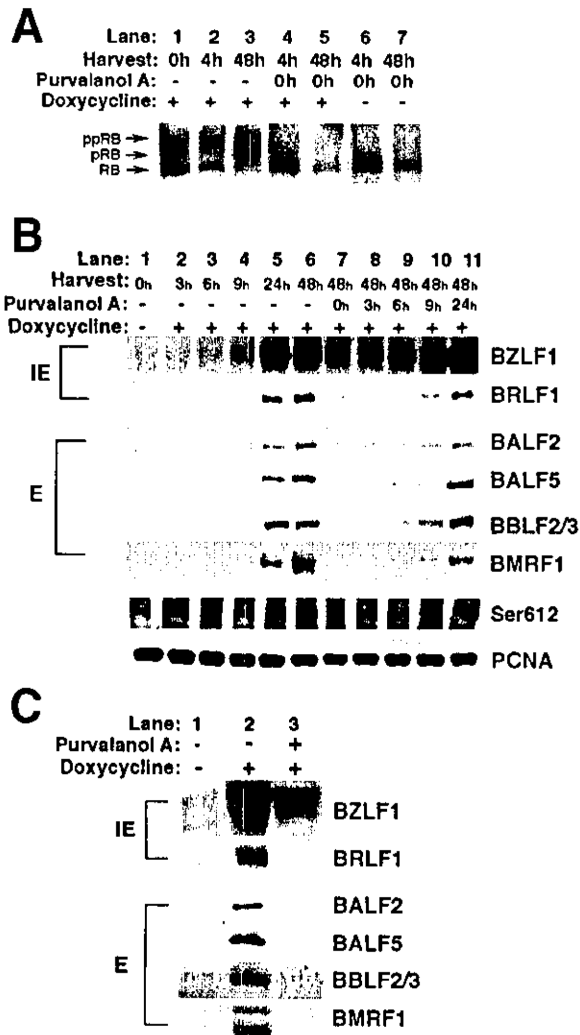

FIG. 5. Effects of purvalanol A on the expression of lytic viral proteins and phosphorylation of Rb protein during EBV lytic infection. (A) Tet-BZLF1/B95-8 cells were treated with (+) or without (−) 5 μg of doxycycline/ml for 48 h and purvalanol A (20 μM) was added to the culture immediately thereafter (0 h). The cells were harvested at the indicated times, and clarified cell lysates were prepared. Phosphorylation states of the Rb protein were analyzed by Western blotting with anti-Rb protein monoclonal antibody. pRB and ppRB are slower-migrating hyperphosphorylated forms of Rb protein. The faster-migrating band is the hypophosphorylated form of Rb protein and is designated as RB. (B) Purvalanol A (20 μM) was added to the culture of doxycycline-treated Tet-BZLF1/B95-8 cells at 0, 3, 6, 9, and 24 h postinduction, and the cells were harvested at the indicated times. Clarified cell lysates were separated by SDS-PAGE and applied for Western blot analyses with each antibody as indicated on the right. The proteins were detected with an enhanced chemiluminescence detection system (Amersham). Images were processed by LumiVision PRO (Aisin/Taitec, Inc.) with a cooled CCD camera and assembled using an Apple G4 computer with Adobe Photoshop 5.0. Signal intensity was quantified with a LumiVision image analyzer as described in Materials and Methods. IE, immediate-early proteins; E, early proteins. (C) Tet-BZLF1/AKATA cells were treated with (+) or without (−) 3 μg of doxycycline/ml and cultured for 48 h in the absence or presence of purvalanol A (5 μM). Lane 1 shows the sample prepared when the cells were treated with doxycycline at time zero. The cells were harvested, and clarified cell lysates were prepared and applied for Western blot analyses with each antibody as indicated on the right.

early proteins were synthesized largely during the first 24 h of induction (Fig. 5B, lane 5), relatively large amounts of viral replication proteins had already been expressed when purvalanol A was added at 24 h postinduction (Fig. 5B, compare lane 6 with lane 11). Purvalanol A had no effects on lytic viral replication 24 h postinduction as shown in Fig. 4A. Thus, purvalanol A does not inhibit lytic EBV replication when added after viral replication proteins are already expressed. These results led us to the conclusion that purvalanol A blocks an (relatively) early stage of viral productive infection due to impairment of viral immediate-early and/or early gene expression. We speculate that the cellular environment in which the Rb protein is underphosphorylated is inappropriate for viral transcription since under such conditions the level of free E2F transcription factor may be very low.

To confirm whether purvalanol A impairs viral immediate-early and/or early gene expression in other EBV latently infected B cells, we examined the effect of the drug in Akata cells, an EBV-positive B-cell line derived from Burkitt's lymphoma. It has previously been reported that induction of lytic replication by anti-immunoglobulin G treatment in Akata cells resulted in hyperphosphorylation of Rb protein, as is the case with Tet-BZLF1/B95-8 cells (33). We also constructed Tet-BZLF1/Akata cells in which the exogenous BZLF1 protein is conditionally expressed under the control of a tetracycline-regulated promoter. As shown in Fig. 5C, lane 2, treatment of the Tet-BZLF1/Akata cells with doxycycline resulted in a significant expression of EBV lytic proteins at 48 h postinduction. In contrast, in the presence of purvalanol A, expression of lytic viral proteins besides the BZLF1 protein was hardly observed (Fig. 5C, lane 3). Thus, it was confirmed that inhibition of S-phase CDK activity impairs viral immediate-early and early gene expression in Akata cells as well as in B95-8 cells.

Purvalanol A inhibits expression of the BRLF1 and viral early proteins even in the presence of sufficient BZLF1 protein. It is technically difficult to evaluate the direct effects of purvalanol A on viral early gene expression by adding the drug at different times after induction because of the overlap in the times of expression of EBV immediate-early and early proteins. The effects of purvalanol A on specific stages of the EBV replication cycle can be determined, however, by blocking the progress of lytic infection by using cycloheximide to inhibit translation of the BZLF1 transcripts and then releasing the block in the presence or absence of purvalanol A. Using the cycloheximide block-and-release experiment, we examined the effects of addition of purvalanol A on the expression of the other immediate-early (BRLF1 protein) and early viral proteins when high levels of the BZLF1 protein had already been expressed (Fig. 6). Cycloheximide is a general inhibitor of translation; hence, during infections in the presence of this drug, the BZLF1 transcripts accumulate but are not translated. Since viral early promoters are not activated, viral early transcripts and proteins are not synthesized and consequently EBV lytic replication does not occur. Further, cycloheximide inhibition is reversible. Thus, if S-phase CDK activity is required for EBV replication functions that occur after accumulation of sufficient BZLF1 protein, purvalanol A would inhibit expression of early viral proteins when added after the reversal of a cycloheximide block.

To test this possibility, cycloheximide was added to culture

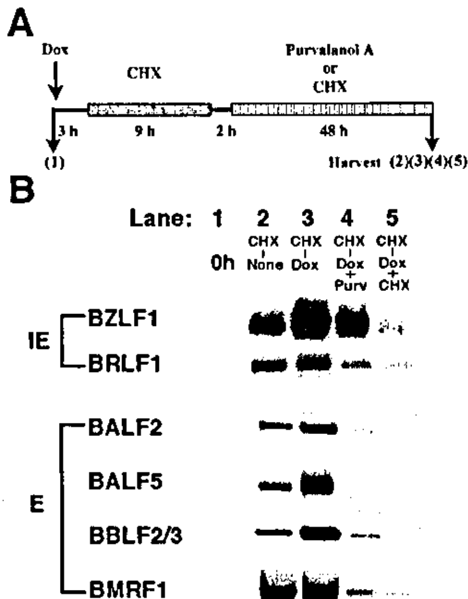

FIG. 6. Effects of purvalanol A on expression of EBV immediate-early and early proteins after release from a cycloheximide block. (A) Illustration of the experimental protocol. Tet-BZLF1/B95-8 cells were treated with doxycycline (1 μg/ml). Three hours later, 50 μg of cycloheximide (CHX)/ml was added to the culture. The cells were incubated for 9 h and then removed from the CHX block by replacing the medium with drug-free medium and culturing for a further 2 h. Subsequently, the medium was replaced with one containing no drug (CHX-None), 1 μg of doxycycline/ml (CHX-Dox), 1 μg of doxycycline/ml plus 15 μM purvalanol A (CHX-Dox+Purv), or 1 μg of doxycycline/ml plus 50 μg of cycloheximide/ml (CHX-Dox+CHX), and the cells were cultured for 48 h and harvested (lanes 2, 3, 4, and 5 of panel B, respectively; lane 1 of panel B represents the sample prepared when the cells were treated with doxycycline at time zero). (B) Clarified lysates were prepared and applied for Western blot analyses with each specific antibody as indicated on the left. The proteins were detected by an enhanced chemiluminescence detection system (Amersham). Images were processed by LumiVision PRO (Aisin/Taitec, Inc.) with a cooled CCD camera and assembled using an Apple G4 computer with Adobe Photoshop 5.0. Signal intensity was quantified with a LumiVision image analyzer as described in Materials and Methods. IE, immediate-early proteins; E, early proteins.

medium of the Tet-BZLF1/B95-8 cells at 3 h postinduction (Fig. 6A), because within 3 h postinduction, no expression of the BZLF1 protein was detected and the expression level of the BZLF1 protein in this case was higher than that seen with simultaneous treatment with cycloheximide and doxycycline (data not shown). Medium was removed 9 h later, and the cells were washed twice with PBS and overlaid with cycloheximide-free fresh medium followed by incubation for 2 h to accumulate sufficient amounts of the BZLF1 proteins in cells. After that, no drug (control), 50 μg of cycloheximide/ml, or 15 μM purvalanol A was added to the culture medium containing doxycycline. At 48 h after the change of medium, cells were harvested, and the cellular extracts were prepared, followed by Western blot analysis (Fig. 6).

Before the Tet-BZLF1/B95-8 cells were treated with doxycycline, no lytic viral protein was detected (Fig. 6B, lane 1). After the cycloheximide block, the cells were cultured in purvalanol A-free medium with doxycycline and several EBV early proteins as well as the BRLF1 protein were fully expressed (Fig. 6B, lane 3). Figure 6B, lane 2, shows the results when the cells were cultured in purvalanol A-free medium without doxycycline after cycloheximide block. Although the level of the BZLF1 protein was low compared with that in the case of lane 3, it should be noted that the expression level was enough for the expression of other immediate-early and early proteins. In contrast, expression of the BRLF1 protein and early viral proteins was drastically prevented in cells released from the 9-h cycloheximide block into purvalanol A-containing medium with doxycycline, although relatively large amounts of the BZLF1 protein accumulated (Fig. 6B, compare lane 4 and lane 2). When cycloheximide was added in place of purvalanol A, the expression level of the BZLF1 was low and there were very low expression levels of lytic viral proteins (Fig. 6B, lane 5). As the BRLF1 protein is also an important transactivator of early genes (33, 41), inhibition of its synthesis by purvalanol A may provide another plausible explanation as to why viral early gene expression is also inhibited. Thus, the inhibitory effect of purvalanol A on EBV replication after reversal of a cycloheximide block could be the result of inhibition of expression of the viral replication proteins.

