Effect of Rous Sarcoma Virus Transformation of Rat-1 Fibroblasts upon Their Growth Factor and Anchorage Requirements in Serum-free Medium

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ABSTRACT

The proliferative response of nontransformed rat embryo (Rat-1) cells and avian sarcoma virus-transformed B31 cells to high-density lipoprotein (HDL), transferrin, insulin, epidermal growth factor (EGF), and fibroblast growth factor has been compared. HDL, added in combination with transferrin, supported the active proliferation of low-density cultures of both Rat-1 and B31 cells. No major difference in the sensitivity of Rat-1 or B31 cells to HDL and transferrin was observed when cells were maintained on dishes coated with an extracellular matrix (ECM) obtained from bovine corneal endothelial cells. The two cell types differed in their response to the other known growth-promoting agents, however. In contrast to Rat-1 cells, transformed B31 cells no longer respond to EGF and fibroblast growth factor and respond only inconsistently to the mitogenic stimulus of insulin.

Nontransformed Rat-1 cells and transformed B31 cells grown in the presence of medium containing, respectively, HDL, transferrin, insulin, EGF, and dexamethasone or HDL, transferrin, and insulin could be subcultured for more than 50 generations in the complete absence of serum without significant alteration in morphology, growth rate, or tumorigenicity (B31 cells). When plastic or collagen-coated dishes were used as the substrate instead of ECM-coated dishes, nontransformed Rat-1 cells grew very slowly in the serum-free medium described above. Dishes coated with collagen were not more efficient than was plastic in supporting growth of Rat-1 cells under these conditions. Coating dishes with fibronectin, however, clearly improved their growth, bringing the final cell density of the cultures up to 50% of that obtained on ECM-coated dishes. In contrast, transformed B31 cells grew significantly in serum-free medium when seeded on plastic or collagen-coated dishes, and the final cell density reached by cells on these substrates was 50% of that of cells maintained on ECM-coated dishes. In addition, B31 cells grew equally well when seeded on fibronectin- or ECM-coated dishes. The transformed cells thus showed less stringent substrate requirements when grown under serum-free conditions than did nontransformed Rat-1 cells. Our data also indicate that HDL, in combination with transferrin, supported efficient anchorage-independent growth of B31 cells. Fibroblast growth factor, but not insulin or EGF, further improved anchorage-independent growth of these cells. The capacity of cells to form colonies in semisolid medium when exposed to HDL and transferrin seems to correlate with high tumorigenic potential.

INTRODUCTION

Previous studies have shown that HDL added in combination with transferrin to synthetic culture medium can support active proliferation of a number of normal cell types in vitro (15, 22, 26, 47). The proliferative response of the cells to HDL depended heavily on the nature of the substratum upon which they rested, since cells divided actively when seeded on plastic dishes coated with an ECM produced by bovine corneal endothelial cells (18, 20) but grew very poorly or not at all when maintained on plastic. It was therefore demonstrated that HDL and transferrin are 2 plasma components the growth-promoting effect of which on normal cells is greatly enhanced by ECM (18, 23). While the presence of only HDL and transferrin in the medium was sufficient to ensure optimal growth of vascular endothelial cells (47), optimal growth of vascular smooth muscle cells (22), corneal endothelial cells (15), and lens epithelial cells (26) required, in addition to HDL and transferrin, the presence of insulin and EGF or FGF.

The growth-promoting effect of HDL is not restricted to normal cells. Indeed, a recent study by Gospodarowicz et al. (25) has demonstrated that HDL is capable of supporting active proliferation of various human tumor cell lines maintained on ECM-coated dishes. For most of these tumor cells, optimal proliferation, as compared to that of cells exposed to serum, was obtained when medium was supplemented with only HDL and transferrin. Such a strong proliferative response of the tumor cells to HDL and transferrin might simply reflect the growth capacity of the tissue from which each tumor derived, or, alternatively, an increased sensitivity to these factors developed during neoplastic transformation. It is well established that neoplastic transformation, whether spontaneous or induced by known oncogenic agent(s), can affect a number of growth-related properties of cultured animal cells (31, 45). Characteristically, transformation may lower the dependence of the cells on growth factor(s) present in serum. It may also decrease their dependence on attachment to a substrate for growth. With that in mind, we became very interested in asking whether neoplastic transformation could affect the growth response of cells to HDL and whether the growth response of normal and transformed cells varies according to their substrate requirements. In this study, we have compared the effect of HDL and other growth-stimulating agents on the proliferation of nontransformed rat embryo (Rat-1) cells and that of their ASV-transformed counterparts, the B31 cells (51). Our results indicate that B31 cells do not differ significantly from Rat-1 cells in their response to HDL but rather suggest that, as a result of a decreased capacity to respond to other growth factor(s), their reliance on HDL for growth in vitro would be increased. We also report that HDL, in combination with transferrin, can support the serum-free growth of the sarcoma virus-transformed cells in suspension.

