Activity of soluble factors produced by Epstein-Barr virus-transformed human B lymphocytes on different cell lines

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Summary. Self-stimulatory growth factors, produced by a human Epstein-Barr virus (EBV)-positive lymphoblastoid B cell line, named BA-D10-4, have been tested for the capacity to induce DNA synthesis in various human and animal cell lines, including lymphoid, either EBV-positive or EBV-negative, and non-lymphoid cell lines. It has been found that BA-D10-4 cells produce growth factors which seem to be essential for their sustained proliferation in vitro, and which increase DNA synthesis in different primate lymphoid cells, independently of the presence of the EBV genome and of the lymphocyte lineage.
Key words. Epstein-Barr virus; growth factors; cell lines.

Epstein-Barr virus (EBV) is a herpesvirus closely associated with the etiology of African Burkitt's lymphoma and nasopharyngeal carcinoma. EBV latently infects human B lymphocytes, inducing lymphoblastoid cell transformation. The entire viral genome persists in infected, growth-transformed lymphocytes either as an episome or integrated into cell DNA [1, 2]. Despite the presence of the complete genome, which encodes over 50 proteins in productive virus infection, only a few genes, which encode six nuclear proteins known as 'Epstein-Barr nuclear antigens' (EBNA-1–6) and a membrane protein known as 'latent membrane protein' (LMP), are expressed in transformed cells [3]. The role of these and, possibly, other viral genes in B cell transformation, as well as the contribution of cellular genes, is incompletely understood. It has been suggested that autocrine growth factor(s) (aGFs) might be involved in the maintenance of EBV-induced B cell transformation. Although first described at the beginning of the 1980s [4, 5], EBV-transformed B cell-derived aGFs are poorly known. So far, the reported EBV-transformed B cell-derived aGFs include two cytokines, interleukin (IL)-1 [6–8] and IL-6 [9, 10], and a B-cell activation membrane antigen, the CD23 (BLAST-2) molecule [11, 12]. The up-regulation of CD23 would be activated, directly or indirectly, by the EBNA-2 gene, thus leading to the shedding of a 25-kilodalton (kDa) molecular form that would act as an aGF [13]. Other unidentified growth factors (GFs) have been also reported [9, 14–16].

In this paper, we have studied a human EBV-positive lymphoblastoid B cell line (EBV-LCL), named BA-D10-4, for the capacity to produce GFs with autocrine activity; moreover, we have tested the BA-D10-4-derived factors for growth-promoting activity on various human and animal cell lines, including lymphoid, either EBV-positive or EBV-negative, and non-lymphoid cell lines.

Materials and methods

The human EBV-positive BA-D10-4 cell line studied in this paper is a cloned line, producing human IgM monoclonal antibody to mycobacterial antigens. It was established in our laboratory by EBV transformation from the peripheral blood cells of a tuberculin skin test-positive healthy donor, according to a method described in detail elsewhere [17]. Two other human EBV-LCLs established in our laboratory by in vitro transformation are coded BA-G2-3 and DG-H-8, and derive respectively from the same and from a different donor of BA-D10-4 cells. Other cell lines used in this study include: Raji, human EBV-positive cells from Burkitt's lymphoma; P3HR-1, human EBV-positive cells from Burkitt's lymphoma, producer of non-transforming virus; Ramos, human EBV-negative cells from Burkitt's lymphoma; B95-8, marmoset EBV-positive cells, producer of transforming virus; Molt-4, human T cells from acute lymphoblastic leukemia; P3.X63.AG8.653 (abbreviated X63.AG8), mouse myeloma cells HGPRT-negative; Hep-2, human cells from laryngeal epidermoid carcinoma; KB, human cells from oral epidermoid carcinoma; Hep-G2, human cells from hepatocellular carcinoma.

All the cell lines were routinely cultured in RPMI-1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone (Flow Lab.). For the production of BA-D10-4-derived GFs, BA-D10-4 cells, cultured in RPMI-1640 with 10% FBS up to a density of 5×10^5/ml, were washed 3 times in serum-free medium and suspended at the concentration of 1×10^6/ml in Dulbecco's modified Iscove's medium (DMIM), containing bovine serum albumin (BSA) and human transferrin (Flow Lab.). The cells were incubated for 3 days at 37 °C in a 5% CO_2 atmosphere and the cell supernatant was collected by centrifugation, filtered through a 0.22 µm membrane, and stored at − 20 °C.

