Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5781–5785, September 1985
Cell Biology

Heparin-treated, v-*myc*-transformed chicken heart mesenchymal cells assume a normal morphology but are hypersensitive to epidermal growth factor (EGF) and brain fibroblast growth factor (bFGF); cells transformed by the v-Ha-*ras* oncogene are refractory to EGF and bFGF but are hypersensitive to insulin-like growth factors

(MC29 virus/platelet-derived growth factor/autonomy/hormone receptors)

SAMUEL D. BALK, THOMAS M. RILEY, HARRIET S. GUNTHER, AND ANDREA MORISI

Department of Pathology and Cancer Research Institute, New England Deaconess Hospital, Boston, MA 02215; and Department of Pathology, Harvard Medical School, Boston, MA 02115

Communicated by Armin C. Braun, May 21, 1985

ABSTRACT **Chicken heart mesenchymal cells do not proliferate in culture medium containing heat-defibrinogenated plasma but proliferate briskly when incubated with epidermal growth factor (EGF) or brain fibroblast growth factor (bFGF) plus insulin-like growth factors (IGFs) or when infected with sarcoma or erythroblastosis viruses. When infected with the retrovirus MC29, which bears a v-*myc* oncogene, chicken heart mesenchymal cells proliferate at a more modest rate and become morphologically transformed. Heparin at 25 μg/ml causes these MC29-transformed cells to become proliferatively quiescent and to assume a normal morphology. Heparin-treated MC29-infected cells are, however, 100 times more sensitive to EGF than are their normal, uninfected counterparts. MC29-infected cells appear, likewise, to be hypersensitive to bFGF and to PDGF preparations but not to insulin. We hypothesize, therefore, (*i*) that heparin prevents the generation by cells of a mitogen from plasma protein precursors in the culture medium; (*ii*) that the v-*myc* oncogene renders cells hypersensitive to EGF, bFGF, PDGF, and the putative plasma-protein-derived mitogen; and (*iii*) that MC29-infected cells must proliferate in order to manifest the transformed morphology. Chicken heart mesenchymal cells infected with a recombinant spleen necrosis virus containing a v-*ras* oncogene are morphologically transformed but proliferate only sluggishly in plasma-containing medium without added mitogenic hormones. Heparin does not significantly affect their behavior. They are refractory to mitogenic stimulation by EGF or bFGF suggesting that ras proteins mediate the effects of receptors for these hormones. The SNV/v-*ras*-infected cells proliferate briskly, however, in response to hyperphysiological concentrations of insulin, an IGF surrogate, and are considerably more sensitive to this IGF mitogenicity than are their normal, uninfected counterparts.**

The acutely transforming avian retrovirus MC29, which bears a v-*myc* oncogene, causes leukemias, carcinomas, and sarcomas in chickens (1). The *myc* protooncogene has been found to be activated by avian leukosis virus promoter insertion in chicken bursal lymphomas and, importantly, may be activated by chromosomal translocation in nonviral tumors, including human tumors (1). Identification of the modification(s) of cellular proliferation caused by the activity of $p110^{gag\text{-}myc}$, the transforming protein encoded by the *myc* oncogene of MC29, will be necessary to an understanding of the contribution of this oncogene to the unregulated cell proliferation that defines neoplasia.

SNV/v-*ras*, a recombinant avian spleen necrosis virus containing a v-Ha-*ras* oncogene, has recently become available (2). Because *ras* oncogenes, first identified in rat sarcoma viruses, have been cloned from a number of human malignancies (3), phenomenological definition of the changes in cell proliferation caused by the v-Ha-*ras*-encoded transforming protein p21 assumes considerable importance.

Chicken heart mesenchymal cells, in a system developed in our laboratory (4, 5), are proliferatively quiescent in low density monolayer cultures in medium containing rooster plasma at 10%. The quiescence of these cells, unlike the proliferative inactivity of fibroblasts in standard culture systems, does not depend on cell-deprivation effects like serum (plasma)-starvation or density-dependent inhibition or on the absence of anchorage. Chicken heart mesenchymal cells proliferate briskly in response to the combination of epidermal growth factor (EGF) or brain fibroblast growth factor (bFGF) plus insulin-like growth factor I (IGF-I), IGF-II, or insulin at hyperphysiological concentrations (6, 7). Insulin, at hyperphysiological concentrations, acts as an IGF surrogate by activating the type-I IGF receptor (7). Chicken heart mesenchymal cells proliferate briskly, likewise, after infection with avian sarcoma or erythroblastosis viruses, whereas they proliferate at more modest rates after infection with MC29 (8). In preliminary experiments, we have observed that the modestly proliferating MC29-infected cells respond to concentrations of EGF (1–10 ng/ml) to which their normal, uninfected counterparts show little or no response. Others have reported that expression of transfected *myc* protooncogenes enhances response to EGF in assays based on [^{3}H]thymidine incorporation of density-arrested 3T3 cells, on clonal growth of 3T3 cells, or on colony formation by Fischer rat fibroblasts in soft agar (9, 10).