MATERIALS AND METHODS

Materials. FGF was purified from bovine brains as described previously (16, 24). Brain FGF yields a single band on polyacrylamide gel...
electrophoresis at pH 4.5 and on an isoelectric focusing column (total volume, 110 ml; pH range, 3.4 to 11.0). All of the activity focused within a single peak with an isoelectric point of 9.6.

Mouse EGF was purified as described by Savage and Cohen (43). Fibronectin was purified from bovine plasma as described by Engvall et al. (14). When analyzed by slab gel-polyacrylamide gel electrophoresis under reduced conditions, the purified fibronectin ran as a doublet with a molecular weight in the range of 220,000. Crystalline bovine serum albumin was obtained from Schwarz/Mann (Orangeburg, N. Y.). Bovine insulin, human transferrin, and dexethemazone were obtained from Sigma Chemical Co. (St. Louis, Mo.). DME, F-12 medium, and Roswell Park Memorial Institute Medium 1640 were obtained from Grand Island Biological Co. (Grand Island, N. Y.). Calf serum and fetal calf serum were obtained from Irvine Serum Co. (Irvine, Calif.). Tissue culture dishes were from Falcon Plastics, gentamicin was from Schering Corp. (Union, N. J.), and Fungizone was from E. R. Squibb & Sons (New York, N. Y.). Agarose (Seakem, Maine) was purchased from Marine Colloids (Rockland, Mass.).

Preparation of LDL and HDL. Human LDL (1.019 < d < 1.063 g/cc cm) and HDL (1.07 < d < 1.21 g/cc cm) were obtained from human plasma by differential ultracentrifugal flotation (28). In order to remove contaminating plasma proteins, the LDL and HDL preparations were washed by reprecipitation in solutions with densities of 1.063 and 1.210, respectively. Protein concentrations were determined as described by Lowry et al. (35) and modified by Maxwell et al. (37).

The purified HDL and LDL preparations were analyzed by double immunodiffusion to determine the degree of cross-contamination of the HDL preparation by LDL and vice versa (47), and it was found to be less than 0.2%. To eliminate the possibility of contamination by plasma proteins, the purity of LDL and HDL preparations was analyzed by slab gel electrophoresis (10 to 18% and 5 to 18%, respectively; exponential polyacrylamide gel gradient containing 0.1% sodium dodecyl sulfate) with or without prior delipidation with tetrathymethylurea (47). When the electrophoretic patterns of HDL and LDL preparations were compared to that of plasma, no contamination by plasma proteins was observable.

Cell Culture Conditions. Cultures of bovine corneal endothelial cells were maintained in the presence of DME supplemented with 500 µg of protein per ml of HDL. The cell zone (2.5 µg/ml). FGF (100 ng/ml) was added every other day until the cultures were nearly confluent.

Preparation of Collagen-, Fibronectin-, or ECM-coated Dishes. Fibronectin-coated dishes were prepared as described by Kramer et al. (33). Plastic dishes coated with an ECM produced by corneal endothelial cells were prepared using either detergent treatment [0.5% Triton in Dulbecco's phosphate-buffered saline (140 mM NaCl, 8 mM Na2HPO4, 3 mM KCl, 1.5 mM KH2PO4, 0.9 mM CaCl2, and 0.5 mM MgCl2)] or base treatment (NH4OH, 0.02 M in distilled water; Ref. (22)]. When base treatment was used, confluent corneal endothelial cell cultures were first washed with distilled water and then exposed to 0.02 M NH4OH in distilled water for 5 min, followed by washing with Dulbecco's phosphate-buffered saline. ECMs treated both ways were equally capable of supporting the growth of various cell types seeded on them (22). Xylose-treated ECM was prepared as above, except that β-d-xyloside (2.5 mM) was added at the time of seeding.4 Previous studies have shown that ECM produced by bovine corneal endothelial cells exposed to xylose (xylose-ECM) contains less than 10% of the amount of glycosaminoglycans normally found in ECM produced by cells which are not exposed to this drug.4 Collagen-coated dishes were prepared as described by Iversen et al. (30) using the air drying technique. The highly purified commercial collagen preparation (Vitrogen; Collagen Corp., Palo Alto, Calif.) from bovine skin was used at the concentration supplied by the manufacturer (2.5 mg/ml). Uniformity of substrate coating with collagen was verified by staining control dishes with Coomassie blue for visualization (30).

