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COMPARISON OF THE EFFECTS OF AURANOFIN AND RETINOIC ACID ON PLASMINOGEN ACTIVATOR ACTIVITY OF PERITONEAL MACROPHAGES AND LEWIS LUNG CARCINOMA CELLS

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Abstract—Urokinase-type plasminogen activator, a neutral proteinase, seems to play a central role in the degradation of the extracellular matrix that accompanies a number of biological phenomena including inflammatory reactions and neoplasia. The effect of auranofin and retinoic acid on the plasminogen activator activity expressed by two cell types, i.e. murine macrophages and Lewis lung carcinoma cells, has been investigated. Low concentrations of both drugs (10^{-6}–10^{-7} M) can inhibit *in vitro* the induction of plasminogen activator in macrophages stimulated by phorbol 12-myristate 13-acetate. This action occurs rapidly (15 min), is irreversible and is independent of a global cytotoxic effect. Auranofin and retinoic acid remain without effect in macrophages when added after stimulation by the phorbol ester. Both drugs are thus potent inhibitors of the induction of plasminogen activator activity in macrophages, possibly through an interaction with the protein kinase C system. The plasminogen activator activity of Lewis lung carcinoma cells, which is apparently not dependent on a protein kinase C pathway, is not influenced by auranofin or retinoic acid. These observations may contribute to explain: (1) the activity of auranofin and retinoic acid in rheumatoid arthritis, and (2) the antitumor promoting activity of retinoic acid. It would be relevant to assess whether auranofin may exhibit, like retinoic acid, an antitumor-promoting activity.

The degradation of connective tissue matrix which occurs in the course of inflammatory diseases like rheumatoid arthritis presents some similarities with the mechanism by which tumor cells invade neighbouring tissues and metastasize. Macrophages, polymorphonuclear leukocytes and synovial cells present in the arthritic joint all express proteolytic activities that are considered responsible for the erosive and invasive nature of the disease. Both in arthritic joints and in tumors, urokinase-type plasminogen activator (PA†), a neutral proteinase (MW 50,000), seems to play a central role in these proteolytic phenomena. High (PA) activity has been found in the synovial fluid of arthritic joints, and PA has been shown to activate latent collagenase produced by inflammatory synovial cells [1]. Similarly, neoplastic tissues contain high PA activity that has been associated with the invasive properties of neoplastic cells and their capacity to form metastasis (for a review see Refs 2 and 3). It has also been reported that PA stimulates cellular growth [4, 5].

Induction of PA activity occurs in different cell types. High PA activity is induced in macrophages activated by various inflammatory stimuli among which phorbol 12-myristate 13-acetate (PMA) [6]. Likewise, PA activity of malignant cells is induced during the promotion stage of the carcinogenic process and PMA is one of the best characterized, tumor promoting agent [6, 7].

PMA is generally recognized to modulate cellular functions by activating a Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C; PK-C) [8]. Under quiescent conditions, PK-C is localized in the cytoplasm and inactive; when the cell is activated, e.g. by PMA, the enzyme is translocated to the plasma membrane where it can actively phosphorylate its substrates at selected threonine and serine residues [9, 10].

Auranofin and retinoic acid, two compounds with anti-inflammatory and/or antitumor-promoting properties can modulate the PK-C activation pathway in a number of cell systems [11–15]. Auranofin (AF) is a lipophilic orally active gold complex used for the treatment of rheumatoid arthritis; its precise mechanism of action is unknown. AF inhibits *in vitro* and *in vivo* several responses of activated macrophages and polymorphonuclear leukocytes including chemotaxis, phagocytosis and interleukin-1 and superoxide production (see Ref. 16). The inhibitory effect of the drug is only observed when the cells are activated by agents, like PMA, involving the PK-C system. Responses elicited by calcium ionophore A23187, which are independent of PK-C, are insensitive to AF [17]. It has also been reported that AF possesses some *in vitro* antitumor activity [16].

