Brief Definitive Report

PROSTAGLANDINS ARE NECESSARY FOR OSTEOCLAST-ACTIVATING FACTOR PRODUCTION BY ACTIVATED PERIPHERAL BLOOD LEUKOCYTES*

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Both osteoclast-activating factor (OAF) and prostaglandins are potent local mediators of bone resorption (1–3). Both of these factors are produced by cells involved in immune responses and are potential mediators of the bone destruction associated with diseases such as rheumatoid arthritis, periodontal disease, and cholesteatoma (4). They may also be produced at the site of metastatic bone lesions. Their interactions at bone resorbing sites therefore may be important in the bone resorption which is associated with a variety of inflammatory and neoplastic diseases. To test if OAF production is dependent on prostaglandins, we have examined the effects of a number of structurally unrelated inhibitors of prostaglandin synthetase on the production of OAF by phytohemagglutinin-(PHA) activated human peripheral blood leukocytes. The production of OAF was inhibited by indomethacin, R020-5720 and flufenamic acid. Inhibition of OAF production by these agents was reversed by adding prostaglandins of the E series back to the leukocyte suspension. These results indicate that prostaglandin synthesis is necessary for OAF production.

Materials and Methods

Peripheral blood leukocytes were obtained from plateletpheresis donations from normal volunteers at the Connecticut Red Cross in Farmington, Conn. 5 ml of 5% dextran was added to the plasma-leukocyte-red mixture and the leukocyte buffy coat was separated from the sedimented erythrocytes. Leukocytes were cultured at a concentration of 1×10^6 cells/ml in BGJ medium (5) (Grand Island Biological Co., Grand Island, N. Y., formula 78-0088) without added serum. PHA-M 1% (Grand Island Biological Co.) was used to stimulate the leukocyte cultures. The leukocytes were cultured for 24–72 h. At the end of the culture, the supernates were removed by gentle aspiration and frozen at −20°C until OAF assay. After the supernates were removed, the leukocytes were pulsed with [^{3}H]thymidine or ^{3}H-amino acids for 4–6 h according to the methods previously described (5). The inhibitors of prostaglandin synthesis which were used were indomethacin, d,-1-6-choromethyl-carbazole-2-acetic acid (R020-5720) (Hoffman-LaRoche, Inc., Nutley, N. J.) and flufenamic acid (Aldrich Chemical Co., Metuchen, N. Y.). Each of these structurally unrelated drugs inhibits prostaglandin synthesis at these concentrations (6–8). These agents were added to the leukocyte culture suspensions 2 h before activation of the leukocytes with PHA.

Bone resorbing activity was assayed in the leukocyte supernates by methods similar to those which have been previously described (5, 9). Pregnant mice at the 16th-day of gestation were

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TABLE I

Effects of Prostaglandin Synthetase Inhibitors on [^{3}H]Thymidine ([^{3}H]TdR) and ^{3}H-Amino Acid (^{3}H-AA) Incorporation and OAF Production by Leukocytes

	PHA	Inhibitors (10^{-5} M)	[^{3}H]TdR incorporation	^{3}H-AA incorporation	OAF production (treated/control ratios of ^{45}Ca release)
	1%		*cpm*	*cpm*	
Exp. 1	+	—	3,094 ± 411*	4,699 ± 744*	1.72 ± 0.13*
	+	Indomethacin	2,928 ± 133*	3,501 ± 468*	1.05 ± 0.12‡
	−	—	1,125 ± 48	1,111 ± 91	1.10 ± 0.11
	−	Indomethacin	1,222 ± 30	974 ± 25	0.80 ± 0.06
Exp. 2	+	—	38,564 ± 2604*	4,008 ± 211*	1.95 ± 0.17*
	+	R020-5720	24,374 ± 2407*	6,063 ± 245*	1.00 ± 0.04‡
	−	—	1,254 ± 43	1,053 ± 11	1.13 ± 0.12
	−	R020-5720	1,185 ± 123	1,651 ± 28	1.18 ± 0.16
Exp. 3	+	—	4,410 ± 263*	6,862 ± 447*	1.76 ± 0.06*
	+	Flufenamic acid	6,708 ± 332*	6,809 ± 417*	1.00 ± 0.05‡
	−	—	1,410 ± 84	2,001 ± 130	0.98 ± 0.03
	−	Flufenamic acid	1,307 ± 52	1,855 ± 53	0.90 ± 0.03

