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ARACHIDONIC ACID METABOLISM IN MURINE FIBROSARCOMA CELLS WITH DIFFERING *IN VIVO* AND *IN VITRO* CHARACTERISTICS

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Arachidonic acid metabolism was examined in a series of strongly malignant murine fibrosarcoma cell lines and in a series of weakly malignant lines isolated from the same tumors. The cells were examined in the unstimulated state and after stimulation with 12-0-tetradecanoyl phorbol acetate (TPA), laminin or fibronectin. All 3 agents were known from previous studies to induce adherence and motility in the murine fibrosarcoma cells. When the cells were prelabelled with ^{3}H-arachidonic acid, all 3 agents stimulated the release of radioactivity into the supernatant fluids. The response to TPA was rapid while the response was slower but sustained when either laminin or fibronectin was used as the stimulating agent. This is of interest because TPA induces a rapid but transient adherence response in the same cells while laminin and fibronectin induce a slow, sustained response. Examination by radioimmunoassay procedures indicated that both control cells and stimulated cells were able to produce a variety of lipoxygenase and cyclooxygenase metabolites. In quantitative terms, the strongly malignant cells were more active than their weakly malignant counterparts. They released greater amounts of radioactivity into the supernatant fluid and produced a greater quantity of arachidonic acid metabolites, particularly prostaglandin E_2, than did the corresponding weakly malignant cells. This is of interest because previous studies have shown that while both the strongly and weakly malignant cells respond in the adherence assay to TPA, laminin and fibronectin, only the strongly malignant cells demonstrate directional motility (chemotaxis and haptotaxis).

Arachidonic acid metabolism accompanies stimulus-coupled responses in several types of cells (Habenicht *et al.*, 1981; Schrey and Rubin, 1979; Butler-Gralla *et al.*, 1983; Wertz and Mueller 1978, 1980; Levine and Hassid, 1977) and has been extensively investigated in leukocytes (see Goldman and Goetzl, 1983 for a review). When leukocytes are stimulated with chemotactic factors, arachidonic acid is released from phospholipid pools and is metabolized through both the lipoxygenase and cyclooxygenase pathways. Products of the lipoxygenase pathway (including monohydroxyeicosatetraenoic acids [HETEs] and leukotrienes [LTs]) are thought to be positive mediators of the chemotactic response as these agents will stimulate cell-to-substrate adherence, cell-to-cell aggregation, enzyme release and chemotaxis in leukocytes (Goldman and Goetzl, 1983). Cyclooxygenase products (*i.e.*, prostaglandins [PGs]), on the other hand, are thought to serve as negative modulators, limiting the cellular response to stimulation (O'Flaherty *et al.*, 1979; Rivkin *et al.*, 1975; Kunkel *et al.*, 1979; Fantone *et al.*, 1983*b*). Arachidonic acid metabolites may play a role in the chemotactic response of non-leukocytic cells as well. Mensing and Czarnetski (1984) showed that LTB_4 was chemotactic for normal fibroblasts and studies in our laboratory showed that the same agent stimulated adhesiveness in Walker carcinosarcoma cells (Varani, 1985). The response in the Walker cells was very similar to that stimulated by chemotactic peptides. Other studies have shown that cyclooxygenase metabolites (*i.e.*, E-series prostaglandins and prostacyclin) inhibit chemotactic factor-induced adherence and chemotaxis in the same Walker cells (Fantone *et al.*, 1983*a*; Mokashi *et al.*, 1983; Varani and Perone, 1985).