RESULTS

Effect of Increasing Concentrations of HDL, LDL, and Transferrin on the Proliferation of Rat-1 and ASV-transformed B31 Cells Maintained on ECM and Exposed to Serum-free Medium. We first compared the proliferative response of Rat-1 cells and ASV-transformed B31 cells exposed to increasing concentrations of human HDL or LDL. Low-density cultures (15 cells/sq mm) seeded on ECM-coated dishes (35 mm) in the absence of serum were exposed to medium supplemented with transferrin (25 µg/ml) and increasing concentrations of either LDL or HDL. As shown in Chart 1, the proliferation of both cell lines was stimulated in the presence of HDL at concentrations lower than 0.08 mM.


*Z. Nevo, R. Gonzalez, and D. Gospodarowicz, unpublished data.
ranging from 25 to 2000 μg of protein per ml. Saturation was observed at concentrations of 1000 and 500 μg of protein per ml for the Rat-1 and B31 cells, respectively. In comparison to HDL, LDL was much less effective in promoting the proliferation of these cells. Maximal stimulation was observed at a concentration of 25 μg of protein per ml of LDL for both cell types, but at concentrations above 50 μg of protein per ml it became toxic for the cells.

The presence of transferrin in the culture medium was absolutely required for the expression of the mitogenic effect of HDL since, in its absence, Rat-1 and B31 cells grew poorly or not at all. Chart 2 shows that in the presence of saturating concentrations of HDL, significant growth of Rat-1 and B31 cells could take place at transferrin concentrations as low as 0.25 to 0.5 μg/ml. Maximal transferrin effects were observed at concentrations of 2.5 to 5 μg/ml, and no cytotoxic effect was detected with concentrations as high as 100 μg/ml. Transferrin had only minor growth-promoting activity when HDL was omitted from the culture medium.

The results presented in Charts 1 and 2 therefore indicate that nontransformed Rat-1 and transformed B31 cells do not differ significantly in their proliferative response to HDL and transferrin. 2 factors routinely provided when cells are grown in the presence of plasma- or serum-supplemented medium.

Effect of Insulin, EGF, and FGF on the Proliferation of Rat-1 and B31 Cells Maintained on ECM and Exposed to Serum-free Medium. Previous studies have shown that insulin, mouse EGF, and FGF can stimulate the proliferation of some (15, 22, 26) but not all (47) normal bovine cells when added to the culture medium in conjunction with HDL and transferrin. Chart 3 compares the effect of these factors, added in conjunction with HDL and transferrin, on the proliferation of Rat-1 and B31 cells after 5 and 6 days of growth, respectively. The concentrations of insulin (5 μg/ml), EGF (25 ng/ml), and FGF (100 ng/ml) used in these experiments had been shown, in preliminary experiments, to be saturating (data not shown).

As mentioned above, Rat-1 and B31 cells grew actively in absence of serum only when HDL and transferrin were added to the culture medium in combination (Chart 3). The addition of insulin together with HDL and transferrin resulted in a small but consistent increase in the final density of Rat-1 cultures; a similar but more irregular effect of insulin on the growth of B31 cells could be observed (Chart 4). Rat-1 and B31 cells differed more markedly in their responses to EGF and FGF. While addition of EGF or FGF to medium already containing HDL, transferrin, and insulin had an obvious effect on the growth of Rat-1 cells, B31 cells displayed much lower sensitivity to these 2 growth factors. Our data suggest that, as a result of their decreased capacity to respond to EGF, FGF, and possibly insulin, the transformed B31 cells may rely more heavily on HDL and transferrin in order to grow actively in vitro than do the nontransformed Rat-1 cells.

Growth Rate and Long-Term Passage of Rat-1 and B31 Cells Maintained on ECM-coated Dishes and Exposed to Serum-free Medium. In the absence of HDL, both nontransformed Rat-1 cells and transformed B31 cells exposed to medium supplemented with, respectively, transferrin, insulin, EGF, and dexamethasone or transferrin and insulin grew actively for about 2 to 3 days after seeding (Chart 4). Proliferation then gradually ceased and never resumed, even when the culture...
medium was frequently renewed. In these conditions, Rat-1 and B31 cells never reached confluence.

