Epstein–Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival

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Epstein–Barr virus (EBV) infects human primary B lymphocytes and induces and maintains proliferation of these cells efficiently in vitro. Mutants of Epstein–Barr virus which express EBV nuclear antigen 2 (EBNA2) in a conditional fashion allow dissection of individual contributions of viral genes to B cell immortalization. EBNA2 is a transcriptional activator of cellular and viral genes, including the viral latent membrane protein 1 (LMP1), which is essential for B cell immortalization and has oncogenic effects in non-lymphoid cells. To analyze the role of this gene in B cell immortalization, LMP1 was constitutively expressed in B cells infected with EBV carrying a conditional EBNA2 allele. In the absence of functional EBNA2, LMP1 was incapable of sustaining B cell proliferation in two independent assays but induced a phenotype consistent with prolonged cell viability. Activation of CD40 displayed a comparable phenotype. These data indicate that both CD40 activation and LMP1 expression may use a common pathway for B cell activation. Proliferation of human B cells, however, requires one or more additional signals triggered by EBNA2.

Keywords: B cell immortalization/CD40/Epstein–Barr virus/latent membrane protein 1

Introduction

Epstein–Barr virus (EBV) is a widespread human herpesvirus. Primary infection in humans is usually asymptomatic or leads to infectious mononucleosis. EBV is also associated with a variety of malignancies that include Burkitt’s lymphoma, B cell lymphomas in immunocompromised hosts, some T cell lymphomas, ~50% of Hodgkin’s disease and nasopharyngeal carcinoma (Zeng et al., 1985; Miller, 1990; Craig et al., 1993; Pallesen et al., 1993).

Experimental infection of resting B cells with EBV efficiently induces and maintains unlimited proliferation of the infected cells in vitro. This process is termed immortalization and is the in vitro model for this virus’s contribution to B lymphoid diseases. EBV is a herpesvirus that encodes ~80 genes, 11 of which are found expressed in in vitro infected B cells; five of these, the Epstein–Barr viral nuclear antigens (EBNA) 1, 2, 3a and 3c and latent membrane protein 1 (LMP1), appear to be absolutely required for B cell immortalization (Cohen et al., 1989; Hammerschmidt and Sudgen, 1989; Kaye et al., 1993; Tomkinson et al., 1993; for a recent review see Farrell, 1995). Two viral proteins, the EBNA leader protein (EBNA-LP) and latent membrane protein 2 (LMP2 or TP), seem to affect the efficiency of the process (Hammerschmidt and Sudgen, 1989; Mannick et al., 1991; Brielmeier et al., 1996). The best characterized latent gene products are EBNA1, EBNA2 and LMP1. EBNA1 is necessary for episomal replication of the viral genome by binding to the viral origin of replication, oriP (Yates et al., 1984). EBNA2 and LMP1 are essential for the immortalization potential of the virus.

EBNA2 acts as a transcriptional activator of cellular and viral genes. It modulates the expression of the B cell activation markers CD21 and CD23 and the tyrosine kinase c-fgr (Calender et al., 1987; Wang,F. et al., 1987; Cordier et al., 1990; Knutson, 1990). In addition, EBNA2 transactivates the viral promoters of three latent membrane antigens (LMP1, LMP2A and LMP2B) and the Cp promoter, which regulates the expression of EBV nuclear antigens (Fahraeus et al., 1990a; Sung et al., 1991; Zimber-Strobl et al., 1991; Laux et al., 1994b). EBNA2 does not bind to DNA directly (Ling et al., 1993; Zimber-Strobl et al., 1993) but interacts with the cellular DNA binding proteins RBP-Jx and PU.1, which bind to EBNA2-responsive promoter elements (Grossman et al., 1994; Henkel et al., 1994; Laux et al., 1994a; Waltzer et al., 1994; Zimber-Strobl et al., 1994).

