Brief Definitive Report

SOLUBLE FACTOR REQUIREMENTS FOR THE AUTOSTIMULATORY GROWTH OF B LYMPHOBLASTS IMMORTALIZED BY EPSTEIN-BARR VIRUS

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Some escape from the usual environmental constraints on growth may be a crucial step in the evolution of a normal to a cancer cell. The possibility that many of the recently identified oncogenes code for proteins intimately involved in the regulation of normal cell growth provides a molecular basis for the appropriate phenotypic change (1-3). Activation of an autocrine loop, whereby a cell both secretes and responds to a soluble growth-promoting factor (4), may be one route to the degree of cellular autonomy necessary for the cancerous state and has been implicated in the continuous growth of a number of cell lines (5-7), including those of lymphoid origin harboring the Epstein-Barr virus (EBV) genome (8). EBV is associated with two human cancers, nasopharyngeal carcinoma and Burkitt’s lymphoma, and cell lines established by EBV transformation of normal B lymphocytes may well represent a preneoplastic state of the latter (9). By generating growth factor activity from such lymphoblastoid cell lines in a defined serum-free medium we have been able to explore the mechanism of autostimulatory growth for virally transformed B lymphoblasts.

Materials and Methods

The establishment, cloning, and maintenance of EBV-transformed cell lines in serum-supplemented RPMI 1640 have been detailed elsewhere (10). For serum-free studies, the basal medium throughout was Iscove’s modified Dulbecco’s medium (Gibco Laboratories, Grand Island, NY). Regular supplements included delipidated bovine serum albumin (Gibco) at 1 mg/ml, soybean lipids (Gibco) at 20 µg/ml, L-glutamine (200 mM), penicillin/streptomycin (50 µg/ml), and 2-mercaptoethanol (5 × 10⁻⁵ M). Transferrin was obtained as an essentially iron-free preparation (Sigma Chemical Co., St. Louis, MO) and used at concentrations indicated in the text.

Conditioned medium (LCL-CM) was collected from lymphoblastoid cells that had been taken from their log phase of growth in serum-supplemented medium and transferred, after extensive washing, to serum-free conditions with the regular supplements but no transferrin. Cells were held at 37°C in a humidified 5% CO₂ atmosphere for 18 h at a concentration of 2-4 × 10⁶/ml before the harvesting of supernatants. Supernatants were passed through a 0.45-µm filter and stored at 4°C before use on the same day.

Target cells for growth factor assays were always used in autologous combinations with LCL-CM and collected by low speed centrifugation from the serum-free conditions. All tests were performed in flat-bottomed 96-well plates (growth area 0.32 cm²) in the
regularly supplemented basal medium. Unless stated otherwise, DNA synthesis was assessed by pulsing wells for 4 h with \([^{3}H]thymidine\) (1 \(\mu\)Ci/well, 25 Ci/mmole) at termination of culture with all determinations performed in quadruplicate. Transferrin receptors were sought using OKT9 (Ortho Diagnostic Systems Inc., Westwoods, MA) in an indirect staining procedure described elsewhere (11). Fluorescently stained cells were analyzed on a Becton-Dickinson FACS IV flow cytometer.

Results

All lymphoblastoid cell lines tested revealed an absolute dependency on transferrin for their growth in serum-free medium. Other additives to the Iscove's basal medium, namely albumin and lipids, were beneficial rather than essential for LCL growth. A sigmoidal dose dependency of LCL growth on transferrin was noted (Fig. 1 a). By analogy with enzyme kinetics, such a relationship between DNA synthesis and concentration of growth-promoting factor has been taken to imply cooperativity in cellular proliferation. As this was noted in the absence of any additional exogenous activity, it implies that the apparent cooperativity relied on an endogenously produced factor.

It can be noted (Fig. 1 a) for cells at low density that no concentration of transferrin was capable of sustaining proliferation, providing further evidence that its growth-promoting effect was dependent on an autogenous product. The results depicted in Fig. 1 b demonstrate that this was a released soluble activity, as LCL-CM was able to replace the high cell density requirement for growth. It was also shown that the growth-promoting action of LCL-CM was, in turn, absolutely dependent on the presence of transferrin for its effect (Fig. 1 b). At a constant transferrin input, \([^{3}H]thymidine\) uptake by lymphoblastoid cells at low density exhibited a sigmoidal dose response to LCL-CM concentration.

