Enhancement of growth factor-induced DNA synthesis by colon tumor-promoting bile acids in Swiss 3T3 cells

Their different mode of action from that of phorbol esters

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In Swiss 3T3 cells, colon tumor-promoting deoxycholate (DOC) enhanced DNA synthesis which was induced by fibroblast growth factor (FGF) in the presence of insulin. This effect was observed only when DOC was added within 10 h after the addition of FGF. DOC by itself did not induce DNA synthesis irrespective of the presence or absence of insulin. Similar results were obtained with other colon tumor-promoting bile acids such as cholate, chenodeoxycholate and taurocholate. In contrast to these bile acids, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced DNA synthesis fully without FGF in the presence of insulin. DOC did not affect TPA-induced DNA synthesis. Prolonged treatment of the cells with phorbol-12,13-dibutyrate caused the down-regulation of the phorbol ester receptor and rendered the cells unresponsive to TPA. In these cells, FGF still induced DNA synthesis in the presence of insulin, but the maximal level was reduced to about one third of that in the control cells. DOC did not enhance this DNA synthesis any more. DOC did not alter the binding of FGF to the cells. These results indicate that colon tumor-promoting bile acids enhance the mitogenic action of FGF and thereby stimulate DNA synthesis, although the phorbol ester substitutes for the mitogenic action of FGF.

Tumor promoter *Bile acid* *Phorbol ester* *DNA synthesis* *(Fibroblast)*

1. INTRODUCTION

Although the initiation-promotion concept of tumorigenesis was developed from experiments with phorbol esters in mouse skin, recent studies have demonstrated that tumor promotion may be operative in other tissues (reviews [1–3]). Epidemiological studies have suggested that bile acids derived directly or indirectly from dietary factors may be responsible for the etiology of colon carcinoma [4,5]. Moreover, it has been demonstrated experimentally that development of colon carcinoma in rat pretreated with some carcinogens was enhanced by administration of bile acids to the colon either medically or surgically [6–12]. Based on these observations, it has been proposed that bile acids may serve as tumor promoters for colon carcinoma [13,14].

Although phorbol esters have been shown to affect a wide variety of biological activities, including stimulation of DNA synthesis and cell proliferation, induction of cell differentiation, stimulation of exocytosis, induction of enzymes and stimulation of lipid metabolism [1–3], the biological activities of bile acids related to tumor

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DOC, deoxycholate; FGF, fibroblast growth factor; PDBu, phorbol-12,13-dibutyrate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PDGF, platelet-derived growth factor

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promotion have not yet been clarified except that they have been shown to induce ornithine decarboxylase in rat colon [15,16]. Among these biological activities of phorbol esters, their mitogenic or comitogenic activities are most commonly observed in many types of cells [1–3]. Therefore, we investigated the effect of colon tumor-promoting bile acids on DNA synthesis in Swiss 3T3 fibroblasts, and compared their mode of action with that of TPA, one of the most potent tumor-promoting phorbol esters [1–3].

This paper describes that colon tumor-promoting bile acids enhance FGF-induced DNA synthesis. This action of the bile acids is totally dependent on the growth factor and the bile acids by themselves are inactive. This is in marked contrast to the action of TPA which by itself substitutes for FGF and induces DNA synthesis. To our knowledge, the agonist-dependent action of the bile acids has not been described in the literature.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

Swiss 3T3 cells, homogeneous bovine pituitary FGF and dehydrocholate were kindly supplied by Dr E. Rozengurt (Imperial Cancer Research Fund, England), Dr D. Gospodarowicz (University of California, San Francisco, USA) and Dr K. Uchida (Shionogi Research Laboratories, Japan), respectively. TPA and PDBu were obtained from CCR. Sodium DOC, cholate, chenodeoxycholate and taurocholate were purchased from Difco, Wako, Aldrich and Sigma, respectively. Other materials and chemicals were obtained from commercial sources.

2.2. *Cell culture*

Stock cultures of Swiss 3T3 cells were maintained at 37°C in a humidified atmosphere of 10% CO_2:90% air in DMEM containing 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were subcultured into a 35 mm dish with the medium containing 10% FCS, and refed with the same medium after 2 days. Such cultures were used 5 days after the last change of the medium. These cells were confluent and quiescent.

2.3. *Assay for DNA synthesis*

DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine into DNA by the method of Dicker and Rozengurt [17] except that the cells were stimulated for 24 h by FGF and exposed to 2 μCi [^{3}H]thymidine for 4 h before termination of the reaction. When bile acids were added to the cells, each bile acid was added to the medium during the first 5 h in a 24 h incubation, cells washed twice with DMEM and incubation continued with the original medium containing FGF.

2.4. *Down-regulation of the phorbol ester receptor*

Quiescent cultures of the cells were incubated with DMEM containing 200 nM PDBu and 0.1% bovine serum albumin for 24 h as described [18]. Under these conditions, [^{3}H]PDBu-binding activity disappeared completely as in [18].

2.5. *Assay for FGF binding*

FGF-binding assay was carried out under the conditions employed for the PDGF-binding assay as in [19] except that ^{125}I-labeled FGF with a specific activity of 1.5 μCi/μg was used. Radioactive FGF was prepared by the method of Bolton and Hunter [20].

3. RESULTS

Incubation of quiescent cultures of Swiss 3T3 cells with various doses of FGF in the presence of insulin markedly stimulated DNA synthesis as shown in fig.1. Either FGF or insulin alone stimulated this reaction to a small extent. Addition of a small amount (100 μM) of DOC to the cells during the first 5 h markedly enhanced DNA synthesis which was induced by FGF in the presence of insulin, particularly at lower doses of FGF. DOC did not affect DNA synthesis induced slightly by FGF in the absence of insulin. The stimulatory effect of DOC on DNA synthesis was dose-dependent, and 25–200 μM DOC was effective in this capacity. DOC by itself in any amount (25–200 μM) did not induce DNA synthesis in the absence of FGF. Incubation with DOC from the 5th to 10th hour after stimulation with FGF was also effective in stimulating DNA synthesis, but the stimulatory effect of DOC was less than that in

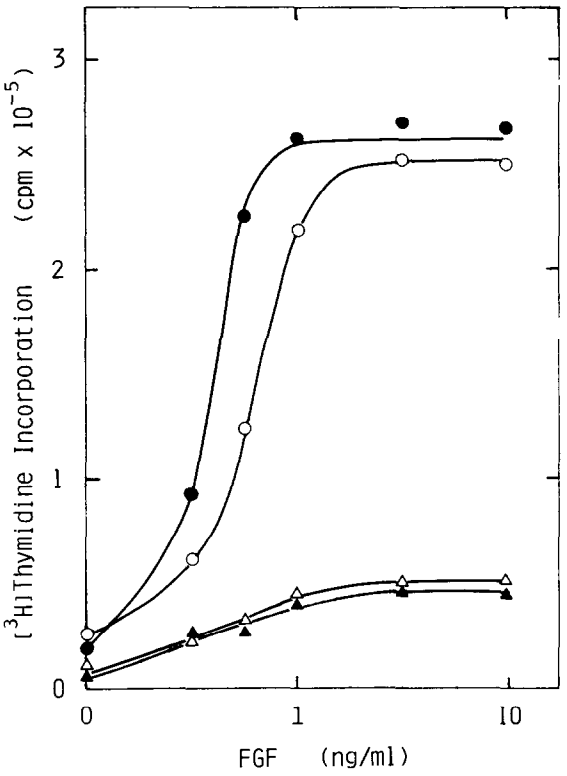

Fig.1. Effect of DOC on FGF-induced DNA synthesis. Quiescent cultures were stimulated by various doses of FGF in the presence or absence of insulin (1 μg/ml). DOC (100 μM) was added during the first 5 h. Other details are described in section 2. (○) Without DOC in the presence of insulin, (●) with DOC in the presence of insulin, (△) without DOC in the absence of insulin, (▲) with DOC in the absence of insulin. Each value is the mean of triplicate determinations.

