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Cover: Brittany-Beagle dog with hereditary canine spinal muscular atrophy (see Cork *et al.*, pp. 69–76). Photograph courtesy of Mr. Victor Raspa.

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Supernumerary Chromosome 1 in Interphase Nuclei of Atypical Germ Cells in Paraffin-Embedded Human Seminiferous Tubules

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The so-called atypical germ cells or cells of carcinoma *in situ* morphologically resemble neoplastic cells in seminoma. Since seminomas show numerical aberrations of chromosome 1 we have used a DNA probe specific for chromosome region 1q12 to determine whether such aberrations can be detected in atypical germ cell nuclei in paraffin-embedded seminiferous tubules as well. One-third of intratubular nuclei, containing atypical germ cells, consistently showed three hybridization signals in contrast to two signals regularly observed in normal intestine and in spermatogonia. We thus show that cytogenetic studies of precancerous cells can be performed directly on the tissue where these cells originate.

Additional key words: Human precancerous testicular germ cells, *In situ* hybridization, Paraffin sections.

Experimental data from animal tumors and from analyses of human teratocarcinoma-derived cell lines suggest that the crucial event in the formation of testicular germ cell tumors is preceeded by the activation of premeiotic germ cells in the seminiferous tubules or in dysgeneic gonads (8). Such activated germ cells, representing stem cells for all kinds of seminomatous and nonseminomatous tumors, are described as atypical germ cells (AGCs, 21) or as cells of carcinoma *in situ* or gonocytoma *in situ*, respectively (27). Some authors have proposed that these cells require a second hit to transform the *in situ* carcinoma into an invasive neoplasm (9). The potential of AGCs to develop into manifestly malignant cells is high, for nearly all patients with such diagnoses will progress into invasive cancer if orchiectomy is not performed (2, 26, 28). AGCs resemble cells of classical seminoma morphologically (25). Furthermore, similar to seminoma AGCs produce human placental-like alkaline phosphatase (13, 15) and show hypotetraploid DNA contents (19). Cytogenetic and DNA flow cytometric studies have shown that cells of seminoma and teratocarcinoma, respectively, have an aneuploid number of chromosomes (1, 10, 20, 22, 27). In cytogenetic preparations of a testicular biopsy from a patient with AGCs, nine mitotic cells were found with numerical and structural chromosome anomalies (17).

For better characterization of early steps in tumorigenesis it is essential to obtain more information about specific chromosome aberrations present in AGCs. Interphase cytogenetics (6, 14) provides a new tool to investigate specific chromosome changes in interphase nuclei in paraffin-embedded tissue sections (11) and eliminates the need for mitotic cells that are indispensable for conventional cytogenetic analyses (7). Both chromosomes 1 and 2 contain the gene loci for alkaline phosphatase isozymes. Recently, we noted overexpression of these isozymes in both seminiferous tubules with AGCs and seminoma cells (13). If these particular chromosomes can be detected in higher numbers in such cells than in normal diploid elements, one could expect enhanced number of copies of the respective genes. Our previous investigations showed an increased number of chromosome 1 in seminoma and nonseminomatous germ cell tumors (10).

Using *in situ* hybridization of a DNA probe specific for chromosome region 1q12 (5), we could detect numerical chromosomal aberrations in interphase nuclei of these precancerous cells in paraffin sections from surgical specimens. Further data suggest that this method could be used to improve the yield of data from biopsies.

EXPERIMENTAL DESIGN

Based on our previous experiments with xenotransplantable human testicular tumors and related cell lines (11), we applied interphase cytogenetics to surgical specimens. This was performed on 6-μm serial sections with

phosphate-buffered 4% formaldehyde and embedded in paraffin. Sections were hybridized with a biotinylated DNA probe for 1q12 (5) that was visualized by a peroxidase reaction.

RESULTS AND DISCUSSION

HISTOLOGY OF SEMINIFEROUS TUBULES WITH AGCs

Seminiferous tubules adjacent to a tumor showed various alterations. Lumina of most tubules, some of them being fibrotic, showed AGCs, Sertoli cells, and occasionally lymphocytic infiltrations. AGCs were clearly recognizable by their increased cell and nuclear size and a particularly bright cytoplasm (Fig. 1). Many spermatogonia in areas with at least partial spermatogenesis were found to be binucleated (Fig. 2).