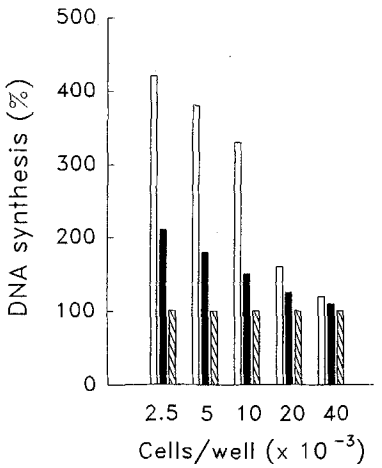
To assay the proliferative activity of BA-D10-4-derived GFs, target cells, cultured in RPMI-1640 supplemented with 10% FBS up to a density of 5×10^5/ml, were washed 3 times in serum-free medium and suspended in DMIM. 100 µl microliter of target cell suspension containing, unless otherwise stated, 10^4 cells was plated in each well of a 96-well plate (Flow Lab.); the cultures then received 100 µl of fresh DMIM or the same volume of BA-D10-4-derived factors. The microcultures were then pulsed, im-

mediately or after 2 days at 37 °C and 5% CO_2, with 0.5 μCi ^{3}H-thymidine (^{3}HTdR; 25 Ci/mMol) in 50 μl fresh DMIM for the last 24 h of culture. The cells were then collected with a semiautomatic cell harvester (Dynatech) and then counted for radioactivity in a Beckman liquid scintillation counter. In experiments employing non-lymphoid, adherent target cells, cultures were trypsinized before collection.

Results

Self-stimulatory activity of BA-D10-4-derived supernatant was first studied. As shown in the figure, DNA synthesis of target cells in the presence of BA-D10-4-derived GFs was higher than in cultures receiving unconditioned medium at all tested cell densities. In particular, the highest percentual increases were observed at low cell density and in the 48–72 h assay. The activity of these self-stimulatory factors also included an increase in viability and cell number in long-term cultures (data not shown).

BA-D10-4-derived factors were then studied for their capacity to induce DNA synthesis in different lymphoid, either EBV-positive or EBV-negative, and non-lymphoid cell lines. As can be seen in the table, in the first 24 h of culture, BA-D10-4 GFs gave rise to a significant increase in DNA synthesis in the other in vitro established EBV-LCLs, i.e., BA-G2-3 and DG-H-8 cells. When assayed between 48 and 72 h of culture, DNA synthesis of the EBV-LCLs cultured in the absence of exogenous GFs was lower than in the first 24 h, but the addition of BA-D10-4-derived supernatant determined an increase in DNA synthesis which was percentually higher than that observed in the first 24 h.



Autocrine activity of BA-D10-4-derived growth factors. BA-D10-4-derived supernatant, obtained by culturing BA-D10-4 cells for 3 days, was tested for the capacity to induce DNA synthesis in BA-D10-4 cells. Results are expressed as percentage of DNA synthesis in the 0–24-h assay (black columns) and in the 48–72-h assay (white columns). DNA synthesis in the presence of medium alone is represented as 100% DNA synthesis at all tested cell densities (diagonal bar columns).

During the first 24 h of culture, BA-D10-4-derived GFs also increased DNA synthesis in cells derived from Burkitt's lymphoma, i.e., Raji, Ramos and P3HR-1, as well as in Molt-4 cells, a line of T-cell lineage, and in the EBV-producer marmoset B95-8 cells. In the 48–72 h assay, DNA synthesis of Raji, Ramos, Molt-4 and B95-8 cells in the presence of GFs derived from BA-D-10-4 cells was higher than that of unstimulated control cultures; however, compared to the results of the 0–24 h assay, the increase was percentually lower for Raji and Ramos cells,

Table 1. Activity of BA-D10-4-derived growth factors (GFs) on different cell lines

