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J Clin Invest. 1994;94(5):2153-2157. https://doi.org/10.1172/JCI117572.

Research Article

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Human Villous Adenomas Engrafted into scid Mice Survive for Prolonged Period without Malignant Transformation

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Abstract

Human villous adenomas are thought to represent premalignancies that subsequently give rise to colorectal adenocarcinomas. Currently there is no in vivo model in which to study the dedifferentiation and malignant transformation of these tumors. We establish here that human villous adenomas can be successfully engrafted into severe combined immunodeficient (scid) mice. Furthermore, these xenografts remain viable for up to 18 mo after either a subcutaneous or intraperitoneal inoculation of the human tissue. Tumors grew slowly and secreted a clear mucinous fluid. Examination of the tumors histologically at 1, 4, and 12 mo after implantation revealed that the villous polypoid structure was maintained and islands of atypical cells were observed within pockets of mucin surrounding the adenomatous tissue. No gross or histologic evidence of malignancy was detected throughout the 20-mo observation period. The human identity of the cells in the graft was confirmed by DNA in situ hybridization with a human-specific probe. We conclude that the human-scid xenograft described here represents a viable animal model with which to study the potential malignant dedifferentiation of villous adenomas over a prolonged period of time and to evaluate the possible contribution of selected oncogenic vectors on the malignant transformation of these adenomas. (*J. Clin. Invest.* 1994. 94:2153–2157.) Key words: colon tumor • polyp • polyposis coli • tumorigenesis • carcinogenesis

Introduction

Dedifferentation of colonic polyps is one mechanism that has been shown to correlate with the generation of colonic adenocarcinoma (1). It has been proposed that a series of gene mutations occurs in this transition from normal colonic mucosa to carcinoma. Though the exact sequence of genetic alterations is not known, a possible model has been constructed through retrospective analyses (2, 3). The frequency of cancer occurrence increases in polyps with a predominant villous histopathology: tubular (5%), tubulovillous (22%), and villous (40%). Because of the absence of an adequate animal model, it has been impossible to observe closely the progression of these polyps over a prolonged period of time. While some very specific oncogenic mutations have been identified in the sequence of events that occur during transition from polyp to carcinoma, many aspects of this transformation remain unanswered. The severe combined immunodeficient (scid)[1] mouse has served as a means for the propagation of a variety of human malignancies (4, 5) as well as several different normal and neonatal tissues (6–8). We report here the successful engraftment and long-term maintenance of human villous adenomas in scid mice. Based upon our findings, it appears that this model offers an opportunity to test directly the proposed notions regarding the transition of colonic polyps from adenoma to carcinoma and ultimately to explore the cause and effect relationship of the known genetic alterations that transpire (3).

Methods

Mice. All CB.17-scid mice used in this study were bred at Roswell Park Cancer Institute. The mice resided in microisolator cages (Lab Products Inc., Maywood, NJ). All food, water, and bedding were sterilized by autoclaving. Animals received standard rodent chow and water ad libitum and were maintained in rooms designated only for scid mice. The investigators dressed in surgical caps, masks, shoe covers, and gowns before entering rooms that housed scid mice. All surgical garb used was autoclaved after unpacking, and gowns were kept strictly sterile. Sterile gloves and laminar flow hoods were used for all mice handling, whether cage changes or surgical procedures. Hands were wet with a liquid sterilant (Exspor; Alcide Co., Norwalk, CT) before making direct contact with the mice. There were no infections in the colony during the study period.

Villous adenoma implantation. Villous adenomas were obtained from three surgical specimens: (*a*) G. W., a 65-yr-old female with a 14-cm rectal villous adenoma displaying moderate to severe atypia and no carcinoma; (*b*) J. K., a 68-yr-old female with a 6.5-cm villous adenoma containing moderately differentiated adenocarcinoma at its base and composing ~ 20% of the polyp; and (*c*) D. N., a 68-yr-old female with a 5.5-cm villotubular adenoma containing focal carcinoma

This study was presented at the American Association for Cancer Research in Orlando, FL on 20 May 1993.

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Received for publication 10 December 1993 and in revised form 16 May 1994.