Purvalanol A inhibits EBV immediate-early and early gene expression at the transcription level. To determine whether purvalanol A inhibits viral gene expression at the transcription level in the presence of a sufficient amount of the BZLF1 protein, we measured the levels of representative viral immediate-early (BZLF1 and BRLF1), early (BALF5 and BMRF1), and cellular housekeeping gene (36B4) transcripts accumulated during the 48 h after the release of the cycloheximide block in the presence of purvalanol A. Total RNAs were extracted from the cells, and the levels of the specific viral and cellular gene transcripts relative to those before treatment with the drugs were compared by Northern blot analyses (Fig. 7A).

It must be noted that the BZLF1 transcripts had already accumulated during the first 9-h cycloheximide block by doxycycline annexation (Fig. 7B, lane 1). As shown in Fig. 7B, when the lytic program-induced cells were released from the cycloheximide block into fresh medium containing only doxycycline, the level of BZLF1 gene transcripts increased fivefold during the 48-h cultivation (Fig. 7C, panel a, column 2). Since the BZLF1 protein itself activates expression of its own gene (2), the total amount of the BZLF1 transcripts would be the sum of the doxycycline-induced transcripts and the endogenously expressed one. When purvalanol A-containing medium was overlayed 2 h after release of the 9-h cycloheximide block, the BZLF1 gene transcripts also accumulated.

Next, the levels of other lytic transcripts were measured. When the cells were released into fresh medium containing only doxycycline after release of the cycloheximide block, BRLF1, BALF5, and BMRF1 transcripts accumulated more than 42-, 430-, and 8-fold, respectively, compared to prerelease levels (Fig. 7C), as expected. After release of the cycloheximide block into purvalanol A-containing medium, the levels of the BRLF1, BALF5, and BMRF1 transcripts were very low.

In contrast to that of viral transcripts, the levels of a cellular housekeeping gene (36B4) transcript were not significantly affected following release of the cycloheximide block into cycloheximide- or purvalanol A-containing medium relative to what

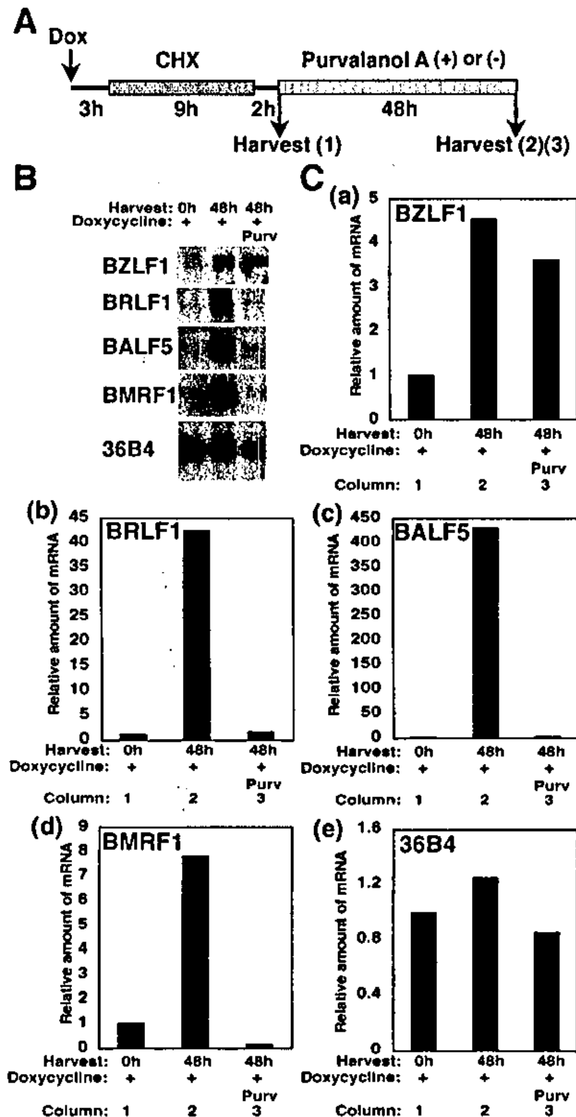

FIG. 7. Effects of purvalanol A on transcription of EBV immediate-early and early genes after release from a cycloheximide block. (A) Illustration of the experimental protocol. Tet-BZLF1/B95-8 cells were treated with doxycycline (1 μg/ml). At 3 h thereafter, 50 μg of cycloheximide (CHX)/ml was added to the culture. The cells were incubated for 9 h and then removed from the CHX block by replacing the medium with drug-free medium and culturing for a further 2 h. Subsequently, the medium was replaced with fresh medium containing 1 μg of doxycycline/ml and cultured for 48 h in the absence or presence of 15 μM purvalanol A [(2) and (3), respectively] and then harvested. As a control, cells were harvested before 48 h cultivation [(1)]. (B) Total RNA was extracted and applied for Northern blot analyses as described in Materials and Methods. (C) Levels of BZLF1-, BRLF1-, BALF5-, BMRF1-, and 36B4-specific transcripts (panels a to e, respectively, in Fig. 7C) were measured by Northern hybridization with ^{32}P-labeled probes obtained by PCR amplification of each viral and cellular DNA. Signal intensity was quantitated with an Image guider (BAS2500; Fujifilm). The levels of the specific viral and cellular gene transcripts relative to the pretreatment values were calculated and plotted in the graphs.

occurred upon release into drug-free medium (Fig. 7C, panel e).

Based on a comparison of the results presented in Fig. 6 and 7, although the level of the BZLF1 protein synthesized in cells released from the 9-h cycloheximide block into purvalanol A-containing medium was higher than in cells released into drug-free medium (Fig. 6B, compare lane 2 with lane 4), viral early transcript accumulation was significantly impaired by purvalanol A under these conditions (Fig. 7C). We conclude that purvalanol A inhibits transcription of immediate-early and early genes even in the presence of sufficient levels of the BZLF1 protein.

Purvalanol A inhibits BZLF1-induced activation of early EBV promoters pBMRF1 and pBHRF1. To determine whether the BZLF1 transactivator function is impaired in purvalanol A-treated cells lacking EBV genome, we performed reporter gene transfection studies with Tet-BZLF1/HeLa cells by using CAT gene constructs driven by two different early EBV promoters: the BMRF1 promoter and the BHRF1 promoter. The BMRF1 promoter drives expression of the BMRF1 gene product, whereas the BHRF1 promoter is within oriLyt. Each of these promoters has been previously shown to be directly bound and transcriptionally activated by the BZLF1 protein (8, 68). In Tet-BZLF1/HeLa cells, the exogenous BZLF1 protein is conditionally expressed under the control of a tetracycline-regulated promoter. As shown in Fig. 8B, the BZLF1 protein was conditionally expressed in the Tet-BZLF1/HeLa cells by the addition of doxycycline in the presence or absence of purvalanol A. As shown in Fig. 8A, the BZLF1 protein produced a stimulatory activation in the constitutive expression of the BMRF1 and BHRF1 promoters in the absence of purvalanol A. However, it was clearly demonstrated that purvalanol A had an inhibitory effect on the BZLF1-induced transcriptional activation of early EBV promoters pBMRF1 and pBHRF1 even in the presence of the BZLF1 protein (Fig. 8A). Further, purvalanol A appeared to inhibit the basal activity of the BMRF1 promoter in HeLa cells (Fig. 8A, compare the left and right columns of the pBMRF1-CAT samples). We speculate that the BHRF1 and BMRF1 promoters might contain binding sites of S-phase CDK-responsive cellular transcription factor(s).

DISCUSSION

The lack of elevation of the cellular CDK inhibitors $p21^{CIP1/WAF1}$ and $p27^{KIP-1}$, and instead the accumulation of hyperphosphorylated forms of Rb protein in EBV productive infection-induced LCLs, indicated that the $p21^{CIP1/WAF1}$- and $p27^{KIP-1}$-sensitive CDKs might play an important role in EBV productive replication. The results of a previous report (30) prompted us to ask whether EBV lytic infection could specifically be inhibited by chemical drugs that function similarly to $p21^{CIP1/WAF1}$ and $p27^{KIP-1}$. Several purine derivative drugs, including roscovitine and purvalanol A, that inhibit specific types of CDK activity have recently been described (21, 37). At low concentrations, their inhibitory effects are highly specific for CDK1/cyclin B, CDK2/cyclin A, and CDK2/cyclin E and none can significantly inhibit CDK4 and CDK6 activities. In this study, it was revealed that these chemical CDK inhibitors block EBV lytic replication significantly, providing evidence

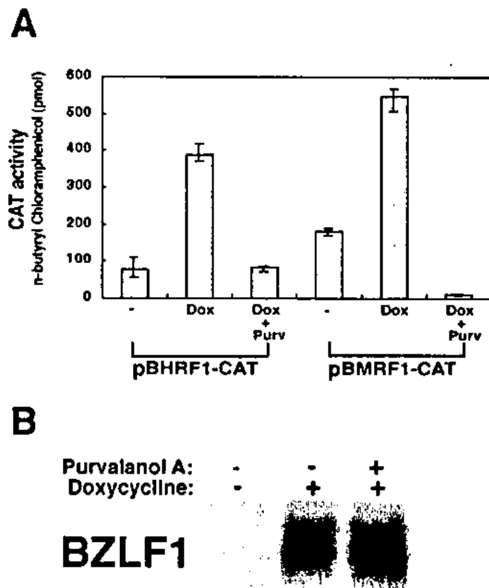

FIG. 8. Purvalanol A prevents BZLF1-induced transcriptional activation of the early EBV promoters pBMRF1 and pBHRF1. Tet-BZLF1/HeLa cells were transfected with 2 μg of pBMRF1-CAT or pBHRF1-CAT and media were replaced with fresh media containing or free of purvalanol A (20 μM) at 16 h posttransfection. Twenty hours posttransfection, doxycycline was added to a final concentration of 5 μg/ml or not. Forty-eight hours after addition of doxycycline, cells were harvested and some of them were used for a CAT enzyme assay. (A) CAT activity was assayed by using the Promega CAT enzyme assay system. The total amount of *n*-butyryl [^{14}C]chloramphenicol (in picomoles) was measured and plotted on the graph. Data represent averages from three independent experiments. (B) The rest of the cells were treated with lysis buffer, and clarified cell lysates were prepared. Clarified cell lysates were separated by SDS-10% PAGE and applied for Western blot analyses with the BZLF1 protein-specific antibody. The proteins were detected by an enhanced chemiluminescence detection system (Amersham). The slower-migrating band is a phosphorylated form of the BZLF1 protein. The faster-migrating band is the hypophosphorylated form.

that G_1 is an unsuitable period in the cell cycle for EBV productive infection and confirming the requirement for the environment of the late G_1/S boundary or beyond.