As shown in Chart 4A, the growth rate of Rat-1 cells seeded on ECM-coated dishes and maintained in the presence of saturating concentrations of HDL and transferrin was similar to that of cultures maintained in the presence of 10% serum. After a lag period of 24 hr, cells maintained under both conditions started to proliferate actively and at a constant rate (doubling time, approximately 20 hr) until cultures reached confluence. The final saturation density of cultures maintained in serum was about 2-fold higher than that of cells maintained in the presence of HDL and transferrin. This difference reflects the larger size of Rat-1 cells when grown in HDL and transferrin as much as the fact that they remain growth density-inhibited. When insulin, EGF (or FGF), and dexamethasone were added together with HDL and transferrin, however, cultures reached a final cell density similar to that of cultures maintained in the presence of serum. Dexamethasone, when assayed in the range of concentrations of 0.1 to 10 µg/ml, neither stimulated nor inhibited the growth of Rat-1 cells exposed to medium supplemented with HDL, transferrin, insulin, and EGF. In some experiments, however, confluent Rat-1 cells maintained in the absence of dexamethasone could suddenly become unstable and lyse within 24 hr, leading to a rapid deterioration of the cultures. This was never observed when dexamethasone was present in the medium. This obser-

Chart 4 shows that the growth rate of B31 cells maintained in the presence of HDL and transferrin was similar to that of cells maintained in the presence of serum. In that particular experiment, the addition of insulin to the medium, in conjunction with HDL and transferrin, had only a minor effect on the growth rate and the final density of the B31 cultures.

As reported previously (51), B31 cells display many of the biological characteristics associated with mammalian cells transformed by ASV. One such characteristic is in morphological transformation, as reflected by the loss of the ability of B31 cells to form a stable monolayer at confluence. When B31 cells exposed to serum or to HDL, transferrin, and insulin reach confluence, they start rounding and piling up, resulting in the formation of progressively growing nodules on top of the monolayer. The fact that this sort of “anchorage-independent growth” (see below) is more rapid in the presence of serum probably explains why cells maintained in the latter condition reach higher cell densities than cells maintained in HDL, transferrin, and insulin (Chart 4B).

As shown in Chart 5, Rat-1 cells maintained on ECM-coated dishes in medium supplemented with HDL, transferrin, insulin,
RSV Transformation and Growth Factor Requirements

EGF, and dexamethasone can be passed repeatedly at low cell density and in the total absence of serum for at least 50 generations. Repeated passage of B31 cells in the absence of serum can similarly be achieved by maintaining and subcultivating them in the presence of HDL, transferrin, and insulin. Despite its inconsistent effect on the growth of B31 cells in short-term assays (see above), insulin was added to the culture medium in long-term growth experiments because it was found to protect B31 cells that had been passaged several times in the absence of serum against their tendency to lyse when reaching confluence. Neither the morphology nor the growth rate of Rat-1 and B31 cells was significantly affected over these periods of growth in the absence of serum. As can be seen in Chart 5, removal of HDL from the culture medium considerably slowed the long-term growth of Rat-1 cells in serum-free medium; unexpectedly, repeated subcultivation of such cultures seemed to ensure a very slow but continued proliferation of these cells in these conditions. B31 cells, on the other hand, stopped dividing definitively after one passage in the absence of HDL.

The tumor-forming ability of B31 cells that had been passaged for more than 30 generations in medium containing HDL, transferrin, and insulin was tested by s.c. injection of 10^6 cells into newborn syngeneic rats. Within 2 weeks, rapidly growing tumors appeared at the site of injection in all test animals, a clear indication that B31 cells had retained their full oncogenic potential (51).

Effect of Substratum on the Growth of Rat-1 and B31 Cells in Serum-free Medium. It is well known that transformation of animal cells by viral agents decreases their dependence on firm attachment to an appropriate substrate in order to grow. To gain more insight into the nature of that decreased requirement for an appropriate substrate, we have compared the growth capacity of transformed Rat-1 cells and ASV-transformed B31 cells when maintained on various substrates and exposed to serum-free conditions. For that purpose, 1.5 x 10^5 Rat-1 or B31 cells were seeded in the complete absence of serum on plastic 35-mm culture dishes or on collagen-, fibronectin-, or ECM-coated culture dishes. Rat-1 cells were then exposed to medium supplemented with HDL, transferrin, insulin, EGF, and dexamethasone, while B31 cells were exposed to medium containing HDL, transferrin, and insulin. Cell density was determined after 5 and 6 days of growth for Rat-1 and B31 cells, respectively.