HYBRIDIZED PARAFFIN SECTIONS

After hybridization treatment, sections were still well preserved. Occasionally, however, delicate elements, such as AGCs or cells of seminoma, displayed disruptures of the cell membrane and/or the cytoplasm. Small intestine mucosa served as normal control and displayed predominantly two spots per cell nucleus (11). The number of chromosome 1q12-targets was aberrant in neoplastic interphase nuclei in sections from both seminoma and teratocarcinoma (Fig. 3). In consecutive sections through seminiferous tubules, interphase nuclei with one, two, three, and four hybridization spots were clearly recognizable (Fig. 4). In 34% of 372 intratubular nuclei analyzed, three spots were found. In 41% two spots and in 24% one spot were noted in nuclei. Four nuclei presented four signals each. In contrast, nuclei with three signals could not be detected in tubuli with normal seminiferous epithelium from patient 2; 121 out of 196 nuclei (62%) were counted with two spots, and 75 (38%) showed only one signal (Table 1). An area with normal spermatogenesis is presented in Figure 5. Spermatogonia showed two spots per nucleus, whereas spermatids only presented one signal. Nuclei from spermatids, being similarly opaque to sperm heads, did not yield a detectable hybridization signal, probably indicating insufficient probe penetration in these cells.

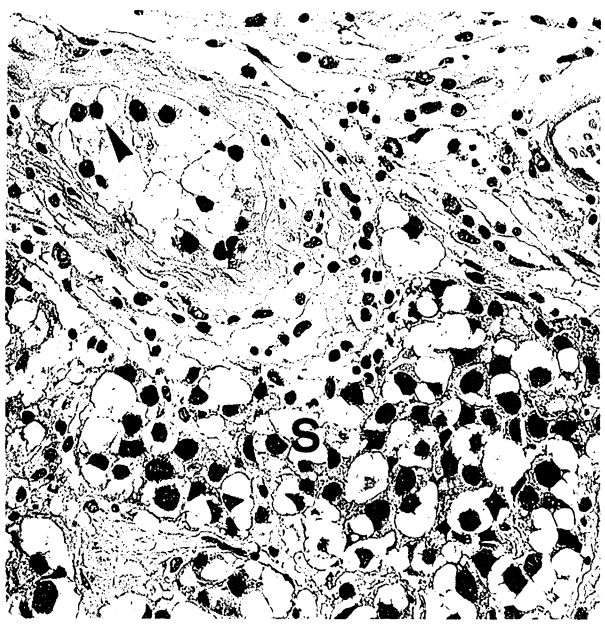

FIG. 1. Seminiferous tubule with impaired spermatogenesis containing AGCs (*arrow*) within a pathologically thickened tubular wall. S, extratubular areas with seminoma cells. Paraffin embedded, hematoxylin and eosin. ×252.

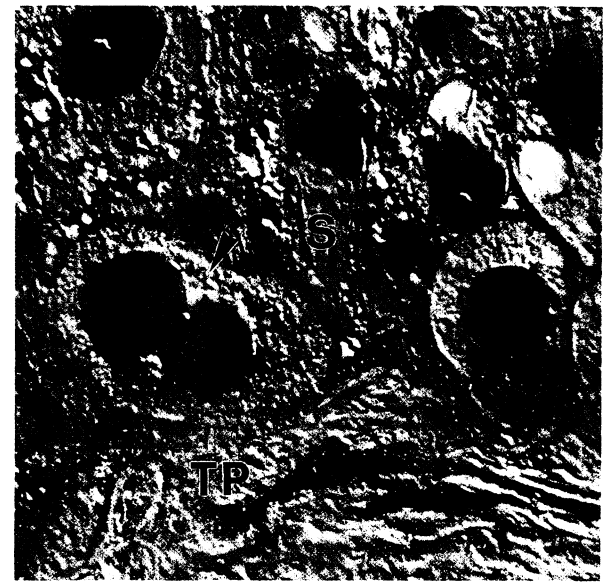

FIG. 2. Binucleated spermatogonium (*arrow*) within germinal epithelium of a patient with a seminoma. This cell borders tunica propria (TP) and a Sertoli cell (S). Embedded in Kulzer Technovit, hematoxylin and eosin, Nomarski-contrast. ×1100.

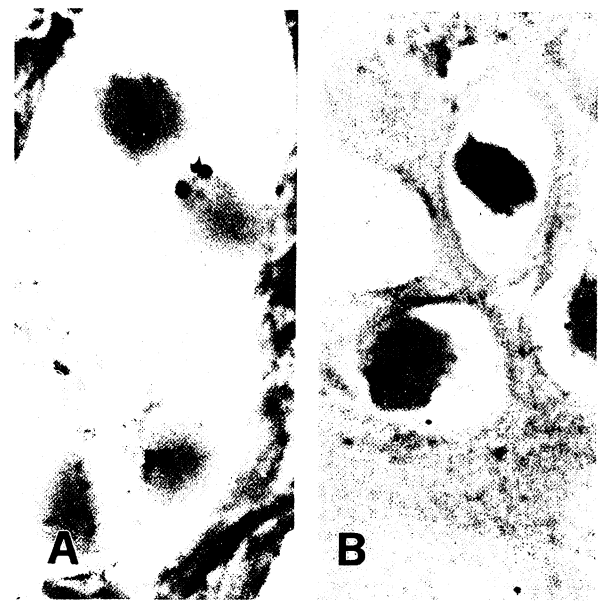

FIG. 3. Area of seminoma (*A*) adjacent to seminiferous tubules with AGCs. *B*, differentiated part (cartilage) of teratocarcinoma of the same patient. Nuclei in *A* and *B* present both two and three spots, indicating chromosome 1. Stained with Mayers hematoxylin. ×1200.

