Eur J Cancer Clin Oncol, Vol. 20, No. 4, pp. 525–533, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Effect of Cytochalasin B on Growth, Multinucleation and Human Chorionic Gonadotropin Secretion in a Human Choriocarcinoma Cell Line*

S. SEKIYA, T. KAIHO, H. IWASAWA, N. INABA, S. SHIROTAKE and H. TAKAMIZAWA
Department of Obstetrics and Gynecology, Chiba University School of Medicine, 1-8-1 Inohana, Chiba 280, Japan

Abstract—*Treatment of human choriocarcinoma cells with cytochalasin B at the doses of inhibiting cell division but not inhibiting nuclear division led to* in vitro *formation of the multinucleated syncytiotrophoblast-like (STL) cells. A concomitant increase in human chorionic gonadotropin (hCG) secretion per cell was noted. Immunocytochemical staining demonstrated the predominant localization of hCG in the STL cells. These results indicate that multinucleation stimulates hCG synthesis and secretion in the choriocarcinoma cells.*

INTRODUCTION

CYTOCHALASIN B (CB) comprises a group of mould metabolites. Although CB inhibits cell division, nuclear division proceeds normally and very large multinucleated cells are produced [1]. Malignant cells become more highly multinucleated than normal diploid cells by exposure to CB [2–6], although the detailed mechanisms remain unknown.

On the other hand, large quantities of hCG are synthesized and secreted by the normal placenta and trophoblastic tumors, but the sites and mechanisms of hCG synthesis and secretion have not yet been fully understood [7]. Immunohistochemical studies revealed that hCG immunoreactivity was found predominantly in syncytial trophoblasts or STL cells [8–10]. The hCG synthesis and secretion in choriocarcinoma cells can be enhanced by addition of various cytotoxic drugs *in vitro* [7]. However, the mechanisms have not been elucidated.

The present report describes the effect of CB on the growth, multinucleation and hCG secretion in a human choriocarcinoma cell line *in vitro*.

Accepted 6 September 1983.
*Supported by a grant from the Ministry of Education, Science and Culture, Japan.

MATERIALS AND METHODS

Cell culture

A human choriocarcinoma cell line, BeWo [11], growing in monolayer was maintained in RPMI 1640 medium supplemented with 10% newborn serum (Flow Lab., McLean, VA, U.S.A.) and antibiotics in humidified 5% CO_2–95% air at 37°C.

Cytochalasin B (CB)

CB (Sigma Chemical Co., St Louis, MI, U.S.A.) was dissolved in 95% ethanol at a concentration of 1 μg/ml. This stock solution was preserved at −20°C and added to the medium to produce specified final concentrations. Less than 1% of ethanol in culture medium had no effect on BeWo cells.

[^{3}H]-Thymidine incorporation

The incorporation of [^{3}H]-thymidine into cellular DNA was assayed as follows. About 10^5 cells in 1 ml of culture medium were incubated in a series of sterile glass scintillation vials. After 48 hr of incubation the cells at exponential growth were exposed to CB at doses of 0.5–8 μg/ml for 96 hr. After collection of culture medium, [6-^{3}H]-thymidine (sp. act. 15 Ci/mmol, New England Nuclear Corp., Boston, MA, U.S.A.) was added to each vial in 1 ml of medium at 1 μCi/ml. After 30 min of incubation

each vial was quenched by addition of 10 ml of ice-cold isotonic saline and the incorporation was counted with a liquid scintillation counter, RackBeta Model 1215 (Wallac Oy, Turk, Finland), according to the method described by Ball and Roynter [12]. All samples were prepared in triplicate.

Growth

Culture dishes 60 mm in diameter (Falcon Plastics, Oxnard, CA, U.S.A.) were seeded with 4 $\times 10^5$ cells and the cells were allowed to attach for 24 hr. The medium was then replaced with 5 ml of medium containing CB at concentrations of 0.5–8 μg/ml. The medium containing CB was changed at 2-day intervals for 6 days. After collection of the culture fluid the cells were harvested with 0.25% trypsin solution and counts were made by hemocytometer after staining with 0.1% crystal violet in 0.1 M citric acid. All samples were prepared in triplicate. Periodically, some cultures were fixed with absolute methanol for enumeration of the nuclei per cell or 50% ethanol for flow cytometry.