The strong interdependence of transferrin and LCL-CM on lymphoblastoid cell growth is reemphasized by the results given in Fig. 2. Here, the influence of the two activities over a wide range of cell densities is compared with the results being expressed in terms of proliferative capacity per cell. It can be seen that the efficient growth-promoting activity of transferrin at high cell numbers declined abruptly as cell density was decreased in the absence of LCL-CM. While LCL-

![Figure 1](image-url)

Figure 1. Requirement for transferrin and an autogenous factor in LCL growth. (a) Cells were incubated serum-free at 10⁵ (●) or 10⁶ (○) per ml for 3 d with varying amounts of transferrin as indicated. DNA synthesis was assessed by a 4-h \([^{3}H]thymidine\) pulse at the termination of culture. (b) Cells at 10⁴ per ml were incubated with varying concentrations of autologous LCL-CM as indicated in the presence (●) or absence (○) of transferrin at 25 \(\mu\)g/ml. DNA synthesis was assessed as described and shown as the mean of quadruplicate determinations that were always within 10% of each other.
CM was unable to provide direct growth support at any cell concentration, it greatly extended the critical cell density for transferrin-dependent growth so that at densities as low as $2 \times 10^3$ cells per ml, significant DNA synthesis was still apparent after 3 d in serum-free conditions.

We designed an experiment in order to ascertain whether the growth-promoting activities of LCL-CM and transferrin were required simultaneously or whether they exerted separate effects sequentially. This necessitated rendering cells essentially growth factor–free. Lymphoblastoid cells taken from their log phase of growth in serum-supplemented medium were manipulated by a series of washings and incubations at low cell density in the absence of transferrin, as detailed in Fig. 3. Such cells, considered “growth factor–free,” were then exposed independently to the growth-promoting activities of LCL-CM and transferrin or simply returned to control serum-free medium lacking both of these (Stage I). Following 16 h of conditioning, cells were washed free of their soluble activities and reseeded at low cell density in the presence or absence of fresh LCL-CM or transferrin (Stage II). Assessing DNA synthesis by a 16-h $[^3H]$thymidine pulse 12 h later revealed, unequivocally, that only when cells had been conditioned in the presence of LCL-CM were they capable of mounting a proliferative response on the addition of transferrin (Fig. 3). Transferrin-conditioned cells showed very little $[^3H]$thymidine incorporation when subsequently incubated with LCL-CM. The growth-promoting activity of LCL-CM therefore appeared to reside in its ability to prime lymphoblastoid cells for responsiveness to transferrin.

It seemed a reasonable proposition that the autostimulatory activity of LCL-CM might act by influencing the expression of transferrin receptors at the lymphoblast surfaces. This assumption was tested by taking cells at Stage I of conditioning and staining their surfaces with anti-transferrin receptor antibody (OKT9). As seen from Fig. 4, cells that had been rendered essentially growth
FIGURE 3. Sequential action of growth-promoting factors on LCL proliferation. Cells were placed at 37°C serum-free in the absence of transferrin at a density of 10^4/ml. At 8, 16, and 24 h cells were washed thoroughly and resuspended in fresh medium. At 36 h from initiation of culture cells were washed and placed at 10^4/ml in either fresh control medium, medium supplemented with 25 μg/ml of transferrin, or 50% LCL-CM. Following 16 h incubation at 37°C (Stage I), cells were washed, resuspended in fresh control medium (without transferrin) at 4 × 10^4/ml and 100 μl plated into microwells. A further 100 μl of either control medium □, 100% LCL-CM □, or medium containing 50 μg/ml of transferrin □ were added in quadruplicate for each condition and the plate was returned to 37°C (Stage II) for 12 h. Wells were then pulsed with [3H]thymidine for 16 h and DNA synthesis assessed (results given as means ± ISD).

