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Human Colon Cells: Culture and in Vitro Transformation

Abstract. *Normal human colon mucosal epithelial cells were cultured in vitro and treated with the oncogenic simian DNA virus (SV40) and the chemical carcinogen azoxymethane. Both SV40 and azoxymethane altered a number of phenotypic characteristics of the normal human colon cells, including their morphology, culture longevity, growth in soft agar, substrate adherence, and peanut agglutinin binding. The SV40 transformants synthesized intranuclear T antigen. These data indicate that normal human colon mucosal cells were transformed toward the malignant phenotype.*

Mechanisms of carcinogenesis are poorly defined not only because of the complexity of the process but also because of the model systems chosen for study. Approximately 80 percent of human neoplasms are carcinomas and originate from epithelial cells. However, most experimental models of neoplastic transformation in vitro are based on the use of fibroblastic cells, because these cells predominate in the outgrowths from most tissue explants and survive subculture under the usual conditions for growing cells in vitro. Consequently, the features of fibroblast transformation and growth are better defined than those of epithelial cells (*1, 2*). Thus there is a need to develop successful models for the initiation and characterization of epithelial cells transformed in vitro.

The incidence of large bowel cancer currently is one of the highest among all human neoplastic diseases in the United States. Although cultured cells have been used to study colon cancer, studies performed in vitro have been limited. Normal human colon mucosal (NCM) epithelial cells have not been used for transformation studies in vitro because only short-term cultures of such gastrointestinal cells have been achieved (*3*). However, we have developed methods that permit longer term culture of human colon and other gastrointestinal epithelial cells (*4, 5*). We now report evidence that NCM cells can be transformed in vitro with the oncogenic simian virus 40 (SV40) or with the chemical carcinogen azoxymethane (Azm). SV40 was chosen because it has been shown to transform a variety of human cells, and many aspects of SV40 genetics and biology are well defined (*6*). Azoxymethane is a direct-acting, carcinogenic derivative of dimethylhydrazine, a potent chemical carcinogen with demonstrated colon specificity in animal model systems (*7*).

The NCM cultures were initiated in vitro (*4*); the culture medium was an enriched L15:S-MEM base medium supplemented with 2 percent fetal bovine serum (MA Bioproducts) (*8*), L broth (*9*), pituitary extract (*10*), and other factors (*11*). The NCM cultures were characterized as epithelial on the basis of several criteria, including morphology, presence of keratin, little or no fibronectin, and synthesis of colon-specific mucins and carcinoembryonic antigen (*4, 12*). The cells were maintained and subcultured in suspension without the use of standard dissociating agents (*4*). At the second subculture, NCM cells were plated (2×10^6 to 3×10^6 cells per flask) into plastic flasks (25 cm^2). Four separate experiments were performed, with three experimental groups in each (controls, SV40-infected, and Azm-treated). Control cultures received 0.1 ml of phosphate-buffered saline. The SV40-treated cells received approximately five virus plaque-forming units (*13*) per cell. Azoxymethane(10 μg/ml in 0.1 ml of phosphate-buffered saline) was added to each appropriate culture for 3 weeks at weekly intervals. Each group had three or four replicate cultures. The suspension cultures were observed daily to assess differences in morphology and growth and were subcultured at weekly intervals (*4*). At various subcultures they were analyzed for altered phenotypic characteristics (Tables 1, 2, and 3).

Significant differences were noted between the viral or chemical carcinogen–treated cells and the control cells. These included changes in cell size, decreased proportions of morphologically differentiated epithelial cells in the population, and altered growth, as evidenced by enhanced adherence to the culture substrate, a reduced requirement for NCM-conditioned medium, the ability to grow in soft agar, and increased culture longevity (Tables 1 and 2). Viability after chemical dissociation and binding of peanut agglutinin lectin (PNA; Sigma) were evident only in the transformants (Table 1). In addition, only SV40-infected cells expressed SV40-specific intranuclear T

Table 1. Phenotypic characteristics displayed by NCM control and transformed cells.