J. Clin. Invest.

0021-9738/94/11/2153/05 $2.00
Volume 94, November 1994, 2153–2157

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; scid, severe combined immunodeficient.

Table I. Villous Adenoma Engraftment into scid Mice

Tumor description*	In vivo passage No.	Site of implantation	No. of tumor growth / No. of inoculated‡	Observation period
				mo
G. W.	1§	SQ	2/2	18
		IP	2/2	17
	2	SQ	2/3	20
		IP	1/2	20
	2	SQ	6/8	7
		IP	5/5	12
	3	SQ	0/0	—
		IP	6/7	4
J. K.	1	SQ	3/3	8
		IP	3/3	8
DN	1	SQ	2/2	7
		IP	1/1	7

SQ, subcutaneous; *IP,* intraperitoneal. * These tumors were taken from surgical specimens of three different patients. ‡ This represents the number of mice that actually had villous adenomas grow versus the number of mice inoculated. § Passage 1 represents the initial passage of tumor into the animal. All these animals have subsequently been killed for repassage and histologic studies.

in situ. Patients G. W. and D. N. had negative family histories for colon cancer. J. K. had a strong family history of cancer, including colon cancer; her grandmother had gastric cancer, her father had prostate cancer, and her sister had colon cancer also at age 68. The presenting complaints in G. W., J. K., and D. N. were those of decreased stool caliber, heme-positive stools, and diarrhea, respectively. The tumor tissues were incubated at room temperature for half an hour in RPMI 1640 culture medium containing penicillin (500 U/ml), streptomycin (250 μg/ml), and fungizone (2 μg/ml). The tissue was then washed × 2 in RPMI medium containing penicillin (100 U/ml) and streptomycin (50 μg/ml) only. It was then cut into 2–4-mm pieces in a Petri dish using a cross-scalpel technique (9). The animals were anesthetized using intraperitoneal injections of 0.5 ml of avertin (2,2,2-tribromoethanol and 2-methyl-2-butanol). After full anesthetization and paralysis, the lower abdomen was sprayed with Exspor solution. A 5-mm incision was made in the skin of the right lower abdomen and 100 mg of tumor tissue was placed subcutaneously. When tissue was to be placed intraperitoneally, the opening was carried through the abdominal wall musculature. Both skin and muscle were closed in a single layer using inert metal clips. The animals were placed under a heat lamp for 10–15 min and then returned to their cages. The animals with subcutaneous implants were observed on a weekly basis for tumor size and viability. Subpassaging was done from these xenografts growing in the subcutaneous space. Since these tumors grew slowly, the volume harvested for inoculation of other mice was 25–50 mg/animal. Tumor was implanted subcutaneously and intraperitoneally in young (1–2-mo-old) mice. Tumor placed intraperitoneally grew much more rapidly to larger volumes, and further subpassaging was usually carried out after ~ 8 mo of in vivo growth. To subpassage from animals harboring intraperitoneal villous adenomas, about 250 mg of solid tumor and 150–200 mg of the mucinous component were removed for each animal to be inoculated. Implantation of tumor was performed immediately after its retrieval from the donor animal. The tumors were not manipulated in any other manner than that described above.

At 1, 4, and 12 mo, tumor was removed from anesthetized animals with subcutaneous implants. Animals with intraperitoneal tumor were killed when specimens were removed. Tumors from both groups were divided and reimplanted in mice to maintain a long-term continuum of tumor growth. Tissues were also prepared by hematoxylin and eosin staining as well as in situ hybridization using a biotinylated probe specific for the alpha-satellite DNA of human chromosome 17 (Oncor Inc., Gaithersburg, MD).

