Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 2008–2012, March 1989
Medical Sciences

Skin fibroblasts from individuals hemizygous for the familial adenopolyposis susceptibility gene show delayed crisis *in vitro*

(human cells/growth control/3T3 passage/proliferative diseases/colon cancer)

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Communicated by Richard Axel, December 15, 1988

ABSTRACT **Normal human fiboblast cells have not been reported to escape crisis—that is, they die after about 24 doublings in culture. We have been studying the growth properties of skin fibroblast cells from persons in families with familial adenopolyposis of the colon (FAP). An individual hemizygous at the FAP locus will develop hyperplasia of the colonic epithelium followed by colonic polyps, both at an early age. Polyps themselves still retain a single functional FAP allele. A mutation or deletion in this allele in a polyp is hypothesized to lead to further loss of growth control; thus, a tumor is formed. We found that the *in vitro* life-span of skin fibroblast cells from FAP individuals and from some asymptomatic children were markedly extended when compared with normal individuals.**

The limited life-span and inexorable death of normal human diploid cells in culture is well documented (1). After about 25 doublings in culture, cells enter a senescence phase during which no increase in cell number is observed. This is followed by cell death (1). The mechanism responsible for the shutdown of cell proliferation is not well understood. Genetic studies with somatic cell hybrids and analysis of heterokaryons generated by fusion of normal replicating cells with quiescent or senescent cells suggest that the phenotype of limited proliferation is dominant (2–4). Add-back experiments also support the notion that senescence is "dominant" over establishment in human fibroblasts. For example, (*i*) microinjection of the human oncogene *HRAS* DNA alone or in conjunction with adenovirus oncogene *E1A* DNA into senescent human cells fails to stimulate the cells to enter into the S phase (4), (*ii*) a membrane-associated protein has been isolated from senescent cells that inhibits DNA synthesis in proliferation-competent cells (5), and (*iii*) microinjection of mRNA isolated from senescent human cells inhibits DNA synthesis in proliferation-competent cells (6). Finally, when genomic DNA isolated from serum-deprived, quiescent human embryo fibroblasts is transfected into HeLa cells, recipient cells show a specific inhibition of DNA synthesis (7). Taken together, these studies suggest that fibroblast growth control is mediated in part by genes whose function is to keep normal fibroblasts from proliferating.

Knudson first proposed that both dominant familial and nonfamilial forms of a particular cancer may be due to mutations in recessive growth-regulating genes in humans (8, 9). According to his hypothesis, the dominant familial pattern of susceptibility to a specific type of cancer results from the inheritance of hemizygosity—that is, the presence of only one functional allele for one such gene. According to this hypothesis, a familial tumor develops from among cells that have suffered a deletion or a somatic mutation in the remaining normal homologous allele, whereas in the sporadic nonfamilial situation, both mutations are presumed to occur somatically. This model has been successful in predicting rates of incidence and genetic linkages in cases of familial and sporadic retinoblastoma, Wilms' tumor, acoustic neuroma, small-cell lung cancer, and adenopolyposis of the colon (10–14). We have studied the cultures of skin fibroblasts of persons in families with familial adenopolyposis of the colon (FAP).

At an early age, an individual hemizygous at the FAP locus will develop colonic hyperplasia and hundreds to thousands of colonic polyps (15, 16). One or more of these polyps can be a direct precursor of a malignant carcinoma (17). Recently, Bodmer and coworkers used restriction fragment-linked polymorphism (RFLP) analysis with the probe $C_{11}P_{11}$ to demonstrate that a gene for FAP susceptibility is located near bands 5q21→q22 on chromosome 5 (18, 19) (assigned the symbol *APC* for adenomatosis polyposis coli). Polyps from individuals heterozygous for this probe remained heterozygous, suggesting that polyp formation did not require deletion of the second allele. Other studies suggest that additional chromosomes may be involved in the development of familial and sporadic colon carcinoma (20, 21). According to Knudson's hypothesis, a defective allele of the FAP susceptibility gene is inherited by FAP individuals, leading to hyperplasia and polyps in the colon, while a mutation in the second allele in a polyp cell leads to the further loss of growth control, resulting in an adenocarcinoma. While the biochemical mechanism driven by these genetic events is obscure, it is reasonable to suppose that in two copies, the FAP gene produces enough of a gene product necessary for the growth control in the colonic cells, that loss of one gene copy leads to partial loss of growth control, and that loss of both copies leads to a tumor.