LMP1 is the only viral protein that is not only essential for B cell transformation but has also transforming effects in non-lymphoid cells. LMP1 acts as an oncogene in established rodent cells (Wang,D. et al., 1985; Baichwal and Sudgen, 1988; Moorthy and Thorley-Lawson, 1993) and blocks differentiation in epithelial cells (Dawson et al., 1990; Fahraeus et al., 1990b; Wilson et al., 1990). In Burkitt’s lymphoma cell lines LMP1 induces the expression of B cell activation markers CD23 and CD40 and cell adhesion molecules ICAM1, LFA1 and LFA3 and stimulates NF-κB activity (Wang,F. et al., 1990; Hammerskjoeld and Simurda, 1992; Laherty et al., 1992; Huen et al., 1995; Mitchell and Sudgen, 1995). In primary human B cells LMP1 similarly stimulates some of the early events of B cell immortalization, like cellular enlargement, up-regulation of B cell markers and adhesion molecules and transient induction of DNA synthesis (Peng and Lundgren, 1992). LMP1 is an integral membrane protein with six transmembrane domains separated by short reverse turns. Both the 23 amino acid N-terminal and 200 amino acid C-terminal parts are cytoplasmic (Fennewald et al., 1984; Liebowitz et al., 1986). Little is
known about LMP1 function, but the recent finding by Mosialos and colleagues (Mosialos et al., 1995) indicates that LMP1 interacts with TRAF1 and TRAF3 (also termed EB16 and LAPI or CRAFI respectively). Both molecules are components of a signal transduction pathway which is used by members of the tumor necrosis factor receptor family. Binding of these TRAF signal mediators suggested that LMP1 could act as a receptor-like molecule which participates in CD40- or TNF-2 receptor-mediated signaling (Rothe et al., 1994, 1995; Cheng et al., 1995).

In this paper we address the question of whether LMP1 supports proliferation of EBV-immortalized B cells in the absence of functional EBNA2. Previously it was demonstrated that EBNA2 is necessary not only for initiation but also for maintenance of B cell immortalization (Kempkes et al., 1995b). Since EBNA2 is one of the transactivators of the LMP1 gene it was possible that the contribution of EBNA2 to B cell proliferation is solely dedicated to up-regulation of the LMP1 gene. To address this hypothesis we analyzed the function of LMP1 in the absence of EBNA2 in EBV-immortalized B cells. We show that constitutive LMP1 expression alone is not sufficient to maintain B cell proliferation but LMP1 enhances the viability and survival of B cells and prolongs the period of DNA synthesis in the absence of EBNA2. Constitutive expression of LMP1 or stimulation of the CD40 receptor resulted in a comparable phenotype, supporting the assumption that both LMP1 and CD40 might engage the same signal transduction pathway to activate B cells.

Results

Uncoupled LMP1 and EBNA2 expression in EBV-immortalized B cells

Proliferation of B cells which are infected with an EBV mutant encoding an EBNA2–estrogen receptor fusion protein (ER–EBNA2) is dependent on estrogen (Kempkes et al., 1995b). This experiment demonstrated that EBNA2 is necessary not only for initiation but also for maintenance of B cell proliferation in vitro. Since genetic and functional evidence indicates that LMP1 is likely to play a central role in B cell immortalization, we asked whether the sole function of EBNA2 required for maintenance of B cell proliferation is to up-regulate LMP1 expression.

To address the question of whether LMP1 maintains B cell proliferation independently of EBNA2 we established a system in which LMP1 is expressed constitutively in EBV-infected B cells. In a first approach, the LMP1 gene expressed from the SV40 early promoter/enhancer was cloned onto an episomal oriP vector encoding the gene for hygromycin phosphotransferase (Sugden et al., 1985). This construct, termed p581, was stably introduced into EREB2-5 cells infected with the P3HR1 strain of EBV and a mini-EBV plasmid encoding the ER–EBNA2 chimeric gene (Kempkes et al., 1995b, 1996). As negative controls, a p581-based plasmid with a truncated nonfunctional LMP1 gene or the parental oriP vector (pHEBo) were introduced in EREB2-5 cells. A number of independent hygromycin-resistant clones were isolated and tested for EBNA2 and LMP1 expression by Western blotting. In EREB2-5 cells transfected with the control plasmids, LMP1 expression was significantly down-regulated 4 days after removal of hormone, indicating that the LMP1 gene is under stringent control of EBNA2, as in the parental EREB2-5 cells (Figure 1A). In contrast, all 10 clones transfected with the wild-type LMP1 gene expressed from the SV40 promoter revealed a level of LMP1 which was not down-regulated after estrogen withdrawal. The LMP1 levels were comparable or even higher than in EREB2-5 cells in the presence of estrogen (Figure 1A; data not shown), which is not unexpected because in the transfectants there is probably expression of LMP1 both from the resident viral genome and from the introduced vector. In
all clones, EBNA2 function was still estrogen dependent, as indicated by autoregulated expression of the ER–EBNA2 protein (Figure 1B) and regulated transactivation of EBNA2-responsive promoter constructs (data not shown).