As shown in Fig. 1A, Rat-1 cells attached to plastic tissue culture dishes and spread on it, albeit to a limited extent. Similar behavior was observed when Rat-1 cells were seeded on dishes coated with collagen (Fig. 1C). On both substrates, Rat-1 cells showed a tendency to form small groups of more or less bipolar cells. As indicated in Table 1, Rat-1 cells grew very slowly on these substrates compared to cells maintained on ECM-coated dishes. Growth and migratory activity of Rat-1 cells was greatly enhanced, however, by coating dishes with fibronectin, and this correlated with more extensive spreading of cells (Fig. 1E). The final density of Rat-1 cultures maintained on fibronectin was one-half of that of cultures grown on ECM-coated dishes (Table 1), thus suggesting that the latter substrate contains additional component(s) which are required by these cells for optimal attachment and/or growth. On both fibronectin- (not shown) and ECM-coated (Fig. 1D) dishes, Rat-1 cells eventually form at confluence a stable monolayer of flat cells that retained density-dependent inhibition of growth.

B31 cells attached but did not spread to a significant extent on uncoated (Fig. 1B) or collagen-coated (Fig. 1D) plastic dishes. In contrast to Rat-1 cells, however, B31 cells did grow actively, although not at a maximal rate, on these surfaces (Table 1). They formed progressively enlarging colonies of round cells that tended to aggregate upon crowding of the available surface. As shown in Fig. 1, F and H, spreading of B31 cells did take place on fibronectin- and ECM-coated dishes, although to a more variable extent among cells seeded on the former substrate. The final density of B31 cultures maintained on fibronectin-coated dishes was the same as that of cells maintained on ECM. Unlike Rat-1 cells, then, the only attachment factor required by B31 cells in order to grow at an optimal rate was fibronectin. Furthermore, B31 cells showed a loss of density-dependent inhibition of growth, even when maintained on ECM. Confluent B31 cells tend to decrease in size and round up, eventually resulting in the formation of nodules progressively growing above the cell monolayer (Fig. 1H). These results indicate that spreading of B31 cells on their substrate is not sufficient to restore to these transformed cells density-dependent inhibition of growth.

The morphology (not shown) and the relative growth (Table 1) of both Rat-1 and B31 cells were the same on ECM free of proteoglycans or on normal ECM. Since ECM prepared in the presence of xyloside is devoid of most of the glycosaminoglycan content normally found in ECM (see "Materials and Methods"), these results suggest that these macromolecular components of ECM are not primarily involved in the control of morphology and growth of these cell types.

HDL and Transferrin Support the Growth of B31 Cells in Semisolid Medium. Previous studies have shown that B31 cells can form colonies with a very high efficiency when grown in soft agar in the presence of serum-supplemented medium (51). We have therefore examined whether B31 cells could grow in suspension when exposed to the serum-free medium defined above. Our results (Table 2) show that when medium containing 0.3% agarose was supplemented with saturating concentrations of HDL and transferrin, 20% of the cells seeded could form progressively growing colonies. The requirement for HDL and trans-
ferrin was absolute, since no colony was ever observed in the absence of one or the other of these factors. Similar results were obtained when 0.3% soft agar was used instead of agarose (data not shown).

The cloning efficiency of B31 cells measured after 14 days of growth was enhanced only slightly or not at all by adding EGF and/or insulin to the medium but, surprisingly, was increased 2-fold by the addition of FGF. In the best conditions, i.e., in the presence of HDL, transferrin, insulin, and FGF, 50% of the cells seeded could give rise to a colony. This value compares well with the cloning efficiency (about 60%) of B31 cells grown in the presence of serum. As shown in Table 2, the average diameter (0.32 mm) of the colonies formed in the presence of serum was 1.5-fold greater than that of cells exposed to HDL, transferrin, insulin, and FGF, however (also see Fig. 2 for the morphological aspect of the colonies formed under these conditions). This indicates that the anchorage-independent cells grow at a faster rate when exposed to serum than when exposed to medium supplemented with HDL and transferrin. In contrast to B31 cells, Rat-1 cells did not give rise to colonies in agarose when exposed to medium containing HDL and transferrin (supplemented or not with insulin, EGF or FGF, and dexamethasone). Thus, none of these factors showed transforming activity under these conditions.

