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Tumor promoter phorbol 12-myristate 13-acetate induces a clastogenic factor in human lymphocytes

(chromosome breakage/active oxygen/arachidonic acid cascade/oxidative burst/anti-inflammatory agent)

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ABSTRACT **The mechanism of the clastogenic action—i.e., the ability to induce chromosomal aberrations—of the tumor promoter phorbol 12-myristate 13-acetate (PMA) was investigated. PMA at 10 and 100 ng/ml induced the formation of a low molecular weight (<10,000) clastogenic factor (CF) in phytohemagglutinin-stimulated human blood and lymphocyte cultures. Bovine erythrocyte Cu–Zn superoxide dismutase strongly inhibited PMA clastogenicity, both the formation of CF and the action of previously formed CF. The nonsteroidal anti-inflammatory agents indomethacin, imidazol, and 5,8,11,14-icosatetraynoic acid inhibited PMA clastogenicity and the clastogenic activity of previously formed CF. These results suggest that superoxide radicals and stimulation of the arachidonic acid cascade play a role in PMA-induced clastogenicity and the mechanism of action of the CF. The CF may relate the initial interaction of PMA with the cell membrane to the genome.**

The mouse skin tumor promoter phorbol 12-myristate 13-acetate (PMA) induces a puzzling variety of biological effects involving the cell membrane and genomic expression (1–3). The mechanisms that may link these domains of action remain largely unknown. While the membrane effects appear to be initiated by interaction with specific surface receptors (4), it is generally accepted that PMA does not form covalent adducts to DNA. However, it induces chromosomal aberrations in human lymphocytes (5) and mouse epidermal cells (6) and mitotic aneuploidy in yeast (7) with considerable efficiency. Since superoxide dismutase inhibited the clastogenic activity of PMA, it was concluded that O_2^- radicals were produced as intermediates—i.e., that PMA induced chromosomal damage via indirect action (5). It is known that phagocytic leukocytes respond to PMA and other promoters with a respiratory burst involving O_2^- formation (8–10).

The central question arises as to the signal that mediates the effects of PMA binding to the cell membrane, the consecutive changes in lipid metabolism, and the respiratory burst to the genome. Clastogenic factors (CF) might be involved. They comprise low molecular weight (<10,000) components of as yet unknown structure that have been discovered in the sera of patients with certain collagen diseases (11) and the chromosomal breakage disorders ataxia telangiectasia (12) and Bloom syndrome (13) as well as in culture media of skin fibroblasts of these patients (14). CF induce chromosomal aberrations in PHA-stimulated lymphocytes from normal donors. In the case of Bloom syndrome, it has been shown that bovine erythrocyte Cu–Zn superoxide dismutase (SOD) decreases the activity of the CF (14). We now demonstrate that PMA induces the formation of a CF. The formation and activity of the CF are inhibited by SOD. Our results with inhibitors of arachidonic acid (Δ_4Ach) metabolism suggest that Δ_4Ach oxidation products play a role in the clastogenic action of PMA.

MATERIALS AND METHODS

Blood was drawn from healthy volunteers recruited among the laboratory personnel or at the Blood Center. The culture medium was TCM 199 (Flow Laboratories, Paris) supplemented with heat-inactivated human AB serum. Each culture flask contained 5 ml of medium, 1.5 ml of serum, and 0.5 ml of blood or 2.5×10^6 lymphocytes separated from heparinized blood by differential centrifugation on Isopaque/Ficoll (Nyegaard, Oslo). Lymphocytes prepared by this procedure are contaminated with monocytes and platelets but are essentially free of granulocytes. The cultures were stimulated to divide with phytohemagglutinin M and P (PHA) (Difco).

PMA or phorbol (CCR, Eden Prairie, MN) dissolved in spectral grade acetone was added to the cultures so that the final concentration of acetone did not exceed 0.1%. In some of the experiments, erythrocyte Cu–Zn SOD (Boehringer Mannheim; final concentration, 10 μg/ml), indomethacin (Merck, Darmstadt, Federal Republic of Germany; from a stock solution in acetone to a final concentration of 10 μg/ml), imidazol (Sigma; from an aqueous stock solution to a final concentration of 50 μg/ml), or 5,8,11,14-icosatetraynoic acid (ETYA) (Hoffmann-La Roche, Basel, Switzerland; from a stock solution in acetone to a final concentration of 10 μg/ml) was added together with PMA at the beginning of the 72-hr incubation period. After incubation, the cultures were centrifuged at 1,000 rpm for 10 min. The media of three cultures were pooled (approximately 20 ml) for the preparation of ultrafiltrates as described below. The cell pellets were suspended in fresh medium containing colchicine and incubated for an additional 2 hr before microscopy slides were prepared for chromosomal analysis according to standard procedures (14). A minimum of 50 mitoses were examined for each experimental point on coded slides.

