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Influence of Adeno-Associated Virus on Adherence and Growth Properties of Normal Cells

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It has been shown previously that infection of newly established cell cultures from malignant human tumors with adeno-associated parvovirus type 2 or type 5 results in growth arrest and cell death. Here we report that the additionally observed antiproliferative effect on diploid human fibroblasts is transient and is connected to a reduced number of cells in S phase. Progression through the cell cycle is disturbed either in G_0/G_1 or at the G_1/S boundary, but an additional arrest in G_2 cannot be excluded. DNA synthesis and cell proliferation are resumed when cells are recultured after loosening of cell-matrix adhesions by trypsin treatment. In contrast, they are not resumed by solely providing growth factors via higher amounts of fetal calf serum. The results suggest that cell adherence is altered in adeno-associated parvovirus-infected human embryo fibroblasts.

Adeno-associated viruses (AAV) are members of the parvovirus group. They depend on coinfection with a viral helper (adenovirus or herpesvirus) for effective productive infection (for reviews, see references 6 and 12); hence, they are also named dependoviruses. The dependence is not absolute, and the viral helper may be replaced to a limited extent in some cell lines by pretreatment of cells with agents that induce cellular stress (2, 4, 30, 40, 41).

Parvoviruses encapsidate single-stranded DNA molecules in icosahedral nonenveloped particles with diameters of 20 to 25 nm (for reviews, see references 6 and 15). The three-dimensional structure of canine parvovirus has been reported recently (36).

AAV particles consist of three capsid proteins that are encoded by the right open reading frame of the parvoviral genome (for reviews, see references 6 and 7). Besides the capsid proteins, AAV DNA encodes proteins with regulatory properties. While autoregulation is both positive and negative (5, 22; reviewed in references 6 and 7), a negative influence of expression from heterologous promoters has been reported (23, 25).

Autonomous parvoviruses are the cause of a number of diseases in humans and animals (7, 32). In contrast, attempts to implicate AAV as an etiological agent of disease have thus far failed (6).

Both virus groups are of interest because they may limit growth of transformed cells in culture and in animals (for a review, see reference 29). The mechanism of the interference is not understood. It appears, however, that the nonstructural proteins play an important role. They interfere with a multitude of cellular processes such as gene expression from heterologous promoters (23, 28), neoplastic transformation (19, 20), and stable transformation of cells by exogenous DNA (23, 25, 27) and may be correlated to killing of transformed cells (11, 13, 14, 37).

AAV oncosuppressive effects have been observed mainly by interference of AAV with the oncogenicity of helper viruses (reviewed in reference 29). No such effects were obtained in otherwise transformed cells of permanent lines unless cells were additionally treated with stress-inducing agents (2, 4, 18). It has, however, been demonstrated recently that infection of newly established cultures of cells derived from malignant human tumors with either AAV-2 or AAV-5 at high multiplicities of infection results in growth arrest and cell death. Growth of normal fibroblasts was reported to be also affected by AAV infection, but the fibroblast cultures appeared able to escape cell death. No helper-independent parvovirus propagation was detectable in either of these cultures. Thus, the influence on cell growth is not due to virus propagation (1). Interferences with cell growth and cell cycle perturbation have been shown by Winocour et al. for presenescent Syrian hamster and human embryo fibroblasts (39). In that study, the impairment is described as being connected to arrest of cell cycle progression during late S or G_2 phase.

Here we report that AAV-induced proliferation arrest in human embryo fibroblasts is connected to a decreasing number of cells in S phase. There is one block in G_0/G_1 or at the G_1/S boundary, but an additional block in G_2 is also consistent with the results. Both reduction of cells in S phase and growth arrest are transient. The antiproliferative activity of AAV cannot be overcome by growth factors alone. Release from the block requires direct loosening of cell-matrix adhesions and reculturing of cells. The results suggest that cell adherence properties of the AAV-infected fibroblasts are altered in a way that leads to firmer cell-matrix attachments.

MATERIALS AND METHODS

Cell culture. Human embryo fibroblasts were obtained by culturing abortion material of a six-week-embryo. They were provided by J. R. Schlehofer. Cells were cultured in Dulbecco's modified Eagle's minimum essential medium as monolayers at 37°C in 6% CO_2. Growth medium was supplemented with glutamine, antibiotics, and 8% fetal calf serum (FCS).