**DISCUSSION**

The results obtained indicate that ASV-transformed B31 cells do not differ significantly from nontransformed Rat-1 cells in their response to HDL. The ability of HDL to stimulate growth of both cell lines is absolutely dependent on the presence of transferrin in the medium. The requirement for transferrin could reflect either its role in delivering essential iron to the cells or its role as a detoxifying protein capable of removing from the medium trace amounts of toxic metals that would otherwise have been removed by serum (1). In addition, when exposed to HDL and transferrin, both cell types maintain a growth rate similar to that observed with cultures exposed to serum. Removal of HDL from the culture medium limited growth of low-density cultures of Rat-1 and B31 cells to the first few days following seeding and considerably lowered, in our experimental conditions, the final density reached by such cultures. Like Rat-1 cells, B31 cells can respond to the mitogenic effect of insulin, although that response was, for unknown reasons, found to be less consistent than that of the nontransformed cells. On the other hand, B31 cells lost the capacity to respond to EGF and FGF. These results suggest that, as a result of this decreased sensitivity to EGF, FGF, and possibly to insulin, B31 cells may rely more heavily on the plasma factors HDL and transferrin when grown in vitro in serum-containing media.

The lack of effect of EGF on the proliferation of B31 cells could be explained by a decrease in their ability to bind the hormone. Indeed, preliminary experiments indicate that the capacity of B31 cells to bind $^{125}$I-EGF is about 100-fold less than that of nontransformed Rat-1 cells. A decrease in EGF binding capacity has been observed previously in animal cells transformed by murine or feline sarcoma viruses (4, 48). Todaro ef al. (48) later showed that murine sarcoma virus-transformed cells release polypeptides into the medium that stimulate cell growth and initiate a phenotypic change in the morphology of untransformed monolayer cell cultures and also induce anchorage-independent cell growth (12, 49). It was later demonstrated that several (12, 13) but not all (41) of these “transforming growth factors” (46, 49) compete with EGF for its membrane receptors. When Rat-1 cells were exposed to culture medium previously conditioned by B31 cells, a decrease in their EGF-binding capacity and morphological transformations similar to those observed in cells exposed to transforming growth factors were observed. Taken together, our results could indicate that B31 cells release into the medium one (or more) transforming growth factor(s). Further experiments will be needed to establish this fact, to determine whether the activities responsible for the decrease in EGF binding and for the induction of morphological transformation can be separated or not, and to evaluate their role in the control of the proliferation of B31 cells in serum-free medium.

Recently, Ozanne ef al. (39) have identified similar transforming growth factors from mouse and rat cells, including Rat-1 cells, transformed by KMSV. The same group (32) later showed that KMSV-transformed cells were capable of continuous proliferation in serum-free medium supplemented with only transferrin and insulin and lacking exogenous growth factors. They also

**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rat-1 (%)</th>
<th>B31 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Plastic</td>
<td>13.3</td>
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</tr>
<tr>
<td>Collagen</td>
<td>8.2</td>
<td>48.4</td>
</tr>
<tr>
<td>Fibronectin</td>
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<td>92.8</td>
</tr>
<tr>
<td>Xyloside-ECM</td>
<td>88.6</td>
<td>93.4</td>
</tr>
</tbody>
</table>

**Table 2**

Plating efficiency of B31 cells in agarose

Cells were seeded at 5 x $10^5$ cells/35-mm dish in growth medium containing 0.3% agarose and supplemented with either 5% calf serum and 5% fetal calf serum or with growth factors added singly or in combination at the following concentrations: HDL, 500 μg/ml; transferrin, 25 μg/ml; insulin, 5 μg/ml; EGF, 25 ng/ml; and FGF, 100 ng/ml. Colonies (≥0.08 mm) were counted after 14 days. Average colony diameter represents the mean of at least 25 randomly chosen colonies, measured at Day 14.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Cloning efficiency (% of plated cells)</th>
<th>Av. colony diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL</td>
<td>&lt;0.02</td>
<td>0.13 ± 0.04 a</td>
</tr>
<tr>
<td>Transferrin</td>
<td>&lt;0.02</td>
<td>0.15 ± 0.06</td>
</tr>
<tr>
<td>Transferrin + insulin + EGF</td>
<td>&lt;0.02</td>
<td>0.15 ± 0.07</td>
</tr>
<tr>
<td>Transferrin + insulin + FGF</td>
<td>&lt;0.02</td>
<td>0.18 ± 0.07</td>
</tr>
<tr>
<td>HDL + transferrin</td>
<td>25</td>
<td>0.16 ± 0.07</td>
</tr>
<tr>
<td>HDL + transferrin + insulin</td>
<td>25</td>
<td>0.19 ± 0.08</td>
</tr>
<tr>
<td>HDL + transferrin + EGF</td>
<td>17</td>
<td>0.20 ± 0.08</td>
</tr>
<tr>
<td>HDL + transferrin + FGF</td>
<td>43</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>HDL + transferrin + insulin + EGF</td>
<td>27</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Serum, 10%</td>
<td>48</td>
<td>0.32 ± 0.23</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
showed that transformed cells did not respond to EGF and FGF. In contrast, nontransformed cells were unable to grow in serum-free, mitogen-free medium unless it was supplemented with EGF or medium conditioned by homologous transformed cells. Continuous growth of transformed cells was shown to be density dependent and to require at clonal density the presence of conditioned medium from transformed cells. The authors proposed that serum-free growth of KiMSV-transformed cells is dependent on ectopically produced growth factors that are intimately involved in reduction of their serum requirement. In preliminary experiments, however, we have been unable to demonstrate any stimulatory effect of crude B31 conditioned medium on the growth of either Rat-1 or B31 cells in serum-free medium; this could result from our use of a low initial cell density and/or an insufficient production of "transforming factor(s)" by B31 cells.