Ultrafiltrates of the media of PMA-, phorbol-, or sham-treated cultures were prepared as described for media from Bloom syndrome fibroblast cultures (14). Diaflo ultrafiltration through UM10 filters and concentration 20 times of the filtrates on UM2 filters was carried out routinely. Aliquots (0.02–0.3 ml) of the ultrafiltrates were tested for their clastogenic potency on standard blood cultures from healthy donors as described above. The effects of Cu–Zn SOD, indomethacin, imidazol, and ETYA on the activity of the ultrafiltrates were determined under the conditions given above.

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Abbreviations: PMA, phorbol 12 myristate 13-acetate; PHA, phytohemagglutinin; SOD, bovine erythrocyte Cu–Zn superoxide dismutase; CF, clastogenic factor(s); Δ_4Ach, arachidonic acid; PG, prostaglandin; ETYA, 5,8,11,14-icosatetraynoic acid.

Conditions for the experiments with [^{3}H]PMA (New England Nuclear; specific activity, 17.2 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) were identical to those with nonradioactive PMA. Aliquots of the original culture media, the ultrafiltrates and the media of the final test blood cultures were assayed after the addition of Aquassure in a Beckman LS 9000 scintillation system.

RESULTS

PMA Induces a Low Molecular Weight Clastogenic Factor. Blood or lymphocyte cultures from normal donors were stimulated with PHA and treated with PMA at 10 or 100 ng/ml for 72 hr. The data (Table 1) show that PMA is strongly clastogenic in human blood or lymphocyte cultures in agreement with our earlier observations (5). The media of these cultures were collected and purified by ultrafiltration as described for Bloom syndrome fibroblast cultures (14). Under these conditions, media components in the 1–10,000 molecular weight range are isolated and concentrated 20 times. Small aliquots of these preparations, which are essentially free of PMA (see below), were tested for their potency to induce aberrations in blood cultures. The data (Table 1) show that ultrafiltrates of the media of these cultures contain the CF. Clastogenic activity was also detectable in the ultrafiltrates of cultures that had been incubated for 24, 48, or 96 hr. For concentrated ultrafiltrates from PMA-treated blood cultures, the aliquots were chosen so that the final concentration of the added media component was the same as that in the cultures from which they were derived. Under these conditions, the aberration frequencies induced by PMA in the first culture were significantly higher than those induced by the clastogenic ultrafiltrates in the test culture. Concentrated ultrafiltrates from PMA-treated lymphocyte rather than blood cultures were considerably more toxic and more clastogenic and therefore the size of the aliquots added to the test cultures was reduced. The concentration range that yielded significant clastogenicity and low toxicity was relatively narrow. The data given in Table 1 were obtained with amounts of ultrafiltrate that were 1/6th of those for the corresponding preparations derived from blood cultures. Clastogenic activity was detectable in media preparations following treatment with PMA at as little as 1 ng/ml (data not shown). No simple dose relationship was observed between the concentration of PMA and the potency of the clastogenic components that were induced. In contrast to PMA, the nonpromoter phorbol was not clastogenic *per se* and did not induce significant clastogenic activity even at concentrations 10 times higher than those for PMA.

Comparison of the type of aberrations induced by PMA and by ultrafiltrates from PMA-treated cultures revealed no significant differences. The aberrations were mainly of the chromatid type while chromosome type aberrations were less than 1% (5). However, there was a striking difference in the incidence of polyploid cells and premature chromosome condensation that was observed in the PMA but not in the ultrafiltrate-treated cultures.

