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Induction of circulating phospholipase A_2 by intravenous administration of recombinant human tumour necrosis factor

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We have examined the effects of intravenous infusion of recombinant human tumour necrosis factor (rh-TNF) on serum activity of phospholipase A_2 (PLA_2) in patients with malignancies. Nine patients received a 24 h continuous intravenous infusion ranging from 1.0×10^5 U/m^2 to 3.0×10^5 U/m^2; 14 patients received a 5 day continuous intravenous infusion ranging from 0.5×10^5 U/m^2/day to 3.0×10^5 U/m^2/day. Twenty one of 23 patients responded with marked increases in serum PLA_2 activity that were detectable 3 h after the beginning of the rh-TNF infusion and reached maximum levels at 18 h with a mean increase of 16.2-fold. In patients receiving a 5 day rh-TNF infusion, the highest levels of PLA_2 were observed after the first day of infusion. Serum PLA_2 activity declined continuously to 2.9-fold above baseline at the end of the infusion. A significant correlation was noted between the dose of infused rh-TNF and the maximum increase in PLA_2 activity. To our knowledge, this is the first time that an association between intravenous TNF administration and induction of circulating PLA_2 in man has been established.

Key words: Cancer patients, Phospholipase A_2, Recombinant human tumour necrosis factor

Introduction

Phospholipase A_2 is a lipolytic enzyme that hydrolyses membrane associated phospholipids of mammalian cells and initiates the arachidonic acid cascade.[1] Several of its end product eicosanoids have well known proinflammatory activity.[2] Recent studies have demonstrated that phospholipase A_2 (PLA_2) is secreted extracellularly in inflammatory sites including synovial fluids in inflammatory arthritides,[3] peritoneal fluid in peritonitis,[4] and sera in septic shock.[5,6] PLA_2 injected into skin,[7] joints[8] or paws[9] of experimental animals elicits a time and dose-dependent inflammatory reaction. We have previously shown that intravenous injection of endotoxin in experimental animals[5] or human volunteers,[10] was associated with a marked increase in circulating PLA_2 activity. Furthermore, intravenous infusion of PLA_2 reproduces many features of endotoxaemia.[5]

Bacterial and toxic insults lead to the rapid synthesis and release of cytokines including IL-1 and TNF.[11–14] We recently demonstrated that these two cytokines markedly enhance the synthesis and extracellular release of PLA_2 from cultured mammalian cells.[15] Infusion of TNF in man elicits clinical and haemodynamic manifestations similar to those caused by endotoxin.[16,17] Since infusion of PLA_2 leads to similar manifestations,[5] it was of significant interest to determine whether recombinant human TNF (rh-TNF), given intravenously, would be followed by intravascular secretion of PLA_2 *in vivo*. The finding that TNF infusions, without prestimulation with endotoxin, cause increase in circulating PLA_2 would reinforce the link between proximal cytokine response and PLA_2 activity, and may provide insights into the mechanisms of the proinflammatory activity of TNF. Such findings may also indicate that TNF has an impact on activation of the PLA_2-initiated arachidonic acid cascade. The present study demonstrates that intravenous administration of TNF in humans leads to a rapid and marked increase in circulating PLA_2 activity.

Materials and Methods

Patient selection and therapy: Twenty-three patients with cancer (Tables 1 and 2) received intravenous infusion of human recombinant tumour necrosis factor (rh-TNF) (Asahi Chemical Industry Company of America, New York, NY). Nine patients (group I) received a 24 h continuous intravenous infusion,[16] and 14 patients (group II) received a 5 day continuous intravenous infusion.[17] In the first group the rh-TNF dose varied from 1.0×10^5 U/m^2/day to 3.0×10^5 U/m^2/day. In the second group the daily dose ranged from 0.5×10^5 U/m^2/day to 3.0×10^5 U/m^2/day. The first group also

received etoposide (Bristol-Myers Oncology, Wallingford, CT), 80 mg.m^{-2} by continuous intravenous infusion for 3 days, starting 24 h prior to rh-TNF infusion. Both groups were pretreated with indomethacin 50 mg on the evening before and q.i.d. thereafter for 24 h. All patients were hospitalized and monitored as described.[16–18] Sodium chloride (0.9%) was infused at 150 ml.h^{1} for 24 h prior to rh-TNF infusion. rh-TNF was diluted in the same buffer and infused at a constant rate (150–300 ml/day). In all patients, previous chemotherapy and/or radiotherapy was discontinued at least 3 weeks prior to rh-TNF infusion.

