Pergamon

Biochemical Pharmacology, Vol. 50, No. 8, pp. 1217–1222, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00261-8

PREFERENTIAL REQUIREMENT FOR PROTEIN TYROSINE PHOSPHATASE ACTIVITY IN THE 12-*O*-TETRADECANOYLPHORBOL-13-ACETATE-INDUCED DIFFERENTIATION OF HUMAN COLON CANCER CELLS

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(*Received* 29 *November* 1994; *accepted* 19 *April* 1995)

Abstract—Some lines of colon cancer cells are forced to undergo differentiation by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The increases in activities of both protein tyrosine phosphatase (PTP) and protein tyrosine kinase (PTK) have been reported to be associated with the TPA-induced differentiation of HL-60 leukemia cells. In the present study, a 2-fold increase in PTP activity was observed in SW620 human colon cancer cells after 30 min of TPA treatment; a maximal level (4- to 5-fold) was reached at 60 min and continued for more than 6 hr. In addition, two TPA-induced differentiated characteristics, morphological alteration and release of cellular surface proteoglycan, were effectively blocked by PTP inhibitors, such as sodium orthovanadate (50 μM), zinc chloride (100 μM), and iodoacetate (250 μM), but not by the protein serine/threonine phosphatase inhibitor okadaic acid (20 nM). On the other hand, although TPA induced a transient slight increase in PTK activity (1.4-fold) at 60 min, four PTK inhibitors (genistein, herbimycin A, tyrphostin-23 and quercetin) had different effects on the TPA-induced release of cell surface proteoglycan. Genistein (60 μM) potentiated this process, but in contrast, quercetin (45 μM) could partially inhibit the TPA effect. Taken together, these observations suggest that both PTP and PTK activities were increased in SW620 cells in response to TPA; however, the activation of PTP seems to be preferentially required for the TPA-induced differentiation of SW620 human colon cancer cells.

Key words: 12-*O*-tetradecanoylphorbol-13-acetate; surface proteoglycan; protein tyrosine phosphatase; protein tyrosine kinase; differentiation; human colon cancer cells

Protein tyrosine phosphorylation is an important cellular event involved in the mediation of a plethora of signal transduction events [1–3]. The level of cellular P-tyr§ is determined by the equilibrium of dynamic actions between PTK, responsible for protein phosphorylation, and PTP, which catalyzes protein dephosphorylation [4, 5]. Recently, a decrease in the fraction of P-tyr was reported to be associated with the TPA-induced differentiation of HL-60 leukemia cells [6–8]. The decrease of P-tyr seemed to be due to the 3- and 11-fold increases of PTK and PTP activities, respectively. Furthermore, TPA could induce PTP 1C activity in HL-60 cells by elevating its gene expression [9]. The time-course increment of PTP 1C activity was closely correlated with the acquisition by HL-60 cells of a macrophage-like phenotype. These data suggest that the reduction of the protein P-tyr level resulting from the activation of PTP is presumably essential for the TPA-induced differentiation process of HL-60 leukemia cells. Interestingly, TPA also induces the differentiation of human colon cancer cells [10–12]. This process included morphological change, cell growth arrest, and release of cell surface heparan sulfate proteoglycans. Although the mechanism by which TPA-induced differentiation in leukemia cells has been investigated extensively, little is known about other tumor cells such as colon cancer.

In this study, we found that TPA rapidly induced the decrease of some cellular phosphotyrosyl proteins by eliciting an elevated and continuing PTP activity in SW620 human colon cancer cells. Moreover, sodium orthovanadate, zinc chloride and iodoacetate, some known PTP inhibitors, could effectively block the TPA-induced differentiated phenotypes, such as morphological change and degradation of surface heparan sulfate proteoglycans. Inhibitors of protein serine/threonine kinase, such as okadaic acid, however, failed to have the same effect. In addition, genistein, herbimycin A, quercetin and tyrphostin-23, which are potent inhibitors of PTK, were examined for their effects on TPA-treated SW620 cells. Only genistein potentiated the TPA-induced differentiation of SW620 cells, even if these inhibitors of PTK may be inducers of differentiation of other cancer cells [13–15]. These results suggest that the activation of PTP also plays an important role in the TPA-induced differentiation of SW620 human colon cancer cells.

MATERIALS AND METHODS

Cell culture

The SW620 colon cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultivated in RPMI 1640 medium supple-

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§ Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; P-tyr, phosphotyrosine; CPC, cetylpyridinium chloride; and GT, poly(glutamine, tyrosine) 4:1.

mented with 10% fetal bovine serum, 2 mM glutamine, and 10 U/mL penicillin.