The Clastogenic Media Ultrafiltrates Are Essentially Free of Residual PMA. It was crucial to show that the clastogenic activity of the media ultrafiltrates was not due to contamination by residual PMA but rather to low molecular weight components released by the cells in response to PMA treatment. For this purpose, several experiments were carried out with radioactive PMA, which allowed determination of the concentration of PMA in the various fractions during the preparation of the clastogenic ultrafiltrates and in the final test culture. PHA-stimulated lymphocyte cultures were treated with [^{3}H]PMA for 72 hr, and the culture media were purified according to our standard procedure. As shown in Table 2, passage through UM10 filters eliminated more than 99% of the radioactivity, indicating that the PMA in the culture media is mostly adsorbed to serum components with molecular weights above 10,000 and the filter membrane itself. Concentration of the ultrafiltrates with UM2 filters and sterilization by passage through Millipore filters further decreased the radioactivity content. The residual PMA concentrations in the media of the test cultures were calculated from their radioactivity content. Depending on the sizes of the aliquots added per 6.5 ml of blood culture, the concentrations varied from 0.02 ng/ml to below detectability. As shown in Table 2, the preparations were strongly clastogenic at residual PMA concentrations as low as 5–8 pg/ml and some clastogenicity was detectable at concentrations 1/5th to 1/3rd of those. PMA in this concentration range is no longer clastogenic for PHA-stimulated human lymphocytes. It is concluded that the clastogenic activity of the ultrafiltrates is produced by the cells in response to PMA treatment and is not due to contamination by residual PMA.

Anticlastogenic Activity of SOD and Nonsteroidal Anti-Inflammatory Agents. Based on our earlier observation that Cu–Zn SOD inhibits the clastogenic activity of PMA (5), we tested the effect of this enzyme on the activity and the formation of the CF. The data (Table 3) confirm our earlier conclusion that SOD inhibits PMA clastogenicity. The data also show that SOD inhibits the activity of CF prepared in its absence. Since bovine erythrocyte Cu–Zn SOD has a molecular weight of approximately 30,000 (15), it is removed by our standard ultrafiltration

Table 1. Clastogenic factor induced by PMA in human blood and lymphocyte cultures

	Conc., ng/ml	Culture type	% mitoses with aberrations	
			PMA	CF
PMA	100	Blood	29.6 ± 12.1 (5)	18.2 ± 3.6 (8)
	100	Lymphocyte	30.0 ± 10.6 (3)	21.0 ± 5.2 (3)
	10	Lymphocyte	16.7 ± 5.0 (3)	24.0 ± 3.5 (3)
Phorbol	1,000	Blood	0 (2)	10.5 (2)
	100	Blood	4.0 (2)	5.0 (2)
Control*		Blood	3.0 ± 2.6 (4)	2.0 ± 2.1 (8)

PMA or phorbol in acetone was added to PHA-stimulated lymphocyte or blood cultures as indicated (final concentration of acetone, 0.1%). After incubation for 72 hr, the cultures were centrifuged and the media of three cultures were pooled for the preparation of clastogenic ultrafiltrates (CF). The cell pellets were suspended in fresh medium containing colchicine and incubated for 2 hr before microscopy slides were prepared. CF from blood cultures: UM10 filtrates were concentrated 20 times on UM2 filters (0.3 ml/6.5 ml of culture); CF from lymphocyte cultures: UM10 filtrates were concentrated on UM2 filters 20 times (0.05 ml/6.5 ml of culture). Results are mean ± SD; values in parentheses are numbers of experiments.

* 0.1% acetone.

Table 2. Purification of the PMA-induced clastogenic factor

	^{3}H, cpm/ml	PMA, ng/ml	% mitoses with aberrations
Experiment 1			
Culture medium 1	1.21×10^6	61	24
UM10 filtrate	2.1×10^3	0.11	—
0.3 ml of UM2 concentrate together with 6.5 ml of culture medium 2	1.7×10^2	0.008	18
0.1 ml of UM2 concentrate together with 6.5 ml of culture medium 2	—	—	10
Experiment 2			
Culture medium 1	1.51×10^6	86	26
UM10 filtrate	3.2×10^3	0.17	—
0.3 ml of UM2 concentrate together with 6.5 ml of culture medium 2	4.0×10^2	0.02	Toxic
0.1 ml of UM2 concentrate together with 6.5 ml of culture medium 2	1.0×10^2	0.005	23
0.02 ml of UM2 concentrate together with 6.5 ml of culture medium 2	—	—	8