In the present paper, we report on the growth properties of skin fibroblast cells from persons with FAP, presumably individuals that are hemizygous for FAP susceptibility gene. Skin fibroblasts from normal individuals and from asymptomatic children of FAP individuals were also studied. All cell culture work was carried out in ignorance of the clinical status of the donor. When the code was broken, we found that the *in vitro* life-span of skin fibroblasts from FAP individuals and from some asymptomatic children was markedly extended.

MATERIALS AND METHODS

All human cells were obtained from M. Lipkin (Sloan–Kettering Institute, New York) at early passage. The number of doublings of cultures obtained from them is calculated from their passage data. All samples were blind-coded and not identified until the end of each experiment. The 3T3 passage protocol for preventing cell–cell contact was essentially the same as described (22). Briefly, the cells are plated

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Abbreviations: FAP, familial adenopolyposis; 1/TD, inverse of the doubling time.

at 3×10^5 per 60-mm plate in Dulbecco-modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum (Hyclone) in an atmosphere of 10% CO_2/90% air at 37°C. Each culture is passaged every 3.5 days until, as result of senescence, there are no longer 3×10^5 viable cells. Skin fibroblasts from each individual were passaged in this fashion in two or three different experiments.

The growth of cells in the 3T3 protocol was calculated in the following way. The fold increase of cells is the number of cells on a plate per number of cells plated out 3.5 days earlier. Doublings are derived from the fold increase by the following formula: fold increase = $2^n/3.5$ days, where n is the number of doublings in a passage. In Figs. 1 and 2, the y axis of the graph is the inverse of the doubling time (1/TD) so that, for example, a value of 0.5 is equivalent to a doubling time of 2 days. As doubling time increases, the y-axis position approaches zero. When there is no increase in cells in 3.5 days, the x-axis position (that is, the total number of doublings) remains the same as the previous passage, and 1/TD is zero. Cultures can recover from such a state. Crisis is complete when there are not enough cells at 3.5 days to be replated at 3×10^5 per 60-mm plate. The number of prior doublings accumulated at M. Lipkin's laboratory was calculated based on growth data obtained from that laboratory. For comparison, some lines were carried according to the Hayflick passage protocol. Here, once the cells became confluent, cultures were trypsinized, counted, and replated at one-fourth confluence as described (1).

Table 1. Human fibroblast cells: Crisis independent of passage protocol

Cells	Exp.	Doublings at crisis, no. 3T3 protocol	Doublings at crisis, no. Hayflick protocol
CCD120	1	26.7	25.8
	2	23.6	
	3	27.1	
CCD29A	1	26.6	
	2	23.0	
	3	26	
CCD12	1	26	26.1
	2	26.7	26
CCD26	1	24.7	
	2	26.2	
CCD18	1	26.3	22
	2	26.7	
CCD87-10	1	23.7	
	2	23.6	
CCD87-11	1	20	19
	2	23.6	
CCD87-13	1	28.3	
	2	28	
CCD87-2	1	26.7	
	2	26.6	
	3	24.0	

RESULTS

Standard Life-Span of Some Cells Regardless of Passage Protocol. Patients in four classes of clinical status provided skin fibroblasts for the present study. The classes are (*i*) familial colon cancer patients, who do not have colon polyps and so are assumed to be homozygous (+/+) for the FAP susceptibility gene; (*ii*) familial polyposis patients with colonic symptoms, who have FAP and so are heterozygous (+/−) for the FAP susceptibility gene; (*iii*) familial polyposis family members without symptoms, who have a 50% chance of being homozygous (+/+) and a 50% chance of being heterozygous (+/−) for the FAP susceptibility gene; and (*iv*) low-risk persons (either a spouse of some of the above persons or a healthy volunteer), who are all homozygous (+/+) for the FAP susceptibility gene.

