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Involvement of phospholipase A2 activation in tumour cell killing by tumour necrosis factor

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SUMMARY

Earlier studies have indicated a possible role for arachidonate metabolism in the direct cytolysis of tumour cells by tumour necrosis factor (TNF) *in vitro*. In this study, the involvement of arachidonate metabolism has been investigated further with the following results: (i) Cytolysis of human U937 tumour cells by recombinant TNF was reduced by dexamethasone and quinacrine, agents which inhibit phospholipase A2. (ii) U937 and L929 cells, which are susceptible to TNF cytolysis, released arachidonic acid and its metabolites within 5 hr of TNF challenge, before cell death was apparent. In contrast, U937/R and L929/R, which are resistant to the cytolytic effects of TNF, did not release arachidonate products on TNF challenge. (iii) rTNF cytolysis of U937 cells was not reduced by inhibitors of the cyclo-oxygenase and lipo-oxygenase pathways of arachidonic acid metabolism. Cytolysis was reduced, however, by inhibitors of the arachidonate metabolic pathway involving cytochrome P450-dependent reductase, but only at reagent concentrations that also inhibited phospholipase A2 activity. Overall, these observations indicate a role for phospholipase A2 but not for arachidonic acid or its metabolites in the direct cytolysis of tumour cell lines by TNF.

INTRODUCTION

Tumour necrosis factor (TNF) is a macrophage product that was first studied because of its anti-tumour effects (Carswell *et al.*, 1975), and subsequently it has been purified, sequenced and cloned. With the advent of the recombinant material it has become apparent that TNF also has a great variety of effects on untransformed cells, and it is now recognized as an important mediator of immunity and inflammation (Beutler & Cerami, 1987; Old, 1987). In addition to growth inhibiting or killing certain tumour cell lines, TNF can stimulate the growth of other tumour cells (Sugarman *et al.*, 1985; Vilcek *et al.*, 1986). Depending upon the tumour, the anti-tumour action of TNF *in vivo* may be indirect (via endothelial and inflammatory cells), direct or both.

From studies on the direct cytolysis of L929 cells *in vitro* by TNF (Matthews, 1983), we speculated that cytolysis may be due to a free radical-induced process (Matthews & Neale, 1987b). A subsequent study gave only limited support for this but did reveal that cytolysis of murine L929 cells by natural rabbit TNF required oxygen and could be inhibited by corticosteroids (Matthews *et al.*, 1987). This indicated a possible role for arachidonic acid metabolism in the cytolytic process.

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In the present study, it is shown that cytolysis of human U937 cells by human recombinant (r) TNF is also inhibited by corticosteroids. Further evidence for arachidonate involvement has also been sought by, firstly, testing the effects on TNF cytolysis of other inhibitors of arachidonate metabolism and, secondly, comparing arachidonic acid release from tumour cell lines which are either killed, unaffected by or growth-stimulated by TNF.

MATERIALS AND METHODS

Tumour cell lines

L929 (mouse fibroblastoid) and a U937 variant (human myelomonocytic) are TNF-susceptible, plastic-adherent cell lines from which TNF-resistant (−/R) sublines have been selected (Matthews, 1984). The strain of U937 cells used in this study was 10 times more susceptible to TNF than the L929 cells (50% cytolysis of U937 with 0·25 ng/ml rTNF after 3 days but 2·5 ng/ml required for L929). P2neoEJ and P2Ki are transformed derivatives of an untransformed rat thyroid fibroblast line, P2. P2neoEJ arose from P2 cells transformed by a plasmid containing Harvey ras, and P2Ki after transformation of P2s with Kirsten MSV/MLV. P2 and P2neoEJ were kindly provided by Dr D. Wynford-Thomas (Dept. Pathology, University Wales College of Medicine) and the Kirsten MSV/MLV by Dr N. Teich (ICRF, London). Both P2neoEJ and P2Ki show an approximately 20% increase in cell number in growth medium

Table 1. Effect of inhibitors of arachidonate metabolism on cytolysis of U937 cells by rTNF

Exp. no.	Inhibitor	Conc.	Mean (absorbance 540nm) × 1000 ± SD −TNF	Mean (absorbance 540nm) × 1000 ± SD +TNF	% cytolysis	% inhibition
1	Medium	—	284 ± 33	63 ± 28	78	—
	Dexamethasone	4 μM	179 ± 18	103 ± 10	42	46
		0·8 μM	192 ± 16	119 ± 11	38	51
		0·16 μM	193 ± 21	117 ± 12	39	50
	BW755C	100 μM	87 ± 9	14 ± 1	84	−7
		50 μM	226 ± 23	21 ± 4	91	−17
		25 μM	257 ± 27	48 ± 9	81	−4
2	Medium	—	365 ± 9	126 ± 15	65	—
	Quinacrine	1·0 μM	157 ± 7	155 ± 21	1	98
		0·25 μM	304 ± 21	215 ± 10	29	55
	SKF525A	20 μM	213 ± 11	188 ± 20	12	82
		10 μM	282 ± 13	227 ± 14	20	69
3	Medium	—	633 ± 9	255 ± 12	60	—
	Benzphetamine	0·4 mM	515 ± 11	339 ± 8	34	43
		0·2 mM	577 ± 18	369 ± 8	36	40
		0·1 mM	623 ± 8	361 ± 8	42	30
	Metyrapone	0·16 mM	657 ± 14	332 ± 3	49	18
		0·04 mM	674 ± 9	319 ± 2	53	12

supplemented with TNF compared with growth medium alone. RPMI-1640 with 5% fetal calf serum (Gibco, Paisley, Renfrewshire) was employed as growth medium throughout.

Reagents

Human rTNF was kindly provided by Dr G. R. Adolf (Ernst Boehringer Institut, Vienna, Austria). SKF525A was obtained from Smith Kline and French Labs. Ltd. (Welwyn Garden City, Herts) and BW755C from Wellcome Research Labs. (Beckenham, Kent). Other reagents were purchased from Sigma Chemical Co. (Poole, Dorset).

TNF cytolysis assay

U937 cells were plated (75 μl of 10^5/ml) in 96-well microtitre trays and the test agent was added in a 75-μl volume, followed by 25 μl TNF (the minimum required to give 60–80% cytolysis—approximately 2·5 ng/ml). Concentrations given refer to the final culture volume of 175 μl. After 3 days at 37°, the remaining adherent viable cells were stained with crystal violet. Dye uptake is proportional to the number of remaining cells and was quantified photometrically by an ELISA reader. The percentage cytolysis was calculated from the formula 100 (a − b)/a where a and b are, respectively, the mean absorbances of triplicate wells without or with TNF. This photometric assay is well established (Matthews, 1983), has been described in detail elsewhere (Matthews & Neale, 1987a), and correlates well with other cytolytic assays (Flick & Gifford, 1984). Inhibition of cytolysis was calculated from the formula 100 (c − d)/c where c and d are, respectively, the percentage cytolysis with medium or drug.

Arachidonate metabolism

Cells were plated in 1-ml volumes of growth medium (4×10^5/ml) in 2·5 cm wells and left at 37° for 6 hr before the addition of 0·1 μCi 5, 6, 8, 9, 11, 12, 14, 15-[^{3}H] arachidonic acid (94·5 Ci/mmol; New England Nuclear, Stevenage, Herts) or 1-14C arachidonic acid (58·3 mCi/mmol, Amersham, Aylesbury, Bucks). After overnight incubation unincorporated arachidonate was removed by three washes with warm growth medium. The cells were refed with 0·5 ml growth medium ± TNF (or with 0·5 ml 0·4 M NaOH to give total releasable arachidonater) and reincubated at 37°. At appropriate times thereafter 50-μl amounts of supernatant were removed for counting to give an indication of the amount of arachidonate breakdown products released from the cell. All cultures were set up in triplicate.