the cells to which DOC was added during the first 5 h, as shown in table 1. Addition of DOC, 10 h after the initial stimulation with FGF, no longer enhanced DNA synthesis. The similar stimulatory effect of DOC on FGF-induced DNA synthesis

Table 1

Chronological analysis of effect of DOC on FGF-induced DNA synthesis

FGF (ng/ml)	[^{3}H]Thymidine incorporation into DNA (cpm × 10^{-3})				
	Period of DOC treatment				
	None	0–5 h	5–10 h	10–15 h[a]	15–20 h[a]
0	23.1	22.2	24.8	16.4	15.7
0.6	130.2	219.4	154.8	92.5	94.6
1.0	218.3	260.5	242.4	143.4	152.9
3.0	247.3	266.1	250.2	217.0	209.7

[a] These experimental values were slightly lower than those of the control experiments. This was due to some toxic effects of DOC which detached part of the cells from the dishes during these periods

Quiescent cultures were stimulated by the indicated doses of FGF in the presence of insulin (1μg/ml). DOC (100 μM) was added in the indicated 5 h period in the 24 h incubation. Other details are described in section 2. Each value is the mean of triplicate determinations

Table 2

Effect of various bile acids on FGF-induced DNA synthesis

Bile acids	[^{3}H]Thymidine incorporation into DNA (cpm × 10^{-3})			
	None	FGF (0.6 ng/ml)	FGF (1.0 ng/ml)	FGF (3.0 ng/ml)
None	23.1	130.2	218.3	247.3
Deoxycholate	22.2	219.4	260.5	266.1
Cholate	23.0	190.7	263.3	250.0
Chenodeoxycholate	21.2	209.3	275.0	269.2
Taurocholate	24.0	203.3	261.2	270.1
Dehydrocholate	20.2	141.0	208.2	243.8

Quiescent cultures were stimulated by indicated doses of FGF in the presence of insulin (1 μg/ml). Each bile acid (100 μM) was added during the first 5 h. Other details are described in section 2. Each value is the mean of triplicate determinations

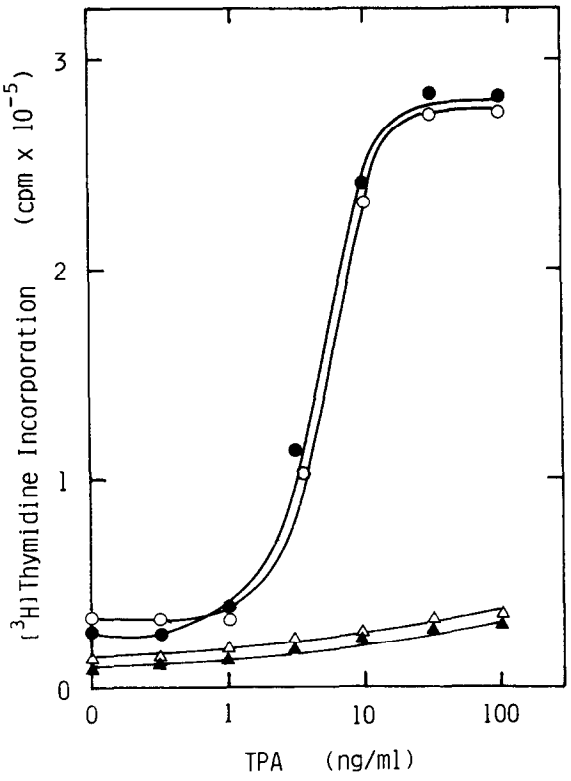

Fig.2. Effect of DOC on TPA-induced DNA synthesis. Quiescent cultures were stimulated by various doses of TPA in the presence or absence of insulin (1 μg/ml). DOC (100 μM) was added during the first 5 h. Other details are described in section 2. (○) Without DOC in the presence of insulin, (●) with DOC in the presence of insulin, (△) without DOC in the absence of insulin, (▲) with DOC in the absence of insulin. Each value is the mean of triplicate determinations.