A loss of EGF requirement in Syrian and Chinese hamster cells transformed by different modes has been reported previously by Cherrington et al. (6). The analysis of these transformed cell lines revealed the existence of a continuum of phenotypes with respect to growth factor requirements and transformation, a continuum going from nontransformed and high EGF requirement to highly tumorigenic (or tumor-derived) and no EGF requirement. Thus, chemically transformed Chinese hamster cells retained a requirement for EGF, but this requirement appeared less stringent than that in nontransformed parental cells. Spontaneously transformed cells, as well as cells derived from tumors induced by injecting chemically transformed cells had, on the other hand, lost the EGF requirement. All of the above transformed or tumor-derived cell lines retained the insulin requirement expressed by the parental line, as indicated by their growth arrest in G1 by insulin deficiency. A loss of EGF requirement was also observed in Syrian hamster cells transformed by polyoma virus, but, in contrast to the transformed Chinese hamster cells, these cells also lost their insulin requirement. Since, in the spontaneously and chemically transformed Chinese hamster cells as well as in the polyoma virus-transformed Syrian hamster cells, a diminished EGF requirement correlated with a reduction in serum requirement, the authors were led to the conclusion that an initial step that reduces serum requirement in culture and in tumorigenesis is relaxation of the growth-regulatory function of EGF. Further studies (40) indicated that, in the transformed Chinese hamster cells, loss of the EGF requirement was not due to secretion of EGF-like growth factor(s) but was associated with low EGF-binding capacity and loss of the thrombin requirement. The exact mechanism by which these cells, which in the transformed state are unusually responsive to the mitogenic effect of thrombin (5), upon transformation lose their requirement for both of these growth factors has not yet been elucidated (40). Sager et al. (42) observed a similar correlation between loss of the EGF or thrombin requirements and gain of tumor-forming ability in a series of mutants derived from the same nontransformed Chinese hamster cells and selected on the basis of their anchorage independence or low serum requirement after chemical mutagenesis.

The use of serum-free growth medium developed for Rat-1 and B31 cells has allowed us to detect subtle differences between these 2 cell types in their substrate requirements for growth. Nontransformed Rat-1 cells were capable of only very limited growth when seeded on uncoated or collagen-coated plastic dishes and exposed to serum-free medium, as compared to cells maintained on ECM-coated dishes. The growth of Rat-1 cells was accelerated, however, when they were seeded on fibronectin-coated dishes and was about 50% of that of cells maintained on ECM. In contrast to Rat-1 cells, transformed B31 cells grew significantly, although not at an optimal rate, when seeded on plastic or collagen-coated dishes and exposed to serum-free medium; the relative growth of B31 cells maintained on the latter substrata was about 50% of that of cells seeded on ECM. Moreover, the relative growth of B31 cells seeded on fibronectin-coated dishes was almost the same as that of cells maintained on ECM. Therefore, the transformed B31 cells seem to have less stringent substrate requirements than do nontransformed Rat-1 cells when grown under serum-free conditions. A similar difference in substrate requirement between transformed Rat-1 cells and KiMSV-transformed derivatives has recently been observed by Kaplan et al. (32). They reported that, unlike KiMSV-transformed cells, nontransformed Rat-1 cells did not grow when seeded directly into serum-free medium supplemented with transferrin, insulin, and EGF or exogenous transforming growth factor(s). Rat-1 cells did proliferate, however, when seeded in the presence of low serum concentration and subsequently exposed to serum-free medium supplemented with the above factors. Kaplan et al. (32) speculated that serum must provide adhesion factor(s) that are required by Rat-1 cells in order to respond to mitogens. Our results indicate that one such factor is fibronectin, which can be isolated from plasma and has been shown to be present in ECM produced by bovine corneal endothelial cells (21).