When passaged by the 3T3 protocol in which cells are kept from confluence (22), cells from nine individuals senesced and died at 20–28 doublings (Table 1). The average number of doublings of these nine lines was 25.4 ± 2.0. A typical plot of doubling time versus the number of doublings is shown in Fig. 1 *Left*, where the terminal doubling passage is indicated

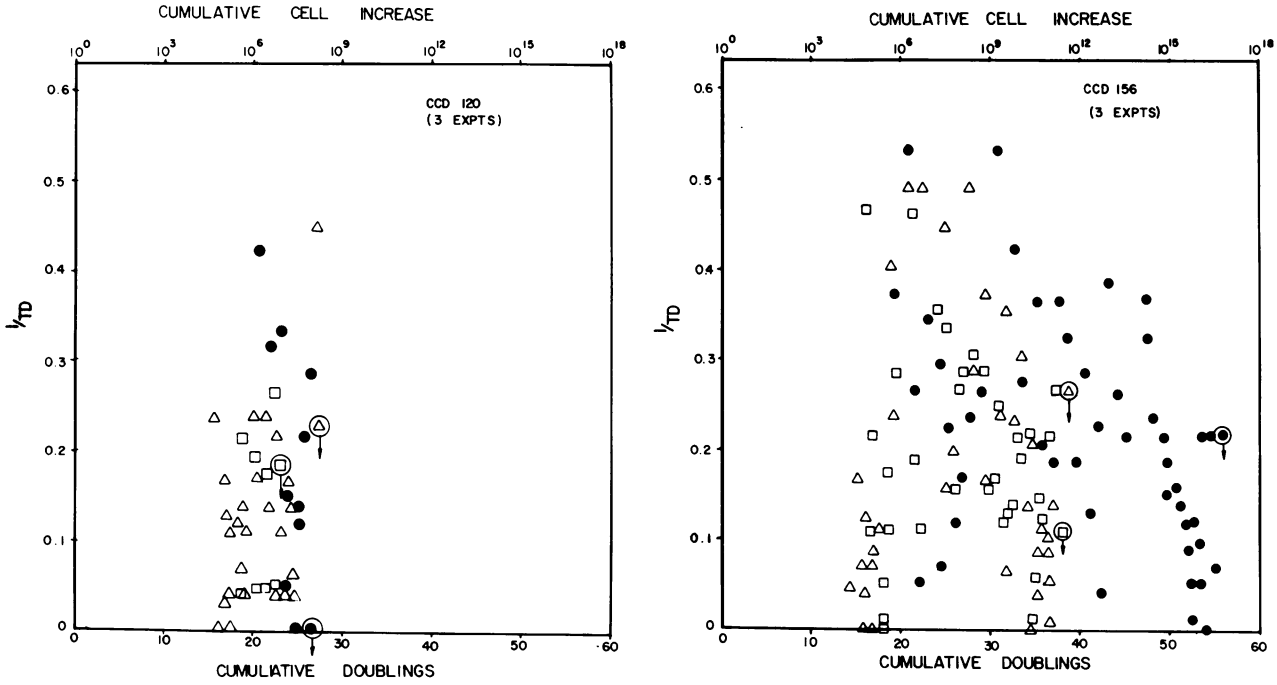

FIG. 1. Growth of human skin fibroblast lines. The inverse of doubling time (days^{-1}) is plotted versus the number of doublings. As doubling time increases, the y axis approaches zero. Doubling time is determined as described in *Materials and Methods*. The last doubling is indicated by a circle with an arrow pointed down. (*A*) CCD120. (*B*) CCD156.