Cell lines	DNA synthesis (cpm × 10^{-3}) 0–24 h			48–72 h		
	–	GFs	(%)	–	GFs	(%)
Lymphoblastoid						
BA-D10-4	5.1 ± 0.3	9.0 ± 0.6	176	2.5 ± 0.3	8.9 ± 0.7	356
BA-G2-3	6.4 ± 1.9	9.2 ± 0.6	144	4.3 ± 0.5	13.3 ± 0.4	309
DG-H-8	7.5 ± 0.7	13.5 ± 1.3	180	3.3 ± 0.2	15.8 ± 1.6	480
Burkitt's and leukemia						
Raji	5.7 ± 1.0	12.7 ± 0.4	223	32.8 ± 2.7	64.7 ± 6.4	197
Ramos	1.0 ± 0.1	2.4 ± 0.2	240	4.4 ± 0.4	6.4 ± 0.8	145
P3HR-1	8.7 ± 1.0	16.0 ± 0.9	184	25.1 ± 1.2	28.0 ± 2.1	112
Molt-4	4.9 ± 0.4	9.1 ± 0.3	186	10.9 ± 1.0	20.1 ± 2.5	184
Marmoset leukocytes						
B95-8	7.9 ± 0.4	11.3 ± 1.8	143	3.9 ± 1.3	9.2 ± 2.2	235
Mouse myeloma						
X63.AG8	33.0 ± 5.9	18.8 ± 2.3	57	98.9 ± 6.8	60.4 ± 4.7	61
Human non-lymphoid						
Hep-2	7.1 ± 1.6	8.3 ± 0.9	117	51.8 ± 9.2	41.6 ± 2.4	80
KB	9.1 ± 1.8	11.0 ± 3.9	121	46.4 ± 7.2	52.4 ± 3.6	113
Hep-G2	8.1 ± 3.8	2.7 ± 1.1	33	13.6 ± 2.5	3.6 ± 0.4	26

Data expressed as mean ± one standard deviation of counts per minute (cpm) of triplicate cultures of 10^4 target cells cultured in the presence of medium alone (–) or BA-D10-4-derived growth factors (GFs). % DNA synthesis was calculated as:

$$\frac{\text{(cpm of cultures in the presence of GFs)} \times 100}{\text{(cpm in the presence of medium alone)}}$$

unchanged for Molt-4 cells, and higher for B95-8 cells; DNA synthesis of P3HR-1 cells, on the other hand, was not statistically changed. In contrast, the mouse myeloma cell line X63.AG8 showed a marked decrease in DNA synthesis when cultured in the presence of BA-D10-4-derived supernatant at 0–24 and 48–72 h of culture. DNA synthesis of the non-lymphoid human cells Hep-2 and KB was not significantly affected, whilst in Hep-G2 cells DNA synthesis was markedly decreased by BA-D10-4-derived factors.

Discussion

The results reported in this paper show that BA-D10-4 cells, i.e., EBV-transformed human B lymphocytes, produce GFs that seem to be essential for their sustained proliferation, and which also increase DNA synthesis in lymphoid cells of B (Raji, P3HR-1, and Ramos) and T cell lineages (Molt-4). The activity of these factors seems to be specific for primate lymphoid cells, since BA-D10-4-derived GFs were active on human and marmoset lymphoid cells, but not on human non-lymphoid cells, such as Hep-2 and KB cells, or on mouse myeloma cells. However, when the responsiveness to BA-D10-4-derived factors of EBV-LCLs is compared with that of Burkitt's lymphoma cells, it seems that the EBV-LCLs are more dependent on these factors than are Burkitt's cells. In fact, in low-density cultures of EBV-transformed B cells, exogenous BA-D10-4-derived factors are required to sustain DNA synthesis, whereas in Burkitt's lymphoma cells exogenous growth factors are not critically required.

Consistently with previous findings showing that inhibitors of DNA synthesis are produced by herpesvirus-transformed cell lines [18], BA-D10-4 cells also produced soluble factor(s) that induced a marked decrease of DNA synthesis in mouse myeloma cells and in human Hep-G2 cells. Experiments in progress indicate a considerable molecular heterogeneity of the factors that affect DNA synthesis in EBV-LCLs and other tumor lymphoid cell lines: some of them resemble IL-1 and IL-6 in their biochemical and biological properties; others, with a strong GF activity, are of low molecular weight (less than 10 kDa) and seem to act synergistically with the higher molecular weight factors (manuscript in preparation).

This study of the activity of BA-D10-4-derived factors on various EBV-positive cell lines, in which the viral genome is differently expressed [19], can provide useful indications about the viral and cellular molecules involved in the responsiveness to EBV-LCL-derived GFs. It is known that EBV-LCLs express the EBNA-2 gene, whose product would lead, directly or indirectly, to the shedding of the 25-kDa molecular form of the membrane CD23, which acts as an aGF [13]. Raji cells, on the other hand, express surface CD23 but not the EBNA-2 gene [19], while P3HR-1 cells contain a deletion in the EBNA-2-encoding region [20, 21] and are CD23-negative. In this context, the responsiveness of both Raji and P3HR-1 cells, as well as EBV-negative Ramos cells, to EBV-LCLs-derived factors, indicates that EBNA-2 and CD23 expression are not critically required for the responsiveness to EBV-LCL-derived GFs, at least in these tumor cells. The present data cannot rule out the involvement of the circuit EBNA-2/CD23 in lymphoblastoid cells, but it seems that the role of soluble CD23 as an aGF in EBV-LCLs, as well as in normal B lymphocytes, has to be reconsidered, since the recombinant molecule failed to stimulate B cell growth [22].