Earlier work suggested that heparin treatment (500 μg/ml), as well as heat-defibrinogenation, of plasma might be necessary to obtain proliferative quiescence of normal chicken heart mesenchymal cells in culture (5). Subsequent studies, however, have indicated that heat-defibrinogenation alone is sufficient, provided that rooster blood collected for plasma is treated with adequate quantities of the calcium chelator EGTA to prevent coagulation (refs. 7, 8, 11 and unpublished

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SNV/v-*ras*, recombinant avian spleen necrosis virus containing a v-*ras* oncogene; RSV, Rous sarcoma virus; EGF, epidermal growth factor; bFGF, brain fibroblast growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; TGF-α, type α transforming growth factor.

results). Balk *et al.* (5) have hypothesized that heat-defibrinogenation of plasma, in addition to rendering plasma noncoagulable, inactivates a cascade that generates mitogens from plasma protein precursors during clotting or during the interaction of plasma-containing medium and cultured cells. Heparin or heparin-like molecules may indeed have, however, a physiological negative regulatory role in the control of cell proliferation (5, 12–14), and the presence of these polyanions in culture medium may be necessary to an experimental formulation of a coherent and physiologically relevant view of the interactions of mitogenic hormones and *onc* genes in the initiation of cell replication. In an attempt to explain the relatively modest proliferative activity of MC29-infected cells, compared to sarcoma and erythroblastosis virus-infected cells, and the apparent hypersensitivity of MC29-infected cells to EGF, as well as to further elucidate the apparent negative role of heparin and heparin-like substances in cell proliferation, we have hypothesized (*i*) that cryptic mitogenic activity, not abolished by heat-defibrinogenation alone, may be generated from plasma protein precursors upon contact with cultured cells and that generation of such a mitogen might be inhibited by addition of heparin to plasma-containing culture medium; (*ii*) that MC29-infected chicken heart mesenchymal cells may be hypersensitive to such a cryptic, plasma protein-derived mitogen and so, unlike normal cells, proliferate at a modest rate in medium containing heat-defibrinogenated plasma; and (*iii*) that MC29-infected cells might become quiescent in heparinized culture medium and there manifest clear hypersensitivity to EGF and to other added mitogens.

We present here the results of experiments done to test this hypothesis. We have also examined the proliferative behavior of chicken heart mesenchymal cells infected with a recombinant avian spleen necrosis virus containing a v-Ha-*ras* oncogene.

MATERIALS AND METHODS

General methods for the preparation of heat-defibrinogenated rooster plasma, synthetic culture medium, and cultures of chicken heart mesenchymal cells have been published (4–8, 11). Rooster blood for the preparation of heat-defibrinogenated plasma was collected using EGTA as anticoagulant. After removal of formed elements by centrifugation, the plasma was heat-defibrinogenated at 57°C for 35 min and then stoichiometrically recalcified with $CaCl_2$.

All cultures were incubated at 42°C in a humidified 95% air/5% CO_2 atmosphere. Primary and secondary stock cultures were prepared, in 60-mm Falcon tissue culture dishes, in synthetic medium with heat-defibrinogenated plasma at 5%, EGF at 100 ng/ml and insulin at 1 μg/ml. On the day after their preparation, primary cultures were inoculated with retroviruses. MC29 (subgroup B) (RAV-2 helper virus) was graciously provided by C. Moscovici (Gainesville, FL). A recombinant avian spleen necrosis virus (GT10) containing a v-Ha-*ras* oncogene (SNV/v-*ras*, reticuloendotheliosis virus-associated helper) was graciously provided by T. Gilmore and H. Temin (2). Schmidt–Ruppin (subgroup A) Rous sarcoma virus (RSV) has been carried in our own laboratory. The primary cultures of normal and retrovirus-infected chicken heart mesenchymal cells were subcultured to yield secondary stock cultures. These were subcultured, in 35-mm Falcon dishes and plasma-containing medium without added mitogenic hormones, to yield replicate tertiary cultures for experiments. The use of secondary stock cultures ensured quantitatively and qualitatively complete transformation of cells with MC29 and SNV/v-*ras*, which are defective retroviruses.

Experiments were begun on the day following subculture (day 0). All experimental culture media contained heparinized, heat-defibrinogenated plasma at 10%. Two milliliters of experimental culture medium were used per 35-mm dish; media were changed on days 2 and 3. Cells were counted with a Coulter electronic cell counter. Cell proliferation was determined over 4-day periods.