Retinoids play an important role in maintaining normal growth and differentiation of epithelial and

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† Abbreviations used: PA, urokinase-type plasminogen activator; PMA, phorbol 12-myristate 13-acetate; PK-C, protein kinase C; AF, auranofin; RA, retinoic acid; LLC, Lewis lung carcinoma cells; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; MT, metallothionein.

mesenchymal tissues; retinoic acid (RA) may be the active metabolite. Since neoplastic transformation results in loss of differentiation and anarchic growth, there is a considerable interest in the use of retinoids for the prophylaxis and possibly the treatment of malignancies. Experimental data have shown that retinoids can prevent the chemical induction of tumors. Retinoids are potent inhibitors of the tumor-promoting effects of phorbol esters and antagonize several of their biochemical effects on cells *in vitro* (for a review, see Ref. 18). Their exact mechanism of action is still unknown; it has been suggested that retinoic acid can inhibit PK-C activity and hence modulates the protein phosphorylation processes controlling tissue growth [14, 15]. RA also possesses anti-inflammatory properties and inhibits collagenase production in synovial cells in culture [19, 20]; its use in inflammatory rheumatisms has been suggested [21]. The purpose of this study was to investigate the *in vitro* effect of AF and RA on the PA activity expressed by two different cell types, namely, PMA-activated macrophages and Lewis lung carcinoma cells (LCC).

MATERIALS AND METHODS

Reagents

Auranofin (((1-thio-β-D-glucopyranose 2,3,4,6-tetraaceto-*S*) (triethyphosphine) gold); AF) was obtained from Smith–Kline and French Lab. (Hertfordshire, U.K.). Dulbecco's modified Eagle medium (DMEM), L-glutamine, sodium pyruvate, lactalbumin hydrolysate, heat inactivated foetal calf serum (FCS) and antibiotics were from GIBCO (Paisley, U.K.). Human plasminogen and S-2251 chromogenic substrate were from Kabi Vitrum (Brussels, Belgium). Phorbol 12-myristate 13-acetate (PMA) and retinoic acid (all-trans) (RA) were purchased from Sigma Chemical Co. (St Louis, MO). Cadmium chloride and all other reagents were from Merck (Darmstadt, F.R.G.).

Culture procedures

Macrophages. Conditions for obtaining and culturing mouse peritoneal macrophages have been previously described [22]. The cells were plated at a density of $3.5–5 \times 10^5$ cells/cm^2 in 24 or 96 well multidishes (Nunc). On the second day of culture, monolayer macrophages were thoroughly washed and stimulated during 4 hr by PMA (10^{-7} M) in DMEM supplemented with 0.1% lactalbumin hydrolysate (stimulation phase).

LLC. Lewis lung carcinoma cells were kindly provided by Dr Y. Eeckhout (ICP, Brussels). After culture for 96 hr in DMEM supplemented with 10% FCS, the cells were plated in 24 or 96 well multidishes at a density of 120×10^3 cells/cm^2 in DMEM supplemented with 20% acid-treated FCS (3 hr).

Exposure of the cells to the drugs

Macrophages. We have followed the same protocol as previously described [22] for assessing the effect of the drugs on PA activity and cell viability. Macrophages were incubated with various concentrations of the drugs during three different periods, i.e. (a) during 15 min preceding the stimulation phase (this incubation was performed in BSS), (b) during the PMA-stimulation phase, and (c) after the stimulation, the drug being then added to the expression medium.

LLC. LLC cells were exposed during 15 min in a medium containing various drug concentrations; the medium was then removed and PA activity and LDH release were determined.

Enzymatic activities

PA activity. PA activity of the cultures was determined after removing the medium and thorough washing of the cells. The system was then incubated for 3 hr in a BSS expression medium containing plasminogen (0.165 CU/ml) and S-2251 as the chromogenic substrate of plasmin. Adequate final concentrations of S-2251 were 0.3 and 0.7 mM for macrophages and LLC, respectively. The amount of *p*-nitroaniline released was determined spectrophotometrically (405 nm). Each assay was run with a plasminogen-free blank. Zymographic analysis according to Granelli-Piperno and Reich [23] only detected a urokinase-type plasminogen activator (MW 47,000) in both cell types.