Thin-layer chromatography (TLC) of arachidonate metabolites

Supernatants of ^{14}C arachidonate-labelled cells were extracted with ethyl acetate, concentrated and separated by TLC using the A9 solvent system (organic phase of ethyl acetate/iso-octane/acetic acid/water) as described by Bednar *et al.* (1984) except that Whatman LK5 silica plates were used. After chromatography, the plates were exposed to Hyperfilm βmax (Amersham) for 3 days. The developed and fixed film was then used to localize the radioactive zones on the TLC plate which were scraped off and immersed in scintillation fluid for beta counting.

RESULTS

Killing of U937 cells by human rTNF is inhibited by dexamethasone (Table 1, Exp. 1), as found earlier for rabbit TNF and murine L929 cells. Corticosteroids induce the synthesis of several proteins, including lipocortin, an inhibitor of phospholipase A2 (PLA2). Protein synthesis is required for dexamethasone inhibition of TNF cytolysis since inhibition was not observed in the presence of the protein synthesis inhibitor emetine (Table 2). It is likely that the inhibitory effect of dexamethasone on TNF killing is due to its effect on PLA2 since another PLA2 inhibitor quinacrine also inhibits TNF killing of U937 cells (Table 1, Exp. 2).

Table 2. Effect of dexamethasone on rTNF cytolysis of U937 cells in the presence of the protein synthesis inhibitor emetine

Exp. no.	Inhibitor	Mean absorbance (540 nm) × 1000 ± SD −TNF	Mean absorbance (540 nm) × 1000 ± SD +TNF	% cytolysis
1	Medium	304 ± 0	56 ± 13	82
	Dexamethasone	305 ± 8	36 ± 2	88
2	Medium	313 ± 10	142 ± 2	55
	Dexamethasone	302 ± 21	123 ± 17	59

Emetine was used at 1 μg/ml and the TNF concentrations were 700 pg/ml in Exp. 1 and 70 pg/ml in Exp. 2. The assay period was 1 day and the cells were plated at 3×10^5/ml rather than 10^5/ml. These modifications were necessary because TNF is more rapidly cytolytic in the presence of protein synthesis inhibitors.

Table 3. Release of radiolabelled arachidonate from TNF-treated cells

Cell line	TNF added	% arachidonate* released after 2 hr	5 hr	21 hr
L929	−	4·1 ± 1·3	2·8 ± 0·9	5·8 ± 1·3
	+	3·0 ± 0·2	5·1 ± 1·1	9·5 ± 1·5
L929/R	−	3·5 ± 0·5	3·7 ± 1·2	4·9 ± 0·7
	+	2·6 ± 0·4	2·8 ± 0·5	3·2 ± 0·3
U937	−	3·1 ± 0·6	4·5 ± 0·6	9·5 ± 1·4
	+	3·1 ± 0·2	30·4 ± 6·1	62·6 ± 2·2
U937/R	−	2·2 ± 0·4	2·2 ± 0·1	5·0 ± 2·3
	+	2·2 ± 0·2	4·0 ± 0·3	5·6 ± 0·2

* Cells were labelled with [^{3}H]arachidonic acid and incorporation was within the range 60–80,000 c.p.m./10^6 cells for all four cell types. TNF was added at 500 ng/ml.

If the cytolytic action of TNF requires PLA2 then arachidonic acid release would be expected on TNF challenge of susceptible cells. Indeed, the susceptible lines U937 and L929, previously loaded with radiolabelled arachidonate, released increased amounts of radiolabel 5 hr after TNF challenge (Table 3). The TNF-induced release of radiolabel from U937 cells was > 80% inhibited by 0·25 μM dexamethasone. By 21 hr L929 and U937 exhibited some cell death on microscopic examination and dye uptake and some of the arachidonate release must be due to cell death. However, this cannot be the sole reason because, for example in the experiment shown in Table 3, with U937 cells there was < 30% cytolysis at 21 hr, as shown by dye uptake, but > 60% arachidonate release. After 5 hr TNF exposure, the U937 and L929 cells showed no evidence of cell death by either phase-contrast microscopy or the standard dye-uptake assay for TNF cytolysis. Studies of TNF involvement in inflammation have shown that TNF can induce arachidonate release and metabolism in skin fibroblasts and synovial cells (Dayer, Beutler & Cerami, 1985; Godfrey, Johnson & Hoffstein, 1987). With the synovial cells, the timing and extent of arachidonate release was similar to that described here.