Grade I, porcine intestinal heparin and crystalline bovine insulin were purchased from Sigma and were dissolved, respectively, at 25 mg/ml in electrolyte solution and 10 mg/ml in 0.02 M HCl. EGF, receptor grade, and platelet-derived growth factor (PDGF) preparation were purchased from Collaborative Research (Waltham, MA), and bFGF preparation was purchased from Biomedical Technologies (Norwood, MA); these polypeptide hormones were dissolved at, respectively, 100 μg/ml, 50 units (equivalent to 2 μg)/ml, and 50 μg/ml in plasma-containing culture medium.

RESULTS AND DISCUSSION

The addition of heparin to plasma-containing culture medium without added mitogenic hormones abolishes the proliferation of MC29-infected chicken heart mesenchymal cells and causes them to assume the morphology of normal, uninfected

NORMAL CELLS

	HEPARIN (μg/ml)							
(ng/ml)	0	200	100	50	25	12	4	1.3
No Additions	15.4±0.2	11.1±0.2	12.3±0.3	11.9±0.4	11.9±0.1	13.2±0.1	13.3±0.3	14.1±0.1
Insulin 10,000	39.3±1.8	21.8±0.4	23.6±0.3	23.8±0.5	25.6±0.4	25.3±0.3	29.4±1.7	32.1±0.9
EGF 1,000	78.5±3.8	50.1±1.0	51.7±1.4	58.5±3.1	56.2±2.0	52.8±1.2	65.2±1.5	65.1±4.6
EGF 100	44.9±0.8	30.8±1.1	35.4±1.0	35.1±0.6	37.2±1.3	39.2±2.0	41.4±1.1	46.2±0.8
EGF 10	18.5±0.3	18.0±0.2	17.8±0.3	18.5±0.4	18.1±0.3	19.5±0.2	20.3±0.4	20.9±0.7
EGF 1	16.4±1.0	14.9±0.1	14.6±0.6	14.9±0.2	14.3±0.3	15.1±0.1	15.6±0.2	16.6±0.0
EGF 1,000 +Insulin 10,000	319±7.6	151±4.0	170±4.6	174±1.2	180±6.4	214±1.6	285±4.7	365±8.9

MC29-INFECTED CELLS

(ng/ml)								
No Additions	72.9±2.9	7.1±0.4	7.6±0.7	7.6±0.5	8.1±1.1	8.1±0.4	10.7±0.3	19.6±0.5
Insulin 10,000	257±7.9	14.4±0.3	19.2±0.3	24.3±1.0	30.1±1.2	38.3±1.2	103±3.0	202±0.9
EGF 1,000	254±10	167±1.4	164±2.8	170±5.3	184±2.7	191±2.5	214±2.1	232±1.1
EGF 100	232±2.5	134±1.1	131±3.2	142±2.8	155±4.1	165±3.8	192±6.4	208±5.2
EGF 10	190±1.4	60.8±0.4	67.5±1.6	77.3±0.5	83.0±2.5	92.6±3.2	105±5.5	132±3.0
EGF 1	117±4.3	19.4±0.1	31.3±0.9	34.8±0.8	43.5±2.1	56.9±1.1	63.9±3.0	79.5±2.7
EGF 1,000 +Insulin 10,000	401±8.8	263±8.0	257±8.1	273±4.8	274±2.4	286±3.4	313±8.2	337±6.6

SNV/v-*ras*-INFECTED CELLS

No Additions	26.1±0.6	18.9±0.2	19.5±0.1	21.2±0.2	20.4±0.7	24.1±0.2	25.7±0.1	25.9±0.7
Insulin 10,000	142±2.2	183±1.2	155±4.8	143±1.2	153±0.5	154±1.4	153±0.5	155±2.8

RSV-INFECTED CELLS

No Additions	509±11	172±1.8	167±2.8	145±1.8	159±2.4	163±2.8	184±7.0	273±3.6

FIG. 1. Results of a single experiment examining the effect of heparin on the proliferative behavior of normal, MC29-infected, SNV/v-*ras*-infected, and RSV-infected chicken heart mesenchymal cells in culture medium, without or with added EGF and/or insulin, containing heat-defibrinogenated chicken plasma at 10%. Each value represents (the mean number of cells ± SEM) × 10^{-4}, from three replicate 35-mm tertiary-culture dishes, counted with a Coulter electronic cell counter after 4 days of incubation. At the beginning of the experiment (day 0), the normal, MC29-infected, SNV/v-*ras*-infected, and RSV-infected cells were present, respectively, at (10.2 ± 0.2) × 10^4, (7.5 ± 0.3) × 10^4, (10.5 ± 0.1) × 10^4, and (7.8 ± 0.3) × 10^4 cells per dish. In all experiments, media were changed on the second and third days of incubation. Each experiment reported in this publication was repeated more than three times, with similar results.