Although the mechanism by which CDK2 and/or CDK1 activate EBV gene expression is unknown at the present time, it has been shown that transcription of the EBV DNA Pol is activated by the E2F transcription factor (33). There are several putative E2F-binding motifs in the promoters of viral genes such as BRLF1 (42), BALF5 (33), BALF4, BRRF2, BVRF1, and BPLF1 (A. Kudoh and T. Tsurumi, unpublished results). Also, E2F putative binding sites have been identified within the promoter of the cellular transcription factor Sp1 (40) and E2F exerts an additive effect on its overexpression. Within the EBV lytic gene promoters are many Sp1 binding sites. CDK2 and/or CDK1 act in part by phosphorylating the Rb protein, which results in the release of the transcription factor E2F (39), which can then transcriptionally activate genes that are required for efficient lytic EBV DNA synthesis. EBV DNA replication is not completely independent of cellular replication proteins; e.g., DNA ligase I and FEN 1 may be required. It is conceivable that the activities of these cellular replication proteins may be promoted under S-phase conditions, when energy generation and other resources may support viral replication.

Purvalanol A induces a reversible arrest in progression through the G_1 and G_2 phases of the cell cycle. Its inhibitory effects are highly specific for CDK1, CDK2, and CDK5. Although CDK5 is a member of the CDK family of cell cycle regulators with important roles in neuronal differentiation, it has no observed function in the regulation of cell proliferation. Its protein kinase activity is only detected in postmitotic neurons. Although influence on CDK7 and CDK9 has not previously been determined, it has been reported recently that roscovitine inhibits CDK7 and CDK9 but not CDK8 (45, 63). The target of CDK7 and -9 is known to be the C-terminal domain of RNA Pol II, which regulates gene transcription. Villerbu et al., however, have reported that purvalanol A does not inhibit transcription under cell-free conditions and that the level of the CDK inhibitory protein p21(WAF1/CIP1) is increased in cells incubated with purvalanol A (62). Remarkably, it has been demonstrated that roscovitine inhibits HSV-1 transcription but not cellular transcription (which requires CDK7 and CDK9) (31, 38, 47, 49), and in this study, purvalanol A did not inhibit the conditional expression of the BZLF1 protein with doxycycline. Thus, purvalanol A does not cause a general inhibition of gene expression and the specificity of action of the drug is supported by the selective inhibition of the phosphorylation of CDK substrates such as Rb protein and cyclin E.

The potential applications of CDK inhibitors have been explored for infections with HSV (47–49), CMV (4), human T-cell leukemia virus type 1 (64), and human immunodeficiency virus (63). Like EBV, these viruses might have evolved various means to perturb the cell cycle to optimize the cellular conditions in favor of their own replication. Although attempts have been made to identify viruses resistant to the CDK inhibitor, almost no resistant HSV-1 or CMV strains could be isolated (4, 47). However, we cannot preclude the possibility that, in addition to cellular CDKs, the inhibitor could inhibit any of the EBV-encoded kinases. Indeed, the EBV BGLF4-encoded protein kinase mediates hyperphosphorylation of cellular elongation factor 1δ (EF-1δ). The BGLF4 protein and CDK1 target the same phosphorylation site (Ser133) in EF-1δ (28). Given that the BGLF4 protein may have the potential to phosphorylate Rb protein followed by release of free E2F, cellular CDKs and viral protein kinase might act to phosphorylate Rb protein synergistically. However, at least cellular CDK activity would be essential for EBV lytic replication since the viral protein kinase(s) belongs to an early or late protein group and is not expressed in the early stage of EBV productive infection.

The present findings clearly demonstrated the requirement for CDK activity in the replication of EBV. Thus, inhibition of CDK activity by drugs blocks EBV replication. We hypothesized that CDK2 in particular may be required to phosphorylate the Rb protein, allowing for release of E2F and subsequently transcription of cellular and/or viral genes required for EBV replication. To investigate this hypothesis further, it will be necessary to determine whether upon phosphorylation of Rb protein, subsequent release of E2F enhances synthesis of viral immediate-early and early proteins. Alternatively, CDK2

activity may also play another role in EBV productive replication. CDK2 activity has been reported to be involved in the initiation of cellular DNA synthesis at origins of replication (25). It is entirely possible that CDK2 activity may also play a similar role in initiating EBV lytic replication. Purvalanol A-sensitive CDKs might possibly regulate BZLF1-mediated transactivation of EBV immediate-early and early genes, as the CDK activity clearly plays a role in BZLF1-mediated transactivation based on the data shown in Fig. 6 and 7. There is a possibility that purvalanol A-sensitive CDKs might directly phosphorylate the BZLF1 protein, which, in turn, is required for its function, and/or regulate downstream effectors of the BZLF1 protein. It should be noted that roscovitine impaired the activities of immediate-early proteins from human CMV (HCMV) and HSV-1 (5, 10, 46), thus supporting one hypothesis that purvalanol A might directly inhibit BZLF1 function. However, when the BZLF1 protein was expressed in Tet-BZLF1/HeLa cells with doxycycline, purvalanol A did not inhibit the phosphorylation of the protein (Fig. 8B). Also, the fact that inhibition of CDK activity by purvalanol A blocks EBV lytic replication sometime after the BZLF1 gene expression, but prior to initiation of viral DNA synthesis, supports the former hypothesis.

A setting similar to the cellular environment evoked by EBV lytic replication has been observed for HCMV infection. A number of studies have revealed that productive HCMV infection stimulates arrested cells to enter the cell cycle (5, 13, 34, 44). Recent studies have confirmed these early observations and demonstrated that productively infected cells traverse the cell cycle through at least late G_1 with induction of cyclin E/CDK2 activity and hyperphosphorylation of Rb protein (4, 5, 26, 65). HCMV infection reduces the abundance of two CDK2 inhibitors (p21WAF1/CIP1 and p27KIP1). In cells stimulated to traverse the cell cycle by serum growth factors, cyclin E/CDK2 acts in part by phosphorylating pRB protein, which results in the release of the transcription factor E2F. These findings suggest an important role for S-phase CDK activity in HCMV replication as well as the lytic phase of EBV DNA replication.

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【上皮系細胞の増殖制御と不死化】

Growth Control and Immortalization of Human Epithelial Cells

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Key words
細胞老化, culture shock,
テロメア, $p16^{INK4a}$

はじめに

ヒト正常細胞は一部の例外を除き，一定回数分裂すると細胞周期が停止する。絶対分裂可能回数を規定しているのは，理論上テロメア長である。事実，皮膚線維芽細胞などではTERTの発現によりテロメラーゼを活性化するだけで無限増殖できるようになる。しかし，多くのヒト上皮系細胞はTERTの導入のみでは不死化できないことが報告されている。また，TERTの導入のみで不死化できるとされたヒト線維芽細胞も由来する組織によってはTERT導入が，十分条件ではないことも分かってきた。これらの細胞では，CDKインヒビターである$p16^{INK4a}$の発現が継代と共に増加するため，RBが活性化し増殖が停止する。そのため，テロメラーゼの活性化に加え，$p16^{INK4a}$/RB経路の不活化が必要である。培養細胞における$p16^{INK4a}$の発現誘導の意義については単に至適でない培養条件が原因であり，細胞老化機構ではないとの議論があるが，発がん抑制機構として機能していることは共通の認識となりつつある。本章では，テロメア短小化による分裂寿命(replicative senescence)に加え，$p16^{INK4a}$の発現誘導による増殖停止を含め，広義の細胞老化(cellular senescence)と呼びその生物学的意義を議論したい。

1．上皮細胞の定義

上皮細胞は，体の外側および表層部に沿って組織を形成している細胞であり，これらの細胞は体表の他，腺および管部分の表層を構成している。したがって，皮膚角化細胞など外胚葉由来のものから，消化管，気管など内胚葉由来のもの，さらには血管，リンパ管の内皮や，泌尿生殖管上皮など中胚葉由来のものまで多種多様である。これらは主に間葉系の細胞との相互作用により異なった方向に分化した細胞集団である。

2．上皮系細胞の増殖制御

上皮系細胞の増殖と生存は，主にインテグリンを介した細胞外マトリクス(extracellular matrix: ECM)との接着と，増殖因子リセプターへのリガンドの結合に依存していると考えられる（図1）。インテグリンを介したECMとの接着は細胞骨格の張力を保つだけでなく，FAK(focal adhesion kinase)などを介して，増殖因子リセプターからのシグナルと同様，細胞の増殖や生存に重要なシグナルを伝える。実際，上皮系細胞では，培養皿に接着できないとanoikisと呼ばれる細胞死が誘導されることが知られている。上皮細胞の増殖，生存に重要なECM蛋白質や増殖因子は主に基底膜を接して近接する間葉系細胞から作られる。初期の上皮系細胞の培養は間葉系細胞との共培養により成功している。単独培養の可能な上皮細胞は全体から見ればまだ一部ではあるが，それらをみると増殖因子の同定に負うところが多い。角化細胞に対するEGF，血管内皮細胞に対するVEGFなどがそうである。また，培養皿の表面にECM蛋白質をコーティングすることにより培養が可能になったものもある。本来，上皮細胞は基底膜と呼ばれる細胞外マトリクスとインテグリンを介し