In the present studies we investigated arachidonic acid metabolism in a series of strongly and weakly malignant murine fibrosarcoma cells. The cells were examined in the unstimulated state and following stimulation with 3 different ligands (*i.e.*, 12-0-tetradecanoyl phorbol acetate [TPA], laminin and fibronectin) that induce adherence and motility in these and other cells (Lowe *et al.*, 1978; Yamasaki *et al.*, 1979; Castagna *et al.*, 1979; Koffler *et al.*, 1981; Schor *et al.*, 1981; Gauss-Muller *et al.*, 1980; McCarthy *et al.*, 1983, 1984; Varani and Fantone, 1982; Lee *et al.*, 1984; Situ *et al.*, 1984; Varani *et al.*, 1985*a,b*). All 3 ligands induced the release of ^{3}H-arachidonic acid from prelabelled cells and a conversion of arachidonic acid into a variety of end products. These results show that in the murine fibrosarcoma cells, as in several other cell types previously investigated (Butler-Gralla *et al.*, 1983; Levine and Hassid, 1977; Wertz and Mueller, 1980) metabolism of arachidonic acid accompanies response to stimulation with TPA. They show, furthermore, that laminin and fibronectin—high-molecular-weight glycoproteins which may have a very different mode of action from TPA—also stimulate arachidonic acid metabolism in responsive cells.

MATERIAL AND METHODS

Cells

The strongly and weakly malignant cell lines used in this study were obtained from three different chemically-induced murine fibrosarcomas. The tumors from which the cell lines arose have been designated as tumors 1.0, 1.1 and 1.2. The strongly malignant cell lines have been designated 1.0/L1, 1.1, 1.1/clone 16, and 1.2. Weakly malignant cell lines were obtained from each of the 3 tumors. These cell lines have been designated as 1.0/anti-B^r, 1.1/anti-B^r, and 1.2/anti-B^r. The isolation and characterization of these populations have been described in previous reports (Varani *et al.*, 1979*a,b*, 1980, 1983*b*). In the present study, all of the cell lines were maintained under identical conditions which included growth in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2/95% air. The cells were subcultured by trypsinization as necessary. Prior to use in these studies all of the cell lines were shown to be free of mycoplasma contamination by incubation in mycoplasma broth and on mycoplasma agar.

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Stimulating agents

Two large glycoprotein molecules, laminin and fibronectin, were used as stimulating agents in this study. Laminin was prepared in our laboratory by the method of Timpl *et al.* (1979) or purchased from Bethesda Research (Gaithersburg, MD). The commercially obtained reagent was also purified by the same method. Purity of the laminin was checked by SDS-polyacrylamide gel (5%) electrophoresis run under reducing conditions. Only 2 major protein bands—at M^r = 200,000 and 400,000—were seen. When examined by means of an enzyme-linked immunosorbent assay (ELISA) (Varani *et al.*, 1983*a*), the laminin reacted with anti-laminin antibodies undiluted or at dilutions up to 1:1,000,000 but did not react with anti-fibronectin antibodies (provided as a gift by Dr. R. Wiggins, Department of Medicine, University of Michigan) or with anti-type IV collagen antibodies (provided as a gift by Dr. H. Furthmayr, Department of Pathology, Yale University). When a cell attachment assay was used as an indicator of biological activity (Varani *et al.*, 1983*a*), the laminin was effective at 0.5-1.0 μg per 35-mm diameter dish.

Human plasma fibronectin was purchased from Bethesda Research or from Sigma (St. Louis, MO). Both commercial preparations showed a single major protein band when examined by SDS-polyacrylamide gel electrophoresis. The fibronectin preparations reacted with anti-fibronectin antibodies by ELISA but did not react with the anti-laminin antibodies. Cell attachment activity was detectable with concentrations as low as 2 μg per 35-mm dish.

We also used the biologically-active phorbol ester, 12-0-tetradecanoyl phorbol acetate (TPA) as a stimulating agent. The TPA was obtained from Sigma and frozen as a 1.6×10^{-2}M solution in dimethylsulfoxide. It was diluted in culture medium at the time of use.