Human DNA identification. In situ hybridization using a biotinylated probe for human chromosome 17 was performed using a modification of techniques reported previously (10). Both immunoperoxidase and immunofluorescent staining were carried out. Frozen section specimens were dried at room temperature and fixed in a 2% paraformaldehyde solution for 10 min and then washed in phosphate buffer solution (PBS) × 2. Nonspecific avidin-biotin binding sites were blocked. Tissue samples were then treated with 0.5% Triton and 0.5% NP-40 for 20 min. The tissue sections underwent prehybridization buffering for 1 h at 55°C in a humidified incubator. The DNA probe was heated for 10 min at 90°C. 20 ng of the heated DNA probe was applied to the tissue sections, and the sections were then heated to 90°C for 10 min. To allow for complete hybridization, the specimens were then incubated at 55°C for

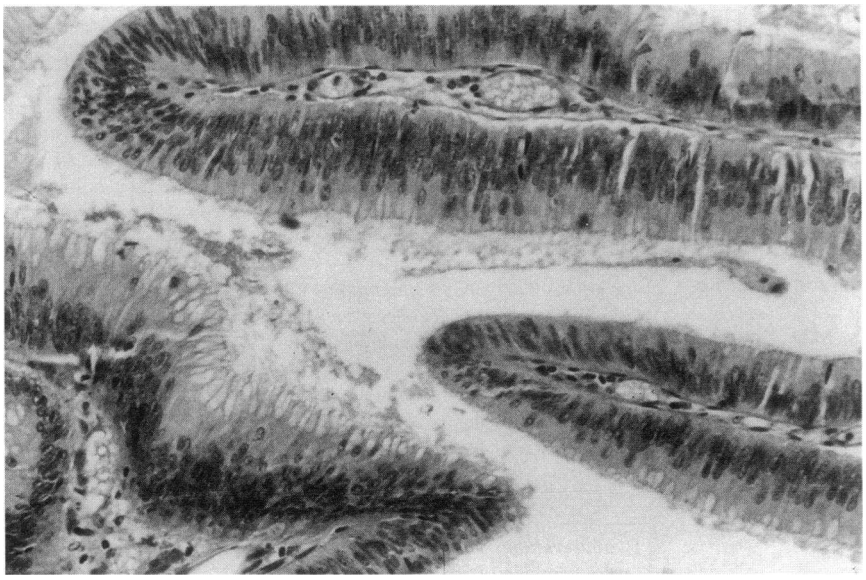

Figure 1. Hematoxylin and eosin–stained human villous adenoma after 4 mo of growth in a scid mouse.