Table2. Human fibroblast cells: Crisis delayed in 3T3 protocol

Cells	Exp.	Doublings at crisis, no. 3T3 protocol	Hayflick protocol
CCD156	1	38.8	23.1
	2	56.4	24.1
	3	38.5	
CCD87-1	1	34.9	
	2	38.9	
CCD87-5	1	55.9	23
	2	40.3	
CCD87-6	1	39.7	22.2
	2	39.5	
	3	35.0	
CCD87-7	1	38.6	
	2	36.6	
	3	38.8	
CCD87-3A	1	43.4	
	2	39.0	
CCD87-4	1	40.1	29.6
	2	45.8	
	3	39.6	
CCD87-8	1	45.4	
	2	40.8	
CCD87-12	1	47.3	
	2	51.4	

by a circle with a downward arrow. Four of these nine populations were also passaged by the Hayflick protocol (1), in which cells are trypsinized only when they have reached confluence. When passaged by the Hayflick protocol, these four cultures, CCD120, CCD12, CCD18, and CCD87-11, reached crisis at doublings 25.8, 26.1, 22, and 19, respectively (Table 1). Thus, for these four cell cultures, crisis was reached at about 25 doublings regardless of the passaging protocol. While Hayflick and Moorhead (1) reported that cells from older persons reached crisis sooner in culture, we did not detect such a relationship.

The cumulative number of cells that could grow from a single cell before senescence can be calculated from the maximum possible number of doublings (Table 1). For these nine cell populations, about 10^7 cells could be grown from a single cell before senescence. Such a "colony" would have a total cellular volume of about 10^2 μl.

Longer Life-Span of Some Cells Is Dependent upon Passage Protocol. Skin fibroblast cell cultures from nine other individuals exhibited markedly different behavior in culture depending upon which cell passage protocol was used. If these cells were passaged only after they became confluent (1), they reached crisis before 30 doublings (Table 2). In distinction, when cultures from this group were kept from confluence by the 3T3 protocol (22), they did not reach crisis until well over 30 doublings (Table 2). The median number of doublings in the 3T3 protocol was 42.0 ± 6.0 for this set. A typical plot of doubling time versus number of doublings for a population of cells of this type is shown in Fig. 1 *Right*. This set of individuals has skin fibroblast cells that can proliferate in culture much more successfully and therefore can produce 10^4 to 10^7 more descendents than cells of the first group (Fig. 1). Here the cumulative cell number from a single cell is 10^{12} to 10^{15}. This means that a single cell would be capable in principle of producing progeny that cumulatively could total more than 10 liters in volume.

Delayed Crisis Phenotype Is Linked to FAP. Under the 3T3 passage protocol, human skin fibroblasts fell into two distinct classes, separated by Student's t test at $P < 10^{-6}$. The first group reached crisis around the 25th doubling. The second group produced 10–25 more doublings or 10^4- to 10^7-fold more cells than the first group (Fig. 1 and Tables 1 and 2). Upon examination of the clinical status of the two groups of donors, we found the two classes to be well correlated with the presumptive number of normal alleles at this FAP locus.

Table 3 compares the donor age, sex, and clinical status with fibroblast life-span results, and Fig. 2 summarizes these data for all 3T3 passage experiments. From Fig. 2 and Table 3 we conclude that, while neither the sex nor the age of the patient is correlated with normal or delayed crisis, persons inheriting heterozygosity for the FAP susceptibility gene have skin fibroblasts that show delayed crisis in culture. Skin fibroblast cells from five FAP patients with colonic symptoms showed delayed crisis in several experiments. Skin fibroblasts from persons of FAP families but without symptoms showed delayed-crisis in four of six cases, and normal