Arachidonic acid metabolism

^{3}H-arachidonic acid (87 Ci/mmol), obtained from New England Nuclear, Boston, MA, was used to measure arachidonic acid release from the control and stimulated cells. Freshly-harvested cells were labelled by preincubation for 2 hr with 1 μCi of the ^{3}H-arachidonic acid per 5×10^7 cells in 5 ml RPMI-1640 culture medium supplemented with 10% fetal bovine serum. Incubation was carried out at 37°C and 5% CO_2. During the labelling procedure, the cells were kept in tubes and maintained in suspension by gentle shaking. The distribution of the radioactivity within the labelled cells was determined by subjecting the labelled cells to a Folch extraction (Folch *et al.*, 1957) and analyzing the chloroform-soluble fraction by thin-layer chromatography using a modification of the solvent system described by Skipski *et al.* (1964). Depending on the cell type and the experiment, we were able to obtain 1-2 $\times 10^5$ counts per minute (CPM) of ^{3}H-arachidonic acid incorporated per 1×10^6 cells. Nearly all of the incorporated material was found in the chloroform-soluble phase and when this material was analyzed, the radioactivity was found in bands co-migrating with phosphatidylcholine, phosphatidylinositol, phosphatidylserine and phosphatidylethanolamine. Less than 10% of the chloroform-soluble radioactivity was in bands which co-migrated with free fatty acids, triglycerides and cholesterol esters. After labelling, the cells were centrifuged and washed twice in culture medium to remove unincorporated ^{3}H-arachidonic acid. The cells were then aliquoted into tubes, stimulated with the appropriate agent and incubated at 37°C and 5% CO_2. At various times thereafter, 200-μl samples (2×10^6 cells) were removed from the tubes. The samples were added to 300 μl of phosphate-buffered saline and the cells separated from the supernatant fluid by centrifugation. Radioactivity in the supernatant fluid was then determined using a β-scintillation counter.

Cyclooxygenase and lipoxygenase metabolites of arachidonic acid were quantitated in the control and stimulated cells. The cyclooxygenase products measured were PGE_2, $PGF_{2\alpha}$, and 6-keto $PGF_{1\alpha}$ (a prostacyclin metabolite). Lipoxygenase products measured included 5-HETE, 12-HETE and LTC_4. For these assays, the cells were harvested, washed 4 times in serum-free culture medium and incubated at 5×10^6 cells per ml of serum-free medium. At various subsequent times, the supernatant fluids (for cyclooxygenase metabolites) or the cells and supernatant fluids (for lipoxygenase products) were harvested, extracted and assayed. The extraction procedure involved precipitation of the protein with acetonitrile, and extraction of most neutral lipids from the acidified acetonitrile fraction with petroleum ether. After removal of the ether layer, the remaining aqueous phase was extracted twice with ethyl acetate and dried in air. The residue was redissolved in phosphate-buffered saline containing 0.1% gelatin and assayed for the various metabolites using radioimmunoassay (RIA) procedures. Assays for cyclooxygenase metabolites were performed with antibodies and labelled ligands obtained from NEN. The limits of sensitivity for the 3 assays were 0.9-3.8 pg/tube. Cross-reactivity of each antibody to related prostaglandins was low (less than 1.8%). The LTC_4 assay was also carried out using a NEN assay kit. The limit of sensitivity for this assay was 3.7 pg/reaction tube. The antibody to LTC_4 cross-reacted with LTD_4 (55%) and LTE_4 (8.6%) but showed low cross-reactivity (less than 1%) with LTB_4, mono-HETEs and various prostaglandins. The 5-HETE and 12-HETE assays were carried out with kits available from Seragen (Boston, MA). The limit of sensitivity of the 5-HETE assay was 8 pg/reaction tube. The antibody used in this assay cross-reacted with 5,15-DiHETE (10%) and to a lesser extent (3-5%) with LTB_4, LTC_4, LTD_4 and LTE_4 but showed low cross-reactivity (less than 0.6%) with other mono-HETEs and various prostaglandins. The 12-HETE assay had a limit of sensitivity of 2 pg/reaction tube but showed extremely low cross-reactivity (less than 0.3%) with other mono-HETES, leukotrienes and prostaglandins. The actual assay procedures were carried out by the Ligand Core Laboratory at the Michigan Diabetes Research and Training Center (Ann Arbor, MI).