LDH activity. Viability of the cells was assessed by measuring LDH release in the medium as described previously [22].

Metallothionein (MT) determination. Cellular MT content was estimated by a latex immunoassay [24] using a rabbit anti-rat MT antibody [25].

Statistics. Statistical analysis was performed using Student's *t*-test, analysis of variance and Scheffé's multiple comparisons test.

RESULTS

1. *Auranofin*

Macrophages. Results obtained after exposing macrophages to AF before, during and after stimulation by PMA are presented in Table 1. Preincubation of the cells during 15 min before stimulation results in a concentration-dependent inhibition of the induction of PA activity. At concentrations of 5×10^{-6} and 10^{-5} M, PA is inhibited by 60 and 90%, respectively. This inhibition is irreversible since it cannot be reversed by washing the cells before PMA stimulation. Cell viability, monitored by LDH leakage in the culture medium, is not altered under these exposure conditions.

The presence of AF during stimulation by PMA causes also a dramatic reduction of PA activity but this is accompanied by a severe cytotoxicity. PA is totally depressed at 10^{-6} M of AF. At this concentration LDH release increases by a factor 2 over the control value. A complete inhibition of PA activity is observed at a concentration (10^{-6} M) 10 times lower than that killing all the cells.

A slight decrease of PA activity is observed when AF is added to the medium after stimulation by PMA, about 80% of the control value still remains at the highest concentration tested. Cell viability is only slightly altered under these conditions. Incubation of purified urokinase during 30 min with 10^{-5} M of AF before substrate addition does not affect its activity (100.0 ± 1.8% of control activity, N = 3).

Lewis lung carcinoma cells. Preliminary experiments were conducted to detect a possible stimulatory effect of PMA on PA activity expressed by LLC. This

Table 1. Effect of auranofin (AF) on plasminogen activator (PA) activity and cytotoxicity in mouse peritoneal macrophages stimulated during 4 hr by phorbol 12-myristate 13-acetate (PMA)

AF (mole/L)	Before stimulation		During stimulation		After stimulation	
	PA activity (% control value)[a]	LDH release (% total)[b]	PA activity (% control value)	LDH release (% total)	PA activity (% control value)	LDH release (% total)
0	100 ± 5.2	13.0 ± 2.4	100 ± 5.4	8.9 ± 1.7	100 ± 9.9	10.9 ± 1.9
10^{-6}	96.4 ± 6.4	9.0 ± 2.8	2.9 ± 0.5**	16.9 ± 2.4**	88.9 ± 5.7	12.7 ± 1.4
5×10^{-6}	39.6 ± 2.0**	19.0 ± 2.8	0	70.7 ± 5.2**	71.4 ± 2.8**	13.8 ± 3.7
10^{-5}	9.0 ± 2.8**	18.6 ± 3.4	0	100**	77.5 ± 4.5**	16.7 ± 2.8*

Means ± SD of three experiments.
[a] Mean PA activity in control stimulated macrophages amounts to 122.5 ± 6.4 nmole *p*-nitroaniline/hr/10^6 cells.
[b] LDH release is expressed as % of total enzyme content determined after cell disruption with Triton X-100.
* Significantly different from corresponding control value ($P < 0.05$).
** Significantly different from corresponding control value ($P < 0.01$).