Drug formulation: The rh-TNF used for this study had a specific activity of 2.3×10^6 U/mg protein. One unit was defined as the amount required to lyse 50% of L-M cells in a 48 h assay. The preparation of TNF used in this study contained less than 100 pg of endotoxin per mg protein, as tested by the Limulus lysate assay.

Enzyme assay: Phospholipase A_2 assay was performed as described, using autoclaved *Escherichia coli* $K_{12}C_{600}$ labelled with [^{14}C]oleic acid as the substrate.[19] Assays were performed in substrate excess, using 2.8×10^8 *E. coli* per assay, corresponding to 5.6 nmol of phospholipid with a specific activity of 4120 cpm/nmol. In conditions of substrate excess, the rate of substrate hydrolysis was linear with reaction times up to 30 min, over a five-fold range of enzyme concentration. One unit of PLA_2 activity is defined as the amount of enzyme activity that hydrolyses 56 pmol of *E. coli* phospholipid in 30 min. Activity of serum PLA_2 after rh-TNF infusion was tested at different pH and calcium concentrations as described previously.[19,20] The effect of neutralizing polyclonal antibody (NPA) against rh-PLA_2 (lot 207, Biogen, Cambridge, MA) on rh-TNF-induced endogenous PLA_2 was tested by incubating NPA with PLA_2-containing sera for 60 min at room temperature, and testing the mixture for residual PLA_2 activity. The direct effect of rh-TNF on PLA_2 activity *in vitro* was tested using *E. coli* phospholipid substrate. rh-TNF in concentrations of up to 1000 U/ml were preincubated with PLA_2 for 30 min at room temperature prior to addition of substrate.

The reference range for normal serum PLA_2 ($n = 143$) is 149 ± 69 (SD) U/ml with a range of 40–365 U/ml. Serum samples from patients infused with rh-TNF were coded and assayed for PLA_2 activity without any knowledge regarding the relationship of the sample to the time, dose or the nature of infusion. The results were simultaneously exchanged and analysed in two centres.

Statistical analysis: Statistical analysis was performed by standard tests including correlation coefficient and Student's *t*-test.

Results

Group I consisted of three men and six women ranging in age from 25 to 73 y (mean 52.2 y). The primary tumours of these patients are summarized in Table 1. Metastases, mainly to the liver and lung, were present in all patients. One patient had a past history of cancer of the breast, one had tuberous sclerosis and one had mild, chronic pancreatitis. None had fever or infection preceding the rh-TNF infusion. White blood cell count varied from 5 to 11×10^{12} / l , with a normal differential count. All patients had normal creatinine.

Before rh-TNF infusion six of the nine patients had normal serum PLA_2. In three patients, two with renal cell carcinoma and one with cancer of the colon, baseline PLA_2 was elevated ranging from 727 U/ml^{-1} to 2297 U / ml . Seven of nine patients responded to rh-TNF infusion with marked increases in PLA_2 (Table 1). Increase in PLA_2 activity was evident at 3 h after beginning the rh-TNF administration, and lasted for the entire period of infusion. Maximal PLA_2 activity (16.2-fold) was observed at 18 h of infusion (Figure 1). Levels of PLA_2 remained elevated (1510–38274 U/ml) in all three patients in whom the serum was tested 24 h after terminating the infusion.

In group II, there were seven men and seven women ranging in age from 37 to 70 y (mean 55.4 y). The diagnoses are summarized in Table 2. Metastases were detected in twelve of 14 patients. No past diseases known to influence PLA_2 activity, such as pancreatitis, rheumatoid arthritis, infection or fever were documented. All patients had normal renal function (creatinine <1.2 mg/dl). Peripheral blood leukocyte count ranged from 4 to 14×10^{12}/l with normal differential counts.