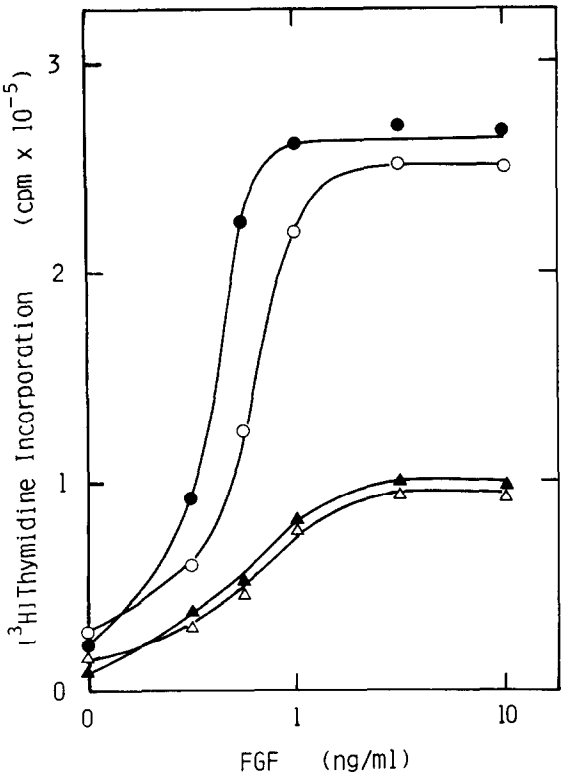

Fig.3. Effect of DOC on FGF-induced DNA synthesis in cells pretreated with PDBu. Quiescent cultures were treated with or without PDBu (200 nM) at 37°C for 24 h. After washing with DMEM, the cultures were stimulated by various doses of FGF in the presence of insulin (1 μg/ml). DOC (100 μM) was added during the first 5 h. Other details are described in section 2. (○) In the absence of DOC without PDBu pretreatment, (●) in the presence of DOC without PDBu pretreatment, (△) in the absence of DOC with PDBu pretreatment, (▲) in the presence of DOC with PDBu pretreatment. Each value is the mean of triplicate determinations.

was also observed with other colon tumor-promoting bile acids such as cholate, chenodeoxycholate and taurocholate as shown in table 2. Dehydrocholate, which is inactive in induction of ornithine decarboxylase in rat colonic epithelia [15], was ineffective at stimulating DNA synthesis.

In contrast to the bile acids, TPA by itself stimulated DNA synthesis without FGF in the presence of insulin as shown in fig.2. DOC did not affect TPA-induced DNA synthesis. Moreover, treatment of the cells with PDBu for 24 h caused the down-regulation and complete disappearance of the phorbol ester receptor and rendered the cells unresponsive to TPA as described in [18]. In these cells, TPA no longer induced DNA synthesis, but FGF still induced DNA synthesis although the level of this reaction was reduced to about one third of that in the control cells. DOC did not enhance FGF-induced DNA synthesis in these cells as shown in fig.3.

In the last set of experiments, the effect of DOC on FGF binding to the cells was examined. As