Enumeration of nuclei

The cells in culture dishes were stained with Giemsa solution after fixation. The number of nuclei per cell was counted with about 200 cells per culture dish under high magnification of microscopy.

Flow cytometry

After the elimination of fixative the cells were incubated for 15 min in 0.1% pepsin solution at 37°C, and then treated with RNase (1 mg/ml) for 30 min. After rinsing with phosphate-buffered saline (PBS) the cells were stained for more than 30 min with ethidium bromide (10 μg/ml in Tris buffer) at room temperature. The cell fluorescence was measured with an Impulse Cytophotometer Type II (PHYWE AG, F.R.G.) after filtering through a 40-μm nylon mesh. Primary peak position of diploid lymphocytes derived from a normal volunteer was adjusted to 2 channel (channel No. 20) of the *x*-axis.

Electron microscopy

The cells were scraped off with a rubber policeman and centrifuged at 1000 rpm for 5 min to obtain cell pellets. The pellets were then fixed with 2.5% glutaraldehyde in PBS solution at 4°C for 2 hr and post-fixed with 1% osmium tetroxide at 4°C for 2 hr. After dehydration in a graded ethanol series, they were embedded in Epon 812. Ultrathin sections were cut with a diamond knife and were double-stained with uranyl acetate followed by Reynold's lead acetate. Observations were made using a Hitachi HS-8-type electron microscope (Hitachi, Tokyo, Japan)

Assay of hCG

The hCG in the culture fluid was assayed using a CEA-IRE-SORIN radioimmunoassay kit (Green Cross Co., Tokyo, Japan).

Peroxidase anti-peroxidase bridge (PAP) method

The rabbit anti-hCG serum was provided by Mochida Pharmaceutical Co. (Tokyo, Japan). The PAP method was performed according to the procedure described elsewhere [13], using the cell pellets described above.

RESULTS

Multinucleation

When BeWo cells were exposed to CB for 96 hr no inhibition of [^{3}H]-thymidine incorporation into cellular DNA was noted at any doses up to 8 μg/ml and the incorporation was not significantly different in each culture (Fig. 1). In contrast, inhibition of cell division was noted on exposure to CB at doses of more than 1 μg/ml, and the inhibition was dose- and time-dependent (Fig. 2). A striking difference in the inhibitory effect was found between 1 μg/ml, which inhibited the cell division slightly, and 2 μg/ml, which inhibited it completely (Fig. 2).

Multinucleation of BeWo cells was studied with CB at concentrations of 1 and 2 μg/ml. The population doubling time of non-treated BeWo cells was about 34 hr. In non-treated controls 96% of the cells were mononuclear in the exponential stage of growth (Figs 3 and 4). On the other hand, only 6% of the cells became multinuclear (more than 3 nuclei per cell) at 96 hr when treated with

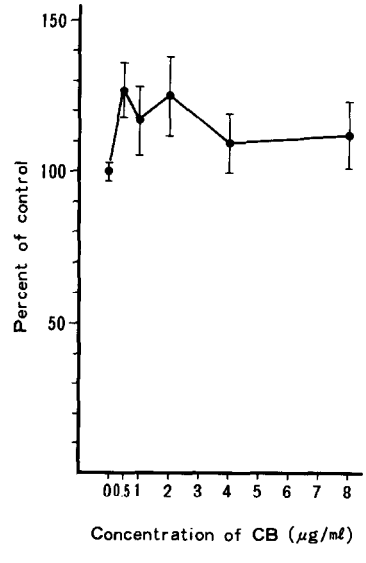

Fig. 1. Effect of CB on [^{3}H]-thymidine incorporation into cellular DNA. Bars, S.D.