Table 3. Clinical status of human fibroblast cells

Donor	Age, yr.	Sex	Colon symptoms	Predicted FAP susceptibility genotype	*In vitro* crisis	Number on Fig. 2
CCD120	29	F	Familial colon cancer	+/+	Normal	1
CCD29A	68	M	Familial colon cancer	+/+	Normal	2
CCD87-13	37	F	Familial colon cancer	+/+	Normal	16
CCD12	39	M	Low risk	+/+	Normal	3
CCD26	58	F	Low risk	+/+	Normal	12
CCD18	32	M	Low risk	+/+	Normal	13
CCD87-11	58	F	Low risk	+/+	Normal	15
CCD87-1	35	F	FAP with symptom	+/−	Delayed	5
CCD87-7	37	M	FAP with symptom	+/−	Delayed	8
CCD87-3A	14	M	FAP with symptom	+/−	Delayed	9
CCD87-8	36	F	FAP with symptom	+/−	Delayed	11
DDC87-12	42	F	FAP with symptom	+/−	Delayed	18
CCD156*†	55	F	FAP without symptom	?	Delayed	4
CCD87-5†	19	M	FAP without symptom	?	Delayed	6
CCD87-6*†	55	F	FAP without symptom	?	Delayed	7
CCD87-4†	20s	M	FAP without symptom	?	Delayed	10
CCD87-10†	18	M	FAP without symptom	?	Normal	14
CCD87-2†	30s	M	FAP without symptom	?	Normal	17

*CCD87-6 and CCD156 are from the same individual. The two cell samples were obtained at different times, and experiments were carried out independently.

†Genotype of the FAP susceptibility gene of these individuals has 50% chance of being +/+ and 50% chance of being +/−.

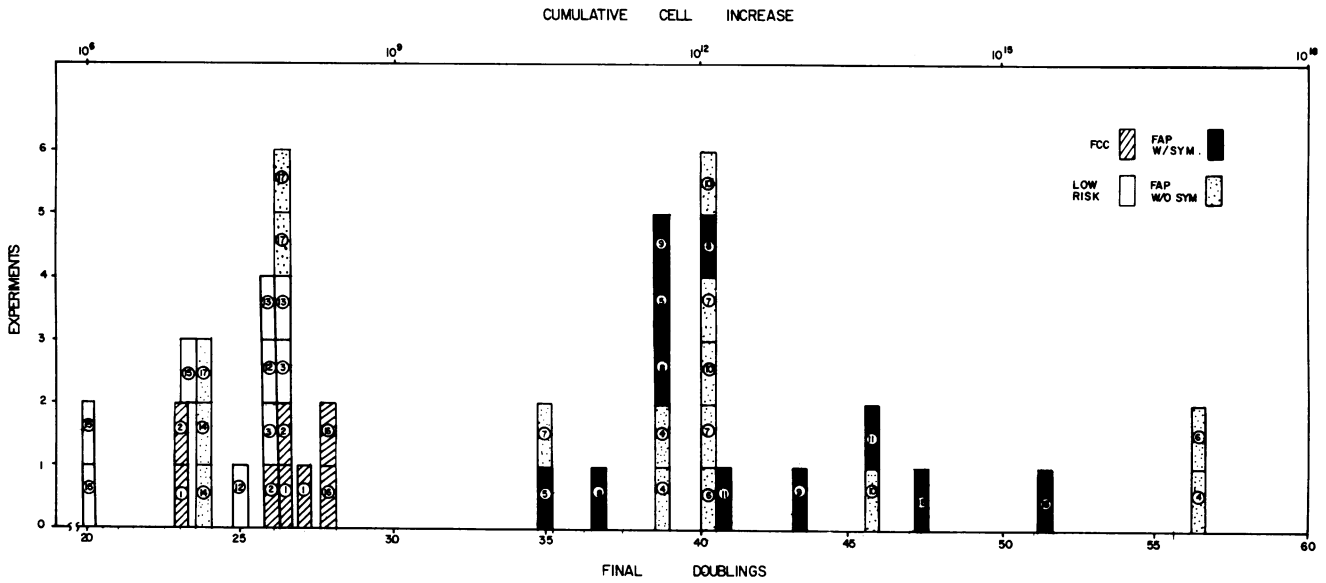

FIG. 2. Distribution of last doublings for human skin fibroblast lines passaged by the 3T3 protocol. Each box is a separate experiment. Cell lines are indicated by numbers as listed in Table 3.