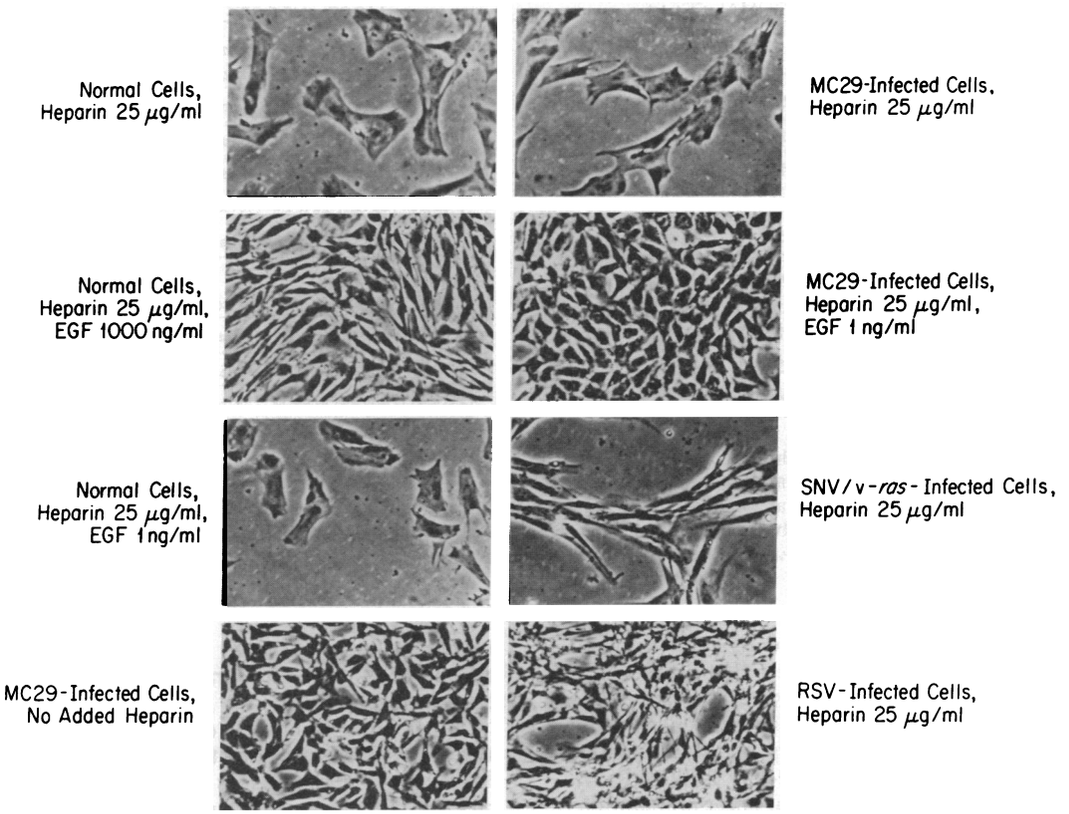

FIG. 2. Phase-contrast photomicrographs of normal, MC29-infected, SNV/v-*ras*-infected, and RSV-infected chicken heart mesenchymal cells in culture medium, with or without added heparin or EGF, containing heat-defibrinogenated chicken plasma at 10%. The cells were inoculated in primary culture, subcultured to obtain quantitatively and qualitatively complete transformation, and photographed after 4 days in tertiary culture. Normal cells were proliferatively quiescent with or without added heparin. (×74.)

cells (Figs. 1 and 2). These effects of heparin on proliferative behavior and cell morphology are complete at a heparin concentration of 25 μg/ml. By comparison with this qualitative effect on the behavior of MC29-infected cells, heparin has but a small quantitative effect on the rate of proliferation of normal cells induced to proliferate with EGF and/or hyperphysiological concentrations of insulin or on the rate of proliferation of RSV-infected cells (Fig. 1). As already noted, insulin at hyperphysiological concentrations acts as a surrogate for IGF-I (somatomedin C) by activating a type-I IGF receptor (7).