For subculturing, cells were routinely treated with trypsin solution containing 0.125% trypsin, 3.35 mM EDTA, 0.7 mM $CaCl_2$, and 0.4 mM $MgSO_4$, phosphate buffered to pH 7. For subcultures after treatment by EDTA alone, monolayers

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TABLE 1. Evidence that infection of subconfluent cultures of human embryo fibroblasts with AAV-2 influences cell proliferation and cell cycle distribution[a]

Expt	Time (h)	Mock infected				AAV infected			
		Relative cell no.	% of cells in:			Relative cell no.	% of cells in:		
			G_0/G_1	S	G_2/M		G_0/G_1	S	G_2/M
1	6	1.00	65.2	12.1	22.7	1.00	64.5	11.5	24.0
	22	1.24	59.2	19.2	21.6	0.98	68.0	6.2	25.8
	35	1.33	64.1	13.2	22.7	1.04	61.8	4.0	34.2
	48	1.87	65.8	14.6	19.6	1.19	65.6	3.1	31.3
	76	2.35	72.9	2.5	24.6	1.17	63.5	2.7	33.8
2	24	1.62	62.0	10.1	27.9	1.19	64.8	3.7	31.6
	50	2.71	59.9	11.8	28.3	1.52	52.2	13.4	34.4
	72	4.14	52.0	26.7	21.3	2.29	52.5	16.3	31.2
	103	8.85	67.2	6.5	26.3	5.10	59.8	9.4	30.8
	168	9.24	60.5	1.4	38.1	5.29	60.9	1.0	38.1
3	53	1.29	ND	ND	ND	1.27	ND	ND	ND
	70	1.89	ND	ND	ND	2.00	ND	ND	ND
	99	2.71	ND	ND	ND	3.07	ND	ND	ND
	120	5.79	ND	ND	ND	6.11	ND	ND	ND

[a] Human embryo fibroblasts were infected at about 40% confluency, cultured, and harvested at various time points after infection (experiment 1). Infected cells were trypsinized, and the same number of cells was seeded and cultured for the periods indicated (experiment 2). Trypsinizing and subculturing of these cells was repeated once (experiment 3). Cell numbers and frequency distribution of cellular DNA were determined at various times after infection in experiment 1, after the first trypsin treatment in experiment 2, and after the second trypsin treatment in experiment 3. Numbers of infected and reseeded cells were set equal to 1. ND, not determined.

were incubated for 30 min at 37°C in a solution of 0.9% NaCl, 1 mM EDTA, and 10 mM KH_2PO_4, pH 7.2.

Virus and virus infection. The titer of AAV-2 was determined by dot blotting of infected HeLa cultures on nitrocellulose filters subsequently hybridized with radiolabeled AAV-2 DNA as described elsewhere (3).

About 4×10^5 to 10^6 fibroblasts were grown in six-well plates (Costar) and were either mock infected or infected with AAV-2 at a multiplicity of infection of 10^3 to 10^4 infectious particles per cell. After 1 h of adsorption, nonadsorbed virus was removed and cells were washed twice with medium. Infected cells were cultured and harvested as described above.

Flow cytometry. Cells were removed from the culture dish by trypsin treatment or by EDTA treatment and were pelleted by low-speed centrifugation. Cell pellets were washed with phosphate-buffered saline (PBS), pelleted as described above, and resuspended. The cell suspension was added to a 10-fold volume of ice-cold ethanol for fixation. After fixation (minimum of 12 h) and centrifugation, the cell pellet was resuspended for staining with the fluorochrome DAPI (4',6-diamidino-2-phenylindole) for DNA and SR101 (to counterstain proteins) as described by Stoehr et al. (34). Flow analyses were carried out by using an argon ion laser (351 to 364 nm) for fluorescence excitation of DAPI. Small amounts of unspecific non-DNA DAPI fluorescence were quenched as a result of energy transfer between DAPI and SR101. Collection of blue DAPI fluorescence was above 450 nm. Further processing and cell cycle analysis of flow cytometric data were done according to Dean and Jett (16), with slight modifications (33).