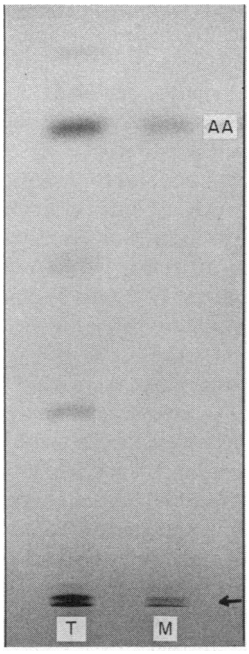

Figure 1. Analysis by TLC/autoradiography of supernatants of ^{14}C arachidonate-labelled U937 cells after treatment for 6 hr with medium alone (M) or with 500 ng/ml rTNF (T). The origin is marked by an arrow and AA indicates arachidonic acid; the major metabolite has tentatively been identified as PGE2.

Intriguingly, the L929/R and U937/R lines selected for resistance to TNF cytolysis took up radiolabelled arachidonic acid as efficiently as their susceptible parents, but did not release arachidonate on TNF challenge (Table 3). In contrast, two cell lines (P2neoEJ and P2Ki), which are growth stimulated by TNF, exhibited increased arachidonate release 2–5 hr after TNF challenge. At 5 hr P2neoEJ had 34·7 ± 2·2% release with TNF and 11·4 ± 2·2% without; with P2Ki the corresponding values were 15·3 ± 0·2% and 6·3 ± 0·2%. From this it seems that TNF-triggered phospholipase A2 activation may be an essential step in both cytolysis and growth stimulation. Tumour cell lines like U937/R and L929/R, which have developed resistance to TNF cytolysis, have lost the capacity to mobilize arachidonic acid on TNF challenge.

In most cells, arachidonic acid released by PLA2 is metabolized to biologically active products along the cyclo-oxygenase and lipo-oxygenase pathways. However, as noted previously with rabbit TNF and L929 cells, neither pathway appears to be involved in the cytolytic process as pharmacological doses of the lipo-oxygenase inhibitor NDGA and the cyclo-oxygenase inhibitors indomethacin and acetylsalicylic acid failed to inhibit TNF killing (Matthews *et al.*, 1987). Similarly, BW755C, an inhibitor of both pathways, failed to inhibit rTNF cytolysis of U937 cells (Table 1, Exp. 1). Indeed, in this experiment and in several others (data not shown), BW755C increased U937 susceptibility to TNF cytolysis suggesting the possible diversion of arachidonate to another metabolic pathway involved in cytolysis. Recently, a third pathway of arachidonate metab-

olism has been recognized—this involves a cytochrome P450-dependent reductase and can be inhibited by SKF525A, benzphetamine and metyrapone (Capdevila *et al.*, 1981; Bednar *et al.*, 1984; Schwartzman *et al.*, 1985). Table 1 (Exps 2 and 3) shows that SKF525A and benzphetamine markedly inhibited TNF cytolysis, with lesser effects being seen with metyrapone. These results raise the possibility that the cytochrome-dependent pathway is the critical route for arachidonate metabolism during the cytolytic process. However, both SKF525A (20 μM) and benzphetamine (0·4 mM) also inhibited TNF-induced arachidonic release from U937 cells by 50–80%, indicating that their effects on cytolysis are probably mediated via phospholipase A2.