Table 2. Effect of AF (15 min) on PA activity and cytotoxicity in LLC cells

AF (mole/L)	PA activity (% control value)[a] ($N = 5$)	LDH release (% total)[b] ($N = 4$)
0	100 ± 7.8	21.2 ± 1.5
5×10^{-6}	96.0 ± 6.6	27.8 ± 4.6
10^{-5}	101.0 ± 5.2	25.8 ± 11.7
10^{-4}	116.8 ± 7.9*	29.0 ± 4.9

Means ± SD.
[a] Control PA activity: 437.5 ± 34 nmole, *p*-nitroaniline/10^6 cells/hr.
[b] LDH release is expressed as % of total enzyme content determined after Triton X-100 disruption.
* Significantly different from control value ($P < 0.05$).

enzymatic activity has remained insensitive to the presence of the phorbol ester; e.g. incubation of LLC with PMA (10^{-7} M) during 4 to 12 hr did not influence the expression of PA activity (102.2 ± 6.2% of control activity after 12 hr incubation with PMA, N = 6). We therefore have studied the influence of AF on the constitutive level of PA activity. The effect of the drug on PA activity and LDH release is presented in Table 2. The LDH release is not affected by AF concentrations as high as 10^{-4} M. At the same concentrations PA activity is not inhibited. On the contrary, a slight but statistically significant increase of activity is observed at 10^{-4} M (116.8 ± 7.9% of the control value). We have verified that this different sensitivity was not due to an absence of gold uptake by LLC. After 15 min of incubation, cellular gold amounted to 1.5 ng/mg proteins both in macrophages and in LLC (atomic absorption spectrometry).

Influence of metallothionein. MT, a low molecular weight protein (MW 6500), rich in cystein which has a high affinity for several metals has been shown to protect some cell lines against the toxicity of AF (see Ref. 16). The synthesis of this cytoplasmic protein can be induced by various metals such as cadmium, copper, zinc, silver, cobalt and mercury.

We have found that LLC has a MT content about twice higher than that of PMA activated macrophages. In order to assess whether this different MT content might explain the different response of macrophages and LLC to AF, macrophages were preincubated during 17 hr with cadmium chloride (10^{-6} M) before being exposed to AF (Table 3). Cadmium chloride alone has no influence on PA activity and cell viability but increases, more than two-fold, MT concentration in macrophages.

This MT induction, however, did not change AF effect on PA activity and cell viability. A statistically significant reduction of LDH release after 5×10^{-6} M AF treatment is observed in cadmium pretreated cells (15.0 ± 0.6 versus 25.7 ± 3.5, $P < 0.05$). This slight protective effect is not found at other AF concentrations and is thus of no biological relevance.

2. *Retinoic acid*

Macrophages. Preincubation of peritoneal macrophages with RA before PMA stimulation causes an important inhibition of the induction of PA activity;

Table 3. Influence of macrophage pretreatment with $CdCl_2$ (10^{-6} M, 17 hr) on cytotoxicity and PA activity inhibition caused by AF

AF[b] (mole/L)	No pretreatment		$CdCl_2$ pretreatment	
	PA activity (% control value)	LDH release (% total)[a]	PA activity (% control value)	LDH release (% total)[a]
Control	100.0 ± 8.5[c]	22.2 ± 8.1	100.0 ± 1.8[c]	14.0 ± 1.7
10^{-6}	68.4 ± 3.2	13.8 ± 1.9	70.0 ± 1.8	19.0 ± 4.4
5×10^{-6}	22.1 ± 1.6	25.7 ± 3.5	19.0 ± 2.1	15.0 ± 0.6*
10^{-5}	12.4 ± 0.2	30.5 ± 5.2	10.3 ± 0.8	21.0 ± 2.1

Mean ± SD of three experiments.

[a] LDH release is expressed as % of total enzyme content determined after Triton X-100 disruption.

[b] The macrophages were challenged with AF during 15 min before stimulation by PMA.

[c] PA activities in untreated control cells and $CdCl_2$ pretreated cells amounted to 209.3 ± 4.2 (N = 3) and 207.3 ± 8.0 nmole *p*-nitroaniline/hr/10^6 cells (N = 3) and were not statistically different (*t*-test = $P > 0.05$).