RESULTS

Arachidonic acid metabolism in the strongly and weakly malignant cells

Cells were prelabelled with ^{3}H-arachidonic acid as described in "Material and Methods." They were then examined for their ability to release radioactivity into the culture medium under unstimulated conditions and after stimulation with laminin, fibronectin or TPA (Fig. 1). In the absence of stimulation there was a slow release of radioactivity into the culture medium from all of the cells. The amount of radioactivity found in

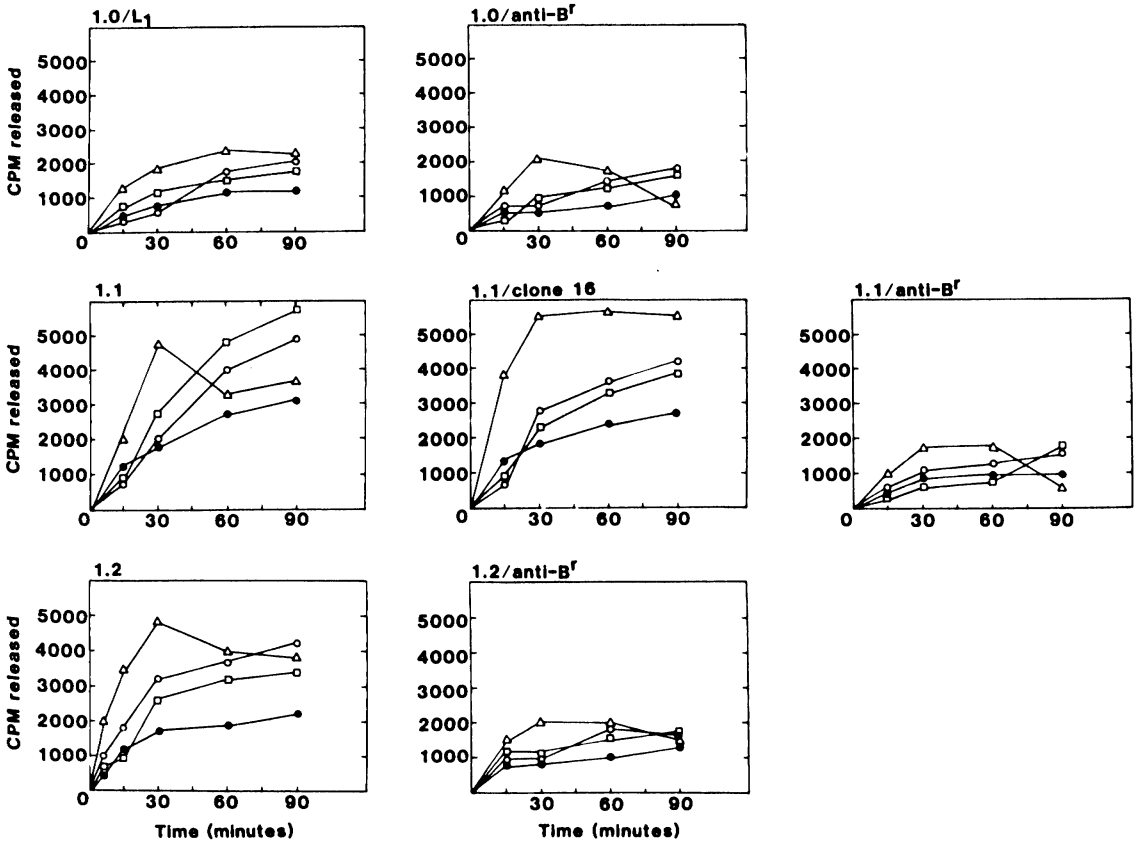

FIGURE 1 – Release of radioactivity from ^{3}H-arachidonic acid-prelabelled cells. The cells were pretreated with ^{3}H-arachidonic acid as described in "Material and Methods" and then incubated in culture medium with and without stimulation. The values shown are the amount of radioactivity recovered from the supernatant fluid at various subsequent times. Values are averages of duplicate tubes in a single experiment where the duplicate values were nearly always within 10% of the mean values. Cells from each tumor were examined together between 3-7 times with very similar results. (Unstimulated, ●—●; 1.6×10^{-8} M TPA, △—△; 100 μg/ml laminin, ○—○; 100 μg/ml fibronectin, □—□).

