MODIFICATIONS OF MICROFILAMENTS AND MICROTUBULES INDUCED BY TWO HEPATIC TUMOR PROMOTERS, PHENOBARBITAL AND BILIVERDIN IN NON-TRANSFORMED AND TRANSFORMED HEPATIC CELL LINES

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Microfilaments and microtubules are components of the cytoskeleton which could be implicated in neoplastic transformation. We studied the effect of two hepatic tumor promoters, phenobarbital (PB) and biliverdin (BV), on microfilaments and microtubules of non-transformed (Cl_3) and transformed (FV) hepatic epithelial cells. Cl_3 non-transformed cells cultured in the presence of $1 \times 10^{-6} M$ BV for 48 h showed a loss of F-actin, fragmentation of actin and the appearance of star-like structures in the cytoplasm, as well as loosening of the peripheral bundle of actin, and some ruffling of cell membranes. In Cl_3 cells exposed to $0.2 \times 10^{-3} M$ PB a similar disappearance of F-actin staining and a very prominent ruffling of cell membrane were observed. BV and PB also produced in these cells modifications of microtubules characterized by a disappearance of centrosome staining in numerous cells, a condensed ring of tubulin around the nucleus and a depolymerized aspect of the microtubular network. All these modifications of microfilaments and microtubules closely resembled those observed in FV transformed cells in the absence of any treatment (Solvent DMSO only). We did not observe an effect of BV and PB on FV cells.

The present data demonstrate that the cytoskeleton of non-transformed epithelial liver cells is sensitive to the action of liver tumor promoters suggesting that it might play a role as to yet be defined in the promotion mechanism.

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2. Key words: liver cells, microfilaments, microtubules, phenobarbital, biliverdin, promoters.

3. Abbreviations: PB: phenobarbital; BV: biliverdin; TPA: 12-0-tetradecanoyl-phorbol 13 acetate; GGT: gamma-glutamyl-transpeptidase; DMSO: dimethylsulfoxyde.

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INTRODUCTION

Microfilaments and microtubules are two major cytoskeletal structures involved in cell morphology, in the maintainance of cell shape, in cell adhesiveness and in transduction of signals through plasma membranes and cytoplasm (Sager et al., 1986). In addition, microtubules are a major component of the mitotic spindle. Moreover, interrelations have been shown between both systems (Pollard et al., 1984).

All these functions concerning cell shape, communication and division are altered during the transformation of a normal cell to a tumoral cell. Relationships have been found between cell transformation induced by viruses or chemical agents and an altered organization of cytoskeleton. Viral transformation of fibroblasts (Fine and Taylor, 1976; Edelman and Yahara, 1976; Carley et al., 1981; Brown et al., 1981) is generally accompanied by a loss of fibrillar actin (F-actin), the constitutive protein of microfilaments and by a more contracted aspect of tubulin network than that observed in normal cells.

Similar disorganization of cytoskeleton, especially at the microfilament level, is induced by the skin tumor promoter, 12-0-tetradecanoyl-phorbol 13 acetate (TPA). Epithelial cells (Schliwa et al., 1984; Kellie et al., 1985) as well as fibroblasts (Rifkin et al., 1979; Delescluse et al., 1988) exposed to low concentrations of TPA for few minutes displayed a loss of F-actin and a reorganization of microfilaments. The microtubule network appeared less extensively disorganized than microfilaments.

The influence on microfilaments and microtubules of other classes of tumor promoters, specifically acting on hepatic cells is, however, not known. Compounds which were demonstrated to act as tumor promoters in rat liver belong to various classes of chemicls such as drugs (phenobarbital), pesticides (DDT), industrial pollutants (dioxine, PCB) or endogenous substances. In the last category, a biliary pigment, biliverdin, is able to promote the neoplastic transformation of hepatic cells initiated with aflatoxin B_1 (Lafarge-Frayssinet et al., 1983).