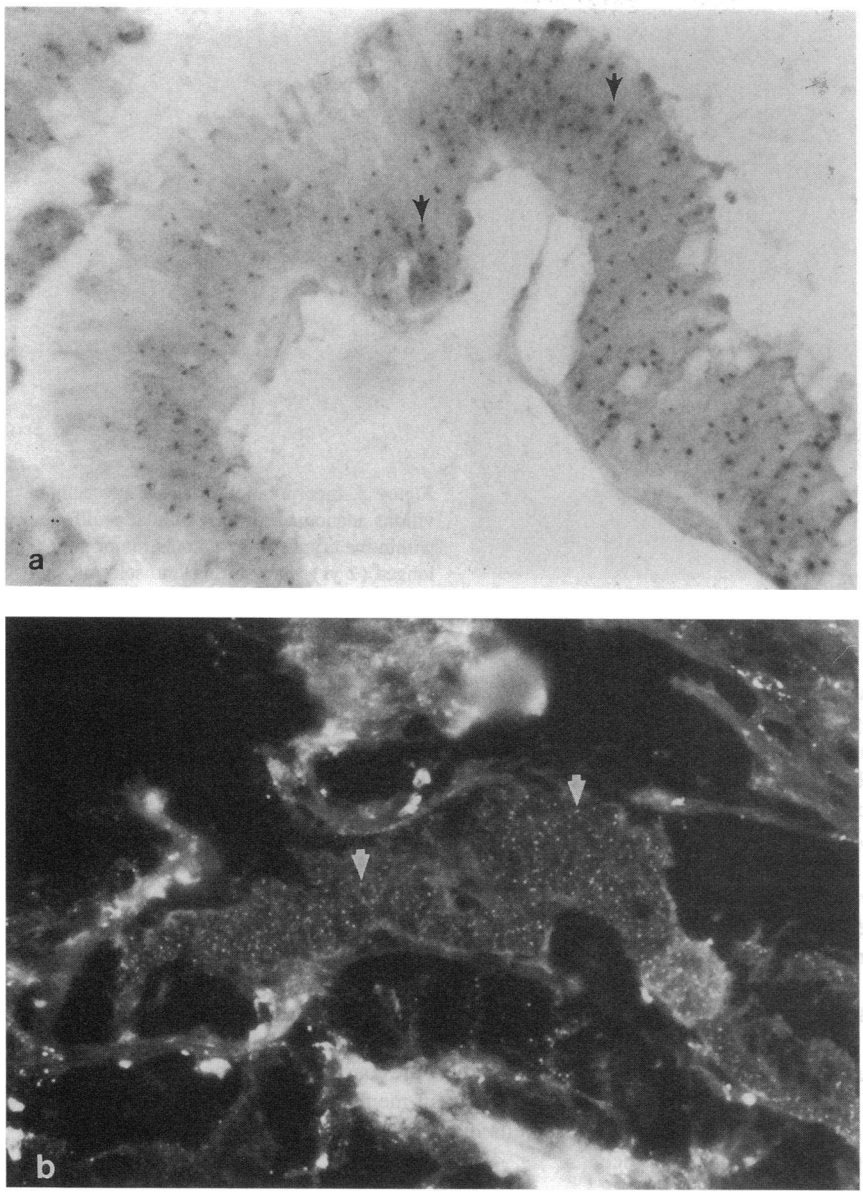

Figure 2. (*a*) After 1 yr of growth in the scid mouse, the adenoma maintains human DNA as demonstrated by DNA in situ hybridization using a human-specific DNA probe, chromosome 17. Arrows indicate hybrid nuclei after immunoperoxidase staining. (*b*) Immunofluorescent staining of this 1-yr-old tumor confirms human genome persistence. Arrows point to the white dots which represent the fluorescence of nuclear hybridization.

18–24 h. These tissues were then washed in serially diluted solutions of sodium chloride/sodium citrate. Before immunofluorescent staining, the specimens were incubated for 1 h at room temperature in a solution of 1% BSA and 0.1% Triton in PBS. Specimens were then exposed to streptavidin-FITC (0.1%) for 45 min. They were washed with PBS and mounted. Tissue sections for immunoperoxidase staining were quenched for endogenous peroxidase for 20 min and then washed × 2 with PBS. Avidin–horseradish peroxidase was incubated with tissue sections for 30 min and then washed × 2 with PBS. The immunoperoxidase specimens were exposed to a substrate of 0.1% diaminobenzidine, counterstained with Mayer's hematoxylin, dehydrated, and mounted.

Bromodeoxyuridine (BrdU) incorporation. To assess cellular proliferation within the adenoma, especially the crypts, BrdU incorporation was evaluated. Mice containing large villous adenomas from patient G. W. were injected by tail vein or subcutaneously with 5 mg BrdU in 0.5 ml PBS. Similar injections were performed for control animals with no tumor. In two separate studies, tissue was harvested 22 or 44 h after BrdU injection. Colon and spleen were removed from control animals, and villous tumor was removed from the study animals. The tissues were fixed in 70% ethanol and stored at 4°C for a minimum of 24 h. Paraffin sections were made according to a routine procedure. DNA was denatured with 4 N HCl. After neutralization with BSA solution (pH 8.5) the sections were blocked with normal goat serum for 45 min. The sections were then exposed to anti-BrdU for 1 h. Specimens were washed overnight in 0.1% Triton/PBS. An affinity-purified biotinylated goat anti–mouse IgG (Zymed Laboratories, Inc., South San Francisco, CA) secondary antibody was used in concentrations of 1:1,000–1:2,000 for 45 min. After a series of washings with PBS, avidin-biotinylated horseradish peroxidase was used for 1 h. The sections were washed and allowed to react with diaminobenzene for 6–10 min and then counterstained with hematoxylin. Next, tissues were dehydrated and mounted.