the supernatant fluid increased over the 90-min observation period and accounted for 0.5-1.5% of the total. With cell lines from 2 of the 3 tumors (1.1 and 1.2) the strongly malignant cells were much more active than their weakly malignant counterparts. With the third tumor (1.0), both the strongly and weakly malignant cells released comparable amounts of radioactivity—similar to the amounts released by the weakly malignant cells from the other 2 tumors.

Stimulation of the prelabelled cells with either laminin, fibronectin or TPA led to an increased release of radioactivity into the supernatant fluids from all cells (Fig. 1). With laminin and fibronectin, significant differences between the treated and control cells were not observed at the earliest time points (5 and 15 min) but were observed after longer incubation (30, 60 and 90 min). TPA also stimulated arachidonic acid release from all of the cell types. The pattern, however, was different from that observed with laminin and fibronectin. The radioactivity released from the TPA-stimulated cells reached a peak early (30 min after stimulation) and then either levelled off or decreased. The decrease in extracellular radioactivity could indicate reincorporation of the released arachidonic acid or a metabolite produced from it.

Radioimmunoassay procedures were used to quantitate arachidonic acid metabolites produced by the various cell lines. For this purpose, the cells were harvested from culture and incubated under serum-free conditions with or without stimulation for 4 hr. The supernatant fluids (for cyclooxygenase metabolites) or the cells and supernatant fluids (for lipoxygenase metabolites) were then extracted as described in "Material and Methods." Cyclooxygenase data are shown in Table I and lipoxygenase data in Table II. To summarize the results of the cyclooxygenase analyses, PGE_2 was the major metabolite identified with all of the cells, although significant amounts of 6-keto $PGF_{1\alpha}$ and $PGF_{2\alpha}$ were also detected. The quantities of metabolites produced by the cells varied with cell type and reflected the amounts of radioactivity released from prelabelled cells (compare Table I and Fig. 1). The strongly malignant cells from tumor 1.0 produced slightly higher levels of prostaglandins than did the weakly malignant cells from the same tumor. The strongly malignant cells from the other 2 tumors produced much higher levels than the corresponding weakly malignant cells.

In regards to lipoxygenase metabolites, there were no consistent or dramatic differences between the strongly and weakly malignant cells (Table II). The amount of LTC_4 obtained from the strongly malignant cells in the unstimulated state was slightly greater than the amount obtained from the corresponding weakly malignant cells. The relative magnitudes of the differences were much smaller than those observed in the