The present work reports the effect of biliverdin (BV) and phenobarbital (PB) as a reference hepatic promoter on the cytoskeleton of two hepatic epithelial cell lines, one presenting a non-transformed, and the other a transformed phenotype. Both cell lines were isolated from 10 day old rats (Morel-Chany et al., 1978). These cells (FV) underwent a neoplastic transformation after a certain number of passages. They became tumorigenic in syngeneic animals and presented the ability to grow in soft agar as well as high gamma-glutamyl-transpeptidase activity (GGT). One clone (Cl_3) was obtained at an early passage of the FV cells and maintained a non-transformed phenotype all along the subcultures with a lack of ability to grow in soft agar and to express GGT.

The effects of BV and PB on microfilaments and microtubules of both cell strains was studied using labeling of F-actin with rhodamine-phalloidin and immunofluorescence staining of tubulin with a specific antibody.

METHODS

Cell culture. Epithelial cell lines, fibroblast free, were initiated from the liver of 10 day-old Fischer rats according to Williams et al. (1971). They were grown in William's medium E (Eurobio, France) supplemented with 10% FBS (Flow, England), glutamine and antibiotics. The cells were usually plated at 5×10^4 cells/ml medium in plastic flasks incubated at 37° in a humidified incubator with 5% CO_2 atmosphere and subcultured weekly. They were periodically screened for mycoplasma contamination and kept frozen for use at sequential passages.

The two cell lines: FV and Cl_3 were respectively subcultured in three groups. One group of cells was cultivated in presence of PB (0.2×10^{-3}M) in solution in dimethylsulfoxide (DMSO) for a short time (48 hours). A second group of cells was cultivated in presence of BV (1×10^{-6}M) in the same solvent and conditions as the first group.

The cells of the third group were used as control and were treated with DMSO alone (less than 0.1%)

F-actin staining: Cells grown on coverslips were quickly rinsed three times with PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-Ca^{++}-Mg^{++}). Fixation was performed in 3.7% formaldehyde solution in PBS for 5–7 minutes at room temperature. After 3 washes in PBS-Ca^{++}-Mg^{++}, cells were permeabilized in cold acetone for 5 minutes at −20° C. Coverslips were air dried and 200 μl of a solution of fluorescent rhodamine-phalloidin (Molecular Probes) was deposited on cells for 20 minutes at room temperature and in the dark. Coverslips were washed 3 times with PBS and mounted in a polyvinylalcohol-glycerol medium (Lennette et al., 1978).

Tubulin immunofluorescence staining: Cells grown on coverslips were quickly rinsed three times in a microtubule stabilizing buffer (PHEM: 45 mM PIPES, 45 mM HEPES, 5 mM $MgCl_2$, 10 mM EGTA, pH = 6.9). They were then permeabilized in a 0.5% Triton-X 100 solution in PHEM buffer for 1 min at room temperature. After 3 washes in PHEM buffer, cells were fixed in cold methanol for 6 min at −20° C. Immunofluorescence staining of tubulin was performed as follows: preincubation in 4% BSA solution in PBS for 30 min at room temperature, 3 washes in PBS, 60 min incubation at 37° C with anti-tubulin (Amersham) diluted in 1% BSA solution in PBS, 3 washes in PBS, 60 min incubation at 37° C with fluorescein-conjugated secondary antibody (Inst. Pasteur) diluted in 1% BSA solution in PBS, 2 washes in PBS, 2 washes in distilled water. Coverslips were mounted in a polyvinylalcohol-glycerol medium.

Preparations were observed with a Zeiss epifluorescence microscope and photographs were taken with Kodak film Tri x Pan 400 ASA.

RESULTS

The morphological features of the cells have been previously described (Lafarge-Frayssinet and Frayssinet, 1989).

Study of actin in microfilaments

F-actin staining by rhodamine-phalloidin in Cl_3 non-transformed cells treated with DMSO alone was characterized by the presence of bright needles in the cytoplasm and of a tight bundle of actin cables at the periphery of cells (Fig. 1a). A few cells appeared to lack intracellular actin fibers, whereas the majority were densely stained; nevertheless, all the cells showed heavy staining of peripheral actin. Intracytoplasmic fibers appeared attached to bundles of actin present at the periphery of cells. Cl_3 cells cultured in the presence of BV for 48 hours presented major alterations of microfilament organization shown in Figure 1b. In most of the cells no F-actin needles could be observed, Intracellular actin, when present, was fragmented. In numerous cells, actin was condensed in star-like structures as shown in Figure 1b and membrane associated actin was characterized by decreased staining, breaks and a loosening of fibers that were tightly bundled in control cells; some ruffling of the membrane was noted.

