# Cell adhesion to substratum and activation of tyrosine kinases are essentially required for G1/S phase transition in BALB/c 3T3 fibroblasts

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## Abstract

Cell adhesion to substratum and activation of tyrosine kinases are essential for the progression of cell cycle through G1 phase in mammalian cells. The kinetic studies of mouse BALB/c 3T3 fibroblasts showed that serum was no longer required for the progression of G1/S phase transition. In contrast, cell adhesion was essentially required in late G1 phase, especially at the period of G1/S transition. Among the kinase inhibitors used to elucidate the signal transduction caused by cell adhesion, tyrosine kinase inhibitors, genistein and herbimycin A, blocked the G1/S transition most effectively when cells were exposed to the inhibitors at the period of G1/S transition. Cell adhesion was not critically required for cells to undergo DNA synthesis once they had passed the G1/S boundary, and the effects of tyrosine kinase inhibitors on the progression of S phase were also not critical. The expressions of histone H2B and dihydrofolate reductase (DHFR) genes (S phase specific genes) and also the transcription factor E2F-1 gene (an activator of DHFR gene) were suppressed when cells were cultured without adhesion or exposed to the tyrosine kinase inhibitors. These results suggest that cell adhesion to substratum plays an important role in the G1/S phase transition of mouse BALB/c 3T3 fibroblasts through the activation of tyrosine kinases other than growth factor receptor-tyrosine kinases.

## 1. Introduction

Both growth factors in serum and cell adhesion to extracellular substratum are the essential factors for the proliferation of fibroblasts in culture. Growth factors are required in facilitating the fibroblast cell cycle through G1 phase up to the restriction point located in late G1 phase [1-3]. Once cells have passed the restriction point, growth factors are no longer required for cells to go through the remaining G1, S, G2, and M phases [1-4]. Intracellular protein tyrosine kinases are important for cell cycle progression, specifically for growth factor-mediated signal

transduction. The activation of growth factor receptor-tyrosine kinases is an initial step in the action of growth factors. The receptor tyrosine kinases autophosphorylate the receptors, which triggers the interaction of Grb2, mSos, and ras proteins through their SH2/3 domains, followed by activation of raf-1, MAP kinase kinase, and MAP kinase [5,6]. And it is also reported that src family protein tyrosine kinases are required for some growth factors to transmit mitogenic responses in their association with growth factor-mediated signal transduction pathways [7].

The requirement of cell adhesion for the growth of non-transformed cells in culture has been known as anchorage dependency for normal cell proliferation [8,9]. Fibroblasts in suspension culture can go through S, G2, and M phases, but they cannot enter into S phase from G1 [4,8]. Recently it has been reported that cell adhesion is required specifically for the G1/S transition of fibroblast cell cycle by affecting the cyclin A expression [10,11]. It is also suggested that tyrosine kinases play an important role in integrin-mediated signal transduction. Integrins located

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in focal adhesion plaques interact with extracellular matrices, followed by the activation of focal adhesion kinase (pp125[FAK], FadK) by the phosphorylation of its tyrosine residues [12,13]. The tyrosine-phosphorylated FAK binds Grb2 and mSos through their SH2/3 domains and leads to the activation of MAP kinase [14]. Src family tyrosine kinases can also phosphorylate FAK on its tyrosine residues, and associate with FAK through their SH2 domains [13,15]. Nevertheless, there is no evidence as to whether integrin-mediated activation of tyrosine kinases is essential for the G1/S phase transition or not.

Given these circumstances, we investigated the requirements for cell adhesion and activation of tyrosine kinases in G1 phase for the G1/S phase transition by kinetic studies. Our results suggest that cell adhesion is required for the G1/S phase transition through activating tyrosine kinases other than growth factor receptor-tyrosine kinases. And we discuss the possible regulatory mechanisms of cell adhesion and tyrosine kinase activation on the S phase entry.

## 2. Materials and methods

### 2.1. Materials

Poly(HEMA) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Genistein, daidzein, herbimycin A, 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7), and N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide (A-3) were purchased from Funakoshi Chemical Co. (Tokyo, Japan). Aphidicolin was from Wako Pure Chemical Industries (Osaka, Japan). [6-$^3$H]Thymidine, [$\alpha$-$^{32}$P]dCTP, and NCS tissue solubilizer were from Amersham Japan (Tokyo, Japan). The random primer DNA labeling kit was from Takara Shuzo Co. (Kyoto, Japan). H-7 and A-3 were dissolved in distilled water, and genistein, daidzein, and herbimycin A were dissolved in DMSO.

### 2.2. Cell culture

Mouse BALB/c 3T3 fibroblast cells (clone A31) were obtained from the Japanese Cancer Research Resources Bank. New cultures were started from the frozen stock once each month to avoid the use of phenotypically changed cells. Cells were cultured with DME/F12 medium supplemented with 200 units/ml penicillin G, 0.1 mg/ml streptomycin sulfate, and 10% FCS and incubated at 37°C in a humidified atmosphere of 5% $CO_2$. To synchronize the cells to quiescent state, cells ($1 \times 10^5$ cells in a 60-mm culture dish or $3 \times 10^5$ cells in a 90-mm culture dish) were cultured for 4–24 h in the medium containing 10% FCS after plating, and then medium was changed to a medium containing 0.3–0.4% FCS, followed by culture for 72 h. To advance cell cycle from quiescent to S phase, quiescent cells were stimulated with 10–15% FCS (10% and 15%

FCS showed the same effect for promoting the cell growth). To culture the cells without adhesion, monolayer cells were detached by trypsinization (0.125% trypsin/0.02% EDTA for 3–5 min at 37°C) and then cultured on plastic dishes entirely coated with poly(HEMA) [4,9]. To prepare the poly(HEMA)-coated dishes, poly(HEMA) was dissolved with 85% ethanol and 15% 1 M Tris-HCl (pH 7.5), and the solution was poured onto plastic culture dishes (poly(HEMA) 2 mg/60-mm dish or 5 mg/90-mm dish). The dishes were evenly coated with poly(HEMA) in the oven with occasional agitation and sterilized by ultraviolet exposure. The coated dishes were rinsed once with PBS before use. The cells cultured in the poly(HEMA)-coated dishes were incapable of forming adhesion plaques and stayed in a round form. The poly(HEMA) itself showed no cytotoxic or cytostatic effects under our experimental conditions so far examined (data not shown). To deprive the serum of the culture medium, the medium containing 10% FCS was changed to the medium containing 0.3–0.4% FCS previously used for preparing quiescent cells. To synchronize the cells at G1/S boundary, aphidicolin (an inhibitor for DNA polymerase-$\alpha$) was added to the culture medium (5 $\mu$g/ml) at 7 h after 10% serum stimulation of quiescent cells and cultured for an additional 17 h [4]. Then, aphidicolin was removed (the medium was changed to the medium containing 10% FCS without aphidicolin) to synchronously start S phase. When cells were treated with genistein, daidzein, and herbimycin A, the cells in the control dishes were treated with the same amount of DMSO as that in the reagents. Viable cell numbers were counted by the 0.15% trypan blue exclusion method with the use of a hemocytometer.

### 2.3. DNA synthesis

The level of DNA synthesis was measured by [6-$^3$H]thymidine incorporation into acid-insoluble materials. Cells were cultured with the medium containing [6-$^3$H]thymidine (0.5 $\mu$Ci/ml, 0.33 Ci/mmol) and 2 $\mu$M of cold thymidine for indicated durations. The difference of specific activity of thymidine between the medium containing 10% serum and serum-free medium was negligible. When cells were harvested, viable cell number in the small aliquot was counted after trypsinization, and the cells in another aliquot were fixed with 7% trichloroacetic acid. The acid-insoluble materials were collected on glass filters. The filters were incubated with NCS tissue solubilizer at 50°C for 16 h, and the radioactivities in the filters were counted with a liquid scintillation counter (Packard Tri-Carb 4530).

### 2.4. Northern blot analysis

The isolation of total cellular RNAs and Northern blot analysis were performed according to the method de-

scribed by Sambrook et al. [16]. Monolayer cultured cells were washed twice with ice-cold PBS and harvested with ice-cold 10 mM EDTA (pH 8.0), 0.5% SDS, followed by the addition of ice-cold 0.1 M sodium acetate (pH 5.2), 10 mM EDTA (pH 8.0). Then RNAs were extracted with water-saturated phenol. The cells cultured in poly(HEMA)-coated dishes were collected by low-speed centrifugation at 4°C, washed once with ice-cold PBS, and then harvested with the same procedures described above. Extracted RNAs were precipitated with ethanol and quantitated by spectrophotometer. Each 7 $\mu$g of total cellular RNA was electrophoresed through 1% agarose gel containing 6% formaldehyde. The RNA was completely transferred to a nylon membrane filter by capillary action. The filter was hybridized with a $^{32}$P-labeled DNA probe in a solution of 50% formamide, 5 × SSPE, 5 × Denhardts' solution, 0.1% SDS, and 20 $\mu$g/ml herring sperm DNA at 42°C for 16 h, followed by washing with 0.1 × SSC, 0.1% SDS at 50°C and then autoradiographed. DNA probes (spec. act. 1–2 × 10$^9$ cpm/$\mu$g DNA) were radiolabeled using the random primer DNA labeling kit with [$\alpha$-$^{32}$P]dCTP. DNA probes used are as follows: chicken histone H2B gene, pKR1a-1.3. [17]; human DHFR pseudo-1 gene, CHB201 [18]; mouse E2F-1 cDNA, pcDNA3-mE2F-1 [19]; rat GAPDH cDNA, pSPGAPDH [20]; and rat ribosomal protein L35a cDNA, pRL35a [21]. The DNA probes were sequentially hybridized to RNAs on the same filter after the removal of previously hybridized probes.
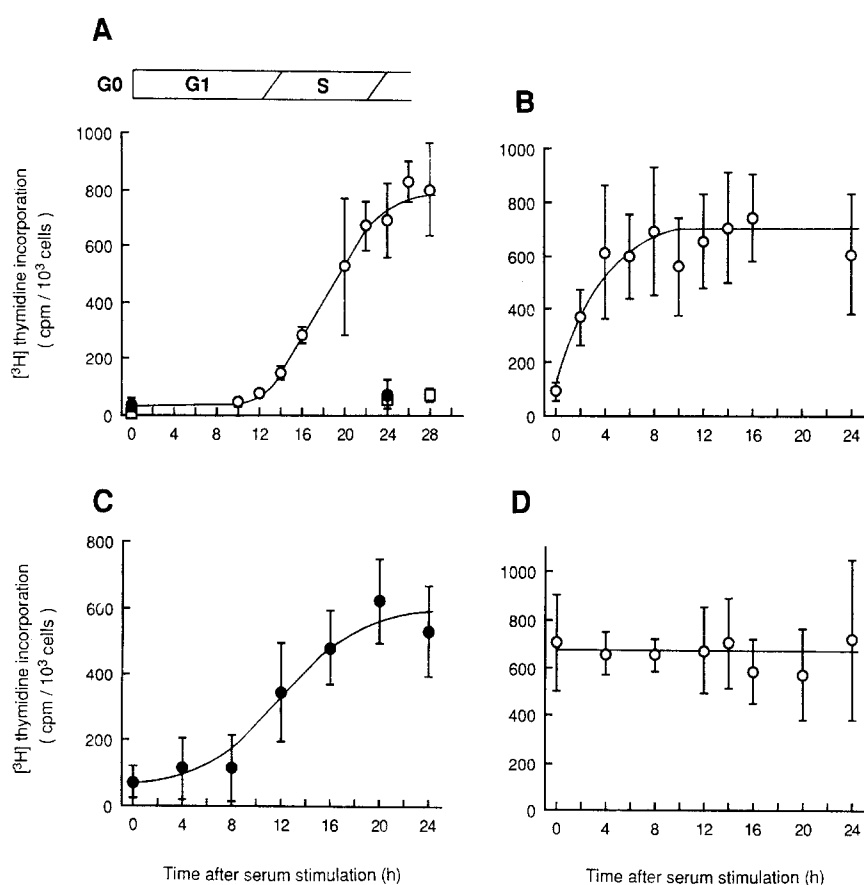


Fig. 1. Serum is required in early G1 phase, and cell adhesion is required at the G1/S phase transition. (A) Serum and cell adhesion are required for the progression of G1 phase. ○, time course of DNA synthesis after serum stimulation. Quiescent BALB/c 3T3 cells in monolayer culture were stimulated with 15% FCS. [$^3$H]Thymidine was added to the medium at the same time that 15% serum was introduced to the cultures. Cells were cultured in the medium containing 10% FCS and [$^3$H]thymidine until each time point when DNA synthesis was measured. ●, quiescent monolayer cells were detached by trypsinization and transferred to poly(HEMA)-coated dishes, and then stimulated with 15% FCS. □, quiescent monolayer cells were not stimulated with 15% FCS. (B) Serum is required in early G1 phase and no longer required at the G1/S transition. Quiescent cells were stimulated with 10% FCS. [$^3$H]Thymidine was added to the medium at the same time that 10% serum was introduced to the cultures. The medium was changed to the serum-deprived medium at each serum-deprivation time point. The cells were cultured in the serum-deprived medium containing [$^3$H]thymidine. DNA synthesis was measured at 24 h point after serum stimulation. The level of DNA synthesis was plotted at each serum-deprivation time point. (C) Cell adhesion is required at the G1/S phase transition. Quiescent monolayer cells were stimulated with 10% FCS. [$^3$H]Thymidine was added to the medium at the same time that 10% serum was introduced to the cultures. Cells were detached by trypsinization at each detachment time point and then cultured without adhesion in poly(HEMA)-coated dishes in the medium containing 10% FCS and [$^3$H]thymidine. DNA synthesis was measured at 24 h point after serum stimulation. The level of DNA synthesis was plotted at each detachment time point. (D) Trypsinization itself has no effect on DNA synthesis. The same procedures described in (C) were performed in this experiment, except that cells were detached by trypsinization at each time examined and immediately re-attached in ordinary culture dishes. All values in the graphs represent means, and T bars represent standard deviations ($n = 6$–12).

## 3. Results

### 3.1. Growth factors in serum are no longer required for the G1 / S phase transition

Mouse BALB/c 3T3 fibroblast cells were synchronized to quiescent state (G0 phase) by the culture in the medium containing 0.3–0.4% FCS for 72 h. When quiescent cells were stimulated with 10–15% FCS, it was necessary for growth factors in serum to act for growth promotion by activating the growth factor receptor-tyrosine kinases. The serum-stimulated cells advanced the cell cycle into S phase with a gap-period (G1 phase) for 12–14 h, and S phase continued for 9–10 h (Fig. 1A, O). The immunohisto-chemical staining for newly synthesized DNA showed that most cells entered into S phase synchronously (data not shown). According to the time course profile of DNA synthesis, the period of G1/S phase transition approximately corresponded to the period of 10–14 h after serum stimulation. If the quiescent cells were not stimulated by serum, cells could not go into S phase (Fig. 1A, □). To determine the period when growth factors exert their effects on the cell cycle, we performed the kinetic experiment for serum action, in which experiment DNA synthesis was measured when cells were exposed to 10% serum for various durations after serum stimulation in the G1 and S phases (Fig. 1B). For example, the level of DNA synthesis of the cells which were exposed to serum for 0–6 h after serum stimulation is shown at 6 h in the graph (the fourth plot from the left in Fig. 1B). The graph clearly shows that the cells demand serum for the progression into S phase for 0–8 h (mostly 0–4 h) after serum stimulation. The result indicates that growth factors in serum are

required in early G1 phase and no longer required at the period of G1/S transition.

### 3.2. Cell adhesion is required in late G1 phase, especially at the period of G1 / S transition

The cells cultured without adhesion in poly(HEMA)-coated dishes could not go into S phase even though they were stimulated by serum (Fig. 1A, ●), which means that cell adhesion to substratum as well as serum is essential for the progression of G1 phase. To determine the period when cell adhesion is required for progression on the cell cycle, we performed a kinetic experiment to determine the requirement of cell adhesion in the G1 and S phases. The experiment was similar to that to determine the requirement of serum. In this experiment, DNA synthesis was measured when cells were cultured with adhesion in ordinary culture dishes for various durations after serum stimulation in the G1 and S phases in the presence of 10% serum (Fig. 1C). For example, the level of DNA synthesis of the cells which were cultured with adhesion for 0–8 h after serum stimulation is shown at 8 h in the graph (the third plot from the left in Fig. 1C). The result was much different from that in Fig. 1B. Contrary to the fact that serum was no longer required at the G1/S phase transition, adhesion was certainly required in late G1 phase, especially at the period of G1/S transition (for 8–14 h after serum stimulation). Since it seemed possible that trypsinization might have some artificial effects in this experiment, we performed a control experiment for Fig. 1C in which the cells were re-attached to ordinary culture dishes immediately after the detachment by trypsinization (Fig. 1D). The result showed that the level of DNA
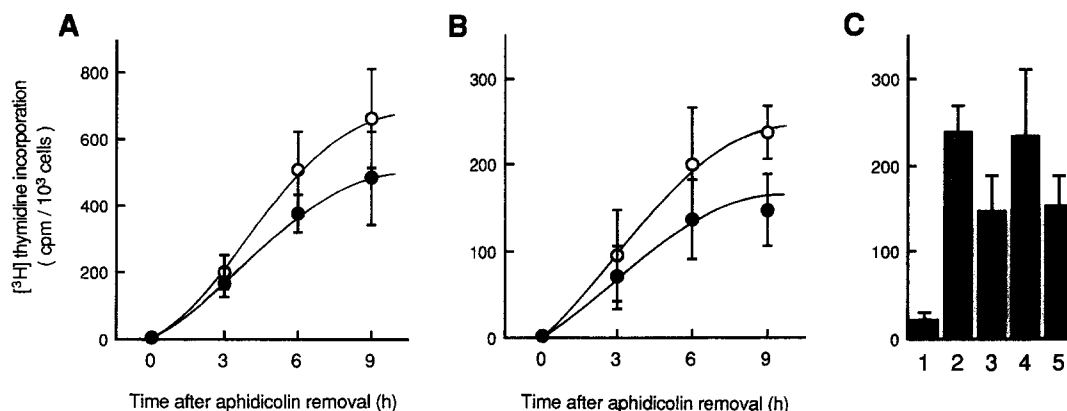


Fig. 2. Cell adhesion and tyrosine kinase inhibitors have no critical effect on S phase progression. BALB/c 3T3 cells were synchronized at G1/S boundary with aphidicolin, and then the aphidicolin was removed to start S phase. (A) Effect of cell adhesion. O, the cells cultured in monolayer in ordinary culture dishes during S phase; ●, the cells cultured without adhesion in poly(HEMA)-coated dishes during S phase. The synchronized cells were detached and transferred to poly(HEMA)-coated dishes filled with 10% FCS without aphidicolin. (B) Effect of genistein. O, the cells cultured in monolayer during S phase in a medium containing DMSO; ●, the cells cultured in monolayer during S phase in a medium containing genistein (15 μg/ml). DMSO and genistein were added to the medium immediately after aphidicolin removal. (C) Effect of herbimycin A. 1, aphidicolin was not removed from the medium. 2–5, aphidicolin was removed and the following reagents were added: 2, DMSO; 3, genistein (15 μg/ml); 4, daidzein (15 μg/ml); 5, herbimycin A (0.2 μg/ml). [³H]Thymidine was added to the medium immediately after removal of aphidicolin, and DNA synthesis was measured at each time point examined (A, B) or at 9 h (C) after aphidicolin removal. All values in these graphs represent means, and T bars represent standard deviations (n = 6–8).

synthesis and cell cycle progression were not affected by the trypsinization procedure.

Furthermore, we examined whether cell adhesion is required to facilitate the S phase progression (Fig. 2A). The cells were synchronously started from G1/S boundary through S phase by removal of aphidicolin from the culture medium. When cells were cultured without adhesion during S phase, the level of DNA synthesis was suppressed to 75–80% of the levels of the control (samples 6 h and 9 h in Fig. 2A; $P < 0.05$, compared to the level of the control; $t$-test). However, when cells were cultured without adhesion from 6 h after serum stimulation, the level of DNA synthesis was suppressed to 10% of the level of the control (Fig. 1C). Therefore, we concluded that cell adhesion in S phase was not critically required for undergoing DNA synthesis. These results indicate that cell adhesion plays an important role in late G1 phase, especially at the period of G1/S transition.

### 3.3. The protein tyrosine kinase inhibitors, genistein and herbimycin A, block the G1 / S phase transition

To investigate the necessity of tyrosine kinases other than growth factor receptor-tyrosine kinases at the period of G1/S transition, we examined the effects of the protein tyrosine kinase inhibitors, genistein and herbimycin A. The cells cultured with serum-free medium from 6 h after serum stimulation were treated with the inhibitors for 6–24 h after serum stimulation (late G1 and S phases). The levels of DNA synthesis in the cells treated with the inhibitors were compared to those in control cells (Fig. 3). Genistein is a member of isoflavone and inhibits tyrosine kinase activity by competing for ATP [22]. Daidzein, which is structurally similar to genistein, but has no inhibitory effect on tyrosine kinases, was used as a control. It was shown that DNA synthesis was suppressed by genistein in a dose-dependent manner, but not by daidzein (Fig. 3A) (samples 5, 6, and 7 in Fig. 3A; $P < 0.01$, compared to the level of sample 3; $t$-test). The effective concentration of genistein was consistent with that of the reported one (half maximal dose for inhibition of tyrosine kinase activities = 6–8 $\mu$g/ml). Herbimycin A, another tyrosine kinase inhibitor, is a benzoquinonoid ansamycin antibiotic and inhibits the activity of tyrosine kinases by its binding to the reactive SH groups essential for the activity [23]. Therefore, sulfhydryl compounds, such as $\beta$-mercaptoethanol, are able to cancel the effect of herbimycin A. Again, it was shown that herbimycin A markedly suppressed DNA synthesis of the cells and its effect was cancelled by $\beta$-mercaptoethanol (Fig. 3B) (samples 4, 5, 6, and 7 in Fig. 3B; $P < 0.01$, compared to the level of sample 3; $t$-test). By checking trypan blue exclusion activity of the cells and the levels of ribosomal protein L35a and GAPDH gene expression (Fig. 5), we confirmed that genistein and herbimycin A had no cytotoxic effect at the concentrations used in this experiment. Furthermore, we examined the effects of two serine/threonine protein ki-

nase inhibitors, H-7 and A-3, on DNA synthesis. H-7, an isoquinolinesulfonamide derivative, is a specific inhibitor of cAMP- and cGMP-dependent protein kinases and protein kinase C and is effective at low concentrations ($K_i = 3.0$–$6.0$ $\mu$M) [24]. A-3, a naphthalene-sulfonamide deriva-
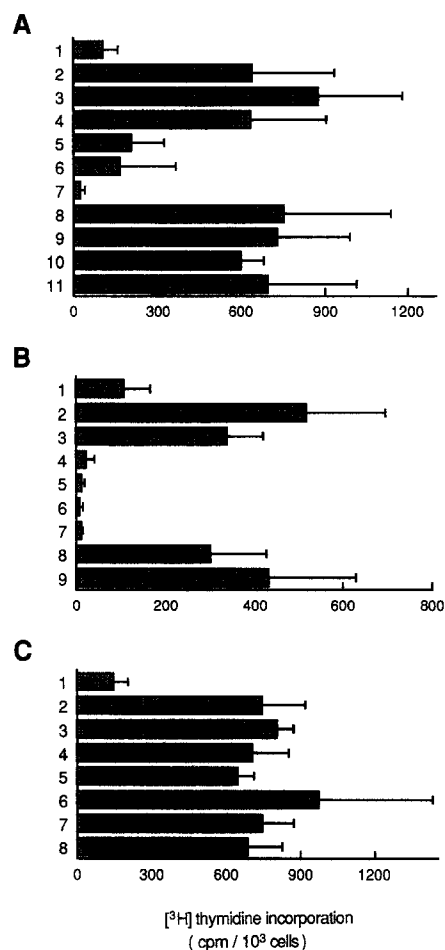


Fig. 3. Tyrosine kinase inhibitors block the G1/S phase transition. Quiescent cells were stimulated with 10% FCS, and then serum was removed at 6 h after serum stimulation. The cells were cultured until 24 h after serum stimulation in a serum-deprived medium. The reagents and [³H]thymidine were added to the serum-deprived medium immediately after serum deprivation (at 6 h). DNA synthesis was measured at 24 h after serum stimulation. (A) Effect of genistein. 1, quiescent cells (not stimulated with serum); 2, serum-stimulated cells with no reagents. 3–11, serum-stimulated cells administered with the following reagents: 3, DMSO; 4, genistein (2 $\mu$g/ml); 5, genistein (5 $\mu$g/ml); 6, genistein (10 $\mu$g/ml); 7, genistein (15 $\mu$g/ml); 8, daidzein (2 $\mu$g/ml); 9, daidzein (5 $\mu$g/ml); 10, daidzein (10 $\mu$g/ml); 11, daidzein (15 $\mu$g/ml). (B) Effect of herbimycin A. 1, quiescent cells (not stimulated with serum); 2, serum-stimulated cells with no reagents. 3–9, serum-stimulated cells administered with the following reagents: 3, DMSO; 4, herbimycin A (0.02 $\mu$g/ml); 5, herbimycin A (0.05 $\mu$g/ml); 6, herbimycin A (0.1 $\mu$g/ml); 7, herbimycin A (0.2 $\mu$g/ml); 8, herbimycin A (0.1 $\mu$g/ml)+ $\beta$-mercaptoethanol (20 $\mu$M); 9, herbimycin A (0.1 $\mu$g/ml)+ $\beta$-mercaptoethanol (60 $\mu$M). (C) Effects of H-7 and A-3. 1, quiescent cells (not stimulated with serum); 2, serum-stimulated cells with no reagents. 3–8, serum-stimulated cells administered with the following reagents: 3, H-7 (5 $\mu$M); 4, H-7 (10 $\mu$M); 5, H-7 (20 $\mu$M); 6, A-3 (5 $\mu$M); 7, A-3 (10 $\mu$M); 8, A-3 (20 $\mu$M). Shaded bars represent means, and T bars represent standard deviations ($n = 6$–12).

tive, also inhibits the activities of cAMP- and cGMP-dependent protein kinases, myosin light chain kinase, and casein kinase II at low concentrations ($K_i$ = 3.8–7.4 $\mu$M) [25]. The result showed that both H-7 and A-3 had no significant effect on DNA synthesis even though the concentration used was as high as 20 $\mu$M (Fig. 3C).

The requirement of tyrosine kinases for S phase progression was examined using the inhibitors in an experiment similar to that used to investigate the requirement of cell adhesion for S phase progression (Fig. 2B). When the cells were treated with genistein (at 15 $\mu$g/ml, which suppressed DNA synthesis to less than 5% of the control when used from 6 h after serum stimulation) during S phase, DNA synthesis was suppressed to 65–70% of the levels of the control (samples 9 h in Fig. 2B; $P < 0.01$, compared to the level of the control; $t$-test). Herbimycin A (0.2 $\mu$g/ml) inhibited DNA synthesis to the same extent as genistein, but daidzein had no effect (Fig. 2C) (samples 3 and 5 in Fig. 2C; $P < 0.01$, compared to the level of sample 2; $t$-test). The result indicates that the effect of the tyrosine kinase inhibitors on S phase progression was much less than that on the G1/S phase transition. Therefore, we concluded that tyrosine kinases other than growth factor receptor-tyrosine kinases were not critically required for S phase progression but were significantly required for cells to advance the G1/S phase transition.
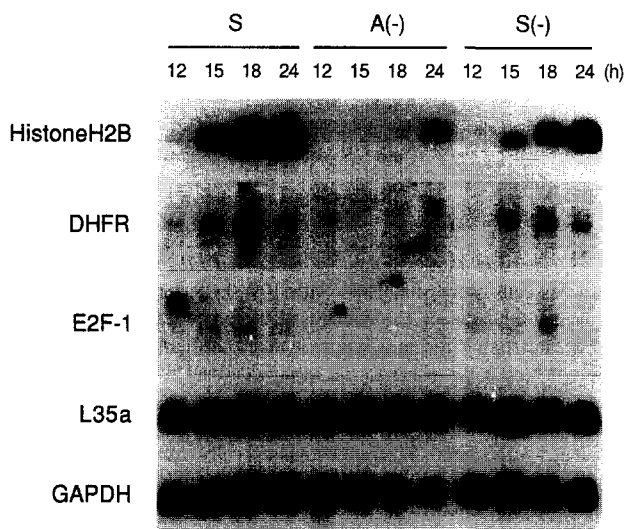


Fig. 4. Northern blot analyses of histone H2B, DHFR, and E2F-1 gene expressions in the cells cultured in poly(HEMA)-coated dishes. RNAs were isolated at each time point examined after serum stimulation and were analyzed by Northern blot analysis. S, A(−), and S(−) on the bar at the top of the figure denote the conditions under which cells were cultured: S, quiescent monolayer cells were stimulated with 10% FCS; A(−), cells were detached by trypsinization at 6 h after serum stimulation, followed by the culture in poly(HEMA)-coated dishes with 10% FCS; S(−), serum was removed at 6 h after serum stimulation, and the culture was continued without serum in the monolayer. Numerals under the bar denote the time periods (hours) after serum stimulation. Expressions of L35a ribosomal protein and GAPDH genes were examined as internal standards.
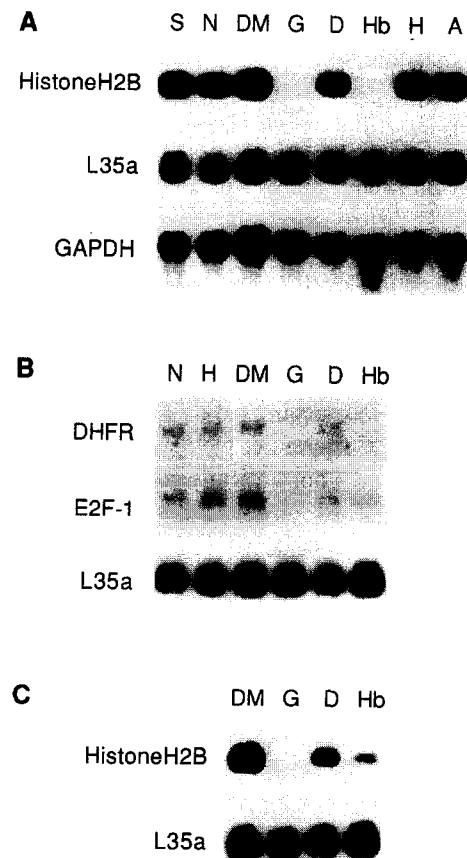


Fig. 5. Northern blot analyses of histone H2B, DHFR, and E2F-1 gene expressions in the cells treated with protein kinase inhibitors. RNAs were isolated at 18 h after serum stimulation and were analyzed by Northern blot analysis. (A) Histone H2B gene expression. Quiescent cells were stimulated with 10% FCS. Serum was removed at 6 h after serum stimulation, and the following reagents were added to the serum-deprived medium immediately after serum deprivation: N, no reagent; DM, DMSO; G, genistein (15 $\mu$g/ml); D, daidzein (15 $\mu$g/ml); Hb, herbimycin A (0.2 $\mu$g/ml); H, H-7 (20 $\mu$M); A, A-3 (20 $\mu$M); S, serum-stimulated cells in which serum was not removed. Expressions of L35a ribosomal protein and GAPDH genes were examined as internal standards. (B) DHFR and E2F-1 gene expressions. Conditions were the same as (A). (C) Histone H2B gene expression. Conditions were the same as (A), except that serum was removed at 10 h after serum stimulation and the reagents were added immediately after serum deprivation.

### 3.4. The expressions of histone H2B, DHFR, and transcription factor E2F-1 genes were suppressed in the cells cultured without adhesion and also in the cells treated with the tyrosine kinase inhibitors

To see the effects of cell adhesion and tyrosine kinase inhibitors on growth-related gene expression, we examined the expressions of histone H2B, DHFR, and transcription factor E2F-1 genes in the cells cultured in poly(HEMA)-coated dishes and treated with the tyrosine kinase inhibitors by Northern blot analysis. The mRNA levels of ribosomal protein L35a and GAPDH were also determined as internal standards. Histone H2B and DHFR genes were expressed in S phase (Fig. 4, S). Histone H2B and DHFR mRNAs hardly accumulated (only 7–10% of the level of

the control by densitometric analysis) when cells were cultured without adhesion from 6 h after serum stimulation (late G1 and S phases) (Fig. 4A( − )). In contrast, when cells were cultured without serum in the late G1 and S phases, both genes were expressed to more than 50–60% of the levels of the control (Fig. 4, S( − )). And also histone H2B and DHFR mRNAs did not accumulate when cells were treated with genistein and herbimycin A, while they accumulated when cells were treated with daidzein, H-7, and A-3 (Fig. 5A,B). Even though the cells were treated with the reagents from as late as 10 h after serum stimulation, genistein and herbimycin A significantly inhibited the histone H2B gene expression (Fig. 5C). The result in Fig. 5C suggests that the tyrosine kinase inhibitors exert their effects most effectively when they are used at the period of G1/S transition.

E2F-1 is known as an important transcription factor for the G1/S transition, as it activates transcription of DHFR gene [26]. The E2F-1 gene was induced at 12 h after serum stimulation, and its expression was the highest at 18 h (Fig. 4, S). The expression of E2F-1 gene was completely inhibited when the cells were cultured without adhesion (Fig. 4, A( − )). In contrast, its expression was not significantly changed when the cells were cultured with serum-free medium (Fig. 4, S( − )). When cells were treated with genistein or herbimycin A, the expression of E2F-1 gene was much suppressed, while H-7 and A-3 showed no significant effect on the expression of E2F-1 gene (Fig. 5B).

All results shown above suggest that the entry into S phase in mouse BALB/c 3T3 fibroblasts is regulated by cell adhesion to substratum through the activation of tyrosine kinases other than growth factor receptor-tyrosine kinases.

## 4. Discussion

In the cultured fibroblasts, progression of G1 phase is critically regulated by growth factors and cell adhesion. Generally, in late G1 phase, especially at the period of G1/S transition, there is a restriction point or a commitment point. These points are reported to be located at 2 h or 3.5 h before or just before starting S phase [1–3]. They are taken as critical points to initiate a new division cycle, and serum is required up to these points for the cells to proceed into S phase [1–4]. In this experiment, we showed that the requirement for serum was during 0–8 h (mostly in early G1 phase (0–4 h)) after serum stimulation. On the other hand, the requirement for adhesion was clearly in late G1 phase (8–14 h), especially at the period of G1/S transition. These results suggest that cell adhesion to substratum directly regulates the S phase entry at the period after the restriction point, which raises the possibility that cell adhesion activates its own signal transduction pathways different from the growth factor-mediated signal transduction pathways for the G1/S phase transition.

It has been reported that genistein and herbimycin A specifically inhibit the activities of protein tyrosine kinases. Genistein inhibits the tyrosine kinase activities of EGF receptor, pp60$^{src}$, and pp110$^{gag-fes}$ in vitro and also in vivo and does not inhibit the serine/threonine kinase activities of protein kinases A and C, phosphorylase kinase, 5'-nucleotidase, phosphodiesterase, and cdc2 kinase [22,27]. Herbimycin A inhibits tyrosine kinase activity of v-src in vitro and suppresses the transforming activity of tyrosine kinase oncogenes including src, yes, fps, ros, abl, and erbB [23]. However, the inhibitors also show some effects on the enzymes other than tyrosine-specific protein kinases. Genistein inhibits the activities of S6 kinase, DNA topoisomerases I and II, and phosphatidylinositol turnover. Herbimycin A also inhibits T-cell receptor-mediated phosphoinositide hydrolysis [22,23]. In this paper, we showed that genistein and herbimycin A blocked the G1/S transition of mouse fibroblasts. Although it is possible that the activity of the important enzymes other than tyrosine kinases are also suppressed by the inhibitors, our results strongly suggest that the activation of tyrosine kinases is required for G1/S transition. The reasons are as follows. (1) Genistein and herbimycin A inhibit the tyrosine kinase activities in completely different ways. Nevertheless, G1/S transition was blocked by both inhibitors. (2) Daidzein, whose structure is very close to that of genistein, did not block the G1/S transition. (3) Herbimycin A did not block the G1/S transition when cells were treated together with a sulfhydryl compound, β-mercaptoethanol, which is known as a compound which cancels the inhibitory effect of herbimycin A on tyrosine kinase activity. (4) Although genistein may inhibit topoisomerase II activity, the expressions of histone H2B, DHFR, and E2F-1 genes were also suppressed by genistein. (5) S6 kinase and phosphatidyl inositol turnover may also be inhibited by genistein, but these are activated immediately at early phase of G1, not at the G1/S transition.

Our observations suggest that cell adhesion plays a critical role in the G1/S phase transition through the activation of tyrosine kinases. One possible mechanism for this is in the signal transduction pathways of adhesion via adhesion plaques, where integrins are located and bind to extracellular matrix such as fibronectins and collagens. Several reports have indicated that phosphotyrosine-containing proteins are concentrated in adhesion plaques and that the binding of integrins to extracellular matrices can alter cellular tyrosine-phosphorylation patterns [28]. This is shown by the facts that focal adhesion kinase, which is localized in adhesion plaques, is tyrosine-phosphorylated by integrin-ligand interaction (caused by adhesion) and that the change in tyrosine-phosphorylation is correlated with an increase in its tyrosine kinase activity [12,13,28]. Additionally, several pieces of evidence suggest that src family tyrosine kinases are located in adhesion plaques and are involved in the signal transduction pathways from integrins [13–15]. The chemical signal

transduction from integrins through the tyrosine kinase activation of these molecules located in adhesion plaques may directly or indirectly regulate the activity of the molecules important for S phase entry.

Another possible mechanism is that the cell shape and internal cellular architecture constructed by cell adhesion support the activation of some tyrosine kinases important for the G1/S transition. It is commonly accepted that cell shapes are important for cell growth and differentiation. Some observations suggest that, in addition to chemical signaling, binding of integrins to extracellular molecules may regulate growth through some form of mechanical signaling [28]. It is reported that the binding of integrin to extracellular molecules is sufficient to induce the G0/G1 transition. However, for S phase entry, the spreading of cells is required [29]. It is not yet clear what kind of tyrosine kinases require cell adhesion for their enzymatic activation. Cdk 2 kinase plays a critical role in S phase initiation function [30]. The activity of cdk2 kinase is regulated by its binding to cyclin E/A and by the phosphorylation of its amino acid residues. Cdk 2 kinase is inactivated by phosphorylation of threonine 14 (Thr 14) and tyrosine 15 (Tyr15) in G1 phase. At the G1/S transition, cdk 2 kinase is activated by dephosphorylation of Thr14 and Tyr15 by cdc 25 phosphatase and also by phosphorylation of Thr160 by CAK kinase [31–33]. As cdk 2 kinase is inactivated by the phosphorylation of its tyrosine residues (Tyr15), it remains unclear whether or not activation of tyrosine kinases is positively required for initiation of S phase at the period of G1/S transition in respect to cdk 2 kinase activation. Further study of G1/S transition at the molecular level is needed to understand the precise regulatory mechanisms of cell adhesion in the G1/S phase transition.

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