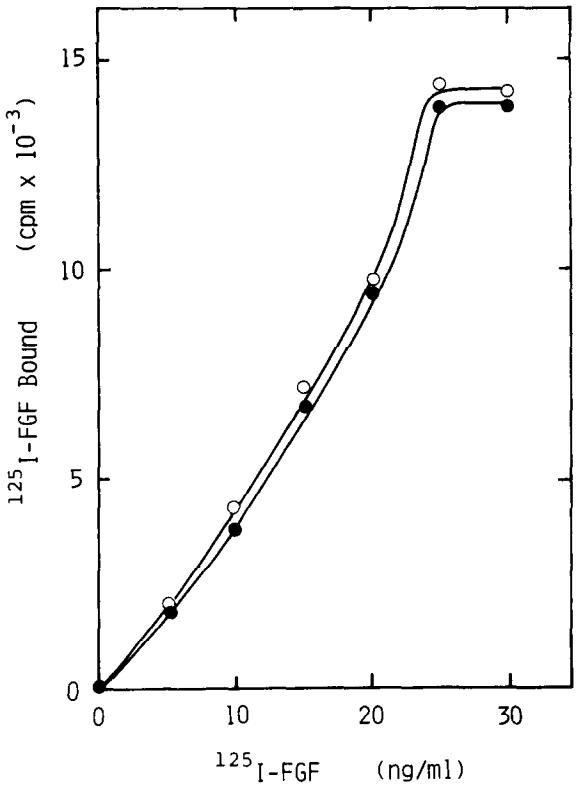

Fig.4. Effect of DOC on ^{125}I-FGF binding to the cells. Quiescent cultures were incubated for 60 min at 37°C with various doses of ^{125}I-FGF in the presence or absence of DOC (100 μM). Other details are described in section 2. Specific binding was calculated by subtraction of nonspecific binding from total binding. Nonspecific binding was defined as tracer binding in the presence of 500 ng/ml of unlabeled FGF and about 60–70% of total binding. (○) Specific binding without DOC, (●) specific binding with DOC. Each value is the mean of triplicate determinations.

shown in fig.4, ^{125}I-labeled FGF bound to the cells in a dose-dependent manner. DOC (100 μM) did not show any effect on this binding of FGF (fig.4).

4. DISCUSSION

Our results clearly indicate that, although bile acids by themselves are not mitogenic, they enhance FGF-induced, insulin-dependent DNA synthesis. This action is in marked contrast to that of TPA. TPA is able to induce DNA synthesis even without FGF. Although it is not known whether the stimulatory effect of bile acids on DNA synthesis is related to their action as detergents, this effect is not nonspecific since bile acids affect neither TPA-induced DNA synthesis nor the binding of FGF to the cells. We have also confirmed that incubation with 100 μM DOC for 5 h does not destroy the cells as judged by a trypan blue exclusion test, although incubation with the same amount of DOC for more than 5 h or incubation with DOC in amounts higher than 200 μM for 5 h shows toxic effects on the cells.

FGF as well as PDGF has been demonstrated to serve as a 'competence factor' in Balb/c 3T3 cells [21]. On the other hand, insulin as well as somatomedin C has been shown to be a 'progression factor' [22]. The competence factor renders the cells 'competent' to leave the G_0 phase of the cell cycle to enter the G_1 phase, whereas the progression factor is essential for the cells, which have become competent, to progress to enter the S phase. Since bile acids enhance FGF-induced DNA synthesis when added within 10 h after the initial stimulation with FGF and are inactive when added more than 10 h after the initial stimulation of FGF, these results strongly suggest that bile acids may potentiate the action of the competence factor rather than that of the progression factor.

We have recently obtained evidence that FGF induces phosphoinositide turnover and subsequent activation of protein kinase C as described for PDGF in Swiss 3T3 cells [23]. Another line of evidence indicates that a phorbol ester receptor may be protein kinase C itself and diverse effects of phorbol esters may be mediated through the activation of this enzyme [24–27]. Here, it is shown that (i) bile acids enhance FGF-induced DNA synthesis but do not enhance TPA-induced DNA synthesis; (ii) bile acids do not enhance FGF-induced DNA synthesis in the cells in which the phorbol ester receptor, namely protein kinase C, is down-regulated by pretreatment with PDBu; (iii) bile acids do not affect the binding of FGF to the cells. These results suggest that bile acids may act on post-receptor and pre-protein kinase C site(s), presumably via the activation of protein kinase C by diacylglycerol. This possibility is now under investigation in our laboratories.

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