Similar alterations of microfilament organization were observed in Cl_3 cells cultured in the presence of PB for 48 hours (Fig. 1c). Intracellular F-actin disappeared and star-like structures were present. The most spectacular modification concerned the structure of peripheral actin which was loosened so as to form a ruffle around the cell.

In the presence of DMSO alone, microfilament organization of FV transformed cells hardly differed from that of Cl_3 non-transformed cells (Figure 2a). Intracellular actin fibers completely disappeared replaced by small granules ("vermiculated" form). Peripheral actin was condensed in some areas around the cell and cables joined these clusters of actin. Instead of tight bundles present in control Cl_3 non-transformed cells, peripheral actin of control FV cells formed ruffling structures showing many extensions outside of the cell. In opposition to Cl_3 non-transformed cells, FV cells were not sensitive to the action of 1×10^{-6}M BV (Fig. 2b) and 0.2×10^{-3}M PB (Fig. 2c) for 48 hours. In these culture conditions the aspect of microfilaments was quite similar to that described in control FV cells.

Study of tubulin in microtubules

Immunofluorescent staining of microtubules by anti α-tubulin antibody in control Cl_3 cells revealed an extended network of thin filaments starting from the heavily

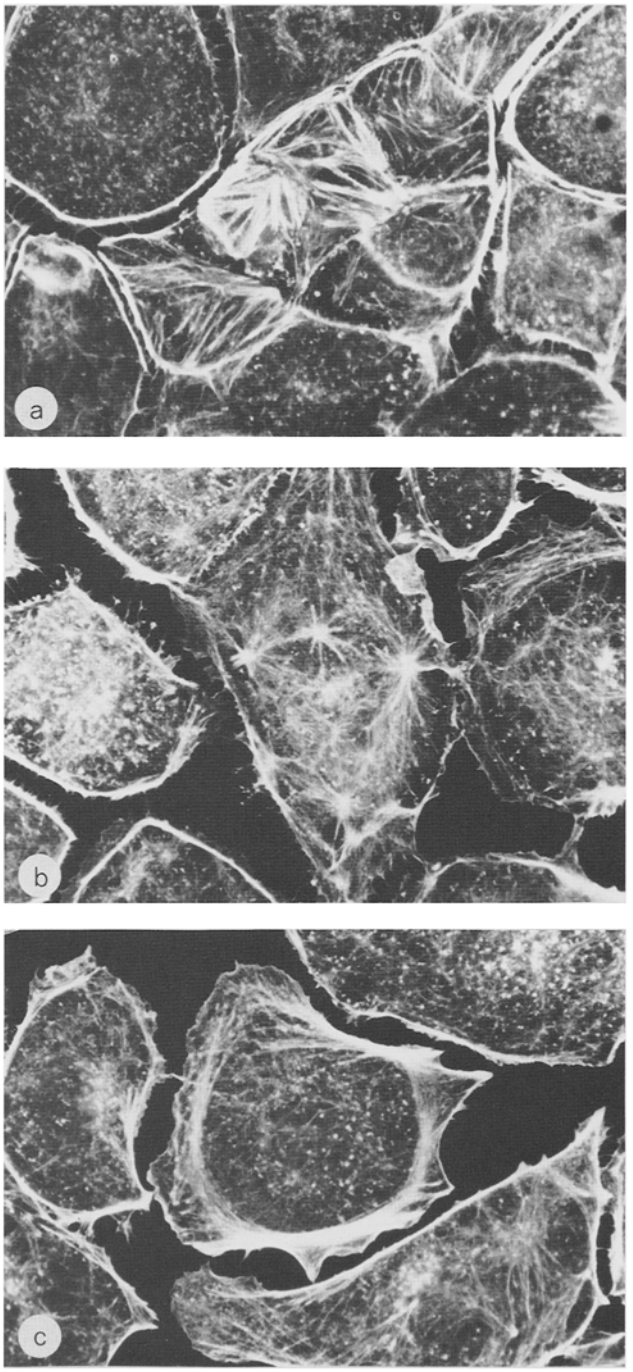

FIGURE 1. Microfilaments of Cl_3 non-transformed cells exposed to biliverdin and phenobarbital. Cells were grown on coverslips in culture medium containing: a) DMSO, b) 1×10^{-6}M/BV, c) 0.2×10^{-3}M PB for 48 h. F-actin was stained with rhodamine-phalloidin. × 1200.

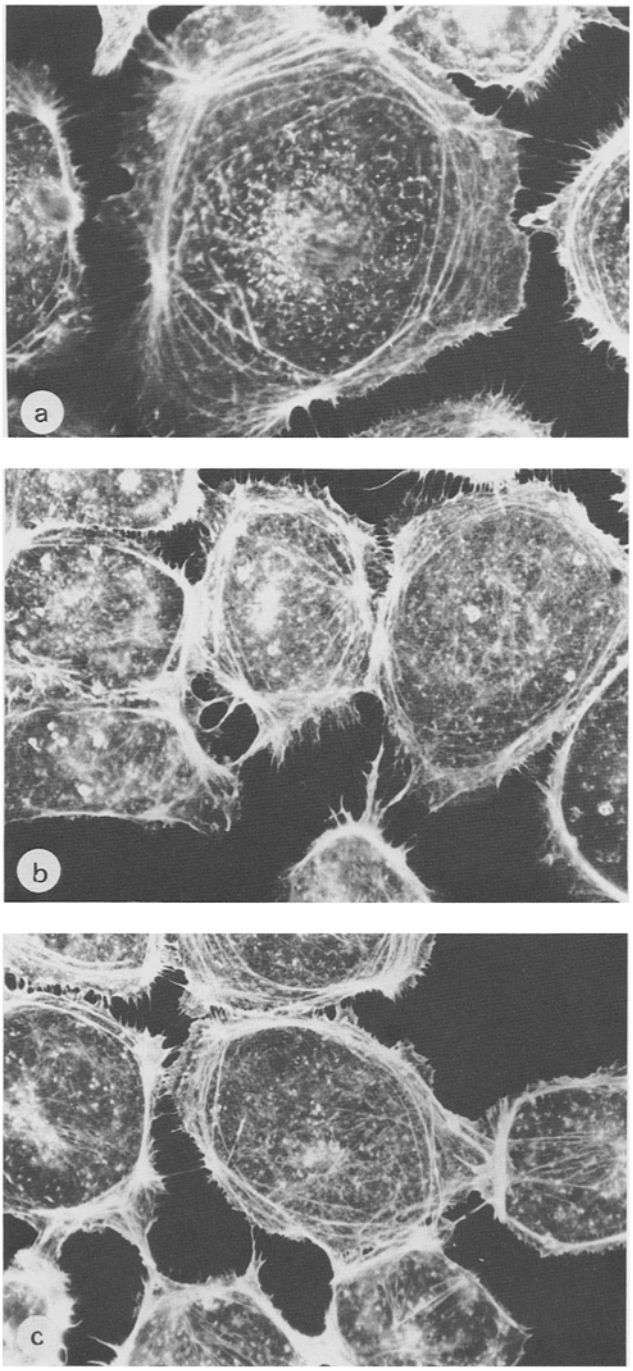

FIGURE 2. Microfilaments of FV transformed cells exposed to biliverdin and phenobarbital. Cells were grown on coverslips in culture medium containing: a) DMSO, b) 1×10^{-6}M BV, c) 0.2 x 10^{-3}M PB for 48 h. F-actin was stained with rhodamine-phalloidin. × 1200.

stained centrosome. Microtubules stretched from the nucleus to the periphery of the cell (Fig. 3a).