* Significantly different from corresponding control value ($P < 0.05$).

more than 50% is inhibited at a concentration of 10^{-7} M (Table 4). At higher concentrations of RA (up to 10^{-5} M), 20–25% of PA remains insensitive to inhibition. The latter is also observed when the compound is added during the PMA stimulation (4 hr); a concentration of 10^{-7} M causes about 70% inhibition of PA activity. No great difference in RA effect is observed whether the compound is present before or during stimulation. As with AF, the action of RA on PA induction is rapid and irreversible. Washing the cell after RA treatment does not restore PA inducibility. On the contrary, addition of RA (up to 10^{-5} M) after the cells have been stimulated remains without influence on the induced PA activity. Likewise, incubation of the purified enzyme during 30 min with 10^{-5} M of RA before substrate addition does not affect its activity (97.5 ± 2.6% of control, N = 3). Monitoring of LDH release (Table 4) does not reveal any important cytotoxic effect of RA.

In order to examine a possible synergistic effect of AF and RA on PA activity, macrophages were incubated during 15 min with AF and RA separately or in combination before stimulation by PMA. The results presented in Table 5 suggest a simple additive effect.

Lewis lung carcinoma cells. The effect of RA on the constitutive PA activity and LDH release in LLC cells is presented in Table 6. The viability of LLC is not affected by RA concentrations as high as 10^{-4} M. At the same concentrations, PA activity is not inhibited.

Incubation of LLC with RA (10^{-6} M) for periods up to 72 hr does not affect PA activity either (not shown).

DISCUSSION

The data presented in this report indicate that AF can completely inhibit the PMA-dependent stimulation of PA activity in macrophages. This action is rapid and irreversible; it occurs after 15 min of contact and persists after the drug is removed and cells are washed; it cannot be prevented by increasing intracellular MT content by $CdCl_2$ pretreatment. Since, as suggested by some reports, membrane proteins may be critical targets for AF (see Ref. 16) it is not surprising that induction of MT, an intracytoplasmic protein, does not protect the cell from AF toxicity. Furthermore, Durnam and Palmiter have reported that certain cell lines overproducing MT by gene amplification do not show increased resistance to several metals even though these metals bind to the protein *in vitro* [26]. Moreover, Monia *et al.* have recently reported that MT protects CHO cells from AF toxicity only to a small extent; they suggest that this may be due to the great instability of the Au–MT complex [27]. Snyder *et al.* [28] have described a sequential thiol exchange mechanism that could explain the molecular process of AF interaction with the macrophage. Membrane-associated thiol groups are believed to represent the rate-limiting step for cellular uptake of the drug. This mechanism might be overwhelmed by phagocytosis and pinocytosis phenomena which are strongly enhanced during stimulation by PMA. This might explain the dramatic toxic effect of AF when present during PMA stimulation (Table 1). Similarly, submicromolar concentrations of RA can also inhibit the stimulation of PA activity in macrophages; this effect also occurs rapidly and is irreversible. After stimulation by PMA, AF and RA have little effect on PA activity, which suggests that both drugs interfere with the mechanisms of cell induction by PMA. This observation is consistent with the hypothesis of an inhibition of the PK-C system that has already been suggested in different cell types both for AF and RA [11–15].

Contrary to AF, inhibition of PA stimulation by RA is not complete even at high (10^{-5} M) concentrations. Likewise, inhibition by RA of superoxide production in leukocytes (which is PK-C dependent) is also submaximal [29]. This suggests that the mechanism of action of both drugs may be partly different. PK-C actually exists not as a single entity, but in a family of isoenzymes; their activation mechanisms are very complex and they may exhibit significant biochemical differences [30, 31]. It has been proposed that retinoic acid directly affects the active site of PK-C and decreases the affinity of PK-C for phosphatidylserine and calcium, indirectly resulting in reduced enzyme activity [14]. Conversely, it has also been reported that retinoic acid directly activates partially purified PK-C [32]. AF, like metals which are known to interact with thiol groups may act via the cystein-rich site

Table 4. Effect of retinoic acid on plasminogen activator (PA) activity and cytotoxicity in mouse peritoneal macrophages stimulated by phorbol 12-myristate 13-acetate (PMA)