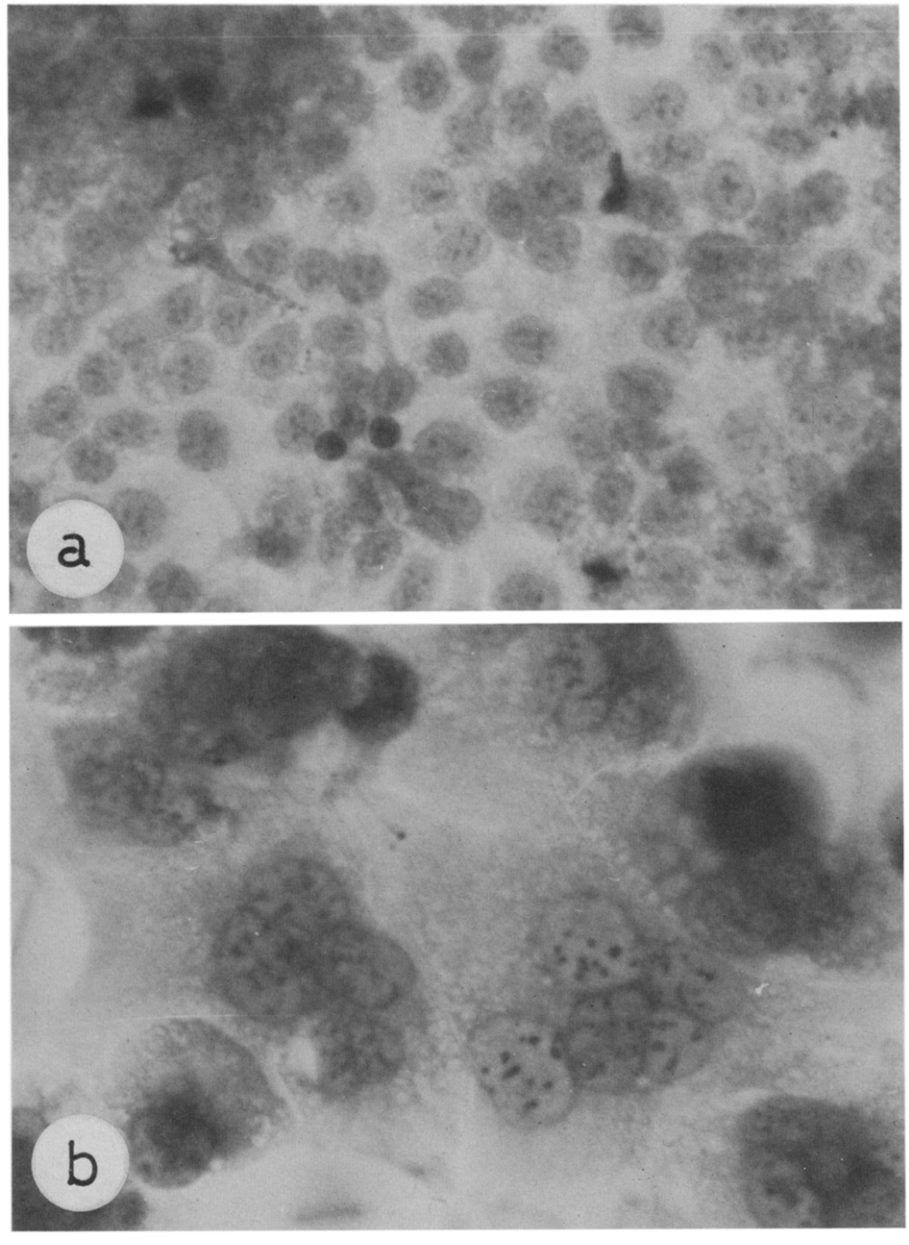

Fig. 4. Nuclei of (a) non-treated control cells and (b) cells treated with 2 μg/ml *CB for* 96 hr. *Giemsa staining,* ×200.