FIGURE 4. Influence of conditioned-medium on the expression of transferrin receptors. Cells from Stage I of conditioning (see Fig. 3) were stained with OKT9 and analyzed on a FACS IV. (□) cells conditioned in the presence of 50% LCL-CM. (◇) cells maintained in control medium. (□□) control staining (i.e., fluorescent conjugate only). This was essentially the same for both lots of cells and only the control for LCL-CM conditioned cells is shown for clarity. Staining intensity is represented by a linear scale.

factor-free and then incubated in the presence of LCL-CM revealed an appreciably stronger reactivity (approximately threefold) with the OKT9 antibody than cells that had been denied this growth-promoting activity.

Discussion

This study confirms the recent findings by Blazar et al. (8) that lymphoblastoid cell lines display autostimulatory growth. We have now extended these observations by identifying the cofactor requirements and suggesting a possible mechanism for LCL growth in serum-free conditions. Attempts to isolate the growth-promoting activity of LCL-CM have so far proved unrewarding, probably due to an extreme inherent stickiness of the factor (unpublished observations, and B. Blazar, personal communication). We also have no molecular characterization save that the activity is retained by dialysis tubing of molecular weight exclusion >2,000. All LCL-CM so far tested do, however, possess potent BCGF (B-cell growth factor) activity (12) as judged by their ability to synergize with Fab′2 fragments of anti-immunoglobulin in stimulating small resting B lymphocytes to DNA synthesis (manuscript in preparation). It is tempting to speculate that it is this same BCGF activity that sustains LCL growth. The EBV could therefore be
envisaged as serving two distinct, but absolutely essential, functions during B lymphocyte transformation. The first of these, by analogy to physiologic (i.e., anti-Ig) activation, would be to induce the appearance of receptors for BCGF at the surfaces of small resting B cells. The second, and possibly the physiologically inappropriate feature of EBV transformation, would be the induction of a BCGF activity either coded directly from the viral genome or through the activation of an otherwise phenotypically silent cellular gene. Experiments designed to explore these possibilities are currently underway in our laboratory.

Autostimulatory growth is clearly not unique to virally transformed B lymphocytes, nor indeed to cells of lymphoid origin (4-7). On the contrary, the induction of an autocrine loop may be a quite regular pathway to cellular immortality, sometimes being reached by viral infection but also via other routes such as the activation of oncogenes coding for autostimulatory products. The relationship between EBV and the development of Burkitt's lymphoma in endemic regions has been extensively covered by Nilsson and Klein in a recent review (9). Our findings offer a functional basis for one of the postulated key steps in the multi-stage evolution of a Burkitt cell. By itself, the endowment of immortality is clearly not sufficient, but is probably a prerequisite, for the neoplastic state. A vital additional element for full tumorigenicity may be some phenotypic alteration that would allow the necessary escape from host regulation.

The observed degree of autonomy in the growth of virally transformed B lymphoblasts was, in fact, quite remarkable. Transferrin was found to be the only essential exogenous factor. The requirement for transferrin in the growth of lymphoid and nonlymphoid cells is well established with the molecule probably involved in complexing and transporting iron into the cell (13, 14). Intriguingly, our observations on LCL growth find close parallel with a recent report on the mitogen-induced activation of human T lymphocytes (15). Here, the authors argued for a sequential appearance of growth factor receptors with the role of interleukin 2 being the induction of transferrin receptors on the mitogen-primed T cells, permitting their entry into the S phase of the cycle on the subsequent binding to transferrin molecules. It remains to be determined whether an analogous pathway exists for the physiological activation of normal B lymphocytes, but our findings clearly point to such a possibility.

Summary

B lymphoblasts immortalized by the Epstein-Barr virus (EBV) exhibit autocrine growth stimulation—that is they release a soluble activity to which they respond by growth. A minimally supplemented serum-free medium conditioned by lymphoblastoid cells in their log phase of growth (LCL-CM) was found to contain autostimulatory activity allowing us to explore the mechanism of autocrine growth for these cell-types in defined conditions. Below cell densities capable of supporting autonomous growth, continued proliferation in serum-free medium was dependent on both added LCL-CM and transferrin. Neither activity alone was capable of sustaining growth. At higher cell densities, transferrin by itself was sufficient to maintain the autocrine loop. The action of the autostimulatory factor appeared to reside in its ability to prime cells continually for a proliferative response to transferrin by enhancing the expression of transferrin receptors at the lymphoblast surfaces. The implications of these findings for normal B cell physiology and their possible relation to oncogenesis are discussed.
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