Retinoic acid (mole/L)	Before stimulation (15 min)		During stimulation (4 hr)		After stimulation	
	PA activity (% control value)	LDH release (% total)[a]	PA activity (% control value)	LDH release (% total)	PA activity (% control value)	LDH release (% total)
0	100.0 ± 20.1	20.7 ± 1.2	100.0 ± 10.6	17.4 ± 0.9	100.0 ± 5.2	24.7 ± 4.7
10^{-7}	46.5 ± 8.2**	15.9 ± 3.9	33.5 ± 3.6**	19.8 ± 0.3	ND	ND
10^{-6}	27.2 ± 2.3**	13.6 ± 2.2*	31.6 ± 1.7**	16.5 ± 7.8	93.3 ± 10.2	35.5 ± 9.9
10^{-5}	25.4 ± 3.4**	12.5 ± 3.9*	18.8 ± 2.5**	17.4 ± 0.9	109.3 ± 4.2	34.7 ± 4.7*

Means ± SD of three experiments.
[a] LDH release is expressed as % of total enzyme content determined after cell disruption with Triton X-100.
* Significantly different from corresponding control value ($P < 0.05$).
** Significantly different from corresponding control value ($P < 0.01$).
ND: not determined.

Table 5. Effect of auranofin (AF) and retinoic acid (RA) alone or in combination on the induction of PA activity in mouse peritoneal macrophages

	PA activity
Control	100 ± 4.3
AF (2×10^{-6} M)	65.6 ± 6.6
RA (10^{-8} M)	52.8 ± 8.2
AF (2×10^{-6} M) + RA (10^{-8} M)	34.2 ± 9.0

Macrophages were incubated during 15 min with the drug(s), stimulated with PMA and assayed for PA activity as described in Materials and Methods. Results are expressed in percentages of the control value (mean ± SD, N = 3).

Table 6. Effect of retinoic acid (RA) (15 min) on PA activity and cytotoxicity in LLC cells

RA (mole/L)	PA activity (% control value) (N = 7)	LDH release (% total)[a] (N = 4)
0	100.0 ± 8.2	20.0 ± 0.6
10^{-6}	105.1 ± 5.4	19.6 ± 0.5
10^{-5}	94.8 ± 15.2	19.6 ± 0.4
10^{-4}	103.5 ± 5.7	19.4 ± 0.9

Means ± SD.
[a] LDH release is expressed as % of total enzyme content determined after cell disruption with Triton X-100.

in the regulatory domain of PK-C. It has also been reported that AF causes a translocation of PK-C from cytoplasm to the particulate subcellular fraction; this translocation, unlike that induced by PMA alone, results in attachment of PK-C to a detergent-insoluble compartment [33].

Brinckerhoff has reported that, in synovial cells in culture, low doses (10^{-10} M) of RA and prednisolone had no effect on the production of collagenase when added alone but had a marked inhibitory effect when added together [19]. We did not find a synergistic effect of RA and AF on PA activity in macrophages.

It is also interesting to note that other drugs used in the treatment of rheumatoid arthritis (indomethacin, *d*-penicillamine and chloroquine) were unable to inhibit PA induction in macrophages (unpublished results).

The PA activity of LLC remains insensitive to AF or RA being thus apparently not dependent on a PK-C pathway. The *in vivo* inhibitory effect of retinoids and AF on established tumors is limited to a few specific conditions, most of the studies performed showed in fact little influence on tumor growth [16]. On the contrary, retinoids are potent inhibitors of the tumor-promotion mechanisms. This is coherent with our finding of its inhibition of PK-C dependent activities which have been suggested to play a role in the promotion process.

The present effects of both drugs occur at concentrations in the micromolar range; it is thus likely that such effects may occur *in vivo* at therapeutically relevant concentrations. The inhibitory effect of AF

on the stimulation of PA activity in macrophages may play a role in the activity of this drug in rheumatoid arthritis. It would be relevant to assess whether like RA, AF may exhibit an antitumor-promoting activity. Similarly, the ability of RA to prevent the stimulation of PA activity in macrophages justifies the study of its efficacy in rheumatoid arthritis [21].

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