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Rapid Communication

Osteosarcomas in Transgenic Mice Expressing an α-Amylase–SV40 T-antigen Hybrid Gene

Barbara B. Knowles,* James McCarrick,* Niles Fox,* Davor Solter,* and Ivan Damjanov†

From The Wistar Institute of Anatomy and Biology; and the Department of Pathology and Cell Biology,† Thomas Jefferson University, Philadelphia, Pennsylvania*

Mice of a transgenic mouse lineage 501, produced by zygotic injection of the parotid α-amylase promoter–SV40 T-antigen hybrid gene, developed osteosarcomas at about 15 months of age. The tumors predominantly involved the axial skeleton, were sometimes multiple, and metastasized to the liver. A cell line derived from a primary tumor produced osteosarcomas on transfer to nude mice. The 501 transgenic lineage thus provides a valuable new model for studying the histogenesis of osteosarcomas. (Am J Pathol 1990, 137:259–262)

Tumors often develop in transgenic mice produced by injecting hybrid genes that consist of the controlling regions of genes expressed in a tissue-specific manner and the coding region of various oncogenes. These tumors usually appear in the targeted organ, although exceptions have been noted.[1,2] For example, transgenic mice carrying a hybrid gene consisting of the α-amylase liver promoter and the SV40 T-antigen coding region, succumb with malignant hibernomas. This paradoxical result was explained by our subsequent discovery of a previously unknown gene activity, ie, α-amylase expression in normal adipose tissues[3] in addition to the previously known distribution of this enzyme in pancreas, salivary glands, and liver.

Transcription of mouse α-amylase gene (*Amy-1*^{a}) is controlled by two different promoters active at different times in development. One, the liver promoter, is already active at birth, whereas the parotid promoter is first activated in mice at 2 to 3 weeks of age.[4] Transgene p600T, consisting of the early promoter of *Amy-1*^{a} combined with SV40 Tantigen, was used to produce the transgenic mice that develop malignant hibernomas.[3] We now report that transgenic mice bearing a hybrid gene (p1.3T) constructed from the α-amylase late promoter and SV40 T antigen develop osteosarcomas late in life.

Materials and Methods

The gene construct p1.3T (provided by Dr. Ueli Schibler, University of Geneva, Geneva Switzerland) was derived by joining a *Pst* I restriction fragment of the *Amy-1*^{a} gene, which contains 1.25 kb of the 5′ flanking region and 30 nucleotides of the untranslated region, and in which the *Pst* I sites were converted to *Eco* RI sites, to the coding region of SV40 Tag (the *Hind* III-*Bam* HI fragment which excludes the 21 and 72 bp repeats) in which the *Hind* III site was converted to an *Eco* RI site, and last cloning it into the plasmid pMLII[5] at the *Hind* III site, which had been converted to an *Eco* RI site. The hybrid gene was excised by partial double digestion with *Eco* RI and *Bam* HI, gel purified, and microinjected into the male pronucleus of C57BL/6J fertilized eggs. Injected eggs were transferred into the oviducts of pseudopregnant outbred CD-1 mice. All mice born were analyzed for the presence of the transgene by Southern blotting and probing with the SV40 T-antigen coding sequences.

Transgenic founder animals were obtained, from which a stable transgenic lineage, 501, was established by mating with C57BL/6J mice, followed by breeding *inter se*. Animals were kept under standard laboratory conditions in The Wistar Institute Animal Facility and examined periodically. Moribund animals or animals with suspected tumors were killed, a complete necropsy was

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Supported by PHS grants CA-10815, 18470, and 21124. Address reprint requests to Barbara B. Knowles, The Wistar Institute, 3601 Spruce Street Philadelphia, PA 19104.

Dr. Fox's current address is E. I. Lilly Research Laboratories, Indianapolis, IA 46285.

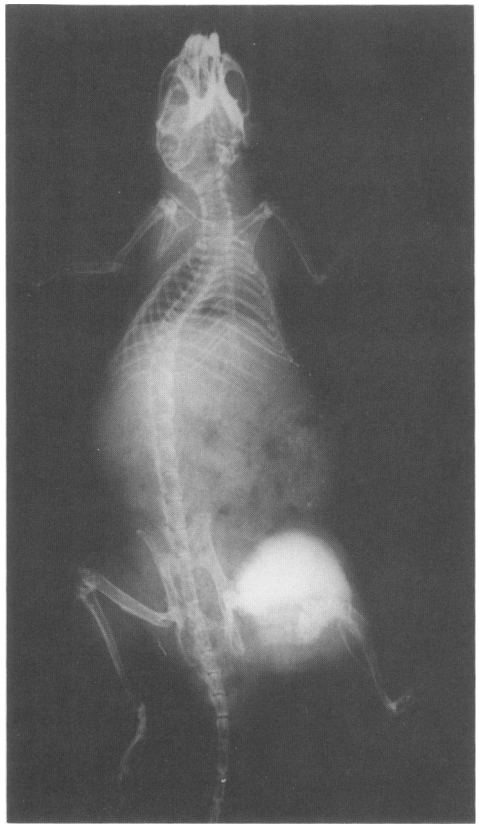

Figure 1. *X-ray photograph of a transgenic mouse with a primary osteosarcoma of the femur.*

performed, and tumor pieces were routinely processed and examined histologically.

One tumor, from animal 2484 of the 501 lineage, was minced under aseptic conditions and placed in Dulbecco's modified minimal essential medium containing 10% fetal bovine serum. Three months after the culture was initiated, islands of proliferating cells were trypsinized and transferred to 96-well plates at approximately 1 cell per well. Two colonies derived from this limiting dilution cloning were established. Nude mice (obtained from Harlan Sprague-Dawley) were inoculated under the kidney capsule with 10^7 cells of the 2484 col two cell line at the ninth passage after cloning.

Tissue samples were snap frozen in liquid nitrogen, sectioned at 6 μm on a crysostat, dried, fixed in 2% paraformaldehyde for 30 minutes, and rinsed in Dulbecco's modified phosphate-buffered saline (PBS). Sections were incubated with mouse monoclonal antibody to SV40 T antigen for 1 hour (supernatant from hybridoma clone PAB 101; TIB 117 ATCC), rinsed twice in PBS, incubated with suitably diluted FITC-labelled anti-mouse immunoglobulin G (Cappell Laboratories, West Chester, PA) for 1 hour, rinsed twice in PBS, mounted in glycerol, and examined with a fluorescence microscope.

Results

Of thirty 501-lineage mice born in five litters between December 1987 and October 1988, 20 developed osteosarcomas between August 1989 and December 1989. At least five of these animals had multiple primary tumors involving anatomically distinct bones. Several tumors of the cervical vertebrae involved the adjacent bones, making it impossible to determine the exact site of origin and whether they represent a single large tumor or confluent multiple tumors. Most of the tumors originated in the axial skeleton and were localized either at the base of the skull, in the vertebral column, shoulder, pelvic girdle, or ribs. In addition, several tumors of the proximal humerus or femur were noted. The distal bones of the extremities were not involved. Visible metastases to the liver were noted in six mice. Age-matched C57B1/6J mice housed in the same setting revealed no such tumors.

On gross examination the primary tumors measured from 0.5 to 3 cm in largest diameter and ranged from those that were entirely of the consistency of bone to those that contained a mixture of hard and soft tissue. Some blood-filled cystic structures also were seen. All tumors were inseparable from bone and had infiltrated but were separable from the adjacent soft tissue. X-ray examination revealed destruction of the bone and replacement by the tumors; the bone and the invaded area appeared mottled due to the irregular calcification within the tumors (Figure 1).