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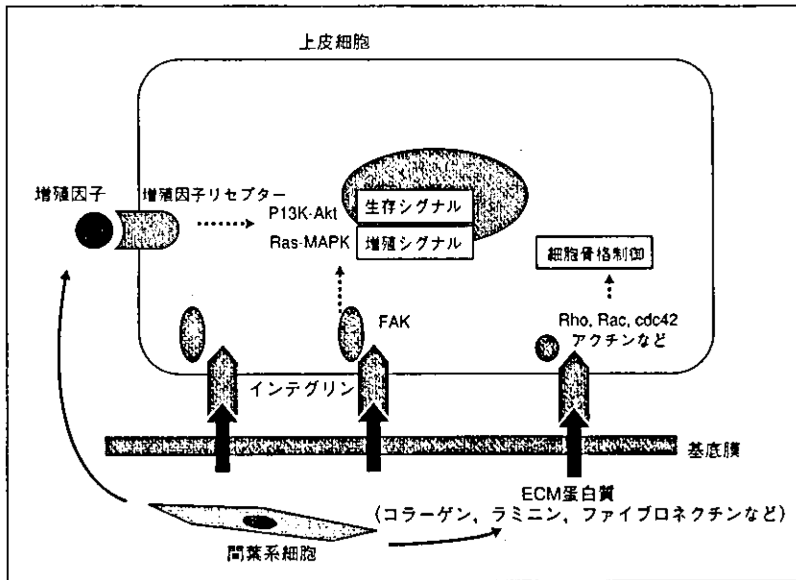

図1　上皮細胞の生存と増殖制御
上皮細胞の増殖はEGFやVEGFなど特異的な増殖因子に依存している。また，上皮組織は基底膜(basal lamina)とよばれる細胞外マトリクス(ECM)と接している。上皮細胞は細胞接着膜リセプター蛋白質インテグリンを介してリガンドであるECM蛋白質と結合する。インテグリンはチロシンキナーゼではないがFAK(focal adhesion kinase)などを介して増殖因子リセプターと同様に増殖。生存シグナルを伝える。インテグリンは細胞骨格，細胞骨格制御蛋白質群とも複合体を形成し細胞骨格の張力を保ち，細胞運動の制御も行っている。ECMや増殖因子は上皮細胞自身が分泌する場合もあるが，多くの場合基底膜を介して存在する間葉系細胞によって分泌され，上皮細胞の分化・生存・増殖を制御していると考えられる。

て接着している。インテグリンは2本のサブユニットからなるヘテロ2量体蛋白質で，多数のα鎖，β鎖の組み合わせにより，多様なECMとの特異的な結合とシグナル伝達を担っている。ECMはコラーゲン，ファイブロネクチン，ラミニンなどの蛋白質に加え，グルコサミノグリカンと呼ばれる高分子多糖の結合したプロテオグリカンなどで構成され多様性に富んでいる。このように，上皮細胞の培養の歴史は，生体内環境の培養皿上での再現に努力が注がれてきたとも言えるが，生体内環境の複雑さから見れば，実際に普及している培養法は単純で改善の余地は多い。

3．上皮系細胞の培養寿命

単独培養が可能となった上皮細胞の培養寿命はテロメアの短小化ではなく，むしろ*p16*^{INK4a}（以下単に*p16*と記す）の発現増加によって規定されている。市販されている上皮細胞と培地を手に入れて培養すると，説明書には通常5継代までは培養を保証するなどの表示がある。細胞種にもよるが，皮膚線維芽細胞のように20継代を越えて増殖停止することなく増え続けるような上皮系の細胞は見あたらない。これらの細胞では，継代と共にCDKインヒビターである*p16*の発現増加が共通して観察される。そして，RBのリン酸化を阻害するレベルにまで*p16*の発現量が増加すると細胞周期は停止してしまう（図2）。乳腺上皮細胞や前立腺細胞などでは，大部分の細胞が増殖停止する一方，*p16*プロモーターがメチル化され*p16*の発現の低い一部の細胞集団が選択的に再増殖し，テロメアが短小化するまで培養できる細胞もある。*p16*の発現増加が実際に上皮細胞の培養可能期間を規定している事をよく示している。

4．“Cultuer shock”と*p16*の発現

マウス胎仔線維芽細胞(MEF)とヒト上皮細胞は，見かけ上同じようにテロメア非依存性の増殖停止が起こる。MEFでは*p16*の増加と同時にみられるp53の増加が，ヒト上皮細胞では*p16*の増加が増殖停止の主因であるという差はあるものの，どちらもプラスチック皿上での培養という“culture shock”によるストレスが原因であるとする説が提唱されている[1]（図1）。角化細胞や乳腺上皮細胞の単独培養では，無血清あるいは低血清培地が使われるが，3T3細胞をfeederとして通常の10%血清を含む培地で培養すると*p16*は増加せず，TERTのみで不死化できるという報告もある[2]。間葉系細胞との共培養の方がより生体環境に近くストレスが少ないためと考察されている。

5．酸化ストレスと細胞寿命

MEFでは酸化ストレスが“culture shock”の原因であるとする論文が昨年報告された[3]。MEFを通常の組織酸素分圧に相当する低酸素(3%)の培養器で培養すると増殖停止は見られず，長期延命できることが報告された[3]（図1）。MEFにおける“culture

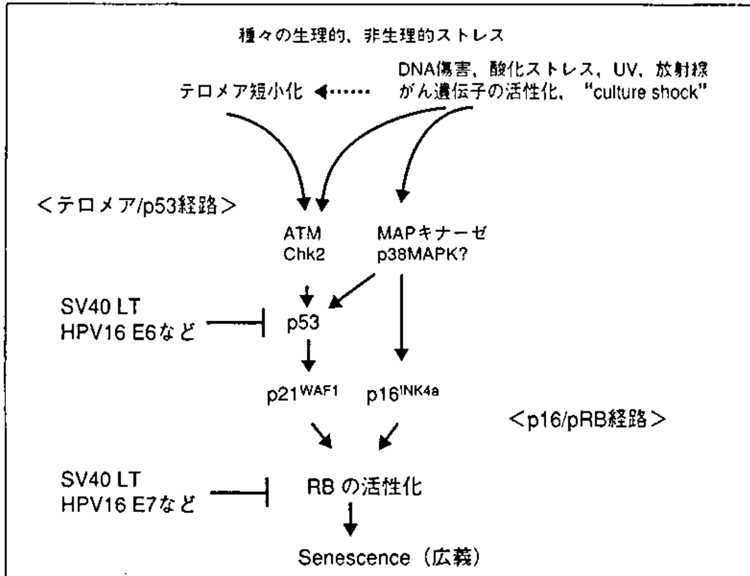

図2　CDKインヒビターとヒト細胞の老化
テロメア短小化はおそらくDNA損傷チェックポイント機構とほぼ同じ機構を介してp53を活性化する（テロメア-p53経路）。一方，ヒト上皮細胞の継代では*p16*が特異的に誘導される（*p16*/RB経路）。結果的にどちらもRBの活性化を引き起こし細胞の老化あるいは老化様変化を引き起こす。両経路の間にはいろいろなレベルでクロストークが見られ，活性酸素など生理的なストレスやDNA損傷誘導薬剤など多くの人工的ストレスでは両経路が同時に活性化されることが多い。

shock"の原因は，大気中に含まれる約20%の過剰な酸素によるDNA傷害であることがほぼ証明された。では，ヒト細胞の場合はどうだろうか？　皮膚線維芽細胞では20%強の酸素存在下でもp53の誘導による増殖停止は見られない。ヒトではDNA修復機構がマウスより発達しており20%強の酸素存在下でも耐えることができるようである。肺由来の線維芽細胞では低酸素(3%)の培養器によりp53の増加による増殖停止を少し抑制することが示されているが，*p16*の増加による増殖停止は抑えられないことが報告されている[4]。間葉系細胞との共培養により"culture shock"が抑制されることから推測すると，ヒト細胞での*p16*の誘導に関わる"culture shock"の本態は不適切なECMによるものかも知れない。

6．ヒト上皮細胞の不死化

継代と共に*p16*が増加する培養条件でヒト上皮細胞を不死化するには，テロメラーゼの活性化に加えp16/RB経路の不活化が必要である[5,6]。*p16*/RB経路の不活化には，種々の方法が試みられている。HPV16などのE7によるRBの不活化や，CDK4の高発現によるRBのリン酸化（不活化）に加え，Bmi-1やId-1によるp16の転写抑制（図3），*p16*に対するアンチセンスRNAやRNAiによる発現抑制などである。しかし，実際にはこれらとTERTの組み合わせのみで高率に不死化できる細胞種は多くない。その場合，HPV16のE6などによってp53も同時に不活化すると不死化の効率は上がることが多い。SV40のLTではRBと同時にp53も不活化するので不死化の効率は高いが，不死化細胞はしばしば造腫瘍性まで獲得してしまう。*p16*/RB経路の不活化によりp14[ARF]の発現が増加しp53が活性化する経路が知られており，この経路が働きやすいか，あるいはp53に対する感受性の高い細胞種では*p16*/RB経路の不活化に加えp53の不活化が必要なのかも知れない。試したことはないが，これらの細胞では低酸素濃度下での培養が有効かも知れない。

7．*p16*の発現制御機構とがん抑制機構

*p16*はDNA傷害を引き起こすさまざまな"ストレス"やがん遺伝子の活性化などによって発現が増加することが知られているが，その分子機構には未知の部分も多い。その中で，最もよく解析されているのが活性型rasによる*p16*の誘導機構である。活性型rasはNIH3T3などに導入するとトランスフォームすることができるが，正常細胞では細胞老化と区別の出来ない老化表現型(premature senescence)を誘導する。ヒト細胞の場合，MAPキナーゼカスケードを介した*p16*の転写活性化によると考えられている[7]（図3）。しかし，この経路も単純にERKがEtsをリン酸化した結果ではなさそうで，p38MAPKの活性化を介しているらしい[8]。一方，ポリコームグループ蛋白質であるBmi-1やCBX7は*p16*プロモーターを負に制御している。こういった*p16*の発現調節機構はがん抑制機構として機能していると考えられるようになった。すなわち，DNA傷害やrasの変