crisis in the other two cases (Fig. 2). Fibroblasts from all persons of the normal, low-risk, and non-FAP colon cancer classes reached crisis at earlier doublings. These data are summarized in Table 4, which shows the correlation of delayed crisis and hemizygosity at FAP.

The association on delayed crisis and FAP hemizygosity seen in Table 4 is consistent with the parsimonious hypothesis that FAP susceptibility and delayed crisis are both the result of loss of the same recessive gene for growth control. One individual with a 50% probability of being hemizygous for FAP has not developed colon polyps at age 55 (Table 3). However, this individual's skin fibroblasts do show delayed crisis (Tables 2 and 3). Restriction fragment length polymorphism analysis will be necessary to distinguish between low penetrance in the colon of the hemizygous FAP state and recombination between two putative growth-control genes. From the sample size of our data (Table 4), any two genes separated by 15 or fewer centimorgans would generate total association at least 5% of the time (binomial distribution).

Hemizygosity of FAP could not be directly detected with the FAP-linked probe $C_{11}p_{11}$ in the limited number of FAP family members available to us (data not shown).

DISCUSSION

Hayflick and Moorhead (1) and others have shown that human skin fibroblasts senesce and die after about 25 confluent passages. We have confirmed this for fibroblasts from persons with FAP susceptibility and for normal controls (Tables 1 and 2). When the Hayflick passage protocols were used, crisis was not affected by age, sex, or clinical status (Table 3). However, when the 3T3 passage protocol (22) was used, cells from persons susceptible to FAP were able to survive an unexpectedly large number of passages (Table 3). We hypothesize that the recessive FAP growth-control gene is either identical or closely linked to a recessive gene that is responsible for early senescence of normal human skin fibroblasts. If these phenotypes are both the result of hemizygosity at the same locus, then the FAP susceptibility gene would code for product(s) that eventually brings about senescence in normal fibroblast cells after approximately 25 doublings in the 3T3 protocol. In any event, heterozygosity at the FAP susceptibility locus is closely linked to a partial loss of growth control. *In situ*, the loss of growth control due to hemizygosity of FAP permits polyps to arise from a hyperplasia in the colon, while *in vitro*, either the same genetic difference or a closely linked second gene shows delayed crisis in skin fibroblast cells. FAP patients without symptoms have 50% chance of being hemizygous at this locus. In our study, we found two of six cases of senescence at the expected time and four of six cases of delayed crisis.

Table 4. Human fibroblast delayed crisis is associated with the absence of one copy of functional FAP gene

FAP gene	Crisis in individuals tested	
	Normal	Delayed
Homozygous	7/7	0/7
Heterozygous	0/5	5/5
50% probability	2/6	4/6

Dominant negative regulation of human cell growth has been shown by studies with gene-transfer, somatic cell hybridization and by the demonstration of inhibitory mRNAs or proteins in quiescent cells (3–7). Knudson's hypothesis (8, 9) suggests that normal cells contain two copies of one or more genes that regulate growth control. We believe that this is the first case of an *in vitro* study in which the loss of growth control is associated with hemizygosity of a gene responsible for normal growth control *in situ*.

We thank Eric Pomert and Richard DeMaio for their excellent technical help, Dr. Donald Melnick for advice on the manuscript, Dr. Martin Lipkin and Peggy Monagham for supplying us with the cells, and Kavitha Subrahmanya and Donna Schwartz for preparation of the manuscript. This work is supported by Grants CA 38883 and CA 33620 from the National Cancer Institute and 00245 from the American Cancer Society.

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