TABLE I - CYCLOOXYGENASE METABOLITES OF ARACHIDONIC ACID PRODUCED BY THE STRONGLY AND WEAKLY MALIGNANT CELLS

Cell line	Stimulation	Amount produced (ng/5 × 10^6 cells/4 hr)[1]		
		PGE_2	6-keto $PGF_{1\alpha}$	$PGF_{2\alpha}$
1.0/L1	None	5.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
	TPA[2]	5.6 ± 0.1	2.5 ± 0.5	0.6 ± 0.1
	Laminin[3]	3.6 ± 0.3	0.5 ± 0.1	0.2 ± 0.2
	Fibronectin[4]	1.5 ± 0.2	0.5 ± 0.1	0.1 ± 0.1
1.0/anti-B^r	None	4.1 ± 0.3	< 0.1	0.4 ± 0.2
	TPA	5.6 ± 0.4	< 0.1	0.6 ± 0.1
	Laminin	12.2 ± 0.6	< 0.1	1.1 ± 0.3
	Fibronectin	14.3 ± 0.4	< 0.1	0.5 ± 0.1
1.1	None	22.3 ± 1.3	1.8 ± 0.1	5.1 ± 0.8
	TPA	21.1 ± 1.0	2.6 ± 0.3	4.8 ± 1.3
	Laminin	18.6 ± 0.9	1.5 ± 0.2	3.8 ± 0.8
	Fibronectin	29.3 ± 0.7	1.8 ± 0.2	3.5 ± 2.0
1.1/clone 16	None	32.0 ± 3.1	2.1 ± 0.3	3.5 ± 0.6
	TPA	35.5 ± 1.9	2.0 ± 0.4	3.8 ± 0.7
	Laminin	39.7 ± 1.6	2.6 ± 0.3	3.7 ± 1.2
	Fibronectin	27.4 ± 1.0	2.4 ± 0.2	4.4 ± 0.7
1.1/anti-B^r	None	3.9 ± 0.9	< 0.1	0.1 ± 0.1
	TPA	4.4 ± 1.7	< 0.1	0.2 ± 0.1
	Laminin	2.3 ± 1.0	< 0.1	0.1 ± 0.1
	Fibronectin	5.0 ± 1.7	< 0.1	0.1 ± 0.1
1.2	None	12.8 ± 1.5	4.2 ± 0.5	0.5 ± 0.1
	TPA	24.4 ± 2.3	1.1 ± 0.1	0.5 ± 0.1
	Laminin	13.0 ± 1.0	1.5 ± 0.1	0.7 ± 0.1
	Fibronectin	11.7 ± 1.3	1.7 ± 0.2	0.5 ± 0.1
1.2/anti-B^r	None	1.4 ± 0.2	< 0.1	0.1 ± 0.1
	TPA	2.3 ± 0.3	< 0.1	0.1 ± 0.1
	Laminin	1.7 ± 0.1	< 0.1	0.2 ± 0.1
	Fibronectin	1.7 ± 0.1	< 0.1	0.2 ± 0.1

[1]Values shown are averages ± standard errors of the mean. PGE_2 values for the 1.2 and 1.2/anti-B^r cells are based on 6 independent experiments. Other values are based on 3 independent experiments.-[2]1.6×10^{-8} M.-[3]100 μg per ml.-[4]100 μg per ml.

TABLE II - LIPOXYGENASE METABOLITES OF ARACHIDONIC ACID PRODUCED BY THE STRONGLY AND WEAKLY MALIGNANT CELLS

Cell line	Stimulation	Amount produced (pg/5 × 10^6 cells/4 hr)[1]		
		5-HETE	12-HETE	LTC_4
1.0/L1	None	ND[5]	ND	196 ± 66
	TPA[2]	ND	ND	172 ± 80
	Laminin[3]	ND	ND	1,790 ± 18
	Fibronectin[4]	ND	ND	1,517 ± 99
1.0/ anti-B^r	None	ND	ND	105 ± 76
	TPA	ND	ND	205 ± 64
	Laminin	ND	ND	752 ± 108
	Fibronectin	ND	ND	2,820 ± 251
1.1	None	ND	ND	263 ± 82
	TPA	ND	ND	177 ± 54
	Laminin	ND	ND	148 ± 44
	Fibronectin	ND	ND	109 ± 70
1.1/ clone 16	None	ND	ND	138 ± 66
	TPA	ND	ND	1,210 ± 132
	Laminin	ND	ND	169 ± 70
	Fibronectin	ND	ND	162 ± 76
1.1/ anti-B^r	None	ND	ND	104 ± 62
	TPA	ND	ND	252 ± 73
	Laminin	ND	ND	266 ± 70
	Fibronectin	ND	ND	1,210 ± 140
1.2	None	31 ± 7	20 ± 1	253 ± 75
	TPA	43 ± 3	45 ± 11	1,070 ± 140
	Laminin	46 ± 2	74 ± 8	212 ± 78
	Fibronectin	ND	ND	175 ± 24
1.2/ anti-B^r	None	51 ± 3	< 10	173 ± 161
	TPA	58 ± 10	< 10	63 ± 71
	Laminin	85 ± 7	30 ± 5	221 ± 73
	Fibronectin	ND	ND	137 ± 72