Normal chicken heart mesenchymal cells in medium with or without heparin show little or no response to EGF at 1–10 ng/ml (Fig. 1). MC29-infected cells whose proliferation has been arrested by heparin at 25 μg/ml, however, increase 10-fold in number over baseline during 4 days of incubation with EGF at 10 ng/ml and 6-fold in the presence of EGF at 1 ng/ml. These hormone-stimulated, MC29-infected cells maintain the transformed morphology in the presence of heparin (Fig. 2). The hypersensitivity to EGF of MC29-infected cells was borne out in a mitogenicity titration of EGF vs. insulin (Fig. 3): Normal chicken heart mesenchymal cells, again, showed little or no significant response to EGF at 1 ng/ml or 10 ng/ml, manifesting a significant response only when the EGF concentration was raised to 100 ng/ml. MC29-infected cells, by comparison, increased 4-fold in number over baseline during 4 days of incubation with EGF at 1 ng/ml and 18-fold in the presence of EGF at 10 ng/ml. In heparin-containing medium, MC29-infected cells can therefore be seen to be ≈100 times as sensitive as normal cells in regard to the threshold concentration of EGF to which they show a significant proliferative response. In the presence of EGF at 100 ng/ml, MC29-infected cells increase ≈40-fold over baseline during 4 days of incubation, whereas normal cells increase only 5-fold.

A profound mitogenic synergy between EGF (or bFGF) and IGFs for normal chicken heart mesenchymal cells has been described (7); we have now explored these interactions for MC29-infected cells as well (Fig. 3). Hyperphysiological concentrations of insulin appear to potentiate the effects of low concentrations of EGF on MC29-infected cells much as they potentiate the effect of high concentrations of EGF on normal cells. Incubation for 4 days with insulin at 10 μg/ml, for example, causes an ≈4-fold increase in numbers of MC29-infected cells stimulated with EGF at 1 ng/ml or of normal cells stimulated with EGF at 1 μg/ml. Acting in the absence of added EGF, insulin at 10 μg/ml causes≈3-fold multiplication of normal cells and ≈7-fold multiplication of MC29-infected cells. Potentiation of the effects of EGF-like mitogens in the plasma-containing culture medium, rather than hypersensitivity to IGFs *per se*, may well explain the greater response of MC29-infected cells to a maximal dose of insulin alone. Our observation that insulin, acting alone for 4 days at 1 μg/ml, causes 2- to 3-fold multiplication of both normal and MC29-infected cells supports the hypothesis that MC29-infected cells, although markedly hypersensitive to EGF, may not be hypersensitive to IGFs.

bFGF preparation at 10 ng/ml does not have a significant mitogenic effect on normal chicken heart mesenchymal cells but causes ≈3-fold multiplication of MC29-infected cells during a 4-day period. The addition of insulin at 100 ng/ml, which acting alone has little effect, to medium with bFGF preparation at 10 ng/ml causes an approximate doubling of normal cells but ≈14-fold multiplication of MC29-infected cells. This further suggests that MC29-infected cells may be hypersensitive to bFGF. Additional insight into this matter

NORMAL CELLS

INSULIN (ng/ml) \ EGF (ng/ml)	0	1000	100	10	1	0.1
0	10.0±0.4	78.5±2.9	51.0±0.6	16.8±0.7	13.6±0.4	12.0±0.2
10,000	28.5±0.7	278±6.6	208±1.9	46.2±0.7	31.5±0.9	27.2±0.5
1,000	22.5±0.1	292±8.2	168±2.2	33.7±0.1	24.9±0.6	22.7±0.1
100	16.6±0.3	168±2.2	85.8±2.8	22.2±0.3	19.0±0.3	17.1±0.4
10	13.5±0.2	106±4.2	51.3±1.4	20.1±1.2	16.1±0.1	15.0±0.3

INSULIN (ng/ml) \ bFGF (ng/ml)	100	10	1
0	67.5±0.5	14.7±0.4	12.5±0.1
10,000	306±9.6	39.1±0.6	28.3±0.6
100	138±1.5	21.9±0.1	16.5±0.2

INSULIN (ng/ml) \ PDGF (ng/ml)	10	1	0.1
0	17.3±0.3	12.7±0.1	11.7±0.4
10,000	34.2±0.7	29.8±0.3	26.7±0.3
100	20.1±0.3	16.7±0.2	15.9±0.1

MC29-INFECTED CELLS

INSULIN (ng/ml) \ EGF (ng/ml)	0	1000	100	10	1	0.1
0	5.9±0.4	200±7.4	222±5.9	112±0.7	25.1±1.3	8.2±0.0
10,000	40.7±2.0	246±3.3	243±6.1	158±2.4	134±4.8	61.7±1.5
1,000	16.7±0.7	255±7.1	228±6.2	173±1.7	118±3.8	26.0±1.3
100	8.9±0.1	254±11	243±11	181±2.5	67.2±2.6	11.1±0.3
10	7.4±0.1	268±8.4	234±3.3	127±1.0	38.6±0.6	8.5±0.3