Group	Phenotypic characteristics									
	Altered morphology	Adherence to culture substrate* (%)	Viability (%)		Total number of subcultures in experiment				Binding of PNA§ (%)	
			After dissociation†	Without NCM-conditioned medium‡	I	II	III	IV	III	IV
Control		5 to 10	0 to 1	0 to 5	5	3	2	4	0 to 10	0 to 10
SV40	±‖	10 to 25	5 to 20	10 to 20	8	8	5	10	60 to 75	75 to 90
Azm	±‖	10 to 20	5 to 20	10 to 20	7	6	5	8	50 to 80	80 to 90

*Under standard culture conditions, substrate uncoated. †Chemical dissociation was accomplished by centrifuging cells (800*g*), resuspending the pellet for 3 to 5 minutes at 37°C in 0.25 percent trypsin–0.02*M* EDTA, then replating cells in complete culture medium. ‡Analyzed by centrifuging cells and replating in complete culture medium without NCM-conditioned medium; viability determined by trypan blue dye exclusion of cells 24 to 48 hours after plating. §Performed by direct assay with fluorescein isothiocyanate (FITC)–labeled PNA (E-Y Laboratories); cells (5 to 8×10^4 per well) were rinsed with phosphate-buffered saline then incubated for 45 minutes at 4°C with a 1:30 dilution of FITC-PNA stock in phosphate-buffered saline. Not done for experiments I and II. ‖±, Similar to controls but cells commonly somewhat larger.

antigen (Table 3). T antigen was detected in cells by the first subculture and was found in an increasing proportion of cells upon continuous culture. Increased culture longevity and growth in soft agar were late phenotypic changes, detected only after two or more subcultures.

As has been observed with other epithelial cells, transformation-associated characteristics found in vitro are often not comparable to those noted for fibroblasts (*1*). For example, colon and many other epithelial cells grow well in low concentrations of serum and have little or no cell surface fibronectin. In contrast, growth in media with low serum and loss of fibronectin are commonly associated with transformation of fibroblasts in vitro. Cell agglutinability or binding by plant lectins has been used for many years as an indication of fibroblast transformation in vitro (*14*). However, lectins (such as concanavalin A) that have been used commonly for determining fibroblast transformation have not necessarily proved valuable for epithelial cells. Since enhanced binding of PNA has been associated with the development of colon cancer (*15*), it seemed logical to determine whether this cell surface change in galactose moieties might be detected in transformed cells (Table 1). This was a discernible change seen by the second subculture of the cells, suggesting that it is a relatively early event in transformation or malignant progression or both.

Another feature of fibroblast transformation, anchorage-independent growth, is commonly assessed by culturing single cells in a semisolid medium such as soft agar (*16*). For fibroblasts, growth in a semisolid medium generally correlates with enhanced ability of the cells to grow in suspension. In contrast, when transformed NCM cultures acquired the ability to grow in soft agar (Table 2), they also displayed a somewhat greater propensity to attach to a substrate. This may suggest that the abilities to grow in suspension or in soft agar are independent phenotypes. Alternatively, it reflects the low viability that is characteristic of dissociated, single NCM cells (*4*). A larger portion of the cells in the transformed cultures remained viable upon dissociation into single cells (Table 1).

Increased culture longevity is often seen upon transformation of fibroblast cultures in vitro. Cell lines have been selected from some of these cultures. Cell line selection has been most common with rodent, infrequent with human, and not possible with chicken fibroblasts. Thus far, although transformed NCM cells showed increased longevity in vitro (Table 1), attempts to establish cell lines either upon harvesting the colonies that grew in soft agar or by continued subculture have been unsuccessful. These observations are in agreement with the general difficulties in establishing human cell lines.

The requirements of NCM cells for conditioned medium, low subculture ratios, and nondisrupted cell-to-cell associations (*4*) suggest that the colon cells may synthesize important paracrine or autocrine growth factors. The transformants were altered in these properties, including a reduced requirement for conditioned medium. Thus, new growth factors might be produced upon transformation of the cells, or the transformed cells may no longer require the same factors for growth stimulation, as has been shown for other transformed and tumor cells (*17*). Further studies concurrently exploring specific growth-factor requirements, transformation in vitro, and differentiation in human colon and other gastrointestinal cells, may define valuable new avenues in gastrointestinal physiology, toxicology, and oncology research.

Table 2. Percent efficiency of colony formation in soft agar. Single cells (dissociated with 0.25 percent trypsin–0.02*M* EDTA) were plated (10^5 cells per well) in semisolid complete medium containing 0.3 percent agarose (*15*); percent efficiency of colony formation was calculated by $(N_c/N_p) \times 100$, where N_c is the number of colonies and N_p is the number of cells plated.

Experiment number	Culture passage number assayed	Experimental group		
		Control	SV40	Azm
I	2	0	0	0
	5	0	0.030	0
	7	NC*	0.040	0.002
II	3	0	0.001	0
	6	NC*	0.004	0.001
III	2	0	0.002	0
	5	NC*	0.040	0.003
IV	4	0	0.004	0.001
	8	NC*	0.010	0.005

*NC, no cultures available; crisis occurred at earlier subculture.

Table 3. Expression of SV40-specific intranuclear T antigen, assayed by indirect immunofluorescence (5×10^4 cells per well) (*17*) with pooled sera from SV40 tumor–bearing hamsters as the primary antibody and goat antibody to hamster immunoglobulin as the secondary antibody. Values indicate percent of cells with intranuclear fluorescence.

Experiment number	Culture passage number assayed	Experimental group		
		Control	SV40	Azm
I	1	0	10 to 25	0
	4	0	80 to 90	0
II	1	0	10 to 20	0
	3	0	75 to 80	0
III	0	0	5 to 10	0
	2	0	40 to 50	0
IV	2	0	45 to 50	0
	4	0	75 to 80	0

MARY PAT MOYER
J. BRADLEY AUST
Department of Surgery, University of Texas Health Science Center at San Antonio 78284

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10. Pituitary extract: three frozen bovine pituitaries (Pel-Freez) were thawed, minced, and homogenized in 50 ml of MEM (AutoPow, Flow Labs) with the use of a stainless steel tissue press (EDCO). After centrifugation (10,000 rev/min) at 4°C for 45 minutes, the clarified supernatant was filter-sterilized through a 0.2-μm Nalgene filter, portioned, and stored frozen at −20°C. This concentrated stock, which contained 8 mg of protein per milliliter, was used at a final concentration of 1 percent by volume.
11. Other factors included as media supplements were: ITS Premix (Collaborative Research), which adds insulin (I), transferrin (T), and selenium (S) to final concentrations of 5 μg/ml, 5 μg/ml, and 5 ng/ml, respectively; hydrocortisone (10 μg/ml; Sigma), pentagastrin (5 μg/ml; Sigma), and NCM-conditioned medium [at least 25 percent of total volume (*4*)]. Antibiotics used were gentamicin (25 μg/ml), penicillin (100 unit/ml), streptomycin (50 μg/ml), and Fungizone (50 μg/ml).
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Duchenne Muscular Dystrophy Involving Translocation of the *dmd* Gene Next to Ribosomal RNA Genes

Abstract. *Duchenne muscular dystrophy (DMD) is a severe X-linked disorder leading to early death of affected males. Females with the disease are rare, but seven are known to be affected because of a chromosomal rearrangement involving a site at or near the* dmd *gene on the X chromosome. One of the seven has a translocation between the X and chromosome 21. The translocation-derived chromosomes from this patient have been isolated, and the translocation is shown to have split the block of genes encoding ribosomal RNA on the short arm of chromosome 21. Thus ribosomal RNA gene probes may be used to identify a junction fragment from the translocation site, allowing access to cloned segments of the X at or near the* dmd *gene and presenting a new approach to the study of this disease.*

Duchenne muscular dystrophy (DMD), the most common and severe of the muscular dystrophies, is an X-linked disorder normally affecting only males. However, the disease has been found in seven females, and in each case an X-autosome translocation is present in which the exchange point in the X is in band Xp21 near the middle of the short arm (*1–7*). As in most X-autosome translocations, the normal X chromosome in these patients is late-replicating and presumably inactive in all or most cells. None of the mothers have been shown to be carriers, and none of the parents carry the translocation. The concurrence of the repeated translocation with the Duchenne phenotype suggests that band Xp21 is the site of the *dmd* gene and that its activity is disrupted by the translocation. The nature of this disruption is unknown; in some cases it may involve direct interruption of the *dmd* gene while in others it may be due to action at a distance, perhaps involving conformational changes in chromatin at the site of the exchange. This, coupled with inactivation of the wild type (*dmd*$^+$) gene on the intact X chromosome, leads to the expression of the disease (*1–7*). This map location for the *dmd* locus is consistent with family studies that have shown linkage of the *dmd* locus to two restriction fragment length polymorphisms (RFLP's) flanking the Xp21 region (*8, 9*).