Results

Human villous adenomas obtained from surgical specimens were implanted either subcutaneously or intraperitoneally into scid mice. The human tissue xenografts grew very slowly in the subcutaneous position, increasing ~ 2 mm in diameter at 6 mo with mucin accounting for a majority of its small volume. Intraperitoneal implants grew at a much faster rate, but also produced an excessively large amount of mucin which ac-

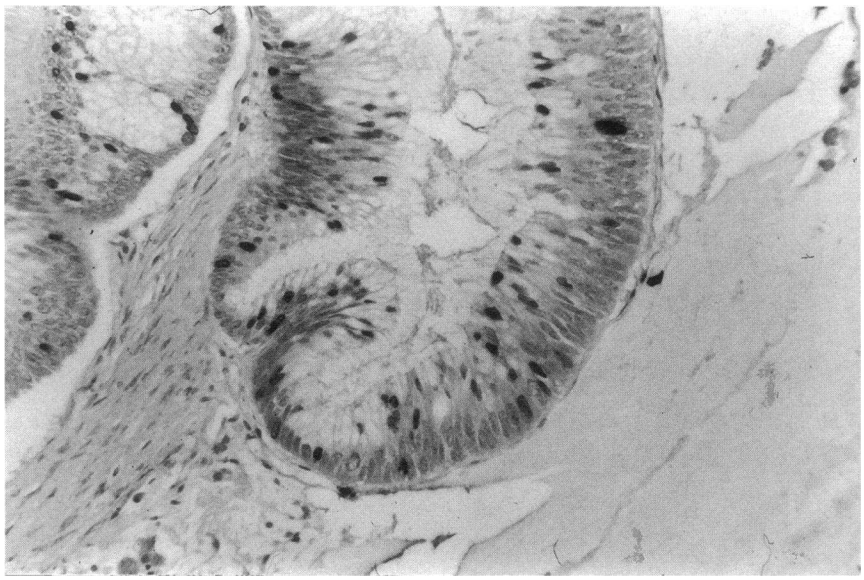

Figure 3. Incorporation of BrdU into cells in villous adenoma indicates cellular proliferation within the crypts of the colonic tumor after prolonged (2 yr) xenografting into scid mice.

counted for an approximate twofold increase in the abdominal girth of the mice after 8 mo. The solid tissue inoculum was noted to have increased in weight 100–250 mg to 4,000–5,000 mg during the time period of observation. The mucin component increased also in similar proportions. The abdominal viscera remained healthy despite the growth and secretory properties of the villous tumors. There were no adhesions between visceral organs or tumor tissue and these organs.

As shown in Table I, tumor specimens were derived from three different patients all of which were successfully engrafted into scid mice. Viable xenografts were maintained for more than 20 mo, and we established that these tumors could be subpassaged from one mouse to another. Implanted human villous tumors growing in scid mice for 4 mo maintained their histologic structure (Fig. 1). The tumor villi are well preserved with pools of mucin surrounding them. The basement membranes remain intact, and the elongated nuclei display stratification toward the cell surface. There are no signs of malignant transformation. In situ hybridization with a human DNA probe 1 yr after in vivo implantation confirmed the human identity of the tissue xenograft (Fig. 2 *a*, *arrowheads*). The black spots in the tissue show hybridization of the alpha-satellite DNA probe to human chromosome 17 within the nuclei of cells. This was further verified with immunofluorescent staining (Fig. 2 *b*) in which the white dots (*arrowheads*) illustrate the chromosomal probe binding to the DNA in the adenomatous tissue. When scid mouse colon was examined using this probe using fluorescent in situ hybridization there was no detectable signal.

To establish that the cells in the xenograft were dividing, scid mice having the human tissue were given BrdU 22 h before killing. Using a BrdU-specific antibody, human cells were found in S phase in all sections of the adenoma evaluated. Most cells incorporating BrdU were located within the crypts of the tissue and near the basement membrane (Fig. 3), although it appears that some cells had begun migrating toward the epithelial surface.