Culture medium 1 was from lymphocyte cultures containing [^{3}H]PMA at 61 or 86 ng/ml. Culture medium 2 consisted of UM10 filtrates concentrated 20 times on UM2 filters.

procedure for the preparation of CF. Therefore, we also compared the clastogenicity of CF preparations induced by PMA in the absence and presence of SOD. The potency of the CF was decreased from 17.3 ± 2.1% to 5.0 ± 3.6% mitoses with aberrations when SOD at 10 μg/ml was present together with PMA at 100 ng/ml (mean ± SD of three experiments). It is concluded that SOD inhibits the clastogenic activity of PMA, formation of the CF, and the activity of previously formed CF.

The nonsteroidal anti-inflammatory agents indomethacin, imidazol, and ETYA were tested for their effects on the clastogenicity of PMA and the potency of PMA-induced CF. The data in Table 3 show that all three drugs were strongly anticlastogenic at concentrations at which they had no significant effects on cell growth (16). They diminished the clastogenicity of PMA and inhibited the activity of the CF. Their inhibitory effect was always more pronounced on the CF relative to PMA itself. Since there is no simple procedure to remove these drugs from the media quantitatively, it could not be tested whether they also inhibited formation of the CF.

DISCUSSION

Our results show that PMA induces a CF in mitogen-stimulated human blood and lymphocyte cultures. From the ultrafiltration procedure used for its isolation, it follows that it possesses a molecular weight of less than 10,000. The experiments with radioactive PMA show that the clastogenic activity of the ultrafiltrates is not due to contamination of these preparations by residual PMA. Removal of PMA from the media was straightforward because it was found that UM10 filtrates were essentially free of PMA. It is evident that PMA in culture media is tightly adsorbed to high molecular weight serum components and retained by the filter membrane itself. From the data in Table 2, it is estimated that the concentration of residual PMA in the test blood cultures was as low as 1 pg/ml in experiments in which PMA at 10 ng/ml was used for induction of the CF. CF preparations derived from PMA-treated lymphocyte cultures were considerably more clastogenic but also more inhibitory to cell growth than the corresponding preparations from whole blood cultures. The concentration range at which the lymphocyte-derived CF exhibited low toxicity but high clastogenicity was relatively narrow and 1/10th to 1/6th of that for CF preparations from blood cultures. It is conceivable that whole blood cultures contain inhibitors of the formation of CF—for example, erythrocyte SOD. Regular lymphocyte preparations of the type used in our experiments are contaminated with other cells, in particular monocytes and residual platelets. Preliminary experiments indicate that PMA is much less active for highly purified T and B lymphocytes, suggesting that the con-

Table 3. Anticlastogenic effects of SOD and anti-inflammatory agents

Addition	Conc., μg/ml	% mitoses with aberrations			
		PMA only	PMA/addition	CF only	CF/addition
SOD	10	36.7 ± 10.3 (3)	5.3 ± 1.1 (3)	24.4 ± 4.6 (5)	4.0 ± 3.7 (5)
Indomethacin	10	31.0 ± 9.7 (6)	9.0 ± 4.9 (6)	18.0 ± 3.6 (6)	4.0 ± 3.1 (6)
Imidazol	50	25.2 ± 5.1 (5)	14.5 ± 3.9 (5)	17.0 ± 4.8 (4)	2.5 ± 3.0 (4)
ETYA	10	26.4 ± 6.0 (12)	17.9 ± 6.2 (12)	15.3 ± 3.0 (3)	4.7 ± 4.2 (3)

Effects on PMA clastogenicity were tested with PMA at 100 ng/ml in the absence or presence of SOD, indomethacin, imidazol, or ETYA in standard blood cultures (13). Effects on activity of the CF were tested on 0.1 ml of 20-times UM2-concentrated UM10 ultrafiltrates from blood cultures treated with PMA at 100 ng/ml and added to standard blood cultures (13) in the absence or presence of the same drugs. Results are mean ± SD; values in parentheses are numbers of experiments. Control values and experimental details are as in Table 1.

taminating cells augment PMA clastogenicity (unpublished data).