Determination of cell numbers. The harvested cells were resuspended in 1 ml of PBS, and aliquots of 100 μl were mixed with 100 μl of trypan blue (0.4% in PBS). Determination of cell numbers was done by counting four times 0.1 μl in a hemocytometer immediately after mixing. Cell counting was performed at least in duplicate, with differences usually being less than ±10%.

RESULTS

Effect of AAV infection on proliferating fibroblasts. Infection of subconfluent cultures of human embryo fibroblasts with AAV-2 leads to interference with cell proliferation. There may be some residual increase in cell numbers in AAV-infected cultures, but then growth is completely inhibited (Table 1, experiment 1). Quantitative analysis of DNA profiles (Fig. 1) representing the frequency distribution of cells in G_0+G_1, S, and G_2+M phases of the cell cycle at various times after infection reveals that the percentage of fibroblasts with a DNA content representing cells in S phase is steadily decreasing (Table 1, experiment 1). Cells fail to enter S phase in the proliferation-arrested cultures, and thus the block should be considered to be in G_0/G_1 or at the onset of S. Since the residual increase in cell numbers in infected cultures may be accounted for by cells that at infection have already passed a cell cycle point critical for division, the results do not exclude an additional block in G_2. (Since mitoses could not be detected, the percentage of cells containing a DNA content corresponding to G_2/M phases can be ascribed to cells in G_2.) This is suggested by the higher percentage of cells with G_2 DNA content in the arrested cultures, which results in an almost constant value for cells in $S+G_2/M$ despite the decreasing percentage of cells in S phase (Table 1, experiment 1).

If cells are trypsinized and the same numbers of cells are plated, the AAV-infected cells reenter S phase and the arrested cultures resume proliferation (Table 1, experiment 2). As should be expected if a pre-S block is removed, the percentage of cells in G_0/G_1 decreases in parallel with an increase of cells in S phase (Table 1, experiment 2). Both resumption of DNA synthesis and increase in cell numbers in AAV-infected cultures occur with a lag compared with controls, but at later times rates are in the range of noninfected cultures (Table 2). However, the lagging behind of cell numbers is not compensated for, since AAV-infected cultures behave like contact-inhibited cultures and stop prolif-

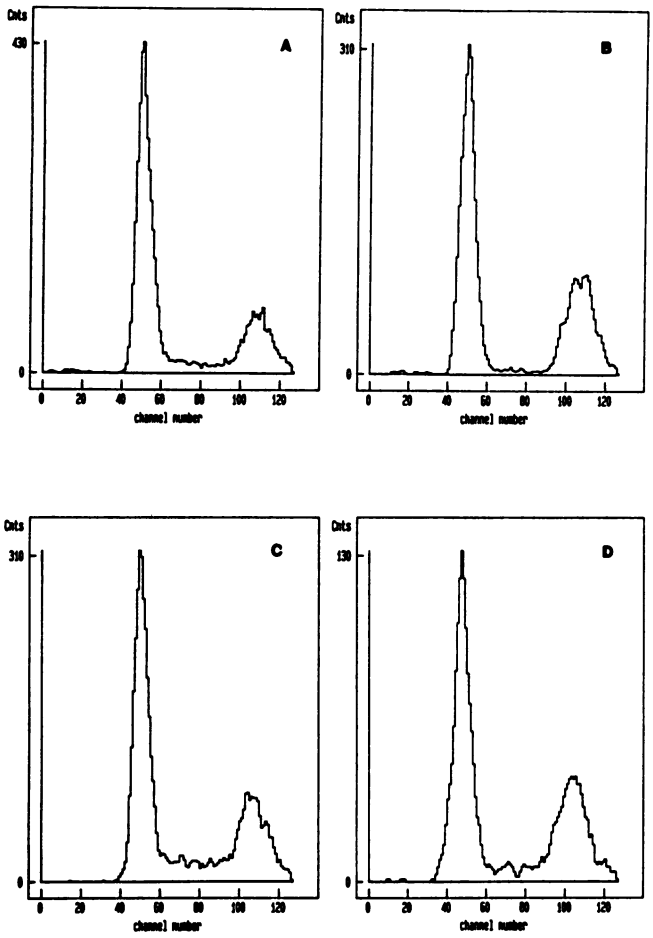

FIG. 1. DNA frequency profiles. Human embryo fibroblasts were either mock infected or infected with AAV-2, cultured, and harvested at various time points after infection. The same number of cells was plated after trypsin treatment, and cell cultures were again harvested at various periods after reseeding. Cells were fixed in ethanol, and the percentage of cells in the phases of the cell cycle (G_0/G_1, S, and G_2/M) was determined by flow cytometry. The left peak represents cells with G_0/G_1 DNA content, and the right peak represents those with G_2/M content. The area between them corresponds to cells in S phase. (A) Mock infected, 35 h p.i.; (B) AAV infected, 35 h p.i.; (C) mock infected, 72 h after trypsin treatment; (D) AAV infected, 72 h after trypsin treatment. The examples shown are from the data in Table 1.

eration at cell densities far below control levels (Fig. 2; Table 1, experiment 2). Comparable observations were made when diploid human foreskin fibroblasts were infected with AAV-5 (data not shown). The premature saturation density arrest suggests some lasting perturbation of cell cycle and thus cell proliferation. Whether it is related to the increased levels of cells with a G_2/M DNA content still seen in the trypsin-subcultured infected cells is not known.

Differences in growth behavior and cell cycle distribution between AAV-infected and control cultures disappear in the following round of culturing. They are not observed again upon further subcultivation (Tables 1 and 3, experiments 3). Thus, AAV-induced growth arrest and cell cycle perturbation are transient in human embryo fibroblasts.

Effect of AAV infection on contact-inhibited fibroblasts. Infection of confluent fibroblast cultures does not, of course, reveal any growth-inhibiting influence of AAV unless the cells are subcultured. However, if the cells are cultured after trypsin treatment, the antiproliferative activity becomes evident (Table 3, experiment 1). In contrast to the effect seen when subconfluent cultures are infected, the arrest is not released, cells do not enter S phase, and no proliferation is observed upon subculturing (Table 3, experiment 1). Another round of trypsin treatment and plating is required to remove the block and to allow cells to enter S phase and resume proliferation (Table 3, experiment 2). Thus, the arrest cannot be avoided solely by trypsinizing the infected cultures; it first must become manifest and only then can be released.

TABLE 2. Relative increase in cell numbers after various time periods of trypsin subculturing of human embryo fibroblasts[a]

Time (h) after cell seeding	Fold increase	
	Mock infected	AAV infected
0	0.00	0.00
24	1.62	1.19
50	1.68	1.28
72	1.53	1.50
103	2.14	2.23
168	1.04	1.04

[a] Subconfluent cultures were either mock infected or infected with AAV-2, and the same number of cells was seeded after trypsin treatment. After the periods indicated, cells were harvested and counted in a hemocytometer, and the relative increase between two time points was determined.

The further stages that the infected cultures pass through when they recover from the block are as described above: resumption of DNA synthesis and proliferation occur with a lag and contact inhibition takes place at lower saturation density (Table 3, experiment 2), while at further passages, the differences in cell numbers and in DNA profiles are abolished (Table 3, experiment 3).

Influence of FCS on AAV-induced growth arrest. To determine whether the AAV-induced growth arrest can be overcome by elevated levels of growth factors, fibroblasts were cultured in the presence of 3, 5, and 15% FCS, with two additional changes of culture medium during the course of the experiment. As can be seen from Table 4, cell proliferation in mock-infected control cultures is influenced by higher amounts of FCS, but the growth inhibitory effect of AAV is not. The residual increase of cell numbers and the proliferation arrest in infected cultures is comparable to those shown in Table 1, experiment 1. Thus, a higher amount of growth factors is not sufficient to overcome the antiproliferative effect of AAV.

Influence of cell detachment on resumption of cell proliferation. It has been described that trypsin treatment stimulates DNA synthesis in cell-free systems (8, 9) and induces DNA synthesis and cell proliferation in contact-inhibited fibroblast cultures (10, 24, 42). Furthermore, it is known that loosening the attachment of cells to their substratum is required for proliferation (10, 24, 42). We therefore investigated whether the resumption of proliferation in subcultured infected cells was due to the influence of trypsin or to the procedure of cell detachment from the culture dish before reseeding. For that purpose, fibroblasts were subcultured 3 days postinfection (p.i.) after treatment either with trypsin-EDTA (containing 3.35 mM EDTA, 0.7 mM Ca^{2+}, and 0.4 mM Mg^{2+}) or with EDTA (1 mM).

It has been observed that detachment of AAV-infected

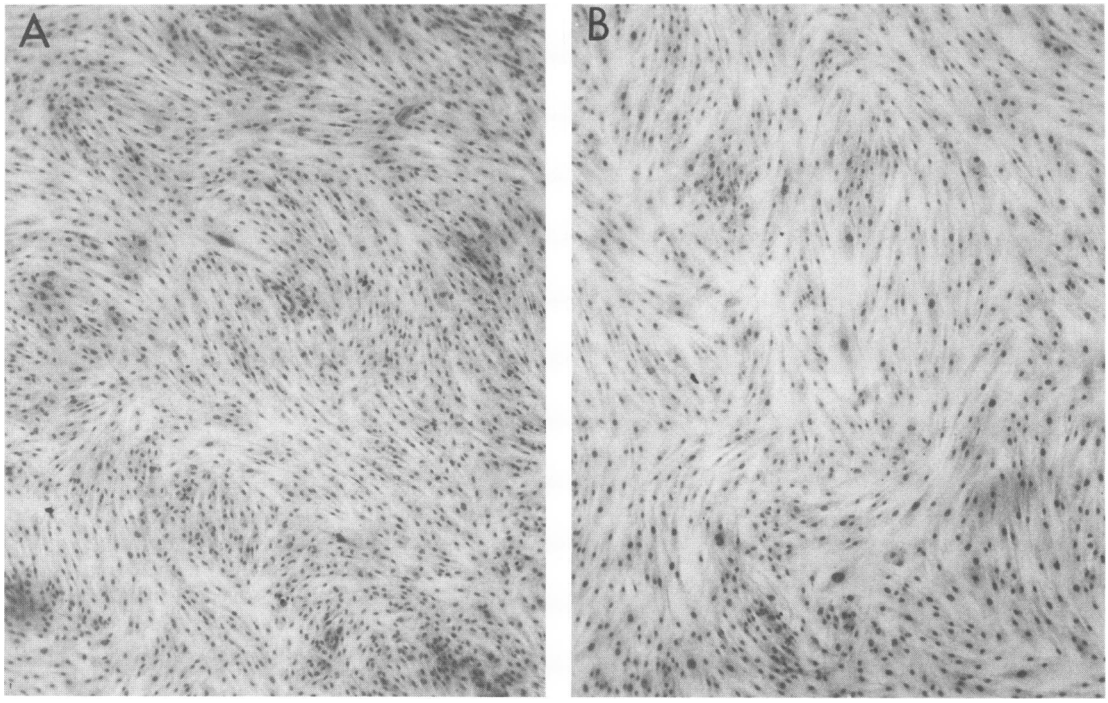

FIG. 2. Micrographs of AAV-infected trypsin-subcultured human fibroblasts. Human fibroblasts were either mock infected (A) or infected with AAV-2 (B) as described in the text. Cells were treated with trypsin and were subcultured at 37°C in 6% CO_2. After 5 days, cells were stained with Giemsa.

cells by trypsin-EDTA is insignificantly prolonged compared with controls (2a). However, when the cell-matrix adhesions were loosened by EDTA alone, the differences between controls and AAV-infected cells became more distinct. After 30 min of EDTA treatment, the amount of detached cells was 0.57-fold that of trypsinized cells in controls (cell counts of 151 versus 263) and 0.39-fold in AAV-infected cultures (cell counts of 62 versus 161). Thus, AAV infection seems to alter cell adherence properties in the sense that cell-matrix attachments are stronger. This notion is supported by results obtained when both the trypsinized and the EDTA-treated cells are further cultured. Mock-infected cultures show comparable growth behaviors (Table 5), and their patterns of frequency distribution of cellular DNA are alike (data not shown) with the exception of amounts of cells in S phase immediately after trypsin or EDTA treatment (16.3 and

TABLE 3. Evidence that infection of confluent cultures of human embryo fibroblasts with AAV-2 influences cell proliferation and cell cycle distribution[a]