Ten of 14 patients had normal serum PLA_2 activity prior to rh-TNF infusion (Table 2). In four patients, initial serum PLA_2 levels were elevated ranging from 604 U/ml to 6397 U/ml. All 14 patients demonstrated an increase in PLA_2 activity after initiation of the rh-TNF infusion (Table 2). The activity was highest after the first day of infusion (13-fold) and it gradually declined to 2.9 times higher than baseline after 5 days of infusion, and two times higher than baseline 24 h after termination of rh-TNF infusion (Fig. 2).

In both groups, there was a significant correlation between the daily dose of rh-TNF infused and the maximum increase in PLA_2 activity ($p < 0.05$) (Fig. 3).

PLA_2 in the sera of rh-TNF infused patients was calcium dependent, with optimal activity at 5 mM and was completely inactivated by 2 mM EDTA. The pH optimum was 7.5. Polyclonal antibodies against human group II PLA_2 completely neu-

Table 1. Clinical/laboratory profile of patients who received continuous 24 h intravenous infusion of rh-TNF

No.	Age	Sex	Diagnosis*	Dose of rh-TNF U/m^2	PLA_2 U/ml pre-TNF	PLA_2 U/ml peak during TNF infusion
1	53	F	Renal cell liver	1×10^5	193	16458
2	62	F	Renal cell liver, lung	1×10^5	2297	2735
3	57	F	Hepatoma lung	1×10^5	105	2935
4	34	F	Colon, lung kidney peritoneum	2×10^5	364	585
5	54	F	Renal cell lung	2×10^5	2093	27022
6	73	M	Colon lung, liver	2×10^5	82	2211
7	25	M	Colon liver	2×10^5	727	3151
8	49	F	Lung contralateral lung	2×10^5	115	11079
9	63	M	Renal cell lung, skeletal	3×10^5	270	38274

* Top line, primary cancer; below, site of metastatic involvement.

Table 2. Clinical/laboratory profile of patients who received 5 day continuous intravenous infusion of rh-TNF

No.	Age	Sex	Diagnosis*	Dose of rh-TNF U/m^2/day	PLA_2 U/ml pre-TNF	PLA_2 U/ml peak during TNF infusion
1	55	F	Lung skeletal	0.5×10^5	263	2191
2	55	M	Carcinoid liver	0.5×10^5	167	485
3	54	F	Skeletal lung, brain	0.5×10^5	158	8676
4	37	F	Ovarian liver	1.0×10^5	106	2505
5	49	F	Breast liver	1.0×10^5	178	9801
6	55	M	Colon liver	1.0×10^5	6397	13045
7	45	M	Renal pancreas	2.0×10^5	601	35056
8	48	M	Colon	2.0×10^5	264	25861
9	58	F	Colon, pleura liver	2.0×10^5	265	7496
10	67	F	Uveal melanoma lung	2.4×10^5	311	18038
11	70	M	Colon	2.4×10^5	604	8950
12	68	M	Lung	2.4×10^5	287	23163
13	56	F	Colon mesenteric	3.0×10^5	224	5596
14	58	M	Renal peritoneum	3.0×10^5	1984	8311

* Top line, primary cancer; below, site of metastatic involvement.

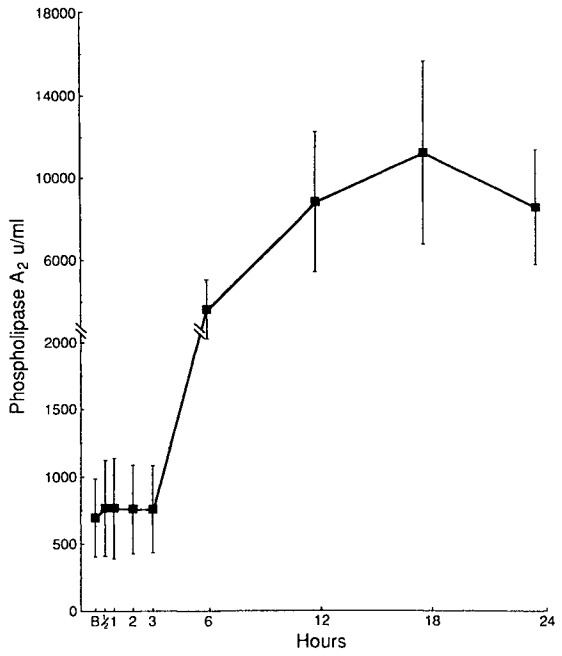

FIG. 1. Phospholipase A_2 in patients who received 24 h continuous intravenous infusion of rh-TNF. Vertical bars, mean ± SEM.

tralized (93%) PLA_2 activity in the sera of rh-TNF infused patients. rh-TNF or etoposide had no detectable effect on the activity of purified PLA_2 *in vitro*.