Numerical aberrations, *i.e.*, gains or losses of whole chromosomes often together with polyploidization events, are the most consistent and probably earliest gross chromosomal abnormalities of malignant human gliomas (3). Similar to malignant gliomas, germ cell tumors show higher than diploid chromosome numbers, indicating the importance of abnormal chromosome segregation events in these tumors as well. Similarly to the data presented by Oosterhuis (22) using DNA flow cytometry our earlier chromosomal analysis suggests that the higher metastatic potential of nonseminomatous tumors is correlated with a range of 55 and 65 chromosomes, whereas

seminomas have generally higher numbers of chromosomes (70–80) (1, 10, 29).

Boveri (4) was the first to predict that segregational errors during mitosis might contribute to tumor formation from single somatic cells. Recent investigations have extended Boveri's early concept to the molecular level and indicated that the loss of certain chromosomes or chromosome segments may unmask recessive mutations at specific loci (12). Conversely, the gain of specific chromosome segments may contribute to the overexpression of dominantly acting tumor-related genes located within these segments, including oncogenes. Similarly, as demonstrated by isoelectric focusing, we found overexpression of the four isozymes of the alkaline phosphatase in germinal epithelium with AGCs and in seminoma, compared with normal tissues and those of nonseminomatous germ cell tumors (13). The genes for these isozymes are located on chromosomes 1 and 2. Whether overexpression of alkaline phosphatase isozymes is directly related to the number of one or both of these chromosomes is not yet clear.

If one speculates that seminoma can progress into nonseminomatous tumors, as suggested by Monaghan and Raghavan (18, 23) and very recently by Oosterhuis (22), this transition should be accompanied by a loss of part of the hypertriploid genome. Considering the high chromosome numbers typical of seminoma and the lower numbers generally found in nonseminoma (1, 10, 29) one might expect further activation through loss of chromosomes during progression to highly metastatic forms. In addition to numerical aberrations structural chromosome aberrations are likely to play an important but even less understood role in these types of tumors.

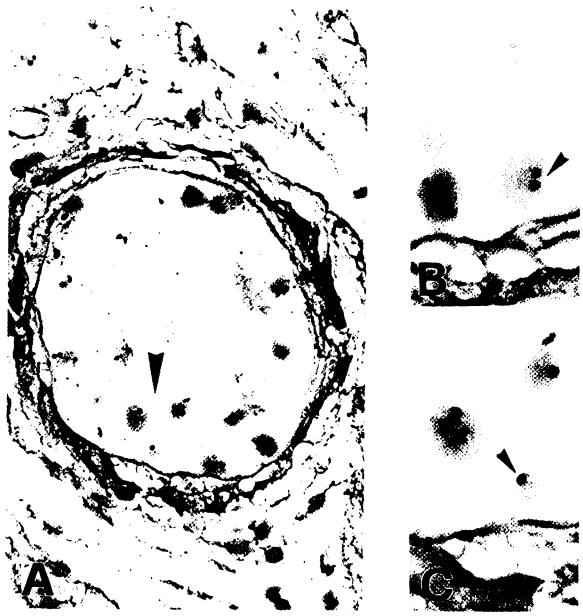

FIG. 4. Seminiferous tubule with AGCs. Nuclei present one, two, three, and four spots, localizing hybridized chromosomes 1. *B* is a consecutive section through the same area in *A* (*arrow*), which is magnified in *C*. Note typical arrangement of chromosomes in nuclei indicated by *small arrows*. In *B*, one chromosome was found in a particular nucleus, whereas in *C* two spots were detected within the consecutive section of the same nucleus. Cell membranes are partially damaged by the hybridization treatment. Stained with Mayers hematoxylin. *A* ×390, *B* and *C* ×890.

METHODS

TISSUE PREPARATION FOR MORPHOLOGY AND *IN SITU* HYBRIDIZATION

Seminiferous epithelium from five patients with a testicular tumor (one seminoma, one teratocarcinoma with prominent portions of seminoma, and three teratocarcinoma without parts of seminoma) and normal intestinal tissue from another patient were fixed conventionally with 4% formaldehyde in phosphate buffer for 5 hours. The latter tissue served as a diploid somatic tissue control. For structural controls, 3-μm sections were produced and others of 6 μm were prepared for *in situ* hybridization by attaching them onto microscope slides pretreated by aminoalkylsilane (24). To allow histological analyses of entire nucleic areas, series of two or three consecutive sections per optical slide were prepared, and identical areas were photographed. In addition, for optimal structural analysis tissues were fixed with a mixture of 2% formaldehyde and 0.2% glutaraldehyde in phosphate buffer and embedded in Kulzer Technovit 7100 (Kulzer, Wehrheim, FRG). Sections of 2 μm were stained with hemalum eosin and with the periodic acid-Schiff reaction.

LABELING PROCEDURE FOR DNA PROBE pUC1.77, PRETREATMENT OF SECTIONS, HYBRIDIZATION, AND PROBE DETECTION

Procedures of this part were identical to those described in detail elsewhere (11). Probe pUC1.77 was a generous gift from Howard Cooke. It was derived from human satellite DNA fraction III and represents a tandemly organized repeated sequence from 1q12 (5). The probe was nick-translated with biotin-11-dUTP as described (16).

Before *in situ* hybridization, a special pretreatment was

TABLE 1. EVALUATION OF NUCLEAR SIGNALS

Patient	Tumor type	*n*	Spots per nucleus			
			1	2	3	4
1	Seminoma	83	32	44	7	
2	Seminoma/teratocarcinoma	123	14	47	60	2
3	Teratocarcinoma	61	15	27	19	
4	Teratocarcinoma	56	17	17	21	1
5	Teratocarcinoma	49	11	17	20	1
Total of all nuclei		372	89	152	127	4
Percent		100	24	41	34	1
Nuclei in normal tubules from patient 2		196	121	75		
Percent		100	62	38		

Nuclear signals within seminiferous tubules were evaluated after *in situ* hybridization of paraffin sections with probe pUC1.77 (peroxidase detection). Seminiferous tubules were adjacent to testicular germ cell tumors from five patients. *n*, Number of evaluated intratubular nuclei.

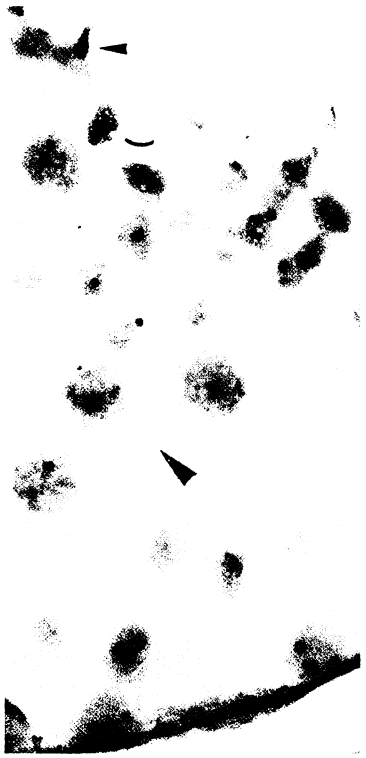

FIG. 5. Segment of seminiferous tubule with intact spermatogenesis. This optical plane shows two nuclei of spermatogonia with two spots near the tubular wall and only one signal in more prominent nuclei of spermatocytes (*arrowhead*) or in smaller ones of spermatids (above). A spermhead is recognizable (*small arrowhead*) but without hybridization signal. Stained with Mayer's hematoxylin. ×790.

applied to tissue sections including five subsequent freezing-thawing steps, overnight incubation at 65°C, removal of paraffin (5 min xylene, 5 min 99% ethanol), 0.2 N HCl for 20 min, and proteinase K digestion (50 μg/ml phosphate buffered saline) at 37°C for 10 min. After the pretreatment sections were dehydrated and finally air dried.

The hybridization mixture containing 60% formamide, 2×SSC, 5% dextrane sulfate, 100 μg/ml herring sperm DNA, and 2 μg/ml labeled probe was applied to the specimen. A coverglass was sealed, and probes and specimen were denaturated simultaneously at 76°C for 10 min in a moist chamber. Hybridization was performed overnight at 40°C. Unbound probe was removed by washing twice in 50% formamide/1×SSC for 15 min at room temperature and once in 0.1×SSC for 30 min at 37°C. Hybridized probes were visualized by a peroxidase reaction, as described previously (11). For counting spots (*i.e.*, peroxidase reaction products at hybridized 1q12 sites), identical areas of three consecutive sections were photographed. Intratubular nuclei of two to ten seminiferous tubules of each patient were evaluated by their hybridization signals and compared with tubules presenting normal spermatogenesis (Table 1). Cells of seminoma and differentiated parts of teratocarcinoma as well as of normal human small intestine were treated identically.

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