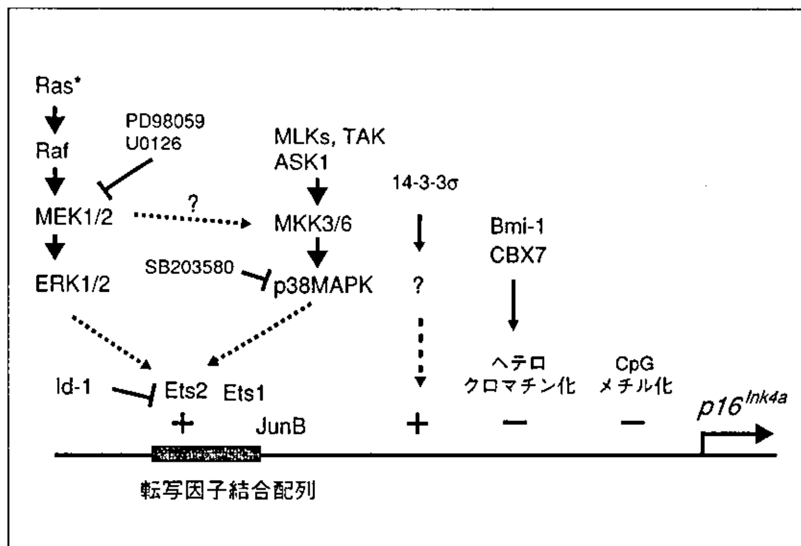

図3　p16Ink4aの転写制御
活性型Ras*によるp16の転写活性化はMAPキナーゼカスケードによる転写因子の活性化によると考えられている。Raf, MEKのクラシカル経路だけでなくMKK3/6, p38MAPKの活性化によっても転写活性化が起きること，MEKの阻害剤だけでなくp38MAPKの阻害剤でもras*による転写活性化はクラシカル経路からp38MAPKへのクロストークを介していると推測されている。Id-1はEtsファミリー蛋白質と結合してその転写活性化を阻害する。その他，p16の転写は14-3-3σによっても活性化することが知られているがその機構は不明である。Bmi-1, CBX7などのポリコームグループ蛋白質はプロモーターをヘテロクロマチン化することで転写を抑制する。多くのがんではプロモーター領域のCpGのメチル化により転写が抑制されている他，Bmi-1が高発現しているがんも見つかっている（本文参照）。

異や，もしかしたら過剰な増殖刺激によるrasの活性化などによるストレスを受けた細胞では，*p16*の発現の増加により不可逆的な増殖停止が誘導されがん化が抑制されるという機構である。*bmi-1*欠損マウスでは造血幹細胞の幹細胞性維持に異常がみられることが報告されているが，このような細胞では生理的に何らかのストレスを受けやすくBmi-1によって*p16*の発現誘導による増殖停止を逃れているのかもしれない。

おわりに

そもそも細胞の不死化とは細胞が死なずに細胞周期が回り続けることである。現在のヒト上皮細胞培養技術でそれを妨げているのは*p16*の増加である。培養法の改良によって，上皮系細胞も皮膚線維芽細胞のようにTERTの導入のみで不死化できる可能性は十分にある。胎生期の細胞では胸腺上皮を除いて*p16*はほとんど発現していないようである。このような生体内環境を培養皿上で再現できれば，*p16*の増加は起きないはずである。一方，培養皿上で見られる*p16*の誘導機構は生体内でも，異常なストレスを受けた細胞の不可逆的増殖停止を誘導するがん抑制機構として機能していることは間違いない。上皮細胞の不死化機構の研究はがん研究のみならず，再生医療においても重要な研究テーマとなっている。

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遺伝子導入によるヒト細胞寿命の延長

Extending life span of human cells by gene transfer

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◎ヒト組織幹細胞を体外で自由に殖やすことができれば細胞移植療法の可能性は大きく広がる．しかし，実際にヒト細胞を培養すると一定期間分裂した後分裂を停止し無限に増殖することはできない．この培養皿上での分裂可能回数を規定している機構にはテロメア依存性のものとテロメア非依存性の2つがある．テロメア依存性のものはテロメア短小化によりチェックポイント機構が働き増殖が停止するもので，狭義の分裂寿命(replicative senescence)とよばれる．しかし，皮膚線維芽細胞を除くほとんどの正常ヒト細胞の培養皿上での分裂可能回数を規定しているのはテロメア短小化によらない$p16^{INK4a}$/RB経路の活性化による増殖停止である．したがって，ほとんどのヒト正常細胞を寿命延長するにはまず$p16^{INK4a}$/RB経路の活性化を止める必要がある．ここでは遺伝子導入による細胞寿命延長法の現状と課題を概説し，今後のヒト細胞の寿命延長法の展望についても述べたい．

Key word：細胞老化，不死化，テロメラーゼ，$p16^{INK4a}$

ヒト初代培養細胞の有限寿命

ヒト正常細胞を培養すると，一定の分裂を繰り返した後，分裂を停止しそれ以上増殖しなくなる．この広義の細胞老化(cellular senescence)は2つの異なる機構によってもたらされる．1つはテロメア短小化による増殖停止であり，狭義の細胞老化(replicative senescence)あるいはM1(mortality stage 1)とよばれる．もうひとつは$p16^{INK4a}$(以下単にp16と記す)の発現増加とRBの活性化により起こる増殖停止であり，ここではM0(mortality stage 0)という用語を使うことにする(「サイドメモ」，図1)．皮膚線維芽細胞にはM1のみがあり，テロメラーゼの触媒サブユニットであるTERTを発現させることで不死化させることができる．しかし，なにもしなくてもこの細胞は50～70回程度分裂できるため，細胞移植に十分な細胞数が得られる．細胞移植に必要な細胞を体外で殖やす際に問題となるのはほとんどの場合，M0である．したがって，TERTを導入して不死化しようとしても不死化できない．まず，M0とM1がどのような機構で細胞老化を引き起こすのかを理解した後，具体的な寿命延長法について述べる．

テロメア短縮による増殖停止(M1)

ヒトをはじめ脊椎動物のテロメアDNAは5′(TTAGGG)3′からなる数百から数千回の繰返し配列(ヒトでは約10 kb)からなる．テロメアはその繰返し配列とテロメア結合蛋白質(TRF1，TRF2)の結合によりt-loop，D-loopとよばれる特殊なクロマチン構造をとり，線状ゲノム末端をDNA断端として認識されることを防ぐとともに，DNA末端どうしの融合，組換えを防いでいると考えられる[1]．しかし，直鎖状DNAをゲノムとしてもつ生物にはいわゆる"末端複製問題"が生じる(図2)．テロメアが一定の長さにまで短縮するとDNA損傷チェックポイント機構とほぼ同じ機構が働き

ATM, p53 依存的に p21$^{WAF1/SDI1/CIP1}$(以下，単に p21 と記す)の増加，高リン酸化型 pRb の消失などが観察される．通常の DNA 損傷によるチェックポイントの活性化は DNA が修復されると解除されるが，テロメア DNA 末端の露出によるものは修復されることがないため不可逆的な増殖停止が誘導される(図 2)．また，テロメア結合蛋白質である TRF2 の機能を変異体の発現によって抑制するとテロメア長が長くてもそのクロマチン構造が保てず正常細胞では senescence や染色体末端融合が，また多くの癌細胞ではアポトーシスが誘導される[1,2]．

サイドメモ

細胞老化(cellular senescence)

ヒト細胞が無限増殖できないことは培養の比較的簡単であった線維芽細胞を用い発見され Hayflick 限界とよばれた．同じ現象論として M1(mortality stage 1)や細胞老化(cellular senescence)という用語が使われていたが，原因論的にテロメアの短小化がこれらの現象を説明できることが推測され，新生児皮膚線維芽細胞などでは証明されたため，Hayflick 限界＝テロメア短小化による増殖停止＝狭義の細胞老化としての分裂寿命(replicative senescence)＝M1(mortality stage 1)として使われることが多い．しかし，現在では線維芽細胞でも皮膚由来のものを除き 2 つの異なる機構で細胞は増殖停止することが明らかになっている．

最初に明らかにされた乳腺上皮細胞では 2 つの増殖停止期がみられるが，後期の増殖停止期が M1 とされ，初期の増殖停止期は無視されていたため後に M0 と命名された．M0 は p16 の増加によって誘導される増殖停止期でテロメア長非依存性である．p16 プロモーターのメチル化により p16 の発現低下した一部の細胞がふたたび増殖するため 2 つの増殖停止期がみられる．

前立腺上皮細胞など一部の細胞では同様の 2 つの増殖停止期がみられるが，多くの細胞種では p16 プロモーターのメチル化された細胞の出現頻度は低く 1 つの増殖停止期しか観察されない．現象論的にははじめての増殖停止期なのでこれも M1 とよぶべきものであるが，原因論的な立場からするとこの増殖停止期は M1 ではなく M0 と同じものである．ここではヒト細胞の通常の培養条件における p16 増加による増殖停止(細胞老化)を乳腺上皮細胞に限らず M0 とよぶことにする．

テロメラーゼの発現調節

テロメラーゼ活性はおもに，*TERT* の転写レベルで調節されている．正の調節因子としては Myc や Ets などが報告されているが，ほとんどのヒト正常体細胞では *TERT* の転写は抑制されており，負の調節因子が重要であると予想される．最近，Mad, SIP1, Menin, Rak1, BRIT1 など複数の因子がこの転写抑制にかかわっていることが報告されている[3]．興味深いことにはその一部を不活化するだけで *TERT* の転写を活性化できるようである．

CDK インヒビター p16 の増加による増殖停止(M0)

さきに述べたようにほとんどのヒト細胞ではおもに p16 の発現増加による p16/RB 経路の活性化により増殖が停止する．この増殖停止機構がテロメア非依存性であることは，乳腺上皮細胞，皮膚角化細胞を用いて明らかにされた．したがって，多くのヒト細胞種を不死化するにはテロメラーゼの活性化のほかに p16/RB 経路の不活化が必要である[4]．

p16 の発現調節

p16 の発現調節もおもに転写レベルで行われている(図 3)．正の転写調節因子として，Ets ファミリーや AP1 が報告されている．また，転写因子ではないが，Polycomb-group genes(PcG)に属する bmi-1 や cbx 7 が p16 の転写を負に制御している．外来性に bmi-1 や CBX7 を高発現してやると，p16 の発現増加は阻止され，細胞増殖停止は抑制される[5,6]．活性化癌遺伝子産物 Ras*(RasV12)が株化細胞を形質転換させることは古くから知られていたが，初代線維芽細胞に対しては不可逆的な増殖停止を誘導する．この Ras*による線維芽細胞の premature senescence には，MAP キナーゼカスケードを介した Ets ファミリー蛋白質のリン酸化がかかわっているが[7]，下流の Erk が直接，転写因子をリン酸化するのではなく p38 MAPK へのク