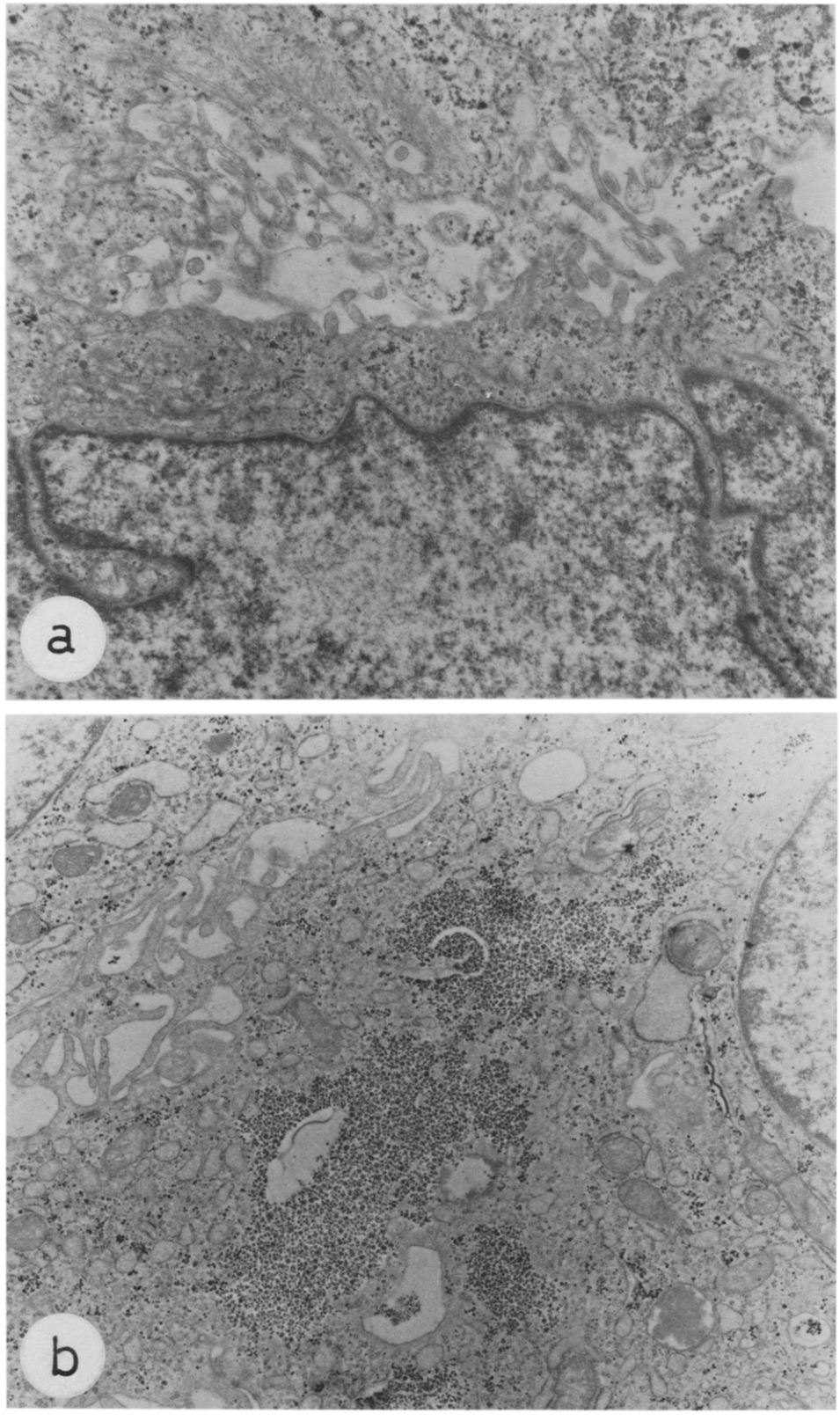

Fig. 5. Electron micrographs of non-treated control cells [(a), ×7000] and cells treated with 2 μg/ml CB for 96 hr [(b), ×9600].