In an alternative approach, the mini-EBV plasmid p1480.40 was constructed, which carries the ER–EBNA2 gene and the SV40 promoter-driven LMP1 gene in the context of an immortalization-competent mini-EBV construct (Figure 2A). The intention of this experiment was to establish a B cell clone in a single step which carried only the SV40 promoter/enhancer-expressed LMP1 allele but lacked LMP1 encoded from the P3HR1 strain of EBV. This experiment should also clarify whether EBNA2-regulated expression of LMP1 is essential for B cell immortalization. Virus stocks were produced containing p1480.40 genomes packaged into an EBV coat to infect and immortalize primary B cells as described (Kempkes et al., 1995a). By PCR analyses, one B cell clone (clone 2 in Figure 2) was found to carry p1480.40 only, whereas other clones were doubly infected with p1480.40 and helper virus (clone 3 in Figure 2; data not shown). This result demonstrated that regulated expression of LMP1 is not mandatory for in vitro B cell immortalization. Two clones (clones 2 and 3) were analyzed in more detail for the expression of LMP1 and ER–EBNA2. As expected, the ER–EBNA2 fusion protein was found to be expressed in a hormone-dependent manner (Figure 2C). In contrast, LMP1 expression levels were independent of EBNA2 and in the same range as in B cells immortalized by wild-type EBV or in EREB2-5 cells in the presence of hormone (Figure 2B).

**Constitutive LMP1 expression is not sufficient to maintain B cell proliferation in the absence of functional EBNA2**

In order to study the phenotype of constitutive LMP1 expression in B cells, proliferation of different B cell clones was analyzed in the presence and absence of estrogen. Two criteria, DNA content as detected by propidium iodide staining and incorporation of bromodeoxyuridine (BrdU), were measured by FACS analysis. In the presence of estrogen no significant differences in the cell cycle profiles were detected in parental EREB2-5 cells compared with EREB2-5 cells stably transfected with the SV40 promoter/enhancer-driven LMP1 plasmid (p581) or the vector control plasmid pHEBo (data not shown). After estrogen removal the number of BrdU-positive cells decreased in all clones, including those expressing high levels of LMP1, concomitant with a higher number of cells in G1 (data not shown). This finding indicated that
LMP1 expression *per se* was not sufficient to maintain B cell proliferation in the absence of functional EBNA2.

**LMP1 expression delays the shut-off of DNA synthesis in the absence of EBNA2**

Cell cycle analyses revealed that the percentage of BrdU-positive cells was less dramatically reduced in LMP1 transfectants 4 days after withdrawal of estrogen compared with the parental or pHBo-transfected EREB2-5 cells (data not shown). To confirm this observation, we measured the kinetics of $[^{3}H]$thymidine uptake in the absence of estrogen in seven cell clones which constitutively expressed LMP1 for 10 days. The kinetics of $[^{3}H]$thymidine incorporation during this period are shown in Figure 3A for two out of seven LMP1 transfectants (clones 2C and 13C), one clone transfected with mutated LMP (clone Mut1) and the untransfected parental cell line EREB2-5. $[^{3}H]$Thymidine incorporation rates on day 4 after withdrawal of estrogen are summarized in Figure 3B for all clones tested. Cells which constitutively express LMP1 showed a significantly higher rate of $[^{3}H]$thymidine incorporation in the absence of hormone than those cells with decreased levels of LMP1. Parental EREB2-5 cells and transfectants with the non-functional truncated LMP1 gene reached $[^{3}H]$thymidine incorporation rates close to background levels 4 days after withdrawal of estrogen. In contrast, LMP1 transfectants clearly incorporated $[^{3}H]$thymidine during the first 7 days and reached background levels finally ~10 days after removal of estrogen.