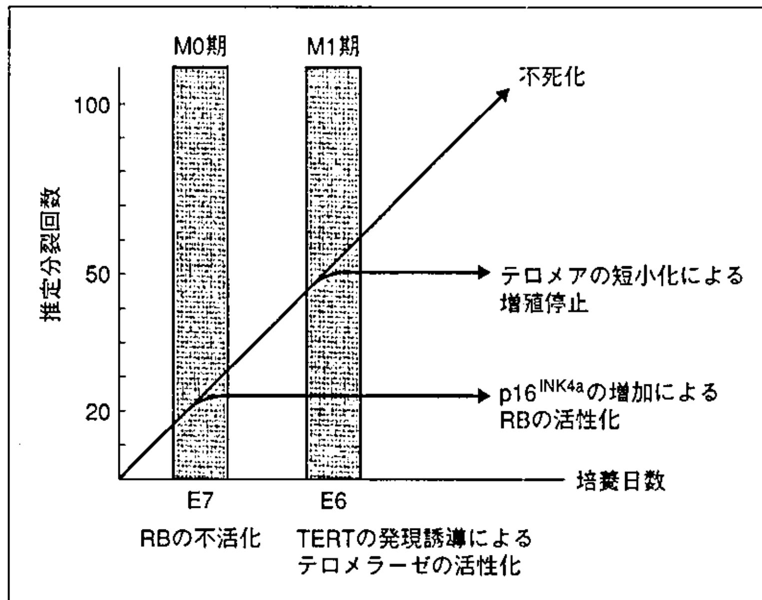

図 1　乳腺上皮細胞の不死化モデル(文献[13]より改変)

ヒト乳腺上皮細胞では約 20PD で最初の senescence(M0 期)を迎える．このとき p16[INK4a]の発現増加がみられる．senescence を逃れたごく一部の細胞では *p16[INK4a]*プロモーターがメチル化により発現が低下しており，増殖を続けるが，約 50PD で senescence(M1 期)を迎える．E7 を発現している細胞では E7 が RB を不活化することで M0 期をバイパスすることができる．一方，E6 あるいは TERT を発現している細胞ではテロメラーゼが活性化されているためテロメア長の短縮によって訪れる M1 期をバイパスすることができる．

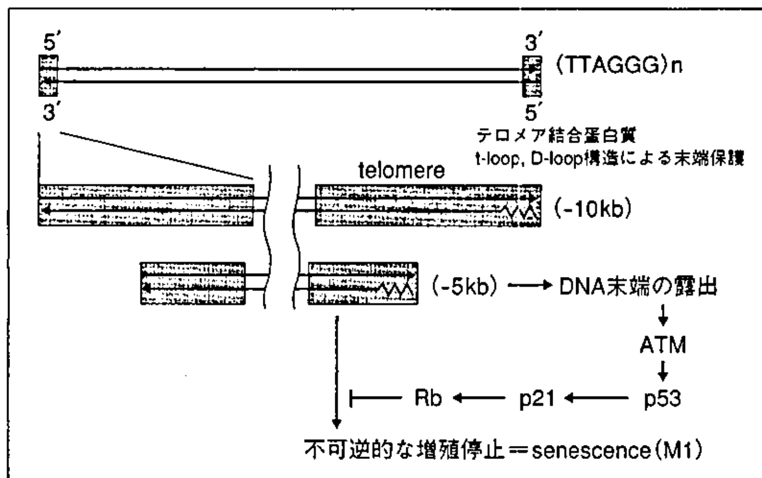

図 2　末端複製問題とテロメア/p53 経路(文献[13]より改変)

線状ゲノムをもつ生物ではゲノム複製の際，新生 DNA 鎖の 5′末端のプライマー RNA 部分およびそれより 5′末端側は DNA に置換されないため，テロメアは複製ごとに短縮される．したがって，テロメラーゼ活性のないヒト体細胞では 1 回の複製(分裂)あたりおよそ 100 塩基ずつテロメア長が短縮する．ヒト体細胞のテロメア長は約 10 kb あるが，50 回分裂して約 5 kb になるとテロメアのクロマチン構造を保てなくなり DNA 末端として露出され，通常の DNA 損傷チェックポイント機構が働くと考えられている．その結果，ATM，p53 が活性化しおもに p21 の増加によって RB のリン酸化が阻害され，細胞周期が停止する．

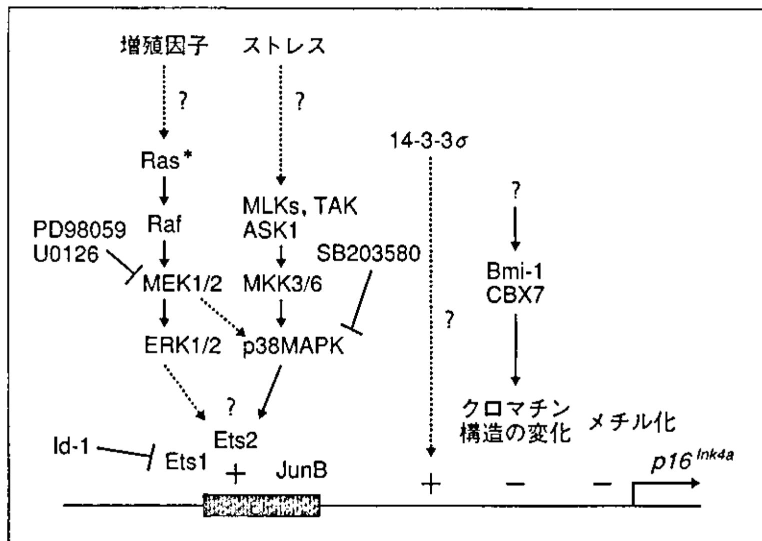

図 3 $p16^{INK4a}$の転写調節

p16 の発現調節もおもに転写レベルで行われている．正の転写調節因子として，Ets ファミリーや JunB が報告されている．一方，Id1 は Ets ファミリーと結合することで転写を負に制御している[5]．また，機構は不明であるが，14-3-3σは p16 の発現を誘導することが報告されている．また，転写因子ではないが，Polycomb-group genes(PcG)に属する bmi-1 や cbx 7 が p16 の転写を負に制御している．活性型 Ras による線維芽細胞の premature senescence には MAP キナーゼカスケードを介した Ets ファミリー蛋白質のリン酸化がかかわっているが，下流の ERK が直接，転写因子をリン酸化するのではなく p38 MAPK へのクロストークを介しているらしいことが報告されている．また，p38 MAPK は種々の"ストレス"によって誘導されるストレスキナーゼとして知られており，SIPS のセンサーとして共通に働いている可能性が示唆されている[8]．

ロストークを介しているらしいことが報告されている．この機構は癌遺伝子活性化による細胞癌化を防ぐ監視システムとして近年注目されている．また，p38 MAPK は，過酸化水素水の添加や，二本鎖 DNA 切断を誘導する薬剤，UV 照射，放射線照射などのさまざまな"ストレス"によっても誘導されることから SIPS(stress-induced premature senescence)のセンサーとして共通に働いている可能性が示唆されている[8](図 3)．

ヒト細胞不死化の現状

現在，著者らはヒト細胞の不死化を図 4 に示すアルゴリズムに従って行っている．その結果，①TERT の導入だけで不死化できるもの(皮膚線維芽細胞)，②TERT に加えて E7 など p16/RB 経路の不活化によって不死化できるもの(皮膚角化細胞など)，③さらに E6 など p53 経路の不活化が不死化に必要なもの(子宮内膜腺細胞など)，に分類される．p53 経路の不活化が必要なものは p16/RB 経路の不活化によって p53 経路が二次的に活性化されたとき感受性の高い細胞だと考えている．なお，それでも不死化に成功していないもの(胃腺細胞)もある．TERT の導入はまだしも，E6 や E7 などウイルス癌遺伝子を導入された細胞が移植医療に適さないのは明らかである．現時点では E7 などの替わりに RNA 干渉法による p16 の発現抑制と TERT 発現ベクターにより皮膚角化細胞や乳腺上皮細胞の不死化に成功している．

ヒト細胞寿命延長の課題と展望

しかし，M0 期の p16 の発現誘導機構が解明されれば，よりよい寿命延長，不死化方法の開発が期待できる．もし M0 が SIPS と同じであるならばそのストレスを軽減する培養法の開発によって p16 の発現誘導そのものをなくすことが可能かもしれない．上皮系の細胞培養は低(無)血清培地が開発

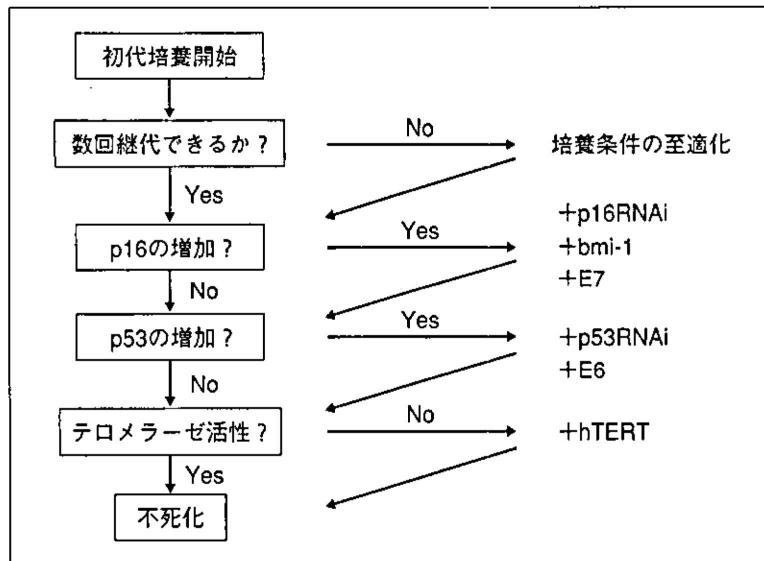

図 4 ヒト細胞不死化のアルゴリズム

まず，初代培養細胞が培養皿上で増殖するか，継代できるかが成否の鍵となる．また，図にはないが，初代培養細胞には目的以外の細胞種の混入は避けられず，培養条件が目的細胞により適したものであることが望まれる．上皮細胞を不死化しようとしたら線維芽細胞ばかり不死化したというようなことが起こりうる．目的の細胞種に特異的なマーカーなどがわかっていれば混在する細胞集団を不死化してから目的の細胞種をクローニングすることも可能である．

初代培養は困難であるが，数週間あるいは1カ月以上たってからコロニーが認められることもある．このような場合，p16 プロモーターのメチル化などの異常が起こっている可能性を検討する必要がある．p16/RB 経路を不活化するには，このなかでは E7 がもっとも強力であるが，その分 p53 経路を活性化させアポトーシスを誘導する可能性もある．材料が限られていて多少細胞の形質が変わってもとにかく不死化したいという場合には，E6 と E7 を同時に導入するのも現実的な選択である．通常テロメア短縮による増殖停止はかなり後期に起こるが，テロメラーゼ活性のないことが自明の細胞には TERT も早めに導入したほうが染色体の安定性が保たれやすい．