An interesting observation resulting from the present study is the ability of HDL to support active growth of B31 cells in agarose. This indicates that HDL is one of the plasma components involved in the control of anchorage-independent growth of transformed cells. As for cells growing in monolayers, the mitogenic effect of HDL on cells suspended in agarose was observed only when the lipoprotein was added in combination with transferrin. In contrast to cells grown in monolayer, however, the growth-promoting effect of HDL was greatly potentiated by FGF. That FGF acts as a potentiating agent is suggested by the fact that B31 cells did not grow in suspension in the absence of HDL.

Since the ability to grow in semisolid medium such as agarose is the in vitro growth property of transformed cells that correlates best with tumorigenicity (7, 31, 45), it is interesting to consider that HDL might play a significant role in tumor formation in vivo. Since demonstration of such a role for HDL in vivo is not easily amenable to direct experimentation, however, an approach that might be meaningful would be to compare the tumor-forming ability of cells transformed by sarcoma virus and other means with their capacity to form colonies in agarose when exposed to HDL and transferrin. That a correlation could exist between these 2 properties is suggested by the fact that the weakly tumorigenic Rat-1 cells (51) cannot form colonies in agarose when exposed to medium containing HDL and transferrin (with or without insulin, EGF, or FGF), while the highly tumorigenic B31 cells can.

The ways in which HDL acts as a growth-promoting agent have not yet been defined. One possibility is that HDL, through its ability to induce 3-hydroxy-3-methylglutaryl-CoA reductase, could increase the synthesis of mevalonate (8, 9, 17). This would in turn lead to increased synthesis of sterol and nonsterol products such as dolichol, ubiquinone, and isopentenyl adenine.
which have been shown to be of importance for cell proliferation (2). Whether stimulation by HDL of anchorage-independent growth of sarcoma virus-transformed cells proceeds by the same mechanism is an additional question raised by the present study. It is generally believed that cellular transformation by ASV is dependent upon the expression of a single gene (src) (53) the product of which is a phosphoprotein with associated protein kinase activity (3, 10, 34, 38). It is therefore of interest to take note of a recent report by Ito et al. (29) showing that anionic phospholipids stimulate the in vitro phosphorylation of vinculin by purified preparation of RSV protein kinase. Since transformation of cells by RSV has been shown to increase phosphorylation of vinculin 8- to 10-fold (44) and to alter markedly the distribution of that cytoskeletal protein (11), the authors raised the possibility that phospholipids modulate vinculin phosphorylation in RSV-transformed cells (29). It would be interesting to determine if HDL, through its phospholipid components, could modulate the src-specific protein kinase in RSV-transformed cells.

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Note Added in Proof

Recently, Molina et al. reported (Cold Spring Harbor Conf. Cell Proliferation, 9: 345-364, 1982) a study of the growth factor requirements of another nontransformed rat embryo cell line (REF52) and of SV40 transformants derived from largely independent of these lipoproteins, although in the clonal assay they used, SV40 transformants retained a requirement for transferrin, but all have obviated ACKNOWLEDGMENTS

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Fig. 1. Morphological appearance of Rat-1 and B31 cells seeded on uncoated plastic dishes (A and B) or plastic dishes coated with collagen (C and D), fibronectin (E and F), or ECM (G and H) and grown in serum-free medium. Rat-1 and B31 cells were seeded as described in the legend of Table 1. Rat-1 cells were grown in medium containing transferrin, HDL, insulin, EGF, and dexamethasone; B31 cells were grown in medium containing transferrin, HDL, and insulin. Concentrations and the schedule of addition of the various factors to the culture media were the same as those described in Chart 4. Photographs of Rat-1 (A, C, E, and G) and B31 (B, D, F, and H) cells were taken on Days 5 and 6, respectively (phase-contrast micrographs, x 100).

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Fig. 2. Growth of B31 cells in agarose. Cells were suspended in DME containing 0.3% agarose and supplemented with HDL (A); transferrin and HDL (B); transferrin, HDL, insulin, and FGF (C); and 5% calf serum plus 5% fetal calf serum (D), as described in “Materials and Methods.” Concentrations of factors added to the suspension medium were as described in the legend of Table 2. Photographs were taken on Day 14 (phase-contrast micrographs, × 100).
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