In a separate experiment, we analyzed the B cell clone infected with the mini-EBV plasmid p1480.40 (clone2/ p1480.40) for its kinetics of $[^{3}H]$thymidine incorporation (Figure 3C). Again, constitutive expression of LMP1 had a similar effect on thymidine incorporation in this clone as in EREB2-5 cells transfected with the LMP1 expression vector p581. Immortalized B cells infected with p1480.40 mini-EBV were generated in a single step and carry only one LMP1 allele expressed from the constitutive SV40 promoter. It is very likely, therefore, that the increase in thymidine incorporation is a specific consequence of LMP1 expression and not an artifact of the selection procedure.

The results indicated that LMP1 expression in the absence of EBNA2 leads to a phenotype which is characterized by an increased and prolonged rate of thymidine incorporation, in contrast to the fast shut-off of DNA synthesis in EREB2-5 cells. Thymidine incorporation correlated with a low but detectable portion of cells in the S phase of the cell cycle (data not shown). Presumably, these cells supported DNA replication for a limited period of time only.

**Constitutive LMP1 expression permits re-induction of DNA synthesis 8 days after withdrawal of estrogen**

In EREB2-5 cells growth arrest in the absence of hormone was reversible for up to 4 days, as indicated by re-induction of DNA synthesis and sequential accumulation and modification of cell cycle-regulating genes (Kempkes et al., 1995b). In order to define the period in which DNA synthesis could be re-induced, seven clones of EREB2-5 cells transfected with the LMP1 expression vector p581 were cultivated in the absence of estrogen for different
time intervals and \(^{3} \text{H}\)-thymidine incorporation was measured 24 h after re-addition of hormone. As controls the parental EREB2-5 cells, cells transfected with the non-functional truncated LMP1 mutant or the pHEBo vector alone were tested in parallel. The rate of thymidine incorporation after 3 and 8 days of estrogen deprivation revealed remarkable differences. In the absence of estrogen, DNA synthesis could be re-induced in all cell lines tested, including the negative controls, after 3 days (Figure 4A). In contrast, all LMP1-expressing cell lines but not the negative controls showed clearly detectable \(^{3} \text{H}\)-thymidine incorporation even when estrogen had been omitted for a period of 8 days (Figure 4B).

**LMP1 expression improves cell survival**

Our results indicated that the expression of LMP1 extended the period during which DNA synthesis could be re-induced by estrogen in EREB2-5 cells. To determine whether this observation was the consequence of a prolonged survival of these cells, we determined the viability of the cells by MTT assay (Mosmann, 1983). Two LMP1 transfectants (clones 2C and 13C), the parental EREB2-5 cells and a pHEBo control transfectant were tested at different time points after removal of estrogen. In three independent experiments the viability was significantly higher only in cells expressing constitutive levels of LMP1 and not in the controls (Figure 5A and B). Withdrawal of estrogen leads to immediate cell death in a certain proportion of EREB2-5 cells, whereas the remaining cells enter a quiescent non-proliferative state (Kempkies et al., 1995b). Therefore, our results suggested that LMP1 expression extends survival of the fraction of non-proliferating B cells in the absence of EBNA2 function.

**CD40 stimulation and LMP1 have comparable effects in EREB2-5 cells lacking functional EBNA2**

Previously it was shown that resting human B cells enter a finite state of sustained cell proliferation by CD40 stimulation through antibody-mediated cross-linking of either CD40 or its ligand, CD40-L (Banchereau et al., 1991; Galibert et al., 1996). CD40 stimulation acts as a survival factor and increases DNA synthesis in tonsillar B cells (Rousset et al., 1991). Since LMP1 expression showed similar effects of cell activation in EREB2-5 cells in the absence of functional EBNA2, we asked whether CD40 activation would mimic the phenotype mediated by constitutive LMP1 expression in these cells. EREB2-5 cells transfected with the LMP1 expression vector p581 (clone 2C and 11C), a control cell line transfected with pHEBo and the parental EREB2-5 cells were kept on CD40-L-expressing fibroblastic L cells in the absence of estrogen for up to 10 days. \(^{3} \text{H}\)-thymidine incorporation was measured and the results indicated that CD40 activation stimulated DNA synthesis in EREB2-5 cells to a certain extent (Figure 6A). Surprisingly, not only the parental EREB2-5 cells and pHEBo-transfected cell line but also the LMP1-expressing clones 2C and 11C showed thymidine incorporation at a rate which was
significantly enhanced by CD40 activation. In addition, we tested the survival of these cells in the absence of estrogen on CD40-L-expressing fibroblasts in MTT assays. The results clearly showed that CD40 activation markedly increased the survival rate of all EREB2-5 clones. Concomitant LMP1 expression did not grossly affect thymidine uptake (Figure 6A) or viability in the MTT read-outs (Figure 6B) in CD40-activated cells as compared with control cells (Figure 6C). It should be noted that irrespective of CD40 stimulation, neither the parental EREB2-5 cells nor their transfectants supported B cell proliferation in the absence of estrogen (compare Figures 5 and 6B) and CD40-activated cells eventually reached the same fate as LMP1 transfectants.