されてからプラスチック皿上での培養が普及した．しかし，線維芽細胞のフィーダー上で血清入り培地で培養することによって皮膚角化細胞や乳腺上皮細胞における p16 の発現誘導は軽減できるとする報告もあるがその機構は不明である[9]．ヒト初代培養細胞への遺伝子導入効率は一般に低いため TERT などはほとんどレトロウイルスベクターにより導入されている．しかし，レトロウイルスベクターは，細胞ゲノムにかなりランダムに組み込まれることから，癌遺伝子の活性化や癌抑制遺伝子の不活化などが起こる可能性がある[10]．また，TERT は癌抑制遺伝子の側面もあるが，逆に癌の 85%で発現していることから，癌遺伝子としての側面もある[11]．したがって，理想的には再生医療に用いる細胞には TERT の遺伝子導入さらにはレトロウイルスベクターの使用もできれば避けるのが望ましい．上述したように，多発性内分泌腫瘍 I 型(MEN-I)の原因遺伝子である menin などが TERT の転写を抑制しており，menin を RNA 干渉法でノックダウンすると TERT が発現し皮膚線維芽細胞を不死化できると報告された[3]．報告ではレトロウイルスベクターによる RNA 干渉法が使われているが，一過的な siRNA の導入でも活性化できる可能性もある．また，PTD(protein transduction domain)との融合蛋白質として種々の遺伝子産物を細胞に供給する方法が応用されはじめている[12]．遺伝子導入に替わる方法として期待できる．

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● お知らせ ●

■第126回日本医学会シンポジウム

アレルギー・アトピー性疾患
日　時：平成16年6月24日(木)10：00～17：00
場　所：日本医師会館大講堂
東京都文京区本駒込2-28-16
TEL：(03)3946-2121(代)

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①アレルギー・アトピー性疾患の疫学と遺伝子解析　白川太郎(京大・健康増進・行動)
②アレルギー・アトピー性疾患における網羅的遺伝子発現解析　斎藤博久(国立成育医療センター研)
③気道のリモデリングと遺伝子多型　大田　健(帝京大・内科)

II．アレルギー性疾患とサイトカイン/転写因子　〈座長〉山本一彦(東大・アレルギー・リウマチ内科)
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④アトピーにおけるIgE受容体発現異常の遺伝子解析　西山千春(順天大・アトピー疾患研究センター)

III．アレルギー性疾患の治療の将来展望　〈座長〉小川秀興(順天大・皮膚科)
①アトピーと皮膚粘膜防御機能について　光石幸市(順天大・アトピー疾患研究センター)
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③DNA免疫法によるアレルギー性疾患の治療　佐野公仁夫(東北大・感染病態)

総合討論　〈司会〉小川秀興(順天大・皮膚科)奥村　康(順天大・免疫)山本一彦(東大・アレルギー・リウマチ内科)

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Observation of osteogenic differentiation cascade of living mesenchymal stem cells on transparent hydroxyapatite ceramics

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Abstract

The use of bioceramics and cultured cells for tissue engineering is a novel approach, which is available in a wide variety of clinical situations. The approach requires apparent verification of the cellular functions occurring on the ceramic surface, and these functions could be monitored by microscopic observation of the cultured living cells on the ceramic material. However, such observation is difficult due to the opaque nature of ordinary ceramics. To overcome this drawback, we used transparent hydroxyapatite (tHA) ceramics as a culture substrate and a transgenic rat having an enhanced green fluorescent protein (EGFP)-expressing gene as the cell source. Marrow mesenchymal stem cells (MSC) were obtained from the rat and cultured on both tHA ceramics and a tissue culture polystyrene (TCPS) dish. One hour after the cell seeding, many MSC had attached and showed initial cell spreading. The attachment and spreading were more obvious 5 h after the seeding. Following the culture in the osteogenic condition, the cells differentiated into osteoblasts, which fabricated bone matrix on the culture substrate. The phenomena were similarly observed on both the tHA ceramics and TCPS substrata. These results confirm the excellent properties of tHA ceramics, which support cell attachment, proliferation, and differentiation. Transparent materials make us know the biological usefulness of ceramics in tissue-engineering field.
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Keywords: Observation; Mesenchymal stem cells (MSC); Transparent hydroxyapatite (tHA) ceramics

1. Introduction

Current technology enables the regeneration of viable tissues or organs by using both cultured cells and suitable scaffolds. Hydroxyapatite (HA) is a well-known scaffold material and plays important roles in the adhesion, proliferation, and differentiation of cultured cells, especially bone-related cells. We have previously reported that mesenchymal stem cells (MSC) derived from bone marrow can be cultured on HA ceramics [1,2]. Significantly, under osteogenic conditions, the cultured MSC further differentiate into osteoblasts, which fabricate bone matrices on the HA ceramic surfaces. The cultured osteoblast/matrix constructs can show in vivo osteogenic capability as evidenced by new bone formation after in vivo implantation [3–5]. Therefore, the tissue-engineered construct fabricated by cultured osteoblasts on a scaffold could be defined as *regenerative cultured bone tissue* [6]. Regenerative cultured bone tissue has already been applied clinically to patients such as in bone tumor cases. Thus, MSC cultured on various ceramics, in particular HA ceramics, has clinical significance. However, due to the non-transparency of the ordinary hydroxyapatite ceramics, analysis of the cellular mechanisms that lead to in vitro bone formation is obscured.

In order to clarify the mechanisms, we tried to use novel materials for cell culture, namely, transparent hydroxyapatite (tHA) ceramics that were made by the

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spark plasma sintering process (SPS). SPS is a new process that enables sintering of materials in short periods by charging the spaces between powder particles with electrical energy and using a high sintering pressure. SPS systems offer many advantages (e.g. rapid sintering, sintering with fewer additives, uniform sintering, low operating expense, easy operation) over conventional systems using hot press (HP) sintering, hot isostatic pressing (HIP), or atmospheric furnaces. The SPS process can be applied to many materials including fine ceramics.

In this report, we have applied cell culture to tHA ceramics. Recently, enhanced green fluorescent protein (EGFP)-expressing transgenic rat has become available; the green fluorescent protein is responsible for the green bioluminescence of the jellyfish, *Aequorea victoria*. The luminescence has caused researchers to use the gene of the protein for real-time bio-imaging monitoring. The cells we used were cultured mesenchymal stem cells derived from the EGFP-expressing transgenic rat bone marrow. As described, because the EGFP-expressing gene affects both fresh as well as cultured cells, the cells fluoresce in green under UV light. This enables clear observation of the shape of cultured cells by using fluorescence microscopy. In this paper, the MSC derived from EGFP transgenic rat were cultured on tHA ceramics as well as on a polystyrene culture dish and were observed under light and fluorescence microscopy. Osteogenic differentiation of the MSC resulting in in vitro bone formation was analyzed both morphologically and biochemically.

2. Materials and methods

2.1. Preparation of ceramics

We previously reported the detailed method of making transparent hydroxyapatite (tHA) ceramics [7]. Briefly, a fine powder of HA (high-purity grade) was utilized as the basic material. One gram of this powder was poured into a graphite mold (inner diameter: 15 mm), then sintered by the spark plasma sintering process (SPS: Dr Sinter-511S, Sumitomo Coal Mining, Tokyo, Japan). The samples were pressed uniaxially under 10 MPa, then heated at 800°C, 900°C, and 1000°C for 10 min at a heating rate of 25°C/min. Each ceramic disk was 5 mm in diameter × 2 mm thick. The ceramic samples were finely polished with a paste containing fine Al_2O_3 particles smaller than 0.5 mm and autoclaved for sterilization at 120°C for 20 min. The microstructure was examined using a scanning electron microscope (SEM). A Fourier transform infrared spectrometer (FT-IR) and X-ray diffractometer (XRD) were also applied for characterization.

2.2. Surface characterization

The sessile contact angles (SCA) of the tHA ceramics and TCPS dishes were determined using Milli-Q water and a goniometer (Face Contact-Angle Meter, Kyowa Kaimenkagaku Co. Ltd., Tokyo, Japan). A probability (p)-value of less than 0.05 was considered significant. To evaluate the surface structures of the materials, a scanning electron microscope (SM-300, TOPCON CORPORATION) was used to analyze the surface of each material. Prior to being placed in the test chamber, the specimens were coated with 200 Å-thick platinum to prevent charging.

2.3. Culture methods

Rat bone marrow cell plugs were obtained from 7-week-old female Sprague–Dawley (SD) rats that were EGFP transgenic rats ("green rat CZ-004", SD TgN (act-EGFP) OsbCZ-004; Japan SCL Inc., Shizuoka, Japan). The EGFP absorbs light in the UV-blue region (400 nm), and emits light in the green (510 nm), facilitating observation of the shape of the transgenic cells using fluorescence microscopy. Preparation and osteogenic differentiation of MSC from the rat were described by Maniatopoulos et al. [8] and modified by Ohgushi et al. [9]. In brief, rat bone marrow cells were flushed out by a culture medium, minimum essential medium (MEM, Nacalai Tesque Inc., Kyoto, Japan) containing 15% fetal bovine serum (FBS, JRH Biosciences Inc., KS, USA) and 1% antibiotics. These bone marrow cells were cultured in a humidified atmosphere of 95% air with 5% CO_2 at 37°C. The adherent mesenchymal stem cells (MSC) from rat bone marrow were initially cultured up to 80% confluence in T-75 flasks (Becton, Dickinson and Company (BD), NJ, USA) and resuspended to 5×10^5 cells/ml in culture medium following harvesting using 0.05% trypsin/ 0.53 mM EDTA. The cell suspension was applied to sterilized, transparent hydroxyapatite (tHA) ceramic disks (5 mm in diameter × 3 mm thick), which were placed into a 24-well plate, and on TCPS dishes (ϕ35 mm, BD) as a control. These cells were cultured with osteogenic medium containing 10 nM dexamethasone (Sigma-Aldrich Corporation, MO, USA), 10 mM β-glycerophosphate (Merck, Darmstadt, Germany) and 0.28 mM ascorbic acid two-phosphate magnesium salt n-hydrate (Sigma-Aldrich Corp.). The culture medium was changed two or three times per week. During the culture period, the cell morphologies were detected by phase-contrast and fluorescence microscopy.

2.4. Alkaline phosphatase (ALP) activity staining

The cultured cells on the tHA ceramics were rinsed with phosphate buffer saline (PBS) and fixed with 4%

paraformaldehyde for 10 min at 4°C. The fixed cells were soaked in 0.1% naphthol AS-MX phosphate and 0.1% fast red violet LB salt in 56 mM 2-amino-2-methyl-1,3-propanediol (pH 9.9) for 10 min at room temperature and washed with PBS [9].

