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Tumour necrosis factor-alpha induces translocation of protein kinase C in tumour necrosis factor-sensitive cell lines

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SUMMARY

In this study we investigated whether the anti-proliferative effect of tumour necrosis factor -alpha (TNF-α) was associated with the activation of protein kinase C (PKC), using PANC-1 cells (TNF-α sensitive) and LoVo cells (TNF-α resistant). In combination with 12-0-tetradecanoylphorbol-13-acetate (TPA), a potent activator of PKC, TNF-α caused marked inhibition of the growth of LoVo cells. Inhibition of PANC-1 cell growth by TNF-α was blocked by pretreatment with TPA for 24 hr, along with down-regulation of PKC activity. Intracellular translocation of PKC from cytosol to membrane was induced by TNF-α treatment in PANC-1 cells but not in LoVo cells.

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α) is a potent cytokine, produced primarily by macrophages and monocytes, that exerts pleiotropic effects on a wide variety of cells and tissues.[1-5] Though it has received considerable attention due to its ability to modulate the growth of both normal and tumour cells in culture, the biochemical basis of sensitivity or resistance to this action of TNF has not yet been defined. Expression of the TNF receptor alone is not sufficient to mediate the cytotoxic and mitotic effects of TNF, and responsiveness to TNF seems to be largely determined at a post-receptor level. However, as yet little is known about the TNF-related signalling mechanisms.

In our previous paper, we reported the possibility that protein kinas C (PKC) may have a key role in the TNF-α signal transduction pathway, because phorbol ester (an activator of PKC) can induce TNF-α-mediated growth inhibition in a dose-dependent manner in LoVo cells, which normally show little sensitivity to this cytokine.[6] In this report, we demonstrate that TNF-α induces PKC activation (translocation from the cytosol to the membrane), which appears to be part of the TNF signalling pathway in sensitive cell lines but not in resistant cell lines.

MATERIALS AND METHODS

Cells

PANC-1 cells derived from human pancreatic cancer were obtained from the American Type Culture Collection (Rockville, MD) and LoVo cells derived from human colon cancer were kindly supplied by Hayashibara Biochemical Laboratories Inc. (Okayama, Japan). The cells were maintained in RPMI-1640 medium (Nissui Pharmacological Co., Tokyo, Japan) supplemented with 10% (v/v) heat-inactivated foetal calf serum (Gibco, Grand Island, NY) at 37° in a humidified 5% CO_2 atmosphere.

Chemicals

Natural human TNF-α was purified from human acute B lymphoblastic leukaemia cells (BALL-1 cells) stimulated by haemagglutinating virus of Japan (HVJ), and was kindly supplied by Hayashibara Biochemical Laboratories Inc. The specific activity of the purified TNF-α was 10^9 U/mg protein. The 12-0-tetradecanoylphorbol-13-acetate (TPA) used was purchased from Sigma Chemical Co. (St Louis, MO).

Determination of cell viability

Cells were plated in 96-well microplates (Falcon Labware, Oxnard, CA) at 5×10^3 cells/well, followed by the addition of various concentrations of natural human TNF-α or TPA. After incubation for 72 hr, the number of viable cells was determined by staining with 0·05% crystal violet in 2% ethanol, followed by elution with Sorenson's buffer (6·1 ml of 0·1 μM disodium citrate, 3·9 ml of 0·1 N HCl, and 10 ml of 95% ethanol).[7] The absorbance at 570 nm was determined with a model FT-2 plate analyser (Toyo Sokki Co., Tokyo, Japan). The number of viable cells was estimated from the absorbance, based on the previously determined correlation between dye elution and cell numbers. Cell growth was calculated according to the following formula: percentage viability = (mean absorbance of the treated group)/(mean absorbance of the control group) × 100. Each assay was performed in at least triplicate, and data were expressed as the mean ± SD.

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Table 1. Effect of TNF-α and TPA on the growth of PANC-1 and LoVo cells

	TNF-α, U/ml		
	10	10^3	10^5
PANC-1			
Control	86·7±1	53·6±1	34·0±1
+TPA	91·0±3	52·5±1	33·3±1
TPA pretreatment	89·2±3	96·8±1**	88·8±2**
LoVo			
Control	93·9±9	89·2±1	80·0±1
+TPA	87·9±2	63·6±1*	54·4±2**
TPA pretreatment	97·9±4	90·2±2	83·3±4

Control: various doses of TNF-α with medium (50 μl) were added to each well. +TPA: 10 μM TPA (50 μl) were added at the same time in combination with each dose of TNF-α. TPA pretreatment: TPA (10 μM) was added 24 hr prior to the addition of TNF-α. The cells were incubated for 72 hr with these agents.