These data indicate that LMP1 expression and CD40 activation had comparable phenotypes in EREB2-5 cells. The differences appear to be quantitative in nature in that CD40 activation was superior to constitutive LMP1 expression. These quantitative differences could possibly be the result of differential expression of CD40 and LMP1. Neither condition, however, supported B cell proliferation, but each prolonged cell survival with no apparent cooperative effect.

**Discussion**

Nearly all B cell-specific effects of LMP1, like up-regulation of adhesion molecules and activation markers or activation of NF-κB, were determined in Burkitt’s lymphoma cell lines after transfection of the LMP1 gene. Because Burkitt’s lymphoma cells are immortalized by a mechanism not requiring expression of LMP1, it is difficult to assess the contribution of LMP1 to growth control of EBV-infected primary human B cells from experiments in Burkitt’s lymphoma cells. To overcome this obstacle, we have established a cell system in which LMP1 function could be studied in the background of an EBV-infected, non-transformed B cell. Two complementing experimental approaches were used: (i) the LMP1 gene driven from the SV40 promoter was stably introduced into EREB2-5 cells
Similarly, it was shown that LMP1 activates the A20 gene, which might also be involved in protection from apoptosis (Laherty et al., 1992). Our experimental approach indicated that bcl-2 expression is not affected by the level of LMP1 gene expression (Figure 7) but should permit exploration of this particularly interesting aspect with other members of this gene family in the future.

Recently, Mosialos and colleagues (Mosialos et al., 1995) demonstrated that LMP1 interacts with LAP1, a member of the TRAF family. It is now known that LAP1, which is also called CRAF1 or TRAF3, specifically interacts with the intracellular domain of CD40, a TNF-R family member also expressed on activated B cells. Stimulation of CD40 by its ligand, CD40-L, activates proliferation and prevents apoptosis of tonsilar B cells. It was therefore tempting to speculate that LMP1 might mimic a constitutively active CD40-like receptor. To test this possibility, we asked whether CD40 stimulation induces a phenotype similar to that of LMP1 expression in estrogen-deprived EREB2-5 cells. Our data demonstrate that CD40 stimulation supported DNA synthesis and prolonged cell survival, as does constitutive LMP1 expression. The phenotype induced by CD40 stimulation was more pronounced than the effect induced by constitutive LMP1 expression but the differences appear to be quantitative. The reason for this phenomenon could be the result of differential expression of LMP1 and CD40 or could reflect differences in their cytoplasmatic C-terminal domains, which are directly involved in signal transduction. In contrast to the CD40 molecule, LMP1 has two NF-kB activating domains, one of which seems to act independently of TRAF3 (Huen et al., 1995; Mitchell and Sugden, 1995; Cheng and Baltimore, 1996). LMP1 is cytotoxic when expressed at high levels (Hammerschmidt et al., 1989) and it is possible that parts of LMP1 resemble the signal transducing domains of FAS/APO-1 or TNF-R1, which induce apoptosis rather than cell survival and proliferation. As a consequence, constitutive LMP1 expression could cause a phenotype which results from interference of apoptotic and anti-apoptotic effects. LMP1 mutants carrying deletions in the C-terminal cytoplasmic part should be informative, as well as constitutive expression of different TRAF family members to elucidate whether LMP1 interacts with the CD40 signal pathway and mimics a constitutive receptor of the TNF-R family.