2.5. Calcium staining by Alizarin Red S

The cultured cells on the ceramics were washed with PBS and fixed with 4% paraformaldehyde for 10 min at 4°C. The fixed cells were soaked in 0.5% Alizarin red S/PBS for 10 min at room temperature and washed with PBS [9].

3. Results

The tissue culture polystyrene (TCPS) dish, which is fabricated from crystal grade polystyrene by a vacuum-gas plasma treatment, shows a slightly hydrophilic nature since the plasma treatment introduces carboxyl and hydroxyl groups onto the surfaces. Under typical culture conditions employing TCPS dishes, a variety of types of cultured cells are able to attach and proliferate on the surfaces, while cells could hardly attach to bare polystyrene because of the high degree of hydrophobicity of the surfaces. Therefore, TCPS is known to be gold standard substratum for cell cultivation. We first analyzed the attachment and spreading of rat mesenchymal stem cells (MSC) on both TCPS and tHA ceramics during early culture periods and then investigated the capacity of MSC for osteogenic differentiation on both substrata during the later stages of culture.

Fig. 1a and b show the X-ray diffraction (XRD) pattern and the Fourier transform infrared spectra (FT-IR) of a spark plasma sintering (SPS) specimen of tHA, respectively. The peaks in both figures clearly show that the specimen we made is typical hydroxyapatite.

It is well known that the roughness and wettability of culture substrata influences cell proliferation and attachment. Therefore, we used scanning electron microscopy (SEM) to analyze the surface of transparent hydroxyapatite (tHA) ceramics before the culture. Although the surfaces of the tHA ceramics were slightly rough compared with the surface of TCPS (Fig. 2b), the grain structure of the surface of the sintered tHA ceramic was fine (less than 1 μm) (Fig. 2a). We also investigated the wettability of tHA ceramics and TCPS, which is represented by sessile contact angles (SCA) (Table 1). The SCA of the tHA ceramics and the TCPS showed no significant difference. The results indicate that the surface configuration is not an important factor in comparing cellular responses on tHA ceramics to that on TCPS.

As tHA ceramic material is highly transparent, the cells cultured on tHA ceramics could be observed by

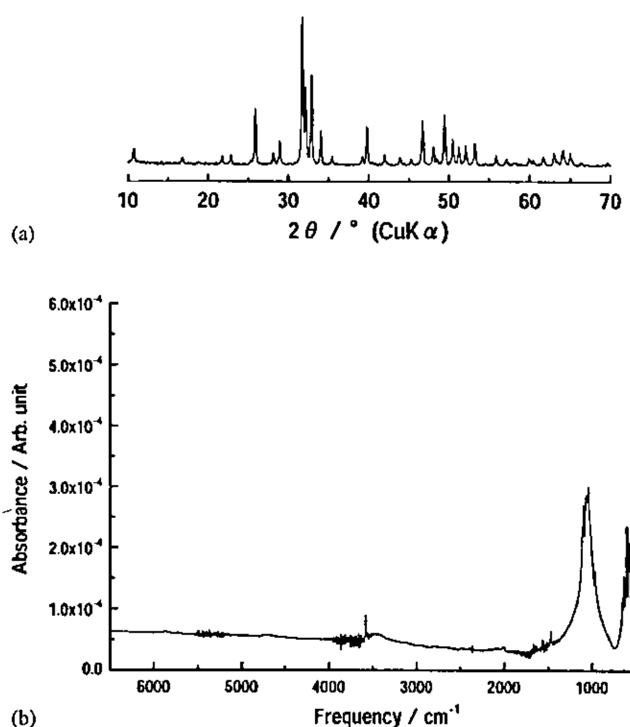

Fig. 1. Powder X-ray diffraction (XRD) pattern (a) and Fourier transform infrared spectra (FT-IR) (b) of transparent hydroxyapatite (tHA) ceramics.

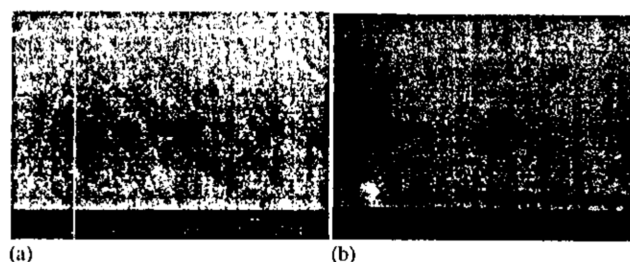

Fig. 2. SEM image of transparent hydroxyapatite (a) and tissue culture polystyrene (TCPS) dish (b). (Original magnification: 5000 ×). Bar: 1 μm. The surface of TCPS dish is very smooth and therefore most of SEM photos were out of focus. To bring the object into focus, we took a photograph of the dish contaminated with very small dust (shown at the lower left) as a pointer.

Table 1
Sessile contact angle (SCA)

	SCA(°) ± SD
tHA	73.13 ± 2.36
TCPS dish	71.00 ± 3.42

Data represent the mean value of eight samples with standard deviation (SD).

phase-contrast and fluorescence microscopy. The shapes of the cultured cells on the tHA ceramics were clearly detected by phase-contrast microscopy; the resolution of the microscopy was almost the same as on the TCPS.

These results indicate that ordinary cells without fluorescent characteristics can be monitored even when the cells are cultured on HA ceramics. To our surprise, at a very early stage of the culture (1 h after cell seeding), many MSC had already attached and showed initial spreading, as evidenced by small round cells on the tHA ceramics (Fig. 3a and b). The attachment and spreading were more obvious 5 h after the seeding (Fig. 3e and f). Most of the cells were able to attach and exhibit the morphological characteristics of mesenchymal types (spindle cell morphology) 1 day after the seeding (Fig. 3i and j). The cascade of the cellular response on the tHA ceramics was similar to that of the cells seeded on the TCPS (Fig. 3c, d, g, h, k and l). It is thus suggested that MSC can attach, spread, and proliferate on tHA ceramics as well as on TCPS.

In order to analyze the osteogenic differentiation of MSC on tHA ceramics, the MSC were cultured on both tHA ceramics and TCPS in the presence or absence of dexamethasone (Dex) for 14 days, and cell morphology was observed by fluorescence and phase-contrast microscopy. Dex is well known as an osteogenic factor and cells were cultured in the presence of β-glycerophosphate, ascorbic acid, and Dex (Dex (+)). The cells differentiated into bone-forming osteoblasts, which fabricate an extracellular mineralized matrix around a clump of the cultured cells. As shown in Fig. 4a–d, the MSC differentiated into osteoblasts and fabricated a

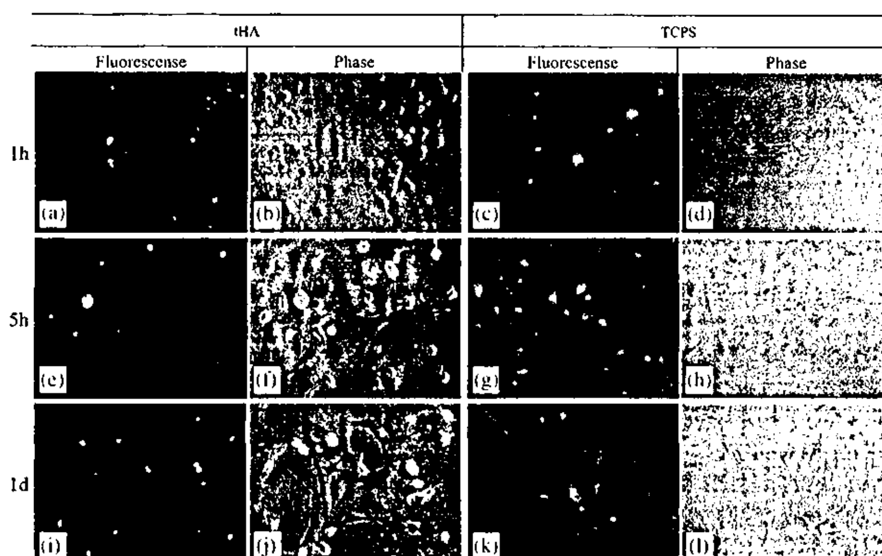

Fig. 3. Cell morphology of rat mesenchymal stem cells (MSC) seeded on transparent hydroxyapatite (tHA) ceramics and tissue culture polystyrene dishes (TCPS). The cells seeded on tHA ceramics are seen in (a), (b), (e), (f), (i) and (j). The cells seeded on TCPS are seen in (c), (d), (g), (h), (k), (l). The cell morphology was observed at 1 h, 5 h and 1 d after MSC were seeded on TCPS dishes and tHA ceramics by fluorescent (a), (c), (e), (g), (i), (k) and phase-contrast (b), (d), (f), (h), (j), (l) microscopy. After 1 h, the cell shape was round (a–d) but after 5 h, the cells began to spread on the surface (e)-(h). Cells on both substrata could spread completely; most cells had a fibroblastic shape after 1 d (i–l). (Original magnification: 200 ×).

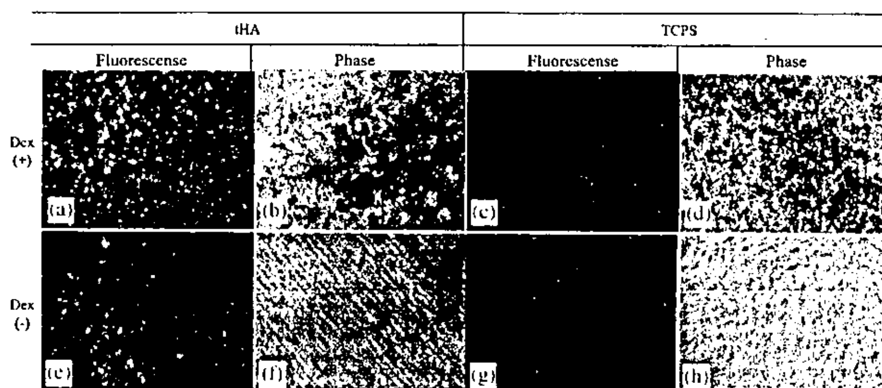

Fig. 4. Cell morphology of rat mesenchymal stem cells (MSC) seeded on transparent hydroxyapatite (tHA) (a, b, e, f) ceramics and tissue culture polystyrene (TCPS) (c, d, g, h) dishes in the presence (+) or absence (-) of dexamethasone (Dex) after day 14 of cultivation. The Dex-treated rMSC differentiated into osteoblasts and formed minerals (asterisk) on those surfaces (a–d) while the MSC not treated with Dex were able to proliferate but not differentiate into osteoblasts (e)–(h). The cell shape can easily be recognized by fluorescent microscopy even after the cells formed minerals (a), (c). (Original magnification: 100 ×).