PTP assay

The PTP activity assay was performed by a non-radioactive tyrosine phosphatase assay kit (Boehringer Mannheim). Cells grown on 10-cm dishes were washed twice with cold phosphate-buffered saline. After that, cells were collected and resuspended in enzyme assay buffer (20 mM Tris-HCl, 0.1% 2-mercaptoethanol, 1 mg/mL bovine serum albumin), sonicated for 30 sec by a Branson sonicator (Danbury, CT), and then centrifuged at 15,000 *g* for 10 min at 4°. The protein concentrations of supernatants were determined by the Bradford method. An equal amount of proteins was used to assay the PTP activity according to a Boehringer Mannheim manual. The concentrations of substrate and enzyme were adapted to the optimal condition to ensure that this assay was in the linear range.

Western blot analysis of P-tyr

Cellular lysates were prepared as described [16]. Fifty micrograms of each lysate was subjected to separation on 10% SDS-polyacrylamide gels, which were then electroblotted onto nitrocellulose filters. After blocking, blots were incubated with anti-P-tyr antibody (UBI) in PBST (phosphate-buffered saline plus 0.05% Tween-20) for 1 hr, followed by two washes (15 min each) of PBST, and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham) for 30 min. After washing, blots were incubated for 1 min with ECL reagent (Amersham), and chemiluminescence was detected by exposure of the filters to Kodak X-Omat films for 10 sec to 10 min.

Proteoglycan release assay

Cells were prelabeled with 5 mCi/mL of [^{35}S]sulfate for 16–18 hr as described previously [17]. Prelabeled cells on a 6-well dish were fed with medium containing 100 nM TPA or 0.05% DMSO as vehicle for the indicated times. The medium was collected and centrifuged at 1000 *g* for 10 min and then mixed with 100 μg of dextran sulfate (M_r 8000; Sigma) as a co-precipitate for the released proteoglycan. Cells were removed from dishes with trypsin-EDTA plus 0.01% (w/v) dextran sulfate S-MEM. An equal volume of a solution containing 0.2% (w/v) cetylpyridinium (CPC), 10 mM EDTA, 10 mM sulfate and 10 mM acetate (pH 6) was added to both sample fractions in siliconized tubes. After incubation at 36° for 60 min, CPC-proteoglycan precipitates were collected on glass fiber filters (Whatman GF-C) and counted for radioactivity by liquid scintillation.

PTK assay

The phosphorylation of the tyrosine residues of artificial substrates was measured by a modification of the method of Braun *et al.* [18]. Proteins (10–20 μg) obtained from cell lysates were incubated with or without GT substrate (1 mg/mL) in tyrosine kinase buffer (20 mM HEPES, pH 7.4, 12 mM $MnCl_2$, 10 μM $ZnCl_2$, 0.5% Nonidet P-40).

The reactions were initiated by the addition of [γ-^{32}P]ATP (3 Ci/mmol) at a final concentration of 25 μM. After incubation at 22° for 3 min, the reaction was terminated by the addition of 7 μL of unlabeled ATP (10 mM); the total reaction volume was 100 μL. Fifty microliters of the mixture was then applied to a 1 cm^2 area of a Whatman 3MM filter, which was washed and counted as described [8]. In each case, radioactivity was determined by liquid scintillation spectrometry, and the real phosphorylation level represented the difference between tubes with or without the GT substrate.

RESULTS

To examine what role protein tyrosine phosphorylation plays in the TPA-induced differentiation process of SW620 cells, we first assayed the PTP activity in SW620 cells treated with or without TPA. As shown in Fig. 1, a 2-fold induction of PTP activity appeared within 30 min of 100 nM TPA treatment, and a maximal level (4- to 5-fold) was reached after a further 30 min. Then the activity of PTP declined gradually to the basal level after 48 hr (data not shown). To determine the significance of stimulated PTP activity in TPA-treated SW620 cells, we performed a P-tyr western blot analysis of total cell lysates. A small increase in band intensities of phosphotyrosyl proteins was observed after TPA treatment for 15 and 30 min (Fig. 2); in contrast, the intensities of phosphotyrosyl protein bands with apparent molecular weights of 85–100 kDa were obviously decreased when cells were exposed to TPA for 60 min. After a longer period (8 hr), additional phosphotyrosyl proteins ranging from 40 to 60 kDa were also attenuated by TPA. Therefore, the activation of PTP is a very important biochemical event at the early stage of cellular response to TPA in SW620 cells.