[1]Values shown are average ± the differences between mean values and individual values in a single experiment. The metabolites were measured on two separate occasions with similar results.-[2]1.6×10^{-8} M.-[3]100 μg per ml.-[4]100 μg per ml.-[5] Not done.

arachidonic acid release studies and assays for prostaglandin production. Likewise, no consistent pattern was seen with any of the 3 stimulating agents. In some instances, there was a dramatic stimulation of LTC_4 production (for example in the 1.0/L1 and 1.0/anti-B^r cells treated with laminin or fibronectin) but in other instances, the same agent had no effect or actually resulted in a decrease in production (for example, in the 1.1 cells).

DISCUSSION

Arachidonic acid metabolism has been extensively investigated in relation to leukocyte chemotaxis. When leukocytes are stimulated with chemotactic factors, there is a release of arachidonic acid from phospholipid pools and a stimulation of arachidonic acid metabolism through the lipoxygenase and cyclooxygenase pathways. Several of the arachidonic acid metabolites produced as a result of stimulation have potent mediator function and are thought to play a role in the biological responses to chemotactic stimulation (Goldman and Goetzl, 1983). Although most other systems have not been as widely investigated as leukocytes, arachidonic acid metabolism is known to accompany stimulus-coupled responses in a variety of cells (Habenicht *et al.*, 1981; Schrey and Rubin, 1979; Butler-Gralla *et al.*, 1983; Wertz and Mueller, 1980; Levine and Hassid, 1977).

In the present study we investigated arachidonic acid metabolism in a series of murine fibrosarcoma cell lines. The cells that were used included 4 strongly malignant lines from 3 different carcinogen-induced tumors and 3 weakly malignant variant lines isolated

from the same 3 tumors. The cells were examined in the unstimulated state and after treatment with laminin, fibronectin or TPA. All 3 agents stimulated increased arachidonic acid release from prelabelled cells. With TPA the response was rapid while a slower but sustained response was observed with laminin and fibronectin. This is of interest because a very similar relationship exists in regard to biological responses. In these tumor cells (Varani *et al.*, 1985*a*) TPA induces a rapid but transient adherence response but has no long-term effect on either cell attachment or spreading. In contrast, both laminin and fibronectin induce a slow but sustained increase in cell attachment and spreading (Varani *et al.*, 1985*a,b*). Thus, the kinetics of the biological response to the stimulating agents and the kinetics of arachidonic acid release stimulated by the same agents are in accord. This allows for the speculation that the 2 are related. Whether specific metabolites of arachidonic acid are essential mediators of the biological responses induced by these 3 agents remains to be seen. This will have to await a more complete qualitative and quantitative assessment of the metabolites produced by the cells and a direct demonstration that these metabolites can elicit biological responses similar to those produced by the stimulating agents. This has not yet been done although previous studies have shown that products of the lipoxygenase system stimulate adhesiveness in the Walker 256 carcinosarcoma cells (Varani, 1985) and chemotaxis in fibroblasts (Mensing and Czarnetski, 1984).

During the course of our studies it became clear that the strongly malignant cells were metabolically more active with regard to arachidonic acid metabolism than their weakly malignant counterparts. What makes these differences of interest is the observation that similar differences exist in regard to cell motility. The strongly malignant cells are much more motile than the weakly malignant ones. This is observed in the absence of a gradient (Situ *et al.*, 1984) as well as in the presence of a chemotactic or haptotactic gradient (Orr *et al.*, 1981; Varani *et al.*, 1985*a*). It is interesting, furthermore, that when the strongly malignant cells from the 3 different tumors are compared among themselves, the 1.0/L1 cells are less motile than the other 3 lines (Situ *et al.*, 1984; Varani *et al.*, 1985*a*). Thus, while there appears to be no precise quantitative relationship between arachidonic acid metabolism and a response in the adherence assay, such a relationship may indeed exist between arachidonic acid metabolism and cell motility.

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