The histologic appearance of tumors was variable, but each contained spindle-shaped cells and larger polygonal and pleomorphic cells with prominent blood vessels. All tumors contained osteoid showing varying degrees of calcification (Figure 2). Woven bone was seen in some of the tumors. Based on the predominant growth pattern, cell morphology, and the amount and nature of the extra-

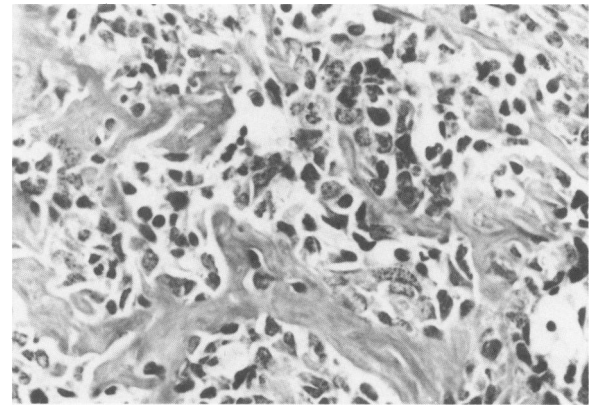

Figure 2. *Histologic appearance of primary osteosarcoma. The tumor is composed of spindle-shaped and polygonal cells surrounding prominent aggregates of osteoid. Hematoxylin and eosin; magnification, ×240.*

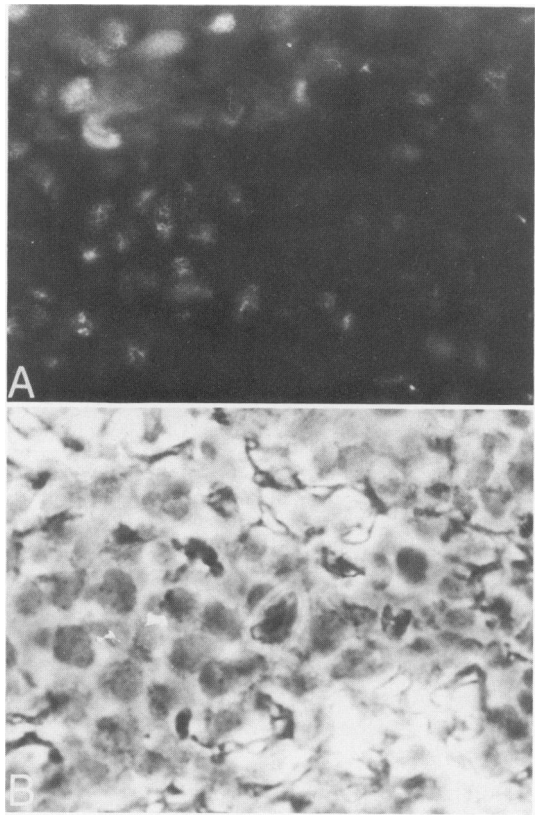

Figure 3. **A:** *Frozen section of the primary osteosarcoma reacted with antibody to SV40 T antigen and FITC-tagged anti-mouse IgG shows nuclear fluorescence in some but not all cells.* **B:** *Phase-contrast photograph of the same field. Immunofluorescence microscopy; magnification ×320.*

cellular matrix,[6] the present tumors fall into the category of osteoblastic osteosarcomas; there is no evidence of enchondral ossification. Metastatic nodules in the liver were histologically composed of the same neoplastic cells and were calcified but contained less osteoid. SV40 T antigen could be demonstrated immunocytochemically in the nuclei of most but not all tumor cells (Figure 3).

The cloned cell lines established were polygonal with a well-developed cytoplasm. When injection into nude mice, the cells formed osteosarcomas that were histologically indistinguishable from the primary tumor (Figure 4).