Expt	Time (h)	Mock infected				AAV infected			
		Relative cell number	% of cells in:			Relative cell number	% of cells in:		
			G_0/G_1	S	G_2/M		G_0/G_1	S	G_2/M
1	45	1.33	66.3	3.2	30.5	1.11	68.5	0.6	30.9
	67	1.50	56.4	11.6	32.0	0.97	70.5	0.6	28.9
	93	2.36	63.7	6.6	29.7	0.95	69.6	0.8	29.6
	128	3.24	71.9	0.9	27.2	0.97	72.7	0.6	26.7
2	36	1.09	73.4	5.7	20.9	1.06	74.0	0.6	25.4
	60	1.59	49.0	33.3	17.7	1.22	69.6	1.2	29.2
	132	7.13	66.1	4.1	29.8	1.72	59.3	11.0	29.7
	165	8.63	67.1	0.6	32.4	2.75	63.5	7.5	29.0
	204	9.00	69.4	0.3	30.4	4.69	62.2	2.8	35.0
3	28	1.16	58.3	4.2	37.5	1.21	60.7	4.1	35.2
	72	1.67	47.0	17.0	36.0	1.79	46.4	19.8	33.8
	96	2.70	52.3	12.2	35.5	2.60	50.6	15.5	33.9
	121	5.23	59.3	9.0	31.7	5.35	56.5	10.5	33.0
	169	6.51	68.1	0.7	31.2	6.60	65.3	0.7	34.0
	193	6.56	65.2	0.8	34.0	6.60	62.6	0.6	36.8

[a] Confluent fibroblasts were infected and harvested 3 days p.i. Equal numbers of trypsinized cells were seeded and cultured for the time periods indicated (experiment 1). Trypsinizing, reseeding, and harvesting of cells were repeated twice (experiments 2 and 3). Cell numbers and frequency distribution of cellular DNA were determined after the first, second, and third trypsin treatments (experiments 1 to 3, respectively). Numbers of reseeded cells were set equal to 1.

TABLE 4. Influence of FCS on AAV-induced growth arrest[a]

Time (h) p.i.	Relative cell no.					
	3% FCS		5% FCS		15% FCS	
	Mock infected	AAV infected	Mock infected	AAV infected	Mock infected	AAV infected
0	1.00	1.00	1.00	1.00	1.00	1.00
46	1.41	1.23	1.50	1.30	1.82	1.39
86	1.55	1.16	1.57	1.35	2.27	1.34
133	1.32	1.16	1.52	1.25	2.43	1.27
239	1.48	1.07	ND	1.27	2.89	1.27

[a] Subconfluent cultures of human fibroblasts were mock infected or infected with AAV and were cultured in the presence of 3, 5, or 15% FCS. At the time points indicated, cells were harvested and counted as usual. Additional changes of culture medium were at 46 and 186 h. ND, not determined.

7.2%, respectively). However, AAV-infected cells that were detached by 30 min of EDTA treatment are to a great extent unable to adhere again to the culture dish and die. Those cells that are able to adhere resume proliferation (Table 5) and have DNA profiles comparable to those of AAV-infected fibroblasts that were detached by trypsin (data not shown). It therefore is the loosening of cell-matrix adhesions that triggers resumption of DNA synthesis and cell proliferation in AAV-induced growth arrest.

DISCUSSION

Infection of presenescent fibroblasts with high infectious doses of AAV has previously been shown to inhibit cell proliferation (1, 39) and to lead to perturbation of the cell cycle in late S or G_2 (39). In contrast to cell cultures that had been newly established from malignant human tumors, the impairment of fibroblast proliferation appeared less severe (1).

The data reported here confirm that the AAV-mediated antiproliferative effect is transient in human embryo fibroblasts and show that the arrest is connected to reduced amounts of cells in S phase. DNA synthesis and cell proliferation are resumed upon trypsin treatment and reculturing of cells. However, resumption is only possible if the arrest has been previously established. Trypsinization of fibroblasts that were infected at density arrest stimulates neither entry into S phase nor proliferation unless the procedure is repeated. This may mean that conditions required for providing the interfering function are cell cycle dependent and favorable only when the density-arrested cultures are stimulated to proliferate. In this context, it may be of interest that preliminary experiments show transient transcription of AAV *rep* RNA (2a) that codes for the viral regulatory proteins. Growth arrest and cell cycle perturbation by influence of the parvoviral regulatory proteins would be in agreement with data that ascribe cell killing to their action (11, 13, 14, 37). However, we have thus far been unable to demonstrate Rep protein by either Western immunoblot or immunofluorescence (2a).