Discussion

Extracellular phospholipase A_2 (PLA_2) has recently been identified in synovial fluids in inflammatory

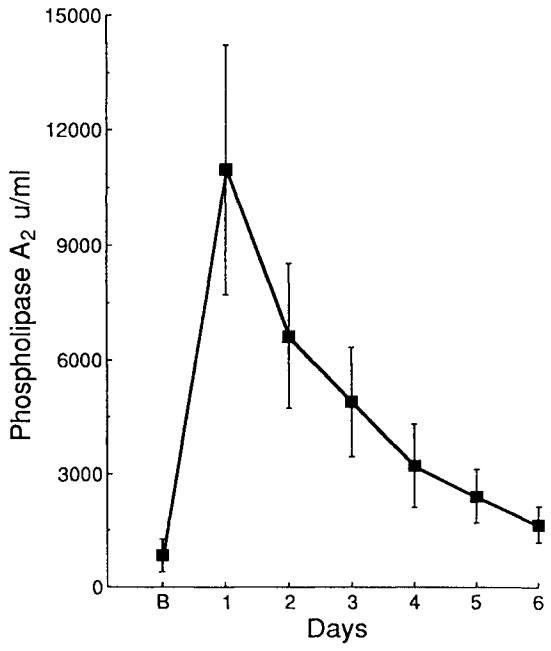

FIG. 2. Phospholipase A_2 in patients who received 5 day continuous intravenous infusion of rh-TNF. Vertical bars, mean ± SEM.

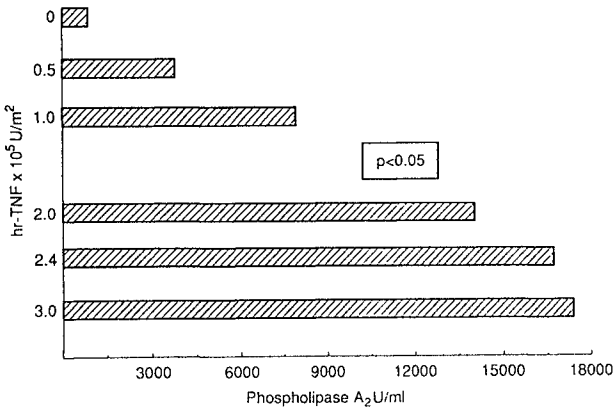

FIG. 3. Correlation between the dose of infused human recombinant TNF and increase of phospholipase A_2. Horizontal bars, means.

arthritides.[3] Subsequently, very high serum activity of PLA_2 was found in systemic inflammatory processes such as septic shock[5,6] and adult respiratory distress syndrome.[6] Purified PLA_2 instilled into the lungs[21] or injected into joints,[8] skin[7] or paws[9] of experimental animals causes marked dose and time-dependent inflammatory reactions that are abolished by inhibitors of this enzyme. Taken together, these results suggest that PLA_2 plays an important role in local and systemic inflammatory processes.[22,23]

Marked increases in circulating PLA_2 have been observed in animals challenged with endotoxin.[5] In such animals, the rise in the serum PLA_2 activity correlates with the fall in the mean arterial blood pressure. When the PLA_2-enriched fraction of septic shock serum is infused into healthy rabbits, it reproduces the clinical and haemodynamic changes induced by endotoxin. Pretreatment of the PLA_2-enriched fraction by the PLA_2 inhibitor, *p*-bromophenacyl bromide, inhibits PLA_2 activity and abrogates the hypotensive effect.[5]

Very high circulating PLA_2 activity has been found in patients with gram-negative septic shock.[6] In both retrospective[6] and prospective[24] studies, the activity of PLA_2 correlates with the Haemodynamic Instability Score ($p < 0.001$). The above studies have shown that PLA_2 fulfils several of Lefer's criteria[25] for a mediator of circulatory shock: (1) a marked increase in circulatory PLA_2 in response to bacteria or their toxins; (2) hypotension caused by PLA_2 in experimental animals; and (3) correlation of endogenous PLA_2 levels with the severity of hypotension in both animals and man. Furthermore, an inhibitor of PLA_2, *p*-bromophenacyl bromide, ameliorates these effects of PLA_2. Taken together, these data suggest that PLA_2 is one of the mediators of septic shock manifestations.

Several studies have recently examined the relationship between PLA_2 and cytokines. TNF

exerts its impact on the cells through its binding to cell surface receptors.[26] The interaction of TNF with the receptors is associated with GTP binding and increase in GTPase activity.[27] Furthermore, the association of TNF and PLA_2 has been reported by several groups.[28–33] TNF stimulates PLA_2 activity in HL-60[32] and Balb/c 3T3 cells[30] and, conversely, PLA_2 activity is required for the transcriptional activation of TNF gene expression.[32,33] Moreover, inhibitors of PLA_2 have been found to interfere with the cytotoxic and cytolytic activity of TNF[28,29] and to block TNF-induced increases in macrophage-specific colony stimulating factor (M-CSF) transcripts.[31]

In human volunteers challenged intravenously with endotoxin, marked increases in circulating PLA_2 followed transient increases in TNF.[10] This temporal relationship is of particular interest, since it has been found that bacterial or toxin challenge in animals or in man leads to a prompt release of cytokines such as IL-1 and TNF. The ensuing clinical and haemodynamic changes, which in the past were assumed to be related to bacterial toxins, have recently been linked to the effects of cytokines. TNF is capable of eliciting most, if not all, effects of endotoxin.[11,12] It was therefore concluded that TNF is one of the endogenous mediators of endotoxic shock.[11,13,14] Recent studies have also suggested that several physiological and metabolic changes that are associated with malignant processes are in fact mediated through TNF.[18]

Several clinical trials with intravenous administration of rh-TNF have been performed in cancer patients.[16,17,34] The cascade of events following TNF infusions has not been elucidated. Transient increases in circulating IL-6 have been observed in cancer patients infused with TNF. The highest level of IL-6 was observed after 3–6 h of TNF infusion, and correlated with TNF dose.[35] We hypothesized that TNF infusion will also lead to increased PLA_2 activity. This postulate was based on the observations that endotoxin infusion leads to increases in TNF followed by high circulating PLA_2[10] and that TNF enhances PLA_2 synthesis and secretion *in vitro*.[15] Many of the effects of TNF depend on the local and systemic activation of the cyclooxygenase pathway.[13] Furthermore, prostaglandins appear to play a role in mediating the effects of TNF.[14] Thus, if the activity of PLA_2 is modulated by TNF, the role of eicosanoids in proximal cytokine mediated reactions would become more apparent.

In the present study we have shown that in 21 of 23 patients infusions of rh-TNF were associated with significant increases in the activity of circulating PLA_2. The earliest serum PLA_2 increases occurred 3 h after beginning the rh-TNF infusion and maximal levels were observed at 18–24 h. Of interest is the fact that in the patients who received 5 day rh-TNF infusion, the level of circulating PLA_2 declined after the first 24 h. This phenomenon may be related to the previously described decline in TNF levels that occurs in patients who received 24 h continuous infusions.[16] Neither the relationship between the saturation of TNF receptors and its impact on PLA_2 release, nor the intra-vascular/extravascular distribution of PLA_2 or its metabolism are known. However, the maximal increase in PLA_2 activity correlated with the daily dose of rh-TNF. Therefore a dose-related link was established between TNF and PLA_2 activation. Thus, PLA_2 is activated by both endotoxin[5,10] and by proximal cytokines. Since PLA_2 is proinflammatory and vasoactive, some manifestations previously attributed to endotoxin and tumour necrosis factor should probably be attributed to PLA_2 as well.

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