Discussion

Spontaneous bone tumors are rare in laboratory mice.[6] In a survey of 12,000 mice kept *ad vitam* under standardized laboratory conditions, Slye et al[7] recorded only 12 osteosarcomas. Innes et al[8] found only 6 osteosarcomas in 19,875 mice kept until they reached 18 months of age, confirming that the incidence of spontaneous bone tumors is less than 1 per 1000 mice. On the other hand, osteogenic sarcomas are readily induced with beta radiation,[9] bone-seeking radionucleotides such as ^{90}Strontium[10] or the FBJ-MSV, a retrovirus that contains the v-*fos* oncogene.[11] Mice transgenic for a human metallothionein, or mouse H-2-promoter c-*fos* construct also develop osteosarcomas provided that the 3′ noncoding region of c-*fos* is replaced by the long terminal repeat (LTR) of FBJ murine osteosarcoma. These data suggest that activated expression of *fos* in the osteoblast is oncogenic.[12,13]

The 501 lineage presents several advantages over these other transgenic mice as models of osteosarcomagenesis. First a very high proportion of these mice develop osteosarcomas, albeit late in life, and consequently each mouse has a good probability of recapitulating the neoplastic process. This contrasts with the human MT c-*fos* LTR transgenic mouse model in which less that 10% of the mice ever develop tumors. Second the age of onset of these tumors, 15 to 20 months, provides a long time span for the occurrence, study, and manipulation of the events that precede tumor emergence. Third, in contrast to the tumors in the aforementioned transgenic mice, the tumors in 501 lineage mice are primarily osteosarcomas, not chondrosarcomas or fibrosarcomas. Furthermore, like their human counterparts, these tumors frequently metastasize. Fourth, because the tumor cells are immortalized by SV40 T antigen, they can be isolated, cloned, and grown *in vitro*, providing an accessible source of the tumor cell itself and of a cell capable of performing its differentiated function, ie, bone formation *in vitro*. Thus the 501 lineage provides a working model for study of a malignant tumor; these endogenous tumors appear late in life in most animals and metastasize.

In humans, osteosarcomas occur at an increased incidence in patients with retinoblastoma[14] who have survived treatment of ocular malignancy. Genetic studies have disclosed that in some cases the osteosarcoma

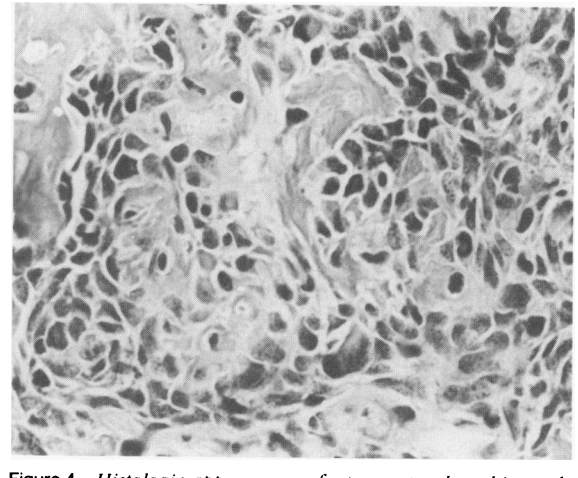

Figure 4. *Histologic appearance of a tumor produced in nude mice by injecting the clonal osteosarcoma cell line. The tumor has the features typical of osteosarcoma and appears indistinguishable from the primary tumor used for isolating the cell line. Hematoxylin and eosin; magnification ×240.*

cells contain defects at the retinoblastoma locus Rb[15,16] on chromosome 13q14.1. Similarly rearrangements of the cellular p53 gene have been reported in human osteosarcomas.[17] Thus *Rb* and related tumor suppressor genes[18–20] might be involved in the pathogenesis of osteosarcoma. SV40 T antigen might function in such a manner in the 501 transgenic mouse lineage because it binds to the p53 and Rb gene products [21] and might thereby prevent the normal cellular function of these products. It remains unclear why osteosarcomas occurred in these particular mice in which the α-amylase promoter should have targeted expression to salivary glands, pancreas, and liver. However, on the basis of our earlier work in which tumor formation in brown fat proved to be an indicator of unsuspected α-amylase expression in adipocytes,[2] we hypothesize that osteoblasts also transcribe the α-amylase gene.

The unexpected discovery of primary osteosarcomas in transgenic mice expressing the α-amylase-SV40 T-antigen hybrid gene provides a new model in which the interactions between endogenous oncogenes and tumor suppressor genes in the histogenesis of bone tumors can be investigated.

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