In the translocation case studied in our laboratory (*1*), the autosomal exchange point was found in the middle of the short arm of chromosome 21, a region known to carry multiple copies of the ribosomal DNA (rDNA) repeat unit coding for 18*S* and 28*S* ribosomal RNA (rRNA). Nucleolus organizer region (NOR) staining revealed that the translocation apparently split the block of ribosomal genes (*1*). We now report the isolation of both chromosomes derived from the translocation in somatic cell hybrids and show by molecular probing with human-specific rDNA sequences that rDNA is present on both translocation-derived chromosomes. This confirms that the rDNA block has been split by the translocation and suggests that rDNA probes might be used to detect molecular clones spanning the translocation site leading to isolation of the *dmd* gene, even though its product is not known.

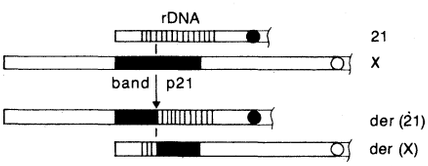

Fig. 1. Schematic of the short arms of chromosome 21, the X chromosome, and the two translocation products der(21) and der(X).

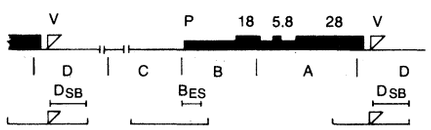

Fig. 2. Schematic of the rDNA repeat unit (*14*), approximately 43 kb in length. The transcribed portion is about 14 kb in length beginning at the promoter P and including the 18*S*, 5.8*S*, and 28*S* rRNA genes. Nontranscribed spacer separates the transcribed units. A variable region V contains one or more copies of a 900-bp internal repeat unit, generating length variability in this region (*15*). The main repeat unit is cut four times with Eco RI, giving four fragments: A (7.3 kb), B (5.7 kb), C (12 kb), and D (19 kb). Below the schematic are the subcloned regions D_{SB} and B_{ES} used as probes. The B_{ES} probe is an Eco RI–Sal I subclone from the B fragment (*16*), and the D_{SB} probe is a Sal I–Bam HI subclone from the D fragment (*15*); both are cloned into the appropriate site in pBR322. At the bottom of the figure are the Bam HI restriction fragments recognized by these probes. The Bam HI fragment recognized by D_{SB} is variable in length because it contains internal repeats.

The subject of our study is a 20-year-old female who was diagnosed at age 8 with Duchenne muscular dystrophy. Details of her phenotype and karyotype have been reported (*1*). In the schematic of her translocation (Fig. 1) we assumed that the ribosomal gene block was split by the translocation.

To test for the presence of rDNA on the translocation-derived chromosomes der(X) and der(21), we first separated them from human chromosomes 13, 14, 15, 21, and 22, each of which carries multiple copies of the rDNA repeat unit. This was accomplished by fusing the patient's cells to a mouse cell line and allowing the human chromosomes to be lost from the hybrid lines.

Hybrids were generated by Sendai virus–induced fusion of the patient's fibroblasts with mouse A9 cells deficient in hypoxanthine phosphoribosyltransferase (HPRT). Hybrids were selected in HAT medium (alpha medium containing 10 μg of hypoxanthine per milliliter, 10 μg of thymidine per milliliter, and 1 μ*M* methotrexate) containing 3 μ*M* ouabain, a dose sufficient to kill human cells but not A9 cells or hybrids (*10*). Colonies were picked from selective medium and grown continuously in HAT medium.

After being cultured for about 1 year in HAT medium, all hybrid lines retained the der(X) chromosome since the only active *hprt*$^+$ gene is on the long arm of this chromosome. Three independent hybrid clones were selected for further study. Hybrids A2 and F1 each contained only the der(X) translocation product. Hybrid C2 contained both der(X) and der(21) plus one or two other nonacrocentric human chromosomes. Subclones A2-4 and F1-3 were generated by plating A2 and F1 in HAT medium, and subclones A2-T4, F1-T5, and C2-T10 were generated by plating in medium containing 6-thioguanine to select for subclones that had lost the der(X) chromosome (*11*). Clones A2-4 and F1-3 contained the der(X); A2-T4 and F1-T5 had no human chromosomes; and C2-T10 contained only the der(21) and in some cells a human chromosome 5.