The capabilities of EBV-transformed B cells for producing self-stimulatory GFs and factors that contribute to proliferation of other lymphoid cells might be relevant in patients who show high numbers of EBV-carrying B lymphocytes, such as patients with the acquired immunodeficiency syndrome (AIDS)[23]. In these patients, EBV-transformed B cells, by means of aGFs, could sustain either self-proliferation or proliferation of other bystanding EBV-transformed B cells, thus providing an expanded additional cell reservoir to support human immunodeficiency virus (HIV) replication [24, 25]. GFs derived from EBV-LCLs could also play a role in the genesis of B cell lymphomas, which occur in AIDS patients with considerable frequency. A large proportion of these lymphomas show the characteristic histopathology of Burkitt's lymphoma and are EBV-carrying [26]. EBV-LCL-derived growth factors could contribute to an increased proliferation of lymphoid cells, which in turn increases the likelihood of these cells undergoing cytogenetic changes, leading to the development of malignant clones [27].

In conclusion, the identification of the exact interplay between the different GFs produced by EBV-transformed B cells could turn out to be useful not only for the understanding of the role of GFs in the induction and maintenance of B cell transformation, but also for a possible control of EBV-related malignancies in patients at risk.

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Zinc increases the longevity of unfertilized sea urchin eggs

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Summary. We have found that Zn^{2+} prevented lysis of unfertilized sea urchin eggs, and the eggs retained the ability to form fertilization membranes and to divide. SDS polyacrylamide gel electrophoresis showed that proteolysis of several proteins accompanied egg lysis, but Zn^{2+} inhibited this proteolysis. Therefore, Zn^{2+} blocks protease activity directly or indirectly and thereby prolongs the longevity of unfertilized sea urchin eggs.
Key words. Longevity; sea urchin eggs; zinc; cell death; protease.

Unfertilized sea urchin eggs have a limited life span. When unfertilized eggs are shed into seawater, they gradually lose the ability to form a fertilization membrane, and undergo disintegration by cytolysis [1, 2]. Many methods had been used to increase their longevity [1-4], but the factor(s) controlling the life span of sea urchin eggs are not known. Recently, we have found that Zn^{2+} increases the longevity of unfertilized eggs and prevents proteolysis of some egg proteins. We regard the lysis of unfertilized eggs as a kind of programmed cell death. Programmed cell death occurs in diverse organisms, but the mechanisms underlying it are unknown [5, 6]. Here we report that Zn^{2+} is useful for the study of the mechanism controlling longevity in unfertilized sea urchin eggs.

Materials and methods

Gametes. Anthocidaris crassispina were collected from the local coast of Toyama Bay and kept in running seawater aquaria at 25 °C. Shedding of gametes was induced by injecting 0.5 M KCl into the coelomic cavity. Eggs were shed into filtered natural seawater (FNSW) and washed three times with FNSW. Sperm was collected 'dry' in plastic dishes and stored at 4 °C until use.

Treatments. Unfertilized eggs were transferred into FNSW containing various concentrations of Zn^{2+} immediately after collecting and washing. In some experiments, eggs were first homogenized in FNSW by 15 strokes of a Teflon homogenizer. All eggs and egg-homogenates were kept in 50-ml plastic tubes rotated at 5 rounds/min (Taitec Rotator RT-50, Taiyo Service Center Co., Ltd., Tokyo) at 25–27 °C (the temperature of the seawater in the spawning season of this sea urchin). Densitiy of eggs was adjusted to about 5% (v/v). A stock solution of $ZnCl_2$ (100 mM) in deionized water was diluted with FNSW to appropriate concentrations.

Measurement of longevity. Aliquots of experimental eggs were taken at suitable time intervals. Some aliquots were fixed immediately in 1% glutaraldehyde in FNSW for analysis of lysed eggs, and some were washed three times with FNSW and inseminated to assay their ability to form a fertilization membrane and to divide. Microscopic observations were made with an inverted microscope (Olympus CK-2).

Gel electrophoresis. Sample eggs were washed three times with FNSW and homogenized in FNSW with a Teflon homogenizer. Protein concentrations of homogenates were measured by the method of Bradford [7] with bovine serum albumin as the standard, and were subjected to SDS polyacrylamide gel electrophoresis according to the method of Laemmli [8]. Gels were stained with silver [9].