasL.

the adenovirus after intratumoral administration, as it is likely to remain within the tumor environment where reduced blood flow and increased interstitial pressure at the tumor site (Zachos et al, 2001) will retain the Ad-FasL at or near the injection (within the tumor), promoting transduction of malignant osteoblasts, endothelial cells, and tumor stroma. As also noted previously, we conducted a preliminary study to determine the safety of FasL gene therapy in tumor-bearing dogs using naked DNA (Bianco et al, 2003). No local or systemic toxicity was seen in any of the five dogs in that study. However, based on the preclinical data described above, we are more likely to achieve therapeutic efficacy with Ad-FasL. The safety and toxicity benchmarks for this product will use a more refined method of development that will allow for examination of possible efficacy/toxicity trade-offs if any local (or systemic) toxic events were identified due to the greater levels of FasL expression achieved using the adenovirus delivery system.

V. Conclusions

Naturally occurring tumors of dogs offer unique models that can complement traditional laboratory rodent models for studies of cancer pathogenesis and for preclinical drug development. The strength of these models is the spontaneous occurrence of tumors with similar etiology in large animals that (1) are physiologically similar to humans, (2) share our environment, (3) can tolerate repeated sampling, and (4) largely show comparable responses to conventional treatments. The recent completion of the canine genome sequence (Lindblad-Toh et al, 2005) provides the resources needed to assess the conservation of genes and proteins that can serve as targets for tailored or molecular approaches to treat cancer. We predict that increasingly, studies in pet dogs will become a standard component in the development process of novel therapies for cancer and other chronic diseases, and that these studies will streamline the selection process to determine compounds that have higher a likelihood of success for treatment of human patients. Unquestionably, these studies will also benefit the pet population and provide potential new markets for manufacturers of novel drug and gene-based therapeutics.

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