The addition of 1×10^{-6}M BV to culture medium of Cl_3 cells for 48 hours produced modifications of microtubules (Fig. 3b). The prominent feature was a disappearance of centrosome staining in several cells and tubulin formed a condensed circular area around the nucleus. A similar pattern of microtubule organization was observed in Cl_3 cells cultured in the presence of 0.2×10^{-3}M PB for 48 hours (Figs. 3c and d).

FV transformed cells cultured with DMSO alone presented a microtubule arrangement lacking a centrosome, with a very thin ring of tubulin around the nucleus and extremely thin and sparse network of microtubules (Fig. 4a), a picture closely resembling that observed in BV and PB treated Cl_3 cells (Figs. 3b and c). Culture of FV cells in a medium containing 1×10^{-6}M BV and 0.2×10^{-3}M PB did not significantly modify the aspect of microtubules (Figs. 4b and c).

DISCUSSION

The present results clearly demonstrate the effect of BV and PB on the structural organization of microfilaments and microtubules in cultured hepatic epithelial cells. The intense and rapid response of microfilaments and microtubules to both compounds which act as *in vivo* and *in vitro* hepatic promoters (Peraino et al., 1971; Lafarge-Frayssinet et al., 1983) might reflect a cellular response to injury rather than an event related to neoplastic transformation. This, however, could not be the case since the present data were obtained in the presence of non-cytotoxic concentrations of BV and PB. BV was used at a very low concentration (1×10^{-6}M) which does not produce any cytotoxic effect on hepatic cells on the basis of growth curve and ^{3}H-thymidine incorporation. Cl_3 non-transformed cells even respond to BV by a stimulation of ^{3}H-thymidine incorporation (Lafarge-Frayssinet et al., 1981) which has been likely related to the promoting effect of BV (Lafarge-Frayssinet et al., 1983). At a concentration of 0.2×10^{-3}M PB, a cytotoxic effect measured by cell counts could not be detected in epithelial cell culture in agreement with data obtained in adult rat hepatocytes which present an increase of lacticdehydrogenase release and a decreased ^{3}H-thymidine incorporation over 1 mM PB only (Decloitre, unpublished results).

Whether the effects of BV and PB on the cytoskeleton might be due to a specific unknown promotional mechanism or to a general disturbance of cell homeostasis could not be presently resolved. The potential mechanism of action of tumor promoters is mainly based upon knowledge of the effects of the skin tumor promoter, TPA. The mode of action of other promoters, showing different organ specificity such as BV and PB is largely unknown, except the involvement of cell-cell communication which has been demonstrated for PB (Williams, 1980; Trosko et al., 1982; Ruch and Klaunig, 1988; Mesnil et al., 1988). One common characteristic

of all the promoters is a reversibility of action after a short-term application. Therefore it is especially relevant to investigate whether the effects observed on the cytoskeleton are dependent on the promoter. Preliminary results of a reversibility study in progress indicate a good reversibility of the PB effect but less so far the BV effect. Further studies are needed to determine the conditions required to reach the complete or partial reversibility of effects. This tends to indicate a different mode of action of PB and BV.

The morphological changes which occurred in microfilaments and microtubules of Cl_3 non-transformed cells treated by BV and PB closely resembled that observed in untreated FV transformed cells. The relation between cytoskeleton anomalies and cell transformation has been already reported in virally transformed fibroblasts (Fine and Taylor, 1976; Edelman and Yahara, 1976; Brown et al., 1981; Carley et al., 1981). Whether this relationship implies cytoskeleton modifications as a primary event in transformation or as a consequence of the development of tumoral phenotype is not yet resolved. Alterations of cytoskeleton, especially microfilaments, similar to those observed in our experiments also occurred in fibroblastic (Rifkin et al., 1979; Delescluse et al., 1988) and epithelial cells (Schliwa et al., 1984; Kellie et al., 1985) treated with the skin tumor promoter, TPA indicating that components of cytoskeleton could be a primary target of TPA. Our data similarly suggest that the cytoskeleton of hepatic cells might be a target for PB and BV which act as *in vivo* and *in vitro* liver promoters. However, experiments have to be performed with a longer exposure of Cl_3 cells to BV and PB to ascertain a possible link between cell transformation and cytoskeletal disorganization.