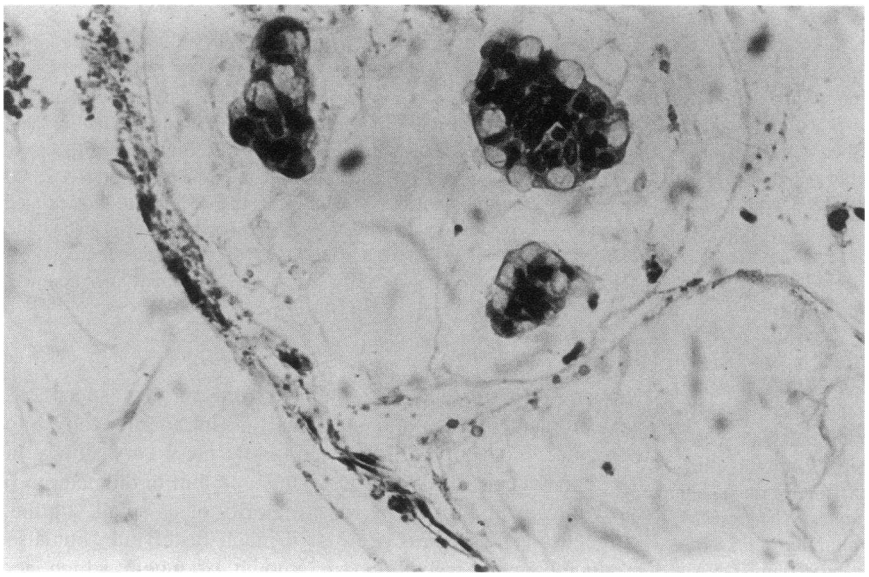

Figure 4. Isolated nests of tumor cells in mucinous pool secreted by villous adenoma.

The mucin was noted to contain isolated nests of large atypical cells with smooth round nuclei and large apical secretory vacuoles (Fig. 4). No cytologic features indicated malignant changes. Attempts to generate in vivo tumors or in vitro cultures from these cells found clustered throughout the mucinous component have been unsuccessful but remain under investigation.

Discussion

We report here for the first time that human colonic adenomas can be successfully engrafted and maintained in scid mice. There has been limited success with the establishment of human colon adenoma cell lines in vitro (11, 12). These researchers have been able to achieve in vitro malignant transformation using various chemical carcinogens. Colonic villous adenomas have a high propensity to naturally progress to a malignant tumor in humans, and we hypothesize that similar characteristics should exist in tumors successfully implanted in scid mice for long periods of time. We have shown that human villous adenomas can be maintained in scid mice for up to 20 mo. Although there is a difference in the growth pattern for tumors implanted subcutaneously and intraperitoneally, inoculation at either site allows for long-term maintenance of healthy tumor tissue. Furthermore, tumors implanted subcutaneously can be reimplanted intraperitoneally and they will conform to the rapid growth pattern noted in tumors initially implanted intraperitoneally.

BrdU is a pyrimidine analogue which labels the nuclei of those cells actively synthesizing DNA. The incorporation of BrdU within the villous adenomas indicated active proliferation of epithelial cells. Cellular proliferation was more pronounced within the crypts of the adenoma. The incorporation of BrdU was fairly uniform within the crypts throughout the adenoma. No areas of the tumor had alterations in proliferation to a degree suggesting possible malignancy. The clusters of cells growing in the mucinous component have often been associated with an increased malignant potential, but this appears to be more anecdotal since studies supporting this theory are not available. After prolonged in vivo growth these isolated clusters of cells do not demonstrate malignant features, though their ability for more aggressive transformation needs to be studied. We now have the capability of evaluating villous adenomas along a continuum of growth in vivo to define alterations in surface proteins and the oncogene expression as these tumors tend toward dedifferentiation. The length of time necessary for a natural progression to malignancy is unknown and therefore makes one aspect of subsequent study long term. As noted above, an array of normal tissues has been engrafted in the scid mice and there was no reason to suspect that normal colonic tissue would not also engraft. However, engrafting normal tissue was outside the scope of this study since we wanted to establish a model of colonic adenomatous tissue growth that would allow study of events leading to malignant transformation. This system will allow for manipulation of these preneoplastic lesions through the insertion of genetic vectors in an attempt to shorten the progression to malignancy. Our human villous adenomas from one patient have greater than 20 mo of in vivo growth in 12 animals, and more recently specimens from 2 other patients have been implanted and have been growing in vivo for up to 8 mo. This indicates a reproducibility of human villous adenoma growth in scid mice.

Acknowledgments

This work was supported by National Institutes of Health grants CA-54491 and CA-57974 and Research for Health in Erie County, Inc.

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