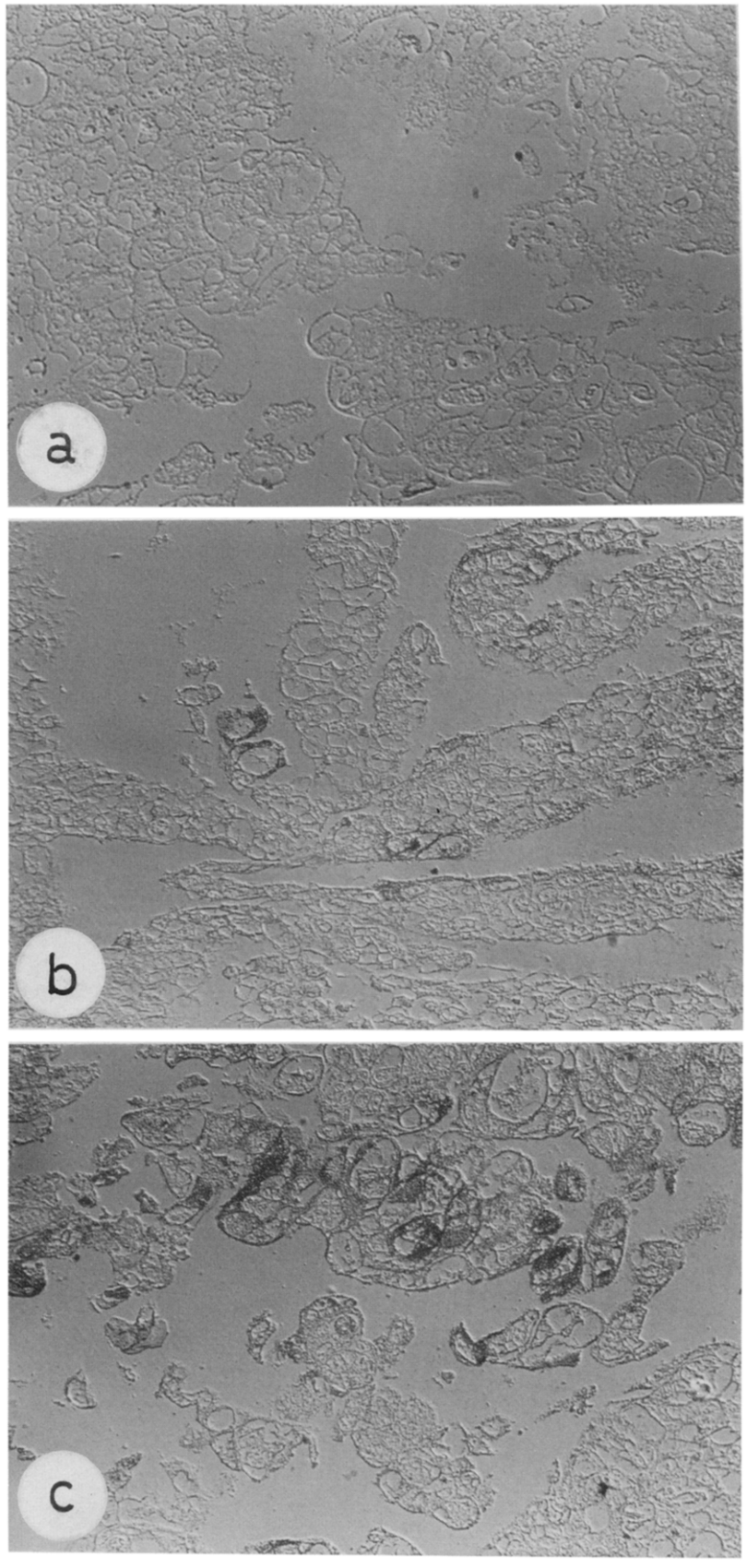

Fig. 8. Localization of hCG by PAP method. (a) Normal rabbit serum; (b) rabbit anti-hCGβ serum, non-treated control cells; (c) rabbit anti-hCGβ serum, cells treated with 2 μg/ml *CB for* 96 hr. *Interference phase-contrast,* ×250.

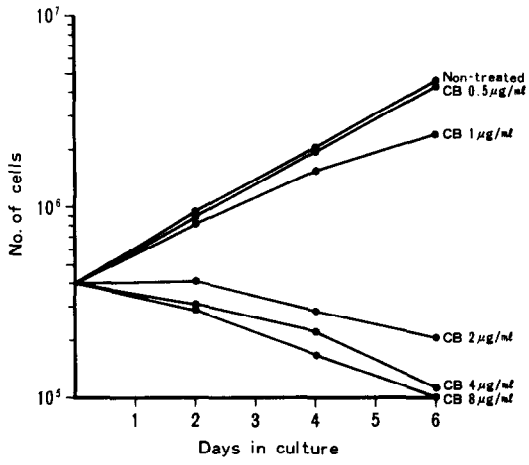

Fig. 2. Effect of CB on cell growth.

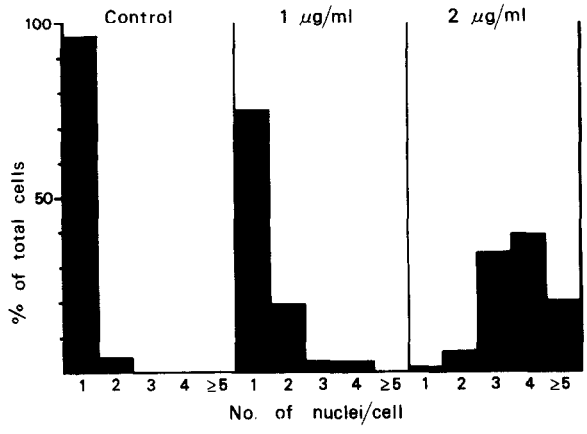

Fig. 3. Distribution of the number of nuclei per cell in the presence of CB at concentrations of 1 *and* 2 μg/ml *for* 96 hr.

1 μg/ml CB, while 93% of them had more than 3 nuclei when treated with 2 μg/ml (Figs 3 and 4). Multinucleation became evident after 48 hr of exposure to CB in coincidence with the inhibition of cell division.

Thus multinucleation is possibly provoked by inhibition of cell division without inhibition of nuclear division. Electron microscopy revealed the decrease of organelles in the cytoplasm such as filaments and increase of aggregated glycogen particles in multinucleated BeWo cells (Fig. 5).

As shown in Fig. 6, a shift of the first peak to hyperploidy (about 3 channel, channel No. 30), wide distribution of nuclear DNA content and S phase accumulation of cells by flow cytometry were characteristic to the asynchronous population of BeWo cells *in vitro*. As compared to non-treated controls, a distinctive change of DNA histogram pattern was not noted in the cultures treated with 1 μg/ml CB for either 48 or 96 hr. Although the first peak of the DNA histogram was found in almost the same channel as that observed in non-treated controls, when treated with 2 μg/ml CB for 48 hr a decrease of S phase accumulation of cells was observed. At 96 hr after treatment with 2 μg/ml CB the first peak shifted to less than 2 channel (channel No. 20), reflecting the death of a part of the cells due to karyorrhexis, and most of the cells contained nuclear DNA, which distributed evenly from 2 channel to 12 channel. These changes in DNA histogram were consistent with the appearance of and increase in multinucleated cells (Figs 3 and 4).

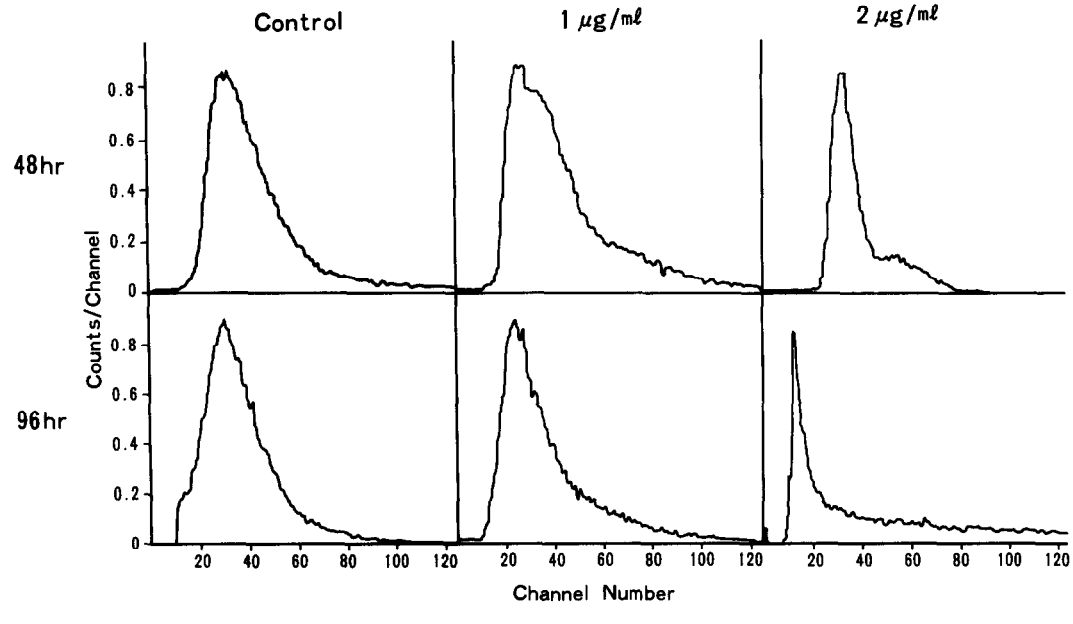

Fig. 6. DNA histogram by flow cytometer of cells in the presence of CB at concentrations of 1 *and* 2 μg/ml *for either* 48 *or* 96 hr.

hCG secretion

Addition of CB at doses of 0.5–8 μg/ml for 96 hr did not lead to a significant increase in the hCG secretion per dish (data not shown). However, if the values were estimated per cell, addition of CB led to an increase in hCG secretion. As shown in Fig. 7, the cultures treated with 1 μg/ml CB for the initial 48 hr secreted about twice as much hCG into the culture medium than the non-treated controls. In both non-treated cultures and those treated with 1 μg/ml CB the secretion tended to decrease during the next 48 hr. In contrast, in cultures treated with 2 μg/ml the hCG secretion was about 7-fold that of non-treated controls after 48 hr and the secretion increased to about 12-fold during the next 48 hr. This increase in hCG secretion per cell correlated with the increase in number of multinucleated cells.

hCG immunoreactivity by the PAP method was found in about 4% of non-treated BeWo cells. When treated with 2 μg/ml CB for 96 hr, about 20% of the cells showed positive findings (Fig. 8).

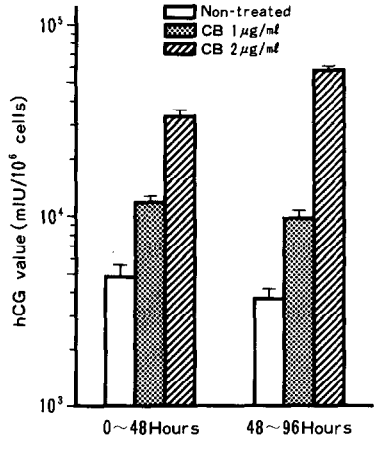

Fig. 7. Effect of CB on hCG secretion per 10^6 cells.

DISCUSSION

The effect of CB on multinucleation has been examined in various types of mammalian cells of normal and malignant natures. Normal diploid cells treated with CB *in vitro* remained mono- or binucleated, whereas almost all the transformed cells became highly multinucleated [2–6]. Although the mechanisms of multinucleation are not fully understood, uncontrolled nuclear division is a common feature of malignant cells and is considered as a cause of the multinucleation [1, 2]. The [^{3}H]-thymidine incorporation into cellular DNA of BeWo cells was not inhibited with CB at concentrations of 0.5–8 μg/ml for 96 hr (Fig. 1). Hirano and Kurimura [14] also reported that DNA synthesis of the normal murine cells was significantly inhibited by CB at concentrations of 0.5–2 μg/ml during 48 hr, whereas that of their virally transformed cells was not inhibited.

In various types of mammalian malignant cells effective CB concentrations to induce multinucleation ranged between 1 and 2 μg/ml [2–5]. The choriocarcinoma cells showed almost the same sensitivity to CB (Fig. 3). The multinucleated BeWo cells with more than 3 nuclei did not seem to further proliferate and treatment of the cells with 2 μg/ml CB for 96 hr caused the death of a part of the cultures (Figs 2 and 6). The higher doses of CB caused nuclear extrusion and enucleation of the cells [1, 2]. Thus multinucleation seems to be induced by inhibition of cell division by CB without inhibition of nuclear division. Inhibitory action on actin network formation, which is a component of cytoskelton, by CB was suggested to be a possible cause of inhibition of cell division [15].

Hussa [16] reported that the stimulation of hCG synthesis and secretion was found in BeWo cells when the cells were treated with 2.1 μM (1 μg/ml) of CB for 24 hr. Similar results were also obtained in this study (Fig. 7). The BeWo cells treated with 1 μg/ml CB for 48 hr secreted about 2-fold hCG, as compared with non-treated cultures. Since almost all the cells were mononuclear at 48 hr after treatment with 1 μg/ml CB (Fig. 3), it is considered that CB may have stimulated the mononuclear cytotrophoblast-like (CTL) cells to synthesize and secrete more hCG. However, the hCG secretion was further enhanced when the cells were treated with 2 μg/ml, which changed almost all the CTL cells to STL cells within 96 hr (Fig. 3). Morphological change from CTL to STL cells was also induced by treatment of the BeWo cells with 10^{-6}M methotrexate (MTX) [17]. A comparable concentration of MTX enhanced the hCG secretion [18]. Although the mechanisms of action may be different between the two drugs, these results suggest that multinucleation of the choriocarcinoma cells may represent a part of the mechanism of hCG synthesis and secretion. In addition, the predominant localization of hCG in the STL cells elucidated by the PAP method further supports the suggestion.

However, other actions of CB on the synthesis and secretion of hCG in the choriocarcinoma cells are not ruled out and the exact mechanisms must be further clarified.

REFERENCES

1. CARTER SB. Effects of cytochalasins on mammalian cells. *Nature* 1967, **213**, 261–264.

2. WRIGHT WE, HAYFLICK L. Formation of anucleate and multinucleate cells in normal and SV_{40} transformed WI-38 cytochalasin B. *Exp Cell Res* 1972, **74,** 187–194.
3. KELLY F, SAMBROOK J. Differential effect of cytochalasin B on normal and tansformed mouse cells. *Nature* 1973, **242,** 217–219.
4. STEINER MR, ALTENBURG B, RICHARDS CS, DUDLEY JP, MEDINA D, BUTEL JS. Differential response of cultured mouse mammary cells of varying tumorigenicity to cytochalasin B. *Cancer Res* 1978, **38,** 2719–2721.
5. SOMERS KD, MURPHEY MM. Multinucleation in response to cytochalasin B: a common feature in several human tumor cell lines. *Cancer Res* 1982, **42,** 2575–2578.
6. ASAGA T, SUZUKI K, TAKEMIYA S, OKAMOTO T, TAMURA N, UMEDA M. Difference in polynucleation of cultured cells from human mammary tumors and normal mammary glands on treatment with cytochalasin B. *Gann* 1983, **74,** 95–99.
7. HUSSA RO. Biosynthesis of human chorionic gonadotropin. *Endocrinol Rev* 1980, **1,** 268–294.
8. GÄRTNER A, LARSSON L-I, SJÖBERG N-O. Immunohistochemical demonstration of chorionic gonadotropin in trophoblastic tumors. *Acta Obstet Gynecol Scand* 1975, **54,** 161–163.
9. TUBBS RR, VELASCO ME, BENJAMIN SP. Immunocytochemical identification of human gonadotropin. *Arch Pathol Lab Med* 1979, **103,** 534–536.
10. YORDE DE, HUSSA RO, GARANCIS JC, PATTILLO RA. Immunocytochemical localization of human choriogonadotropin (hCG) in human malignant trophoblast. *Lab Invest* 1979, **40,** 391–398.
11. PATTILLO RA, GEY GO. The establishment of a cell line of human hormone-synthesizing trophoblast cells *in vitro*. *Cancer Res* 1968, **28,** 1231–1236.
12. BALL CR, ROYNTER RW. A nobel method for measuring incorporation of radioactive precursors into nuclei acids and proteins of cells in monolayer cuture. *Anal Biochem* 1972, **46,** 101–107.
13. INABA N, RENK T, BOHN H. immunohistochemical location of placental proteins (PP8, 9, 10, 11, 12) in human term placentae. *Arch Gynecol* 1980, **230,** 109–121.
14. HIRANO A, KURIMURA T. Virally transformed cells and cytochalasin B. I. The effect of cytochalasin B on cytokinesis, karyokinesis and DNA synthesis in cells. *Exp Cell Res* 1974, **89,** 111–120.
15. BRAY D. Cytochalasin action. *Nature* 1979, **282,** 671.
16. HUSSA RO. Effects of antimicrotuble agents, potassium and inhibitors of energy production on hCG secretion. *In Vitro* 1979, **15,** 237–245.
17. FRIEDMAN SJ, SKEHAN P. Morphological differentiation of human choriocarcinoma cells by methotrexate. *Cancer Res* 1979, **39,** 1960–1967.
18. SPEEG KV JR, AZIZKHAN JC, STROMBERG K. The stimulation by methotrexate of human chorionic gonadotropin and placental alkaline phosphatase in cultured choriocarcinoma cells. *Cancer Res* 1976, **36,** 4570–4576.