In addition, our data demonstrated that control FV transformed cells showing loss and fragmentation of cytoplasmic actin, high disturbance of peripheral actin and apparent depolymerization of tubulin were not sensitive to BV and PB treatment. Some parallelism may be drawn between the effect of hepatic tumor promoters on the cytoskeleton of hepatic cells and on oncogene expression which has been implied in the neoplastic transformation. Ki-*ras*, *fos* and *myc* proto-oncogenes are over expressed in Cl_3 non-transformed cells exposed to the same BV and PB treatment used in the present study while FV transformed cells do not respond to the same treatment (Lafarge-Frayssinet and Frayssinet, 1989).

On another hand, alterations in microfilaments organization (actin depolymerization, ruffling) and an increased level of Ha-*ras* oncogene protein are both involved in the cellular response to a proliferative stimulus such as growth factors and TPA (Bar Sagi and Feramisco, 1986; Takahashi et al., 1986; Mellstrom et al., 1988). Our data demonstrate that the treatment of non-transformed epithelial hepatic cells by BV and PB, both compounds having promoting properties, deeply modify the structural organization of the microfilament-microtubule system while an over-expression of proto-oncogenes has been reported elsewhere. The relevance of these

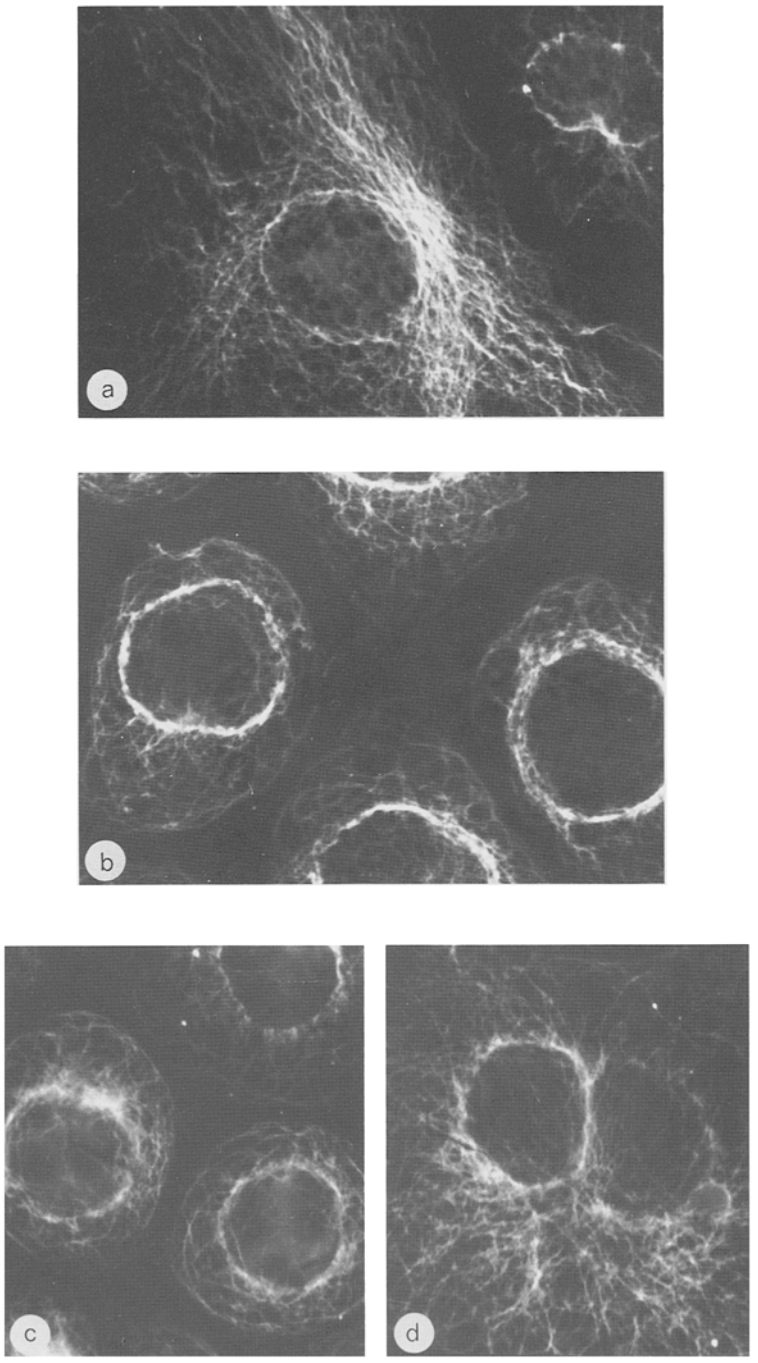

FIGURE 3. Microtubules of Cl_3 non-transformed cells exposed to biliverdin and phenobarbital. Cells were grown on coverslips in culture medium containing: a) DMSO, b) 1×10^{-6}M BV, c) and d) 0.2×10^{-3}M PB for 48 h. Tubulin staining was examined by indirect immunofluorescence. $\times$ 1200.

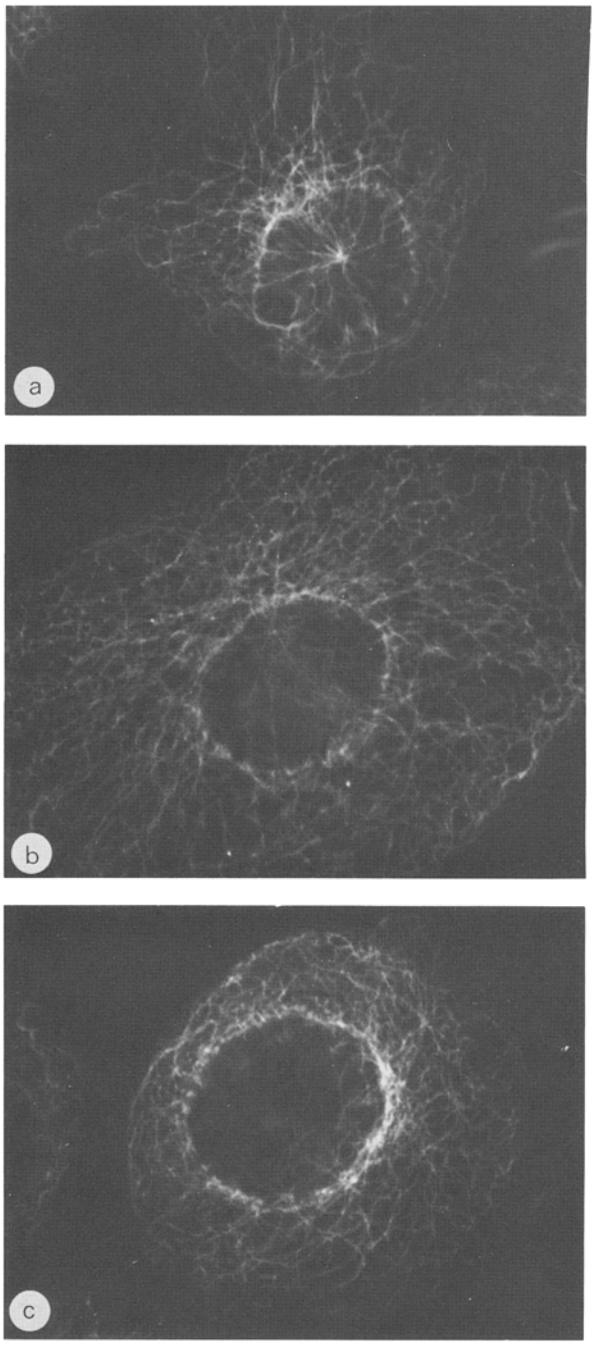

FIGURE 4. Microtubules of FV transformed cells exposed to biliverdin and phenobarbital. Cells were grown on coverslips in culture medium containing: a) DMSO, b) 1×10^{-6}M BV, c) 0.2×10^{-3}M PB for 48 h. Tubulin staining was examined by indirect immunofluorescence. × 1200.

observations to the possible promoting mechanism of actin of BV and PB require further studies.

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