INSULIN (ng/ml) \ bFGF (ng/ml)	100	10	1
0	120±0.5	22.1±0.9	7.9±0.4
10,000	182±6.9	151±1.4	83.1±2.4
100	215±4.1	79.5±4.4	10.9±0.2

INSULIN (ng/ml) \ PDGF (ng/ml)	10	1	0.1
0	43.7±1.8	8.7±0.3	7.1±0.1
10,000	122±0.5	54.1±1.0	40.5±0.7
100	59.5±1.1	11.5±0.2	10.0±0.2

FIG. 3. A single experiment examining the effect of EGF, bFGF, PDGF, and insulin (at hyperphysiological concentrations; i.e., an IGF surrogate) on proliferative behavior of normal and MC29-infected chicken heart mesenchymal cells in culture medium containing heat-defibrinogenated plasma at 10% and heparin at 25 μg/ml. Each value represents (the mean number of cells ± SEM) × 10^{-4}, from three tertiary cultures after 4 days of incubation. At the beginning of the experiment (day 0), there were (9.2 ± 0.0) × 10^4 normal or (6.5 ± 0.2) × 10^4 MC29-infected cells per dish. RSV-infected cells in this experiment increased in number from (10.2 ± 0.2) × 10^4 per dish on day 0 to (158 ± 2.4) × 10^4 per dish on day 4.

must await definitive identification of the active principle of bFGF (15). Crude PDGF preparation, tested at a concentration containing ≈10 ng of the pure mitogen per ml, caused less than doubling of normal cells during a 4-day period but caused an ≈8-fold increase in numbers of MC29-infected cells, a difference that was not amplified by insulin. Provisional upon the commercial availability of purer preparations of PDGF, these results suggest that MC29-infected cells may be hypersensitive to PDGF, as well as to EGF and bFGF. Alternatively, the report that PDGF induces human diploid fibroblasts to secrete IGF-I (16) suggests that autocrine secretion of IGF-I, by potentiating the effects of EGF-like mitogens present in the plasma component of the culture medium, may contribute to the apparent hypersensitivity of MC29-infected chicken heart mesenchymal cells to PDGF.

We hypothesize, in accordance with the results of these experiments, (*i*) that heparin prevents the generation by cultured cells of a mitogen from protein precursors in medium containing heat-defibrinogenated plasma; (*ii*) that the activity of the protein product of the *myc* oncogene of the MC29 virus renders cells hypersensitive to EGF, to bFGF, to the putative mitogen released by cultured cells from plasma protein precursors, and, possibly, to PDGF; and (*iii*) that MC29-infected cells must proliferate in order to manifest a transformed morphology.

The abolition by heparin of proliferation of MC29-infected cells might be explained by the prevention of generation from plasma protein precursors of a mitogen to which MC29-infected cells were considerably more sensitive than normal cells. The small effects of heparin on the rate of proliferation of EGF- and insulin-stimulated normal cells and on the rate of proliferation of mitogen-independent RSV-infected cells (11), as well as the published work of others (12–14), suggests that heparin or heparin-like substances may exert physiologically regulatory, negative effects on cell proliferation in addition to or as alternatives to inhibition of generation of a cryptic mitogen from plasma (serum) proteins in culture medium.

Induction of hypersensitivity to mitogenic hormones might explain a significant portion of the contribution of an active *myc* oncogene to the excessive (autonomous) cell proliferation that defines the neoplastic state. Such abnormally sensitive cells might proliferate in response to physiological baseline concentrations of plasma and extracellular fluid

NORMAL CELLS

INSULIN (ng/ml) \ EGF (ng/ml)	0	1000	100	10	1
0	11.7±0.2	99.8±1.9	41.1±0.9	17.9±0.2	14.3±0.6
10,000	34.5±0.8	267±4.2	206±4.6	60.1±1.0	36.3±0.8
1,000	25.0±0.8	298±0.9	154±1.0	44.7±0.1	29.7±0.6
100	20.3±0.6	235±4.2	85.1±2.2	31.4±0.7	22.9±0.3
10	15.5±0.5	152±0.0	58.9±1.3	23.8±0.4	18.1±0.3
1	15.8±0.9	122±3.7	49.2±0.5	22.1±0.3	16.3±0.2

INSULIN (ng/ml) \ bFGF (ng/ml)	500	50	5
0	85.3±1.1	40.5±0.4	14.5±0.3
10,000	287±4.2	241±2.0	34.6±0.3
100	297±11	87.5±1.8	23.1±0.4