The inhibitors were added to the leukocyte cultures 2 h before PHA. The leukocytes were then cultured for 48 h. Values for [^{3}H]TdR and ^{3}H-AA incorporation are means ± standard errors for three leukocyte cultures. Values for OAF production are means ± standard errors for four bone cultures.

* Significantly greater than corresponding control, $P < 0.05$.

‡ Significantly different from stimulated cultures without inhibitor, $P < 0.05$.

injected subcutaneously with 0.05 mCi of ^{45}Ca. The next day the fetuses were removed and the mineralized shafts of the radii and ulnae were dissected free from surrounding subcutaneous soft tissue and the cartilagenous ends. The bones were cultured in control media for 24 h to allow for exchange of loosely complexed ^{45}Ca with stable calcium in the medium. The bones were then cultured with the leukocyte culture supernates for 72 h and bone resorbing activity was assessed as the percent of total radioactivity released from the bones into the culture medium during the period of culture. The leukocyte culture supernates were diluted 1:1 with fresh medium before assay for bone resorbing activity. Bone resorbing activity was also expressed as ratios of ^{45}Ca release from the test bones compared with corresponding paired bones cultured in control media. Four bones were used in each test group and differences were assessed using Student's *t* test.

Results and Discussion

Each of the structurally unrelated inhibitors of prostaglandin synthetase inhibited the production of OAF by leukocytes at 10^{-5} M (Table I). In the same experiments, these drugs did not inhibit protein or DNA synthesis by the leukocytes as measured by the incorporation of ^{3}H-amino acids of [^{3}H]thymidine into the cells. The inhibitory effects of these drugs on OAF production were obtained with concentrations as low as 10^{-7} M (data not shown). Although each of these drugs inhibited OAF production, they had no effect on the biological activity due to OAF when they were added to OAF containing media in concentrations of 10^{-5} M. OAF production was restored by adding prostaglandins of the E series together with indomethacin to the leukocytes before the leukocytes were activated with PHA (Table II). Similar effects were seen with both prostaglandin-E_1 and prostaglandin-E_2. However, prostaglandin-F_{1_α} and -F_{2_α} had no significant effect on restoration of OAF activity. Prostaglandins were

TABLE II

Restoration of Leukocyte Production of OAF by Addition of Prostaglandin E_1 (PGE_1) to the Cultures Treated with Indomethacin (Indo), an Inhibitor of Prostaglandins Synthesis

Source of media applied to bone cultures				Bone resorbing activity (treated/control ratios)	
				Before diafiltration	After diafiltration
Indo, 10^{-5} M	PGE_1, 10^{-6} M	PGE_1, 10^{-7} M	PHA-act lk		
−	−	−	+	1.82 ± 0.37*	1.54 ± 0.08*
+	−	−	+	0.89 ± 0.02	1.13 ± 0.05
+	+	−	+	2.47 ± 0.43*	1.75 ± 0.06*
+	−	+	+	1.84 ± 0.41*	1.68 ± 0.09*
−	+	−	−	2.81 ± 0.48*	0.99 ± 0.02
−	−	+	−	1.97 ± 0.02*	1.06 ± 0.03

The prostaglandins and indo were added to the leukocytes 2 h before the addition of PHA. The leukocytes (lk) were then cultured for 48 h. The lk culture supernates were assayed for bone resorping activity before and after diafiltration to remove the prostaglandins.

Values are means ± SEM for four pairs of bone cultures.

* Significantly greater than 1.0, $P < 0.05$.

removed from the leukocyte-conditioned media before assay for bone resorbing activity by diafiltration (ultