Induction of In Vitro Differentiation of Mouse Embryonal Carcinoma (F9) and Erythroleukemia (MEL) Cells by Herbimycin A, an Inhibitor of Protein Phosphorylation

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Abstract. Herbimycin A is one of the benzenoid ansamycin antibiotics isolated from a culture of a Streptomyces species (Omura, S., A. Nakagawa, and N. Sadakane. 1979. Tetrahedron Lett. 1979: 4323–4326). Recent studies have shown that the antibiotic not only inhibits the phosphorylation of p60src in Rous sarcoma virus– (RSV) infected cells, but also reverses the cellular phenotypes acquired by transfection with tyrosine kinase oncogenes (Uehara, Y., M. Hori, T. Takeuchi, and H. Umezawa. 1985. Jpn. J. Cancer Res. 76:672–675; Uehara, Y., M. Hori, T. Takeuchi, and H. Umezawa. 1986. Mol. Cell. Biol. 6:2198–2206; Uehara, Y., Y. Murakami, S. Mizuno, and S. Kawai. 1988. Virology. 164:294–298). These studies and other evidence indicate that the antibiotic inhibits a reaction(s) closely associated with the function of cellular tyrosine kinases.

We have found that herbimycin A is an effective inducing agent capable of triggering differentiation in two typical mouse in vitro differentiation systems, which have been considered to be quite different in their mechanism of induction: endoderm differentiation of embryonal carcinoma (F9) cells and terminal erythroid differentiation of erythroleukemia (MEL) cells. The results suggest that there is a common step in the intracellular differentiation cascade which is, directly or indirectly, associated with phosphorylation at specific (tyrosine) residues of cellular proteins. The significance of this finding with respect to the molecular mechanism of in vitro differentiation is discussed.

There are a number of agents that trigger in vitro differentiation in established mammalian cell lines. Since the morphological and biochemical changes induced by such inducing agents are often quite analogous to those observed in vivo, in vitro differentiation has been used widely as a model to study the molecular mechanism of differentiation. One of the interesting but puzzling problems of in vitro differentiation is that compounds which are structurally and presumably functionally unrelated are often equally effective in inducing a specific differentiation. For example, more than a dozen compounds with a wide spectrum of structures ranging from a simple compound such as dimethyl sulfoxide (DMSO) (Friend et al., 1971) to more complex proteins like proteases (Scher et al., 1982) induce the differentiation of mouse erythroleukemia (MEL) cells (Friend et al., 1966; Marks and Rifkind, 1989) to erythroid cells. One possible explanation is that the intracellular molecular cascades leading to differentiation are quite diversified at the initial stage, but eventually converge to a single critical step (or reaction), although it may be naive to believe that such a step exists universally among all or most differentiation processes.

Herbimycin A is one of the benzenoid ansamycin antibiotics isolated from a culture of a Streptomyces species (Omura et al., 1979; Uehara et al., 1985). The antibiotic was shown to reduce the phosphorylation of p60Src in a rat kidney cell line infected with Rous sarcoma virus (RSV) (Uehara et al., 1986). Tyrosine kinase activity associated with p60Src in the antibiotic-treated cell extracts was found to be inactivated. Furthermore, herbimycin A reversed the morphological changes in chicken and mammalian cells transformed by tyrosine kinase oncogenes such as src, yes, fps, ros, abl, and erbb, but had no effect on the changes induced by nontyrosine kinase oncogenes such as ras, raf, or myc (Uehara et al., 1988; Murakami et al., 1988). These results strongly suggest that herbimycin A inhibits, or interferes with, a reaction(s) closely associated with the maintenance of functional integrity of tyrosine kinase in the cells.

To obtain a clue to the possible common reaction underlying a variety of types of in vitro differentiation, we examined the effect of herbimycin A on two typical in vitro mouse cell differentiation systems. One is that of embryonal carcinoma (F9) cells (Bernstein et al., 1973), which show retinoic acid–induced differentiation into endoderm cells (Strickland and Mahdavi, 1978) and the other is that of MEL cells (Friend et al., 1966), which undergo terminal differentiation...
into erythroid cells upon induction by DMSO (Friend et al., 1971) or hexamethylenebisacetamide (HMBA) (Reuben et al., 1976). We report here that herbinycin A is an effective inducing agent which triggers differentiation of both F9 and MEL cells. We also discuss the biological significance of the results with respect to the possible involvement of protein phosphorylation (or dephosphorylation) as a common and critical factor in vitro differentiation.

**Materials and Methods**

**Materials**

Herbinycin A was isolated as described previously (Uehara et al., 1985). Retinoic acid and dibutyryl cyclic AMP were purchased from Sigma Chemical Co. (St. Louis, MO), human plasminogen was from Green Cross Co. (Osaka, Japan), FITC-conjugated goat anti-rabbit IgG and rabbit anti-mouse IgG were from Cappel Laboratories (Malvern, PA) and skim milk, gelatin, and agar noble were from Difco Laboratories Inc. (Detroit, MI). HMBA and PMA (phorbol 12-myristate 13-acetate) were gifts from T. Yamane (Bell Laboratories, Murray Hill, NJ) and M. Terada (National Cancer Institute, Tokyo), respectively. Anti-laminin antibody was purchased from Bioscience Products AG (Emmenbrücke, Switzerland). Anti-stage-specific embryonic antigen-1 (SSEA-1) was provided by D. Solter (Wistar Institute, Philadelphia, PA) through T. Muramatsu (Kagoshima University, Kagoshima, Japan). All other agents were reagent grade. MEM, DME, and ES medium used for assaying the colony-forming ability of MEL cells, DME (OSaka, Japan) and MEL (Friend) cells (745A, DSI9) by M. Terada (National Cancer Institute, Tokyo), respectively. Anti-laminin antibody was purchased from Sigma Chemical Co. (St. Louis, MO).

**Cells and Cell Culture**

F9 cells were supplied by Y. Nishimune (Institute of Microbial Diseases, Osaka University, Osaka) and MEL (Friend) cells (745A, DS19) by M. Terada (National Cancer Center Institute, Tokyo). F9 and MEL cells were cultured at 37°C in a CO2 (5%) incubator in ES medium supplemented with FCS (10% [vol/vol]) and MEM with FCS (12% [vol/vol]), respectively. For F9 cells, plastic tissue culture dishes (Falcon Labware, Oxnard, CA) were coated with gelatin solution (0.4% [wt/vol]) before use. In the case of the medium used for assaying the colony-forming ability of MEL cells, DME was supplemented with FCS (15% [vol/vol]) and methylcellulose (1.5% [wt/vol]).

**Assays for Plasminogen Activator (F9 Cells), SSEA-1 (F9 Cells), Laminin (F9 Cells), and Hemoglobin (MEL Cells)**

Plasminogen activator was assayed as described by Nishimune et al. (1983). In essence, F9 cells, grown in ES with FCS (10% [vol/vol]) on gelatin-coated plastic Petri dishes (Falcon Labware), were overlayed with the ES medium containing agar noble (0.75% [wt/vol]), skim milk (2.5% [wt/vol]), and human plasminogen (0.2 casein U/ml). After 24 h of incubation at 37°C in a CO2 incubator, the haloes developed were counted. Usually over 200 colonies per plate (in duplicate) were examined.

Laminin was detected by staining the cells with antibody against laminin. For this, F9 cells grown on plastic Petri dishes (35 mm diam) were fixed with methanol-acetic acid mixture (2:1) (15 min at room temperature) and washed with 0.05% (vol/vol) Tween 20 (Wako Junyaku, Tokyo) in PBS. Anti-laminin antibody (100 µl, diluted 100-fold from the original sample) was then added to the dish and left for 1 h at 37°C. After washing twice with the Tween solution, FITC-conjugated goat anti-rabbit IgG (100 µl, diluted 500-fold from the original sample) was added and the samples were left at 37°C for 1 h. They were then washed with the Tween solution, and after addition of one drop of NaCO3-NaHCO3 (0.05 M) buffer (pH 9.5) in glycerol, photographs were taken using a Nikon Optiphot epifluorescence microscope with a B-2 filter equipped with a Microflex UFX-II photomicrographic attachment.

1. **Abbreviations used in this paper:** HMBA, hexamethylenebisacetamide; PMA, phorbol 12-myristate 13-acetate; SSEA-1, stage-specific embryonic antigen-1.

**Results**

**Induction of Differentiation of F9 Cells**

F9 cells, an established cell line of mouse embryonal carcinoma cells (Bernstein et al., 1973), differentiate in vitro into endoderm cells upon induction by retinoic acid (Strickland and Mahdavi, 1978). The presence of dibutyryl cyclic AMP along with retinoic acid further stimulates the differentiation, and the cells that differentiate under this condition exhibit biochemical and morphological characteristics of parietal endoderm cells (Strickland and Mahdavi, 1978; Strickland et al., 1980; Hogan et al., 1981).

Before we examined the effect of herbinycin A on F9 differentiation, we had confirmed the inhibitory effect of herbinycin A on protein phosphorylation at tyrosine residues. For this, F9 cells were labeled in ES medium (with limited phosphate) with 32P-phosphate (1 mCi/ml) in the presence of different concentrations of herbinycin A and the immunoprecipitates with phosphotyrosine specific antibody (Ohtsuka et al., 1984) from the cell extracts were analyzed by autoradiography and densitometry after SDS-PAGE. Consistent with the previous finding (Uehara et al., 1986), incorporation of 32P-phosphate into proteins reacted with the antibody was uniformly reduced by >98% at 0.5 µg/ml of herbinycin A concentration when incorporation into the total protein was reduced by 25.5% (data not shown).

When F9 cells were incubated with herbinycin A (0.5 µg/ml) for 4 d, the drug induced changes in the morphology of the cells (Fig. 1 D). Since the changes were quite similar to those induced by retinoic acid plus dibutyryl cyclic AMP (Fig. 1 C), the antibiotic seemed to have triggered differentiation of F9 cells into endoderm-like cells (Strickland et al., 1981; Hogan et al., 1981). The morphological changes were irreversible because the cells still maintained their changed morphology even after removal of the drug (data not shown).

To confirm the effect of herbinycin A on F9 cells, we treated the cells with the antibiotic for 2 d and overlaid them...
Figure 1. Changes in morphology of herbimycin A-treated F9 cells. F9 cells (≈10⁵ cells per dish) were plated on gelatin-coated Petri dishes (Falcon Labware; 60 mm diam). Retinoic acid (1 μM) and retinoic acid (1 μM) plus dibutyryl cyclic AMP (1 mM) or herbimycin A (0.5 μg/ml) was then added and, after 4 d incubation in a CO₂ incubator at 37°C, pictures were taken using a microscope (Olympus IMT-2). A, control; B, retinoic acid; C, retinoic acid plus dibutyryl cyclic AMP; and D, herbimycin A-treated cells, respectively. All pictures were taken at the same magnification. Bar, 0.1 mm.

Figure 2. Induction of plasminogen activator by herbimycin A. F9 cells (≈2 × 10⁵ cells per dish) were plated on gelatin-coated plastic Petri dishes (Falcon Labware; 35 mm diam) and incubated for 2 d in a CO₂ incubator at 37°C. Herbimycin A (0.5 μg/ml) was then added and the cells incubated for a further 2 d. The cells were then overlaid with a solution containing skim milk and plasminogen (see Materials and Methods) and photographs were taken 24 h later. A, control cells; and B, herbimycin A-treated cells.
plus dibutyryl cyclic AMP (1 mM) or herbimycin A (0.5 μg/ml). After 5 d of incubation, laminin was stained with FITC-labeled antibody as described in Materials and Methods. The photographs were taken using a Nikon Optiphot epifluorescence microscope without (phase microscopy) or with (fluorescent microscopy) a B-2 filter equipped with Microflex UFX-II photomicrographic attachment. A and B, cells incubated without the drugs (control cells); C and D, cells incubated with retinoic acid plus dibutyryl cyclic AMP; E and F, cells incubated with herbimycin A. A, C, and E, phase microscopy; B, D, and F, fluorescent microscopy. Bar, 0.1 mm.

**Figure 4.** Immunofluorescence staining of laminin in F9 cells. F9 cells were incubated in the absence or presence of retinoic acid (1 μM) plus dibutyryl cyclic AMP (1 mM) or herbimycin A (0.5 μg/ml). After 5 d of incubation, laminin was stained with FITC-labeled antibody as described in Materials and Methods. The photographs were taken using a Nikon Optiphot epifluorescence microscope without (phase microscopy) or with (fluorescent microscopy) a B-2 filter equipped with Microflex UFX-II photomicrographic attachment. A and B, cells incubated without the drugs (control cells); C and D, cells incubated with retinoic acid plus dibutyryl cyclic AMP; E and F, cells incubated with herbimycin A. A, C, and E, phase microscopy; B, D, and F, fluorescent microscopy. Bar, 0.1 mm.

with a medium containing skim milk and plasminogen to assay the production of plasminogen activator, an indicator of endodermal differentiation of F9 cells (Strickland and Mahdavi, 1978; Strickland et al., 1981). A considerable number of haloes emerged after the herbimycin treatment (Fig. 2). A (0.5 μg/ml) was then added and incubation of the cells was continued. Every day (0–5 d) after addition of the drug, samples were subjected to the assay of plasminogen activator production described above. The percentage of PA⁺ colonies induced by retinoic acid (1 μM) at the fifth day was 86%.