We also determined whether the activation of PTP was involved in the differentiation of SW620 cells induced by TPA. To address this, three known PTP inhibitors, sodium orthovanadate, zinc chloride and iodoace-

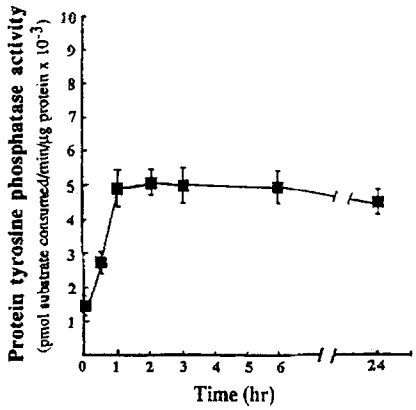

Fig. 1. Time-dependent increase of PTP activity in TPA-stimulated SW620 cells. SW620 cells were treated with 100 nM TPA for the indicated times. PTP activity was assayed as described in Materials and Methods. Each value is the mean ± SD of triplicate determinations. The result was reproducible in two separate experiments. By calculating the amount of substrate turn-over from a phosphopeptide standard curve, enzyme activity can be expressed as pmol of dephosphorylated substrate per min per μg of protein.

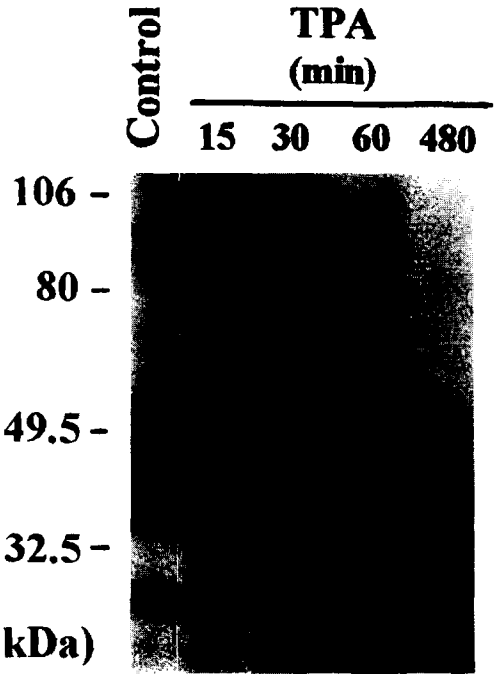

Fig. 2. Attenuation of protein phosphotyrosine in SW620 cells treated with TPA. Cell lysates were prepared and immunoblotted with anti-P-tyr antibody as described in Materials and Methods. The data are representative of at least five similar experiments. The numbers on the left indicate the positions of molecular weight standards.

tate [19–21], were examined for their effects on TPA-induced differentiation characteristics. If SW620 cells were treated with TPA for 24 hr, the original aggregative epithelioid morphology changed into extraordinary flattened differentiated cells with a marked increase of the intercellular space (Fig. 3, A and B). The remarkable morphological alteration induced by TPA was abolished completely by sodium orthovanadate at a concentration of 50 μM (Fig. 3C). In contrast, the well-known protein serine/threonine phosphatase inhibitor okadaic acid (20 nM) did not interfere with the TPA effect (Fig. 3D), even if its concentration was increased up to a cytotoxic dose of 50 nM. Two other PTP inhibitors, zinc chloride (100 μM) and iodoacetate (250 μM), also had the ability to inhibit the TPA-induced morphological alteration significantly (data not shown). Of all the PTP inhibitors tested, at the same concentration, sodium orthovanadate exhibited the most inhibitory activity on the morphological change induced by TPA. In addition, we examined the release of cell surface proteoglycan, which also has been used as a differentiation marker. As shown in Fig. 4, TPA induced a rapid degradation of the [^{35}S]sulfate proteoglycan from the cell surface within 30 min, and this effect lasted for 120 min. Consistent with the observation on cell morphology, we found that all three PTP inhibitors effectively prevented the releasing process, even though okadaic acid failed to have the same effect (Fig. 4). These results indicated that PTP activity is preferentially required for the TPA-induced differentiation of SW620 colon cancer cells. However, the protein kinase C inhibitor staurosporine also dramatically decreased the release process induced by TPA.

To check whether PTK is also essential for the TPA-induced differentiation of SW620 cells, we examined the PTK activities in cells exposed to a time-course treatment of TPA. It was observed that TPA induced slight increases of PTK activity after 30 and 60 min (1.2- and 1.4-fold, respectively) (Fig. 5). However, the PTK activity rapidly returned to basal level if cells were treated for more than 3 hr. Moreover, four PTK inhibitors,

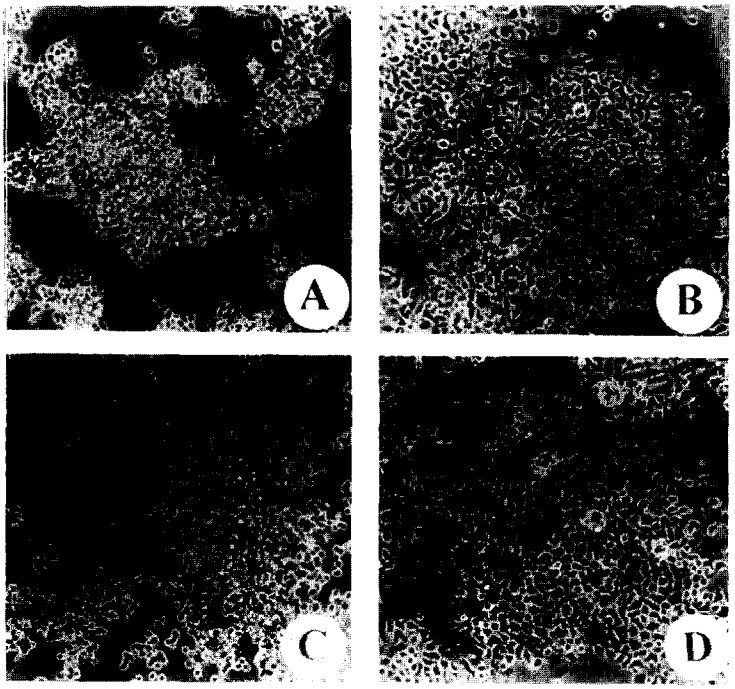

Fig. 3. Effects of sodium orthovanadate and okadaic acid on the TPA-induced differentiated morphologic features of SW620 human colon cancer cells. Cells in the exponential growth phase were cultured in the absence (A) or the presence of 100 nM TPA (B), TPA plus 50 μM sodium orthovanadate (C) or TPA plus 20 nM okadaic acid (D). The photographs were taken 24 hr later. Magnification: 60×.

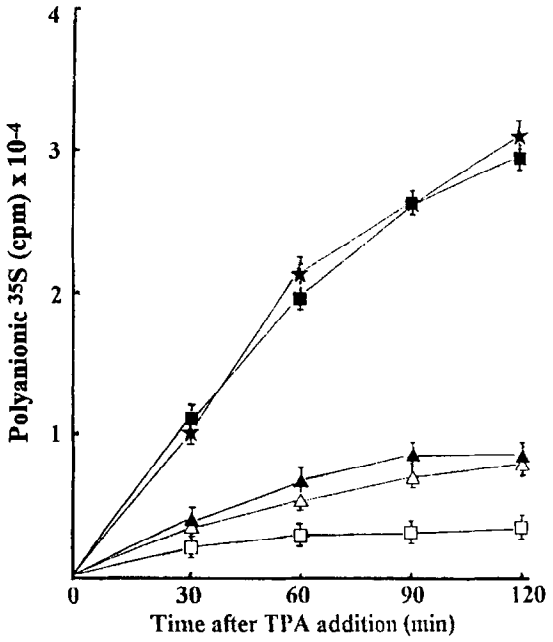

Fig. 4. Suppressive effect of PTP inhibitors on the release of cell surface proteoglycan from differentiating colon cancer cells induced by TPA. Cells were prelabeled with [^{35}S]sulfate, as described in Materials and Methods, and refed with fresh medium at 37° in the presence of 100 nM TPA (★) or plus 50 μM sodium orthovanadate (□), 100 μM zinc chloride (▲), 250 μM iodoacetate (△), and 20 nM okadaic acid (■). The total cell-associated CPC precipitated level at zero time was 87,500 cpm. Data represent CPC precipitable label in culture medium (means ± SD; N = 3). Values on the y-axis represent the amount of [^{35}S]proteoglycan present in the culture medium that was precipitated by CPC.

genistein, herbimycin A, tyrphostin-23 and quercetin, were used to treat SW620 cells concomitantly with TPA. All four compounds were able to inhibit PTK activity in SW620 cells (Table 1). In the proteoglycan release assay, only genistein potentiated the inductive effect of TPA; herbimycin A and tyrphostin-23 had no effect (Table 1). In contrast, quercetin could partially block the releasing process stimulated by TPA. In cellular morphological observations, concomitant treatment of SW620 cells with TPA and genistein (30 μM) for 24 hr resulted in more flattened and large intercellular-space differentiated cells (Fig. 6C) as compared with TPA-treated cells (Fig. 6B). Consistent with the results obtained in the proteoglycan release assay, herbimycin A and tyrphostin-23 did not affect any morphological change induced by TPA, even at a higher concentration (data not shown). All these PTK inhibitors by themselves did not cause the differentiation-like morphology of SW620 cells (not shown).

DISCUSSION

The data presented here demonstrate that the activities of both PTP and PTK are increased when colon cancer cells are forced by TPA to undergo the differentiation process. Although the induction of PTP and PTK was found in the differentiation of HL-60 cells initiated by TPA and the combination of α-interferon and tumor necrosis factor [7, 22], the experiments described in this paper extend these findings into TPA-induced colon cancer cell differentiation. Since protein tyrosine phosphorylation was mostly linked to cellular proliferation, and differentiation was accompanied by the programmed shutdown of the cell cycle, the decrease in P-tyr content of cellular proteins that occurs with maturation was not surprising. Moreover, specific PTP inhibitors could dramatically block the TPA effect, suggesting that the elevated PTP activity is essential for the TPA-induced differentiation of colon cancer cells. However, the increase of PTK activity was transient and occurred at an early stage of TPA treatment. It is more likely that the slight induction of PTK could play a role in the immediate early signal transduction in response to TPA, rather than being related to the differentiation process as observed in the HL-60 cells.

In this study, the release of cell surface proteoglycan was used as a biochemical marker to monitor the differentiation. Several cancer cells have been reported to release surface heparan sulfate to the culture medium as they respond to various differentiation inducers [23, 24]. The release process initiated by a novel membrane-localized protease did not require *de novo* protein synthesis, but depended on PKC activity [25]. Our data also suggest that both PTP and PKC are involved in this process, but the relationship between PTP and PKC is not clear yet. However, it has been reported that the phosphorylation may provide a mechanism for controlling PTP activity. For example, TPA induces a rapid phosphorylation of CD45 in T cells [26], and the phosphorylation of PTP1B in HeLa cells is also enhanced by TPA [27]. Therefore, it is possible that the TPA-induced release of proteoglycan is via a preliminary signalling by PKC, subsequent PTP phosphorylation, and finally the activation of membrane-associated protease.

Of the PTK inhibitors tested, only genistein significantly potentiated the TPA-induced differentiation of SW620 cells. Since tyrphostin-23, quercetin, and herbimycin A abolished the TPA-induced PTK activity as effectively as genistein did, the potentiation of differentiation by genistein seems to result from some other mechanism, such as the inhibition of topoisomerase II

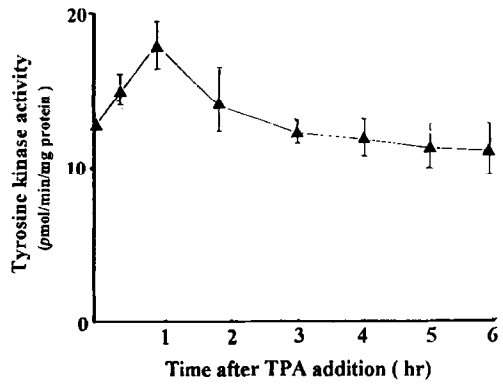

Fig. 5. Kinetic study of protein tyrosine kinase activity induced by TPA in SW620 cells. Cells were treated with 100 nM TPA for the indicated time. PTK activity from cell extracts was determined as described in Materials and Methods. Each value is the mean ± SD of triplicate determinations.

Table 1. Effects of protein tyrosine kinase inhibitors on TPA-induced proteoglycan release and tyrosine kinase activity

Compounds	Concentration*	Proteoglycan released† (cpm)	Protein tyrosine kinase activity‡ (pmol/min/mg protein)
None		2,800 ± 570	11.4 ± 0.41
TPA	100 nM	32,300 ± 890	15.8 ± 0.35
+Genistein	60 μM	46,920 ± 910§	10.7 ± 0.15‖
+Tyrphostin-23	60 μM	31,660 ± 690	9.9 ± 0.38‖
+Quercetin	45 μM	28,900 ± 970	9.6 ± 0.35‖
+Herbimycin A	3 ng/mL	31,300 ± 890	11.4 ± 0.16‖

* The concentrations of these protein kinase inhibitors used here were equitoxic (LC_{37}).