**$P<0.01$: significantly different from control by Student's t-test.

The growth of PANC-1 cells was determined by crystal violet staining as described in the Materials and Methods. Results represent the mean±SD of triplicate determinations.

Protein kinase C assay

The cell pellet of 5×10^7 PANC-1 or LoVo cells was resuspended in 4 ml of ice-cold buffer A [20 mM Tris-HCl (pH 7·5), 5 ml NaCl, 0·5 mM $MgCl_2$, 1 mM dithiothreitol, 1× protease inhibitor mixture (10 μg/ml of pepstatin, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 25 μg/ml soybean trypsin inhibitor)] after homogenization and centrifugation at 100,000 *g* for 1 hr. The supernatant (designated as the cytosol fraction) were kept on ice, while the pellets were extracted in ice-cold buffer A containing 1% Triton X-100. This suspension was then recentrifuged at 100,000 *g* for 1 hr, and the supernatant was designated as the membrane fraction. Samples of both the cytosolic and membrane fractions were applied to a DEAE–cellulose column equilibrated with buffer B [20 mM Tris-HCl (pH 7·5), 2 mM EDTA, 5 mM EGTA, 2 mM dithiothreitol). After the column was washed with 10 vol of buffer B, PKC was eluted with buffer B containing 100 mM NaCl.[8] The sample was then kept at 0° in the presence of 1× protease inhibitor mixture, and PKC activity was assayed within 24 hr using an Amersham PKC assay kit (Amersham, Bucks, U.K.)

RESULTS

Effect of TPA administration on PANC-1 and LoVo cells

The effect of TNF-α on the growth of the two tumour cell lines was examined after incubation with 10^5 U/ml of TNF-α for 72 hr. PANC-1 cells were sensitive to the anti-proliferative action of TNF-α, but LoVo cells showed low sensitivity to TNF-α (Table 1). The effect of TPA treatment on the response to TNF-α of these cell lines was also examined. When LoVo cells were exposed concurrently to 10 μM TPA and 10^5 U/ml of TNF-α for 72 hr, TPA increased the sensitivity to TNF-α. The percentage viability in the absence of TPA was 80·0±0% (SD), whereas in the presence of TPA it was 55·4±2% (SD). When LoVo cells were treated with a 24-hr exposure to 10 μM TPA followed by exposure to TNF-α for 72 hr, which resulted in a 95% loss of enzyme activity (data not shown), no increase in sensitivity to TNF-α was observed compared with when TNF-α was administered to LoVo cells alone. TPA at this concentration did not have any cytotoxicity in itself.

Table 2. Localization and level of protein kinase C activity of PANC-1 and LoVo cells after exposure of TNF-α and TPA

	PKC activity (pmoles ^{32}P transferred/min/mg/protein)	
	Cytosol	Membrane
PANC-1		
Control	2·1±0·2	1·59±0·2
TNF-α	0·73±0·09**	2·44±0·4*
TPA	1·8±0·07	3·31±0·3**
LoVo		
Control	0·91±0·3	0·48±0·1
TNF-α	0·60±0·2	0·53±0·1
TNF-α+TPA	0·68±0·1	0·82±0·1*

TNF-α (10^5 U/ml) or TPA (10 μM) was added to PANC-1 cells for 20 min, and then the PKC activity was measured as described in the Materials and Methods. TNF-α (10^5 U/ml) was added to LoVo cells alone or with TPA (10 μM) for 20 min, and then the PKC activity was measured. The activity is expressed as pmoles [^{32}P] incorporated/min/mg protein.