The induction of differentiation by herbimycin A in F9 cells was also confirmed by assaying the disappearance of an antigen specific to embryonal cells. We monitored the stage-specific embryonic antigen-1 (SSEA-1) during the incubation of F9 cells after herbimycin A treatment. The antigen is specifically present in embryonal cells and disappears in the process of in vitro differentiation induced by retinoic acid (Solter and Knowles, 1978; Moore et al., 1986). As shown in Fig. 5, SSEA-1, detected by FITC-labeled antibody, disappeared after incubation of the cells with herbimycin A for 5 d. This indicates that the F9 cells lost the antigen specific to the embryo as they underwent morphological and biochemical alterations in response to herbimycin A.

These results indicate that herbimycin A induces morphological and biochemical changes in F9 cells which are at least ostensibly similar to, though may not be identical with, those exhibited in the retinoic acid-induced differentiation.

**Induction of Terminal Differentiation of MEL Cells**

MEL cells (Friend et al., 1966), which normally exhibit characteristics of proerythroblast cells, undergo biochemical and morphological changes in response to inducing agents such as DMSO (Friend et al., 1971) or HMBA (Reuben et al., 1976). The major changes include accumulation of hemoglobin (Friend et al., 1971) and loss of proliferation capacity (Gusella et al., 1976; Fibach et al., 1977), typical characteristics of erythroid cells.

In Fig. 6, we show that exposure of MEL cells to herbimycin A induced intracellular accumulation of hemoglobin. In this experiment, the MEL cells were exposed to herbimycin A at different concentrations for 2 d, the antibiotic removed, and the cells incubated for a further 3 d; the cells which had accumulated hemoglobin were assayed by benzidine staining (Orkin et al., 1975). As shown in the figure, ~70–80% of the herbimycin A-treated cells became reactive to benzidine (B⁺ cells) at a drug concentration of 0.5 μg/ml (0.87 μM). Higher concentrations drastically reduced induction (Fig. 6). Herbimycin A apparently induces differentiation very efficiently because the optimum concentration (0.87 μM) for induction was much lower (less than one-thousandth) than cyclic AMP was observed on the herbimycin A-induced differentiation (data not shown).

To further confirm the effect of herbimycin A on F9 differentiation, we examined the appearance of laminin using antibody against laminin. Laminin is one of the markers specific to endodermal differentiation of F9 cells induced by retinoic acid (Knowles et al., 1980; Moore et al., 1986). As seen in Fig. 4, herbimycin A induced a more rapid expression of laminin in the cells to the level ever higher than that induced by retinoic acid (plus dibutyryl cyclic AMP). The induction of laminin was further confirmed by SDS-PAGE electrophoresis of the immunoprecipitates of proteins labeled with [35S]methionine after herbimycin A treatment (data not shown).

The induction of differentiation by herbimycin A in F9 cells was also confirmed by assaying the disappearance of an antigen specific to embryonal cells. We monitored the stage-specific embryonic antigen-1 (SSEA-1) during the incubation of F9 cells after herbimycin A treatment. The antigen is specifically present in embryonal cells and disappears in the process of in vitro differentiation induced by retinoic acid (Solter and Knowles, 1978; Moore et al., 1986). As shown in Fig. 5, SSEA-1, detected by FITC-labeled antibody, disappeared after incubation of the cells with herbimycin A for 5 d. This indicates that the F9 cells lost the antigen specific to the embryo as they underwent morphological and biochemical alterations in response to herbimycin A.

These results indicate that herbimycin A induces morphological and biochemical changes in F9 cells which are at least ostensibly similar to, though may not be identical with, those exhibited in the retinoic acid-induced differentiation.
that of DMSO or HMBA, for which ~280 mM (DMSO) or 5 mM (HMBA) is required to obtain the same level of erythroid induction. The continuous presence of herbimycin A throughout the incubation (5 d) gave essentially the same results (data not shown) as those shown in Fig. 6, suggesting that the first 2 d of exposure of MEL cells to herbimycin A was sufficient to induce hemoglobin at the later stage (after third day) of incubation.