INSULIN (ng/ml) \ PDGF (ng/ml)	30	3	0.3
0	33.3±0.5	15.0±0.4	12.1±0.2
10,000	54.3±3.2	31.1±1.1	30.0±1.1
100	37.5±2.3	22.8±1.1	19.1±0.9

SNV/v-*ras*-INFECTED CELLS

INSULIN (ng/ml) \ EGF (ng/ml)	0	1000	100	10	1
0	22.7±0.4	253±1.0	261±0.5	245±0.5	263±1.7
10,000	160±2.9	188±0.9	163±4.8	178±1.7	169±2.6
1,000	129±2.2	148±1.0	133±1.7	141±1.9	144±2.5
100	66.8±1.5	72.2±1.6	72.0±3.3	74.9±1.1	74.3±1.7
10	33.2±1.2	39.1±1.6	38.7±0.7	36.3±1.2	35.6±0.6
1	30.5±0.6	30.5±0.7	32.4±0.6	29.3 0.9	27.5±0.1

INSULIN (ng/ml) \ bFGF (ng/ml)	500	50	5
0	32.1±0.2	28.3±0.7	22.8±0.5
10,000	261±2.4	205±3.5	158±1.9
100	140±10	93.3±5.3	72.1±4.5

INSULIN (ng/ml) \ PDGF (ng/ml)	30	3	0.3
0	190±3.7	38.2±0.2	25.0±0.7
10,000	254±5.7	198±1.9	188±1.2
100	216±10.3	100±4.8	78.5±2.7

FIG. 4. A single experiment examining the effect of EGF, bFGF, PDGF, and hyperphysiological insulin on proliferative behavior of normal and SNV/v-*ras*-infected chicken heart mesenchymal cells in culture medium containing heat-defibrinogenated plasma at 10% and heparin at 25 μg/ml. Each value represents (the mean number of cells ± SEM) × 10^{-4}, from three tertiary cultures after 4 days of incubation. At the beginning of the experiment (day 0), there were (9.7 ± 0.2) × 10^4 normal and (10.5 ± 0.1) × 10^4 SNV/v-*ras*-infected cells per dish. RSV-infected cells in this experiment increased in number from (8.1 ± 0.1) × 10^4 per dish on day 0 to (324 ± 2.5) × 10^4 per dish on day 4.

hormones that are not mitogenic for normal cells. In renewal ("turnover") tissues such as bone marrow, skin, and gut, similarly, cells hypersensitive to mitogenic hormones might well proliferate in excess of their normal counterparts and so yield a tumor. The transforming protein of the *myc* oncogene may confer hypersensitivity to mitogenic hormones on cells by increasing the numbers or sensitivity of hormone receptors (17) or via a postreceptor mechanism.

MC29-infected cells become proliferatively quiescent and revert to a normal morphology when heparin is added to plasma-containing culture medium without mitogenic hormones but maintain the transformed morphology when stimulated with mitogenic hormones. This observation suggests that reversion of MC29-infected cells to a normal morphology is a consequence of proliferative quiescence rather than a response to heparin *per se*.

Chicken heart mesenchymal cells infected with SNV/v-*ras* proliferate only sluggishly in plasma-containing medium without added mitogenic hormones, increasing 2-fold in number during 4 days of incubation (Figs. 1 and 4), and assume a spindle-shaped transformed morphology (Fig. 2). The proliferative behavior and transformed morphology of SNV/v-*ras*-infected cells are not significantly affected by addition of heparin to plasma-containing medium without added hormones or with added insulin, our IGF surrogate (Fig. 1). Unlike MC29-infected cells, which are hypersensitive to EGF and bFGF and normally sensitive to IGFs, SNV/v-*ras*-infected cells are refractory to EGF and bFGF and hypersensitive to IGFs: EGF at 1 μg/ml and bFGF at 500 ng/ml, while causing, respectively, 8-fold and 7-fold increases in number of normal chicken heart mesenchymal cells during 4 days of incubation, have no stimulatory effect on the proliferation of cells infected with SNV/v-*ras*. By contrast, although insulin at 10, 1, and 0.1 μg/ml causes, respectively, approximately 3-, 2-, and 1.5-fold increases in number of normal cells, these same concentrations of insulin cause, respectively, approximately 7-, 6-, and 3-fold increases in number of SNV/v-*ras*-infected cells (Fig. 4).