As reported previously, the frequency of aberrations induced by PMA was concentration dependent (5). However, no simple relationship was observed between the concentration of PMA and the potency of the CF that was induced. A systematic study of this question was precluded because of the narrow concentration range in which the CF preparations had to be tested to avoid substantial toxicity.

Insight into the mechanism of the formation and action of the PMA-induced CF was obtained from the experiments with bovine Cu–Zn SOD. SOD inhibited the clastogenicity of PMA as well as the formation and activity of the CF. Precluding any unknown side effects of this enzyme, these results implicate O_2^- radicals in all three reactions. The clastogenic action of PMA itself may be mediated by the CF. If this is the case, the effect of SOD on PMA clastogenicity is due to the inhibition of CF formation. The observation that SOD also inhibited the activity of previously formed CF is reminiscent of our results with a similar CF isolated from Bloom syndrome fibroblast cultures (14). No consistent results could be obtained with catalase.

The following observations reported in the literature suggested that oxidation products of membrane lipids might play a role in PMA clastogenicity and formed the basis for the experiments with nonsteroidal anti-inflammatory agents. PMA is known to stimulate the oxidation of Δ_4Ach and this effect may be related to its activity as a tumor promoter. PMA stimulates the synthesis of prostaglandin (PG) E_2 in mouse epidermis (17, 18), PGE and PGF, hydroperoxy- and hydroxy-Δ_4Ach in mouse skin (19, 20, ‡), and PGE_2 and PGF_2 in dog kidney cells (21). In Δ_4Ach-labeled human lymphocytes it mostly induces the release of free Δ_4Ach and the formation of a polar Δ_4Ach derivative that has not yet been identified structurally. This polar material is not produced in response to PHA and the release of Δ_4Ach is much lower than for PMA (unpublished work; cf. ref. 22). PHA is not clastogenic under our standard conditions. The strong inhibitory activity of indomethacin, imidazol, and ETYA observed in our experiments (see Table 3) support the hypothesis that products of the Δ_4Ach cascade are involved in PMA clastogenicity. Indomethacin preferentially inhibits Δ_4Ach cyclooxygenase and imidazol thromboxane synthetase (23). ETYA is an analogue of Δ_4Ach that inhibits both the cyclooxygenase and lipoxygenase pathways (24). Preliminary results indicate that the anti-inflammatory agents nordihydroguaiaretic acid (at 10 μg/ml) and flufenamic acid (at 1–10 μg/ml) are also anticlastogenic (unpublished data). Because of the limited selectivity of all these drugs for a specific step in the Δ_4Ach cascade (25–28), no safe conclusion can be reached concerning the structural identity of the CF. It is also conceivable that these drugs influence the radical-scavenging potential of the cell and some of them may act as metal chelators.

On the basis of our present cytogenetic results and the preliminary chromatographic data mentioned above, we speculate that the CF consists of lipid hydroperoxides together with free Δ_4Ach and that platelets in our regular blood and lymphocyte cultures assist in the metabolism of Δ_4Ach. PGG_2 (29) and hydroperoxy-Δ_4Ach are probably clastogenic since they release active oxygen on transformation to more stable derivatives. Malondialdehyde and other aldehydes produced in fragmentation reactions of lipid peroxides may also be clastogenic. It has been speculated that malondialdehyde is an intermediate in radiation carcinogenesis (30) but it should be noted that the chemically pure substance is only weakly mutagenic (31). Regardless of the exact biochemical identity of the PMA-induced CF, our data suggest that it originates in membrane lipids. Therefore, it relates the initial interaction of PMA with the cell membrane (1–4) to the genome. This does not imply that the CF represents a signal that is specific for tumor promotion, of course.

The CF induced by PMA in human lymphocytes resembles the CF detected in the serum of patients with certain collagen diseases (11, 32, 33) and the hereditary diseases with increased cancer incidence Bloom syndrome (14, 34) and ataxia telangiectasia (12). It is also similar to the CF in the serum of New Zealand Black mice (35, 36), which are considered an animal model for autoimmune diseases. Since the CF in Bloom syndrome and ataxia telangiectasia were also produced by cultured fibroblasts, they are not lymphokines. The PMA-induced CF also differs from a mitogenic factor described by Sundar *et al.* (37) that possesses a molecular weight greater than 10,000. Only further biochemical characterization will clarify the relationship among the CF from different sources.

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