To examine whether the accumulation of hemoglobin by herbimycin A (assayed by benzidine staining) accompanies an increase of RNA transcripts specific to ß globin, RNA was isolated from MEL cells which had been incubated with herbimycin A for 3 d and, after electrophoresis, analyzed by Northern hybridization using a cloned ß globin gene DNA probe. Fig. 7 shows that ß globin transcripts were increased several-fold by herbimycin A treatment and that the increased level was equivalent to that observed after DMSO treatment. This shows that the effect of herbimycin A occurs at least at the transcription level, which is similar to the effect induced by DMSO.

Table I shows that the induction of hemoglobin accumulation by herbimycin A was inhibited by phorbol 12-myristate 13-acetate (PMA) (Yamasaki et al., 1977; Rovera et al., 1977) and dexamethasone (Scher et al., 1978; Chen et al., 1982), specific inhibitors of MEL cell differentiation induced by DMSO or HMBA. In this experiment, during the first 2 d of incubation with herbimycin A, PMA or dexamethasone was added and the effect of the inhibitors on erythroid differentiation was examined 3 d later after removal of herbimycin A and the inhibitors. It is quite clear that PMA and dexamethasone also inhibited erythroid induction by herbimycin A, as they did for induction by DMSO and HMBA (Table I). These results suggest that herbimycin A-induced erythroid differentiation shares a common reaction(s) with that in the differentiation cascade triggered by DMSO or HMBA. An early inducible reaction sensitive to PMA was implicated in MEL cell differentiation by the previous cell and cytoplast fusion experiments (Nomura and Oishi, 1983; Kaneko et al., 1984; Watanabe et al., 1985).

We also examined whether the accumulation of hemoglobin by herbimycin A accompanied the loss of colony-forming ability, another characteristic of MEL cell differentiation (Gusella et al., 1976; Fibach et al., 1977). As shown in Fig. 8, the appearance of B+ cells accompanied the loss of the
Discussion

This paper presents experimental evidence that herbimycin A induces both differentiation of mouse embryonal carcinoma (F9) cells into endoderm-like cells and terminal differentiation of erythroleukemia (MEL) cells into erythroid cells. This is the first demonstration that a single agent acts as a common inducer for the two differentiation systems, which seemed to be quite different in their mechanism of induction. MEL cells are induced to differentiate into erythroid cells by a number of compounds including DMSO (Friend et al., 1971), HMBA (Reuben et al., 1976), butyric acid (Leder and Leder, 1975; Takahashi et al., 1975), but none of these compounds induces F9 differentiation. In contrast, retinoic acid, which induces F9 cells, has no effect on MEL cell differentiation.

How and why does herbimycin A effectively induce these two different kinds of cell, whose differentiation seems to be executed by apparently different molecular mechanisms? One straightforward answer is that herbimycin A acts on an intracellular reaction (or reaction product) essential for triggering differentiation, which is common to the endoderm differentiation from the embryonic (F9) cells and the terminal differentiation of MEL cells. If this is the case, intracellular processes or cascades leading to the common reaction would be quite diversified at the initial stage and dependent upon the inducing agents and the cell types, but would eventually converge to a single common intracellular reaction which is closely related to the cellular commitment to differentiate. Alternatively, it is also possible that the induction of differentiation by herbimycin A is executed through an entirely independent cascade from those functioning in the differentiation triggered by conventional inducing agents. In any event, it is reasonable to conclude that there is a common step between F9 cells and MEL cells which is responsive to herbimycin A.

Previous experimental results strongly suggest that herbimycin A interferes with protein phosphorylation by p60tyr and other tyrosine kinases. Herbimycin A not only reduces phosphorylation by p60tyr (Uehara et al., 1986), but also specifically reverses the phenotypic changes caused by transfection of tyrosine kinase oncogenes (Uehara et al., 1988, Murakami et al., 1988). If the site of the action of herbimycin A is limited to tyrosine residues or closely associated reactions as we now believe, the hypothetical common step in differentiation is likely to be related to phosphorylation of tyrosine residues in cellular proteins. In this respect, it may be worth mentioning that ST-638, a recently synthesized phosphotyrosine analogue, and genistein, an antibiotic, which inhibit protein phosphorylating activity of tyrosine kinase in vitro (Shiraishi et al., 1987, Akiyama et al., 1987), also induces erythroid differentiation of MEL cells under certain conditions (Watanabe, T., T. Shiraishi, H. Sasaki, and M. Oishi, manuscript submitted for publication). Taken together, one could argue that specific inhibition of protein colony-forming ability of MEL cells, as observed in the differentiation induced by DMSO or HMBA. Taken together, the various results indicate that herbimycin A induces erythroid differentiation in MEL cells in a manner indistinguishable from that observed with typical erythroid-inducing agents such as DMSO or HMBA.

Figure 6. Erythroid differentiation induced by herbimycin A as a function of the drug concentration. Herbimycin A at different concentrations (shown in the figure) was added to cultures of MEL cells (2 × 10⁶ cells/ml) and after 2 d of incubation at 37°C the drug was removed by repeated (twice) washing with PBS. The cells continued to be incubated for another 3 d and benzidine-reactive (B⁺) cells were counted. For details, see Materials and Methods.

Figure 7. Increase of β globin-specific transcripts by herbimycin A. MEL cells, cultured to confluent growth (2 × 10⁶/ml) in MEM supplemented with 12% FCS, were diluted with fresh medium to a cell density of 1.5 × 10⁶/ml. The culture was divided into three (each 12 ml) and incubation was continued in the absence of drug, or presence of DMSO (280 mM) or herbimycin A (0.5 μg/ml). After 3 d of incubation, 10-ml aliquots of the cultures were withdrawn and RNA (80 μg) was extracted by the phenol procedure. The RNA (20 μg each) was then electrophoresed and subjected to Northern hybridization using cloned mouse β globin DNA (pMβGA) and chicken β actin DNA (pAβI) (control) as probes. Lane 1, control cells; lane 2, DMSO-treated cells; lane 3, herbimycin A-treated cells. Levels of erythroid differentiation in each preparation (after 5 d of incubation) were 0% (control), 75% (DMSO-treated cells), and 75% (herbimycin A-treated). For details, see Materials and Methods.
phosphorylation at tyrosine residues makes the cells, directly or indirectly, less proliferative and more susceptible to physiological conditions favoring differentiation, although it is still possible that the induction by herbimycin A was caused by an as yet unidentified effect of the drug (other than the inhibitory effect on protein phosphorylation) on mammalian cells.

At present, it is not clear whether differentiation triggered by agents which reduce phosphorylated tyrosine residues in cellular proteins is a phenomenon limited to specific cell lines as reported here, or reflects a more universal one involved in all or most types of in vivo differentiation. Closer examination of the mechanism of inhibition of tyrosine phosphorylation by herbimycin A and the search for a protein dephosphorylating activity in the cytoplasmic differentiation-inducing factors for MEL cell differentiation (Nomura et al., 1986; Watanabe and Oishi, 1987) are the obvious steps to be undertaken to answer many questions regarding the biochemical nature of differentiation cascades.

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### Table I. Effect of PMA and Dexamethasone on Erythroid Differentiation by Herbimycin A

| Addition        | Herbimycin A | DMSO | HMBA |
|-----------------|--------------|------|------|
|                  | %            | %    | %    |
| No addition     | 74.5         | 81.2 | 82.5 |
| PMA             | 0.0          | 3.3  | 5.6  |
| Dexamethasone   | 1.1          | 1.0  | 4.7  |

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PM (100 ng/ml) or dexamethasone (10 μM) was present for the first 2 d incubation with herbimycin A, DMSO, or HMBA as described in the legend of Fig. 6. The concentrations of herbimycin A, DMSO, and HMBA were 0.87 μM (0.5 μg/ml), 280 mM, and 5 mM, respectively.

Figure 6. Effect of herbimycin A on colony-forming ability of MEL cells. MEL cells cultured to confluence were diluted and incubated in the presence of different concentrations of herbimycin A as shown in the legend of Fig. 6. After 2 d of incubation, the cells were washed with PBS and divided into two. One part was plated on a semi-solid medium after appropriate dilutions and colonies were counted after 2 wk of incubation at 37°C. The percentage of colony formation, compared to the number of colonies formed by the control cells (without herbimycin A treatment) is shown in the figure (right-hand scale). The colony-forming efficiency of the control cells was ~63%. The other half of the cells after the initial incubation was incubated in liquid medium as done for the experiments shown in Fig. 1 and benzidine-reactive (B+) cells were counted after 3 d of incubation (left scale). Benzidine-reactive (B+) cells (●). Colony formation (○).
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