Some cells bearing v-*ras* oncogenes have been reported to secrete type α transforming growth factor (TGF-α), which binds to EGF receptors and, purportedly, blocks the binding of EGF (17). TGF-α has not been reported to bind to bFGF receptors. Furthermore, it is unlikely that TGF-α would bind to EGF or bFGF receptors with sufficient avidity to render SNV/v-*ras*-infected cells refractory to EGF and bFGF without itself being potently mitogenic. Other workers have suggested that proto-*ras*-encoded proteins mediate the effects of activated EGF receptors and that the presence in cells of activated ras proteins might cause down-regulation of EGF receptors (18) and, by inference, of bFGF receptors. An assumption that such mediation occurs in normal cells would suggest that chicken heart mesenchymal cells infected heavily enough with SNV/v-*ras* to render them refractory to EGF and bFGF would proliferate actively, unless the action of EGF and bFGF receptors involved cellular mediators, as yet undefined, in addition to proto-ras proteins. Generally speaking, the hypersensitivity to insulin of chicken heart mesenchymal cells bearing a v-*ras* oncogene, which may constitutively activate some cellular functions normally activated by EGF and bFGF receptors, may parallel the strong synergistic relationship (7) between EGF or bFGF on the one hand and IGF-I or IGF-II on the other.

SNV/v-*ras*-infected chicken heart mesenchymal cells appear to be more sensitive to the activity of PDGF than their normal counterparts (Fig. 4). PDGF preparation at a concentration equivalent to 30 ng of the pure mitogen per ml causes an ≈8-fold increase in SNV/v-*ras*-infected cells during a 4-day period while causing only a 3-fold increase in normal cells (Fig. 4). Chicken heart mesenchymal cells bearing an active *ras* oncogene appear to be hypersensitive to IGFs, and it is possible that PDGF-induced autocrine secretion of IGF-I, as reported in human fibroblasts (16), underlies the apparent hypersensitivity of SNV/v-*ras*-infected cells to PDGF.

We thank Mrs. Ruth Dziadul for expert preparation of the manuscript. This work was supported by Grant 1536 from the Council for Tobacco Research. This is manuscript no. 771 from the Cancer Research Institute of the New England Deaconess Hospital.

1. Papas, T. S., Kan, N. K., Watson, D. K., Flordellis, C. S., Psallidopoulos, M. C., Lautenberger, J., Samuel, K. P. & Duesberg, P. (1984) in *Cancer Cells*, eds. Vande Woude, G. F., Topp, W. C. & Watson, J. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 2, pp. 153–163.
2. Wilhelmsen, K. C., Tarpley, W. G. & Temin, H. (1984) in *Cancer Cells*, eds. Vande Woude, G. F., Topp, W. C. & Watson, J. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 2, pp. 303–308.
3. Wigler, M., Fasano, O., Taparowsky, E., Powers, S., Kataoka, T., Birnbaum, D. & Goldfarb, M. (1984) in *Cancer Cells*, eds. Vande Woude, G. F., Topp, W. C. & Watson, J. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 2, pp. 419–423.
4. Balk, S. D. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6606–6610.
5. Balk, S. D., Levine, S. P., Young, L. L., LaFleur, M. M. & Raymond, N. M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5656–5660.
6. Balk, S. D., Shiu, R. P. C., LaFleur, M. M. & Young, L. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1154–1157.
7. Balk, S. D., Morisi, A., Gunther, H. S., Svoboda, M. F., Van Wyk, J. J., Nissley, S. P. & Scanes, C. G. (1984) *Life Sci.* **35**, 335–346.
8. Balk, S. D., Gunther, H. S. & Morisi, A. (1984) *Life Sci.* **35**, 1157–1171.
9. Armelin, H. A., Armelin, M. C. S., Kelly, K., Stewart, T., Leder, P., Cochran, B. H. & Stiles, C. D. (1984) *Nature (London)* **310**, 655–660.
10. Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche, N. S., Stern, D. F. & Sporn, M. B. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 119–123.
11. Balk, S. D., Morisi, A. & Gunther, H. S. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6418–6421.
12. Clowes, A. W. & Karnowsky, M. J. (1977) *Nature (London)* **265**, 625–626.
13. Castellot, J. J., Jr., Beeler, D. L., Rosenberg, R. D. & Karnowsky, M. J. (1984) *J. Cell. Physiol.* **120**, 315–320.
14. Fritze, L. M., Reilly, C. F. & Rosenberg, R. D. (1985) *J. Cell Biol.* **100**, 1041–1049.
15. Gospodarowicz, D., Cheng, J., Lui, G., Baird, A. & Bohlent, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6963–6967.
16. Clemmons, D. R., Underwood, L. E. & Van Wyk, J. J. (1981) *J. Clin. Invest.* **67**, 10–19.
17. Sporn, M. B. & Roberts, A. B. (1985) *Nature (London)* **313**, 745–747.
18. Kamata, T. & Feramisco, J. R. (1984) in *Cancer Cells*, eds. Levine, A., Vande Woude, G. F., Topp, W. C. & Watson, J. D. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 1, pp. 11–16.