Because of the possible involvement of an unusual metabolic pathway we looked for novel arachidonate metabolites in TNF-treated cells. U937 cells were labelled with ^{14}C arachidonic acid, incubated with or without TNF for 6 hr, and arachidonate metabolites were extracted from the supernatants and analysed by TLC (Fig. 1). In unstimulated cell supernatants there was one major and one minor metabolite in addition to arachidonic acid: supernatants of TNF-treated cells had increased amounts of these three products but no new metabolites were seen. The major metabolite comprised $13·7 \pm 5·5\%$, the minor metabolite $< 5\%$ and arachidonic acid $80·0 \pm 12·2\%$ in unstimulated cells (mean + SD, Exp. 3). In TNF-treated cells the major metabolite comprised $20·7 \pm 7·1\%$, the minor metabolite $< 5\%$ and arachidonic acid $75·3 \pm 5·5\%$. Production of both metabolites was prevented by 5 μM indomethacin or 100 μM BW755C but not by 20 μM SKF525A. Since SKF525A inhibits cytolysis but these doses of indomethacin and BW755C do not, the metabolites seen on TLC cannot be involved in the cytolytic process. If cytochrome P450-dependent reductase products are involved in cytolysis they are either trace products of arachidonic acid metabolism or very reactive molecules which bind to cellular structures and are not released into the culture supernatant. To test the latter possibility, U937 cells loaded with radiolabelled arachidonic acid were treated with TNF for 6–24 hr and cellular proteins were precipitated with 5% TCA and washed with acetone. Insignificant amounts of radiolabel were detected in the protein fraction.

DISCUSSION

In the present study several lines of evidence support a role for phospholipase A2 in the cytolytic process. Firstly, cytolysis is reduced by dexamethasone and quinacrine, agents which inhibit phospholipase A2. Secondly, TNF-susceptible cells exhibit increased release of arachidonic acid and its metabolites on TNF challenge, indicating activation of phospholipase A2. Thirdly, tumour cell lines selected for TNF resistance lose the capacity to mobilize arachidonic acid on TNF challenge.

In our earlier study (Matthews *et al.*, 1987) we noted that promethazine, which was employed as a free radical scavenger, inhibited TNF cytolysis. We are aware that this compound has a variety of other effects on cells and recently have found that it is as effective as dexamethasone in preventing arachidonate release from TNF-treated U937 cells; this action rather than free-radical scavenging may account for its inhibition of TNF cytolysis. Similarly, although SKF525A and benzphetamine are nominally inhibitors of cytochrome P450-dependent arachidonate metabolism they also prevented arachidonic acid release from U937 cells and this latter mechanism probably accounts for their inhibition of TNF cytolysis. We do not know whether promethazine, SKF525A and benzphetamine act directly on phospholipase A2 or at a step preceding it, but all three reagents have structural features in common with each other and with quinacrine, a known inhibitor of phospholipase A2.

In TNF-treated susceptible cells, in addition to arachidonic acid in the supernatant, two metabolites were also detected. Production of both metabolites could be prevented by BW755C and indomethacin but not by SKF525A, indicating that if there are cytochrome P450-dependent products they are either a minor proportion of the metabolic products or highly reactive and bind to cellular structures and therefore remain within the cell. Epoxide metabolites generated by the cytochrome-dependent pathway are potentially highly reactive species (Needleman *et al.*, 1986) but we could find no evidence of arachidonic acid metabolites being bound to cellular proteins. Also, addition of exogenous arachidonic acid failed to increase the susceptibility to TNF cytolysis of L929, L929/R, U937 or U937/R cells (M. L. Neale and N. Matthews, unpublished observations). From the above it seems unlikely that arachidonic acid or its metabolites are involved in the cytolytic process, therefore suggesting that lysophospholipids or their products may be implicated; this is currently being investigated. Lysophospholipids can be toxic to cells (Weltzien, 1979) and it may be that cells that are susceptible to TNF cytolysis lack acyl transferases or other enzymes which detoxify these substances.

Two tumour cells lines which are growth stimulated by TNF also mobilized arachidonic acid on TNF challenge. It may be that phospholipase A2 activation is also a critical step in TNF-induced growth stimulation but obviously much more work is required to confirm this. The TNF-resistant cell lines L929/R and U937/R retained TNF receptors (Matthews, 1984 and unpublished observations) but did not release arachidonate on TNF challenge, indicating that these cells have lost a critical link in the pathway leading to TNF-induced phospholipase A2 activation. This differs from tumour lines which have developed resistance to other cytotoxic agents. For example, in methotrexate or 'multi-drug' resistance there is over-expression of certain proteins rather than loss of function (Melera *et al.*, 1982; Roninson *et al.*, 1984).

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