TABLE 5. Influence of cell detachment by trypsin-EDTA or EDTA treatment on growth of subcultured fibroblasts[a]

Time (h) after cell seeding	Relative cell no.			
	Mock infected		AAV infected	
	Trypsin-EDTA	EDTA	Trypsin-EDTA	EDTA
0	1.00	1.00	1.00	1.00
24	1.60	1.60	1.14	?[b]
54	2.57	2.07	1.06	0.60
72	3.77	3.43	1.49	0.83
96	5.80	6.00	2.34	1.10
120	7.66	7.73	5.11	1.90

[a] Human embryo fibroblasts were mock infected or infected with AAV-2 and were treated for subculturing either with trypsin-EDTA or with EDTA alone 3 days p.i. At the time points indicated, subcultured cells were harvested, and cell numbers were determined in a hemocytometer as described in the text.

[b] Because of the great amount of dead material, cell numbers could not be determined reliably.

AAV-mediated proliferation arrest is due to a block at G_0/G_1 or the G_1/S boundary. An additional block is likely to be in G_2. Thus, besides the block in late S or G_2 described by Winocour et al. (39), an additional point of perturbation exists. The possibility of a stop in late G_1 that prevents cells from entering S phase may be considered in the light of several reports on AAV-mediated reduction of initiator-induced simian virus 40 or bovine papillomavirus DNA amplification (3, 17, 30, 31).

The growth arrest cannot be overcome solely by providing higher amounts of growth factors by FCS. Instead, direct loosening of cell-matrix adhesions is required for stimulation. It has been known for many years that loosening of adhesions between cells and substratum stimulates entry into S phase and proliferation (10, 24, 42). However, the pathway by which alteration of extracellular and cell surface components generates the intracellular stimulating signals remains obscure. Since treatment with the chelating agent EDTA alone has a detrimental effect on AAV-infected fibroblasts, it seems noteworthy that a decrease in intracellular Ca^{2+} concentration or antagonism of the intracellular Ca^{2+} receptor calmodulin blocks progression through the cell cycle at the G_1/S boundary and during G_2/M (26). Furthermore, cell cycle arrest by blocking of calmodulin may result in differentiation (38), and induction of cell differentiation by AAV infection has indeed been observed in cells of human and mouse origin (21).

Release from the block occurs with a lag. Thus, recovery is not a rapid event but may involve more complex activities. Since cell proliferation stops at reduced saturation density, recovery is not complete unless a further round of stimulation takes place. Whether this is connected to the existence of a second block in G_2 is not known.

AAV infection alters cell adherence properties such that fibroblasts attach more firmly to the culture dish. Whether the effect is due to the binding of AAV to its membrane receptor or whether it is brought about more indirectly by AAV-mediated interference with cellular processes is not known. Winocour et al. observe decreased plating efficiency

also with UV-inactivated virus, and the authors postulate interaction of the incoming virus with a cellular target (39). It should, however, be noted that the aforementioned transcription of *rep* RNA takes place in the virtual absence of AAV DNA synthesis (the increase in AAV DNA being less than twice the amount of input [2a]). If RNA synthesis originated from the incoming DNA template, this might provide an explanation for the high virus doses required for the effect and might suggest that AAV could have a harmful potential comparable to that of autonomous parvoviruses if conditions were to allow accumulation of some of its products.

Unlike normal cells, cancer cells usually proliferate without requiring adhesion to the extracellular matrix, and the loss of growth control in cancer cells is often associated with reduction of cell adhesiveness. If transformed chicken embryo fibroblasts are forced to attach to the culture dish more firmly, they behave like normal cells and stop dividing upon reaching confluency (35). Thus, altering cellular adhesive properties in a way that makes cells attach more firmly to the extracellular matrix evidently can result in more restricted growth control. If cellular adhesion in newly established cell cultures of malignant cells is altered by AAV infection in a way similar to that described here, it is not surprising that AAV has a lethal effect in these systems, and AAV interference with oncogenicity may well be related to modified cell adhesion.

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