**$P<0.01$, *<0.05: significantly different from the control by Student's t-test.

The effect of TNF-α on the growth of PANC-1 cells is also shown in Table 1. PANC-1 cells demonstrated sensitivity to the anti-proliferative effect of TNF-α. When 10 μM TPA were given to PANC-1 cells concurrently with TNF-α, there was no change in their sensitivity. However, when PANC-1 cells were treated with a 24-hr exposure to 10 μM TPA, which resulted in a 97% loss of enzyme activity (data not shown), followed by exposure to TNF-α, there was no growth inhibitory effect such as was observed with TNF-α treatment alone.

We further examined the effect of TPA on the TNF-α-induced anti-proliferative effect of four other cancer cell lines (the human MIA-PaCa-2, BxPC-3 and TuP-1 pancreatic cancer and human RPMI 4788 colon cancer cell lines), for which TNF-α showed growth-inhibiting effects of various degrees. Administration of TPA at the same time or 24 hr prior to TNF-α showed the same modulatory effects as seen in LoVo and PANC-1 cells on the sensitivity of each cells (data not shown).

PKC activity in PANC cells and LoVo cells

To confirm that PKC plays a key role in regulating cellular sensitivity to the anti-proliferative effect of TNF-α, the enzymatic activity of PKC was assessed. As shown in Table 2, enhancement of the specific activity of PKC in the membrane fraction and a concomitant down-regulation of cytosolic PKC activity was observed in TNF-α-treated PANC-1 cells. TPA activated PKC activity in a manner similar to TNF-α, causing

translocation in PANC-1 cells. TNF-α treatment at 10^5 U/ml of LoVo cells followed by partial purification of the enzyme caused no change in the activity of the cytosolic and membrane PKC fractions (Table 2). However, when TNF-α and TPA were concomitantly administered, the membrane PKC activity increased from $0{\cdot}53 \pm 0{\cdot}1$ to $0{\cdot}82 \pm 0{\cdot}1$ pmoles [^{32}P] transferred/min/mg protein.

DISCUSSION

To understand the molecular actions of TNF, it is important to examine the early biochemical changes which occur subsequent to binding to the TNF receptor. The present study provides three lines of evidence that PKC is involved in TNF-mediated signal transduction. (i) TPA, which has the principal action in cells of activating PKC, induced sensitivity to TNF in LoVo cells (Table 1). (ii) Intracellular translocation of PKC from the cytosolic to the membrane fraction was induced by TNF-α treatment of PANC-1 cells (TNF-sensitive cells), but not by the treatment of LoVo cells (TNF-resistant cells) (Table 2). (iii) Induction of sensitivity to TNF-α in LoVo cells by TPA was blocked by preincubation with TPA for 24 hr, and growth inhibition of PANC-1 cells by TNF-α was also blocked by preincubation with TPA. Both changes were due to down-regulation of PKC (Table 1).

The target protein of PKC phosphorylation is unknown. Previous studies have shown that TNF-α stimulates the phosphorylation of a 26,000 MW protein in U937 cells, a 27,000 MW protein in human dermal fibroblasts, HeLa cells and Me-180 cells, and a 28,000 MW protein in ME-180 human cervical cancer cells.[9-12] These proteins were found to be heat-shock protein (27,000 MW) and cap-binding protein (28,000 MW). Phosphorylation of cap-binding protein is decreased by heat shock,[13] which correlates with the inhibition of protein synthesis. Thus, these proteins may be involved in the translation control of cellular protein synthesis, though whether or not PKC actually phosphorylates them remains unclear.

There are also some reports that TNF induces the modulation of epidermal growth factor receptor expression via a unique mechanism and that this plays a role in the cytotoxic action of TNF.[14-16]

The molecular mechanisms underlying TNF-induced PKC activation remain unclear. So far, none of the classical second messenger systems has been identified as being involved in TNF signalling. A number of genes are known to be transcriptionally induced by phorbol esters, including the cellular protoncogenes c-fos, c-myc and c-sis, as well as genes encoding collagenase, stromelysin and methallothionein IIa. Some of these genes can also be activated by TNF. All these TPA-inducible genes contain a conserved cis-element that is recognized by transcription factor AP-1, the major component of which (jun/AP-1) is actually encoded by the genes themselves.[17-19]

TNF appears to act at the surface of tumour cells to alter the membrane structure, producing changes of membrane fluidity and chemical composition as well as of functional distribution and/or association of membrane components with various membrane-associated proteins. It will be of interest to investigate whether PKC mediates these membrane-associated changes induced by TNF.

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