† Cells were prelabeled with [^{35}S]sulfate, as described in Materials and Methods, and refed with fresh medium at 37° in the presence of 100 nM TPA or TPA plus the above-indicated tyrosine kinase inhibitors for 2 hr. The total cell-precipitable level in culture medium is shown (means ± SD; N = 3).

‡ Cell extracts of untreated or treated cells were used to measure tyrosine kinase activity, as described in Materials and Methods. Each value is expressed as the mean ± SD of triplicate determinations. PTK activity was measured in this assay at 1 hr of TPA or TPA plus inhibitor treatment. Statistical analysis was carried out using Student's *t*-test.

§ Significantly different from the TPA-treated group, $P < 0.001$.

‖ Significantly different from the TPA-treated group, $P < 0.05$.

activity [28]. Perhaps, this may reflect on the selectivity of actions of different PTK inhibitors. For example, owing to a higher binding affinity, genistein exhibits a more specific inhibition of epidermal growth factor (EGF) receptor than do other inhibitors [29, 30]. Interestingly, EGF receptor and $p60^{src}$ were found to be highly expressed and probably responsible for retaining the characteristics of numerous colon cancer cell lines [31, 32]. On the other hand, the flavonoid quercetin was reported to inhibit not only PTK but also serine/threonine kinase like PKC [33]. It is well known that some novel PKC isoforms are involved in the TPA-induced differentiation of leukemia cells [34]. This may explain why quercetin can partially inhibit the TPA-induced proteoglycan release. To study what role PKC isoforms play in signal transduction will provide a clue to understanding the mechanism of the TPA-induced differentiation of colon cancer cells.

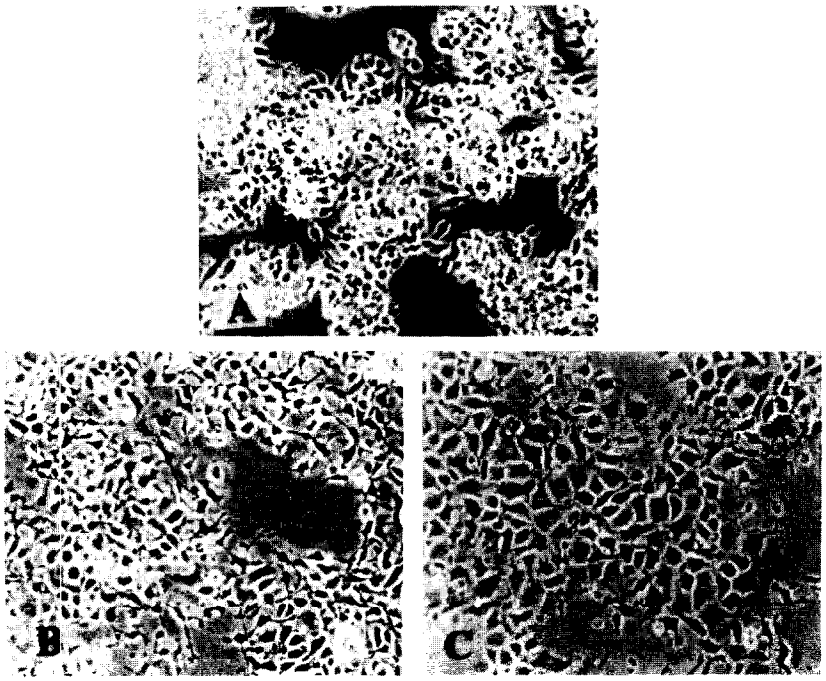

Fig. 6. Effects of protein tyrosine kinase inhibitors on the TPA-induced differentiated morphology of SW620 human colon cancer cells. Cells in the exponential growth phase were cultured in the absence (A) or the presence of 100 nM TPA (B), or TPA plus 30 μM genistein (C). The morphology of SW620 cells that were treated with genistein alone was identical to that observed in panel A. The photographs were taken 24 hr later. Magnification: 60×.

Acknowledgements—This study was supported by the National Science Council, NSC 84-0412-B002-033 and NSC 83-0412-B002-103, Taipei, Taiwan, R.O.C.

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