**Materials and methods**

**Cell lines**

All cell lines were grown in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO₂. EREB2-5 is a lymphoblastoid cell line generated by infection of CD19-enriched human B cells with an Epstein–Barr virus mutant conditional for EBNA2 (Kempkes et al., 1995b). The cell line was diluted 1:2 twice a week in standard RPMI 1640 medium supplemented with 1 μM estrogen. HH514 is a single cell clone of the Burkitt’s lymphoma cell line P3HR1 (Rabson et al., 1982). MRC5, a human embryonic lung fibroblast cell line, was obtained from the American Type Culture Collection. Primary human B lymphocytes were isolated from routine tonsillectomies by generating single cell suspensions and T cell depletion by rosetting with whole sheep blood. The preparations were analyzed by FACS® analysis and were found to be >95% positive for the pan-B marker CD19 (DAKO, Hamburg, Germany), CD40L- and CD32-expressing L cells (Banchereau and Rousset, 1991) were diluted 1:4 twice a week.
Plasmids

The expression plasmid for LMP1, p58L, contains the Msrl-Smal fragment (nucleotide coordinates 169 476–167 920) of the EBV genome B95.8 under the control of the SV40 early promoter/enhancer in the oriP construct pBHeo, which also encodes for resistance against the drug hygromycin B (Sugden et al., 1985). The construct which served as a negative control for LMP1 expression is a derivative of p58L in which the LMP1 gene was truncated after amino acid position 25 of LMP1 by an incorrect out-of-frame deletion between the Stul and MscI sites in LMP1. The mini-EV plasmid p1480.40 is based on the plasmid pL244.8a-8.4, which can induce and maintain in vitro B cell immortalization (Kempkes et al., 1995a). p1244.8a-8.4 was modified in pXL1114 by allelic conversion (O’Connor et al., 1989) to include an SV40 early promoter/enhancer-expressed LMP1 gene and the chimeric ER-EBNA2 gene, which consists of the ligand binding domain of the human estrogen receptor fused to the open reading frame of EBNA2 (Kempkes et al., 1995b).

Transformation of primary B cells

HH514 cells (1 × 10⁵) were co-transfected with 10 μg pCMV-BZLFl and 10 μg mini-EV plasmid p1480.40. Electroporation was performed with a Bio-Rad gene pulser at 960 μF and 250 V in a total volume of 250 μl RPMI 1640. The harvest of the virus stocks and drug hygromycin B (Sugden et al., 1985). The construct which consists of the ligand binding domain of the human estrogen receptor fused to the open reading frame of EBNA2 (Kempkes et al., 1995b).

Stable transfection of EREB2-5 cells

The EREB2-5 cell clone (Kempkes et al., 1995b) was transfected by electroporation using a Bio-Rad gene pulser at 960 μF and 230 V. After transfection the cells were seeded in 96-well flat bottom plates and selected in RPMI medium supplemented with β-estradiol and hygromycin B.

Protein immunoblot

For Western blot analyses cellular extracts were prepared by sonification in H8 lysis buffer (20 mM Tris, pH 7.0, 2 mM EGTA, 2 mM EDTA, 6 mM β-mercaptoethanol, 50 mM NaF, 100 mM NaCl, 1% SDS). The protein concentration was determined and equal amounts of protein were separated on Laemmli 8 or 10% polyacrylamide–SDS gels. Proteins were transferred onto nitrocellulose filters (Amersham Hybond ECL) and protein expression was analyzed with monoclonal antibodies directed against LMP1 (S12; kindly provided by Dr David Thorley-Lawson), bcl-2 (Santa Cruz Biotechnology Inc.) or EBNA2 (anti-EBNA2-R3), or monoclonal antibodies conjugated with fluorescein isothiocyanate and propidium iodide as described (Lowe et al., 1993). The stained cells were analyzed on a Becton Dickinson FACS analyzer.

3HThymidine incorporation

For determination of DNA synthesis 5×10⁴ cells in 100 μl well were seeded into 96-well cluster plates in triplicate and kept in the absence or presence of estrogen for the time indicated. [3H]Thymidine at 0.5 μCi/well was added for 4 h and incorporation of radioactivity was determined in a scintillation counter after lysis of cells in water.

MTT assays

Samples of 3×10⁴ cells were seeded in 100 μl cell culture medium in triplicate in the absence or presence of estrogen for the indicated time. After incubation with MTT [3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyl tetrazolium bromide; 0.5 mg/ml] for 4 h, MTT conversion, which correlates with the number of living cells in the assay, was determined in an ELISA reader as described (Mosmann, 1983). Each experiment was repeated at least twice. To compare different experiments the OD on day 1 was set to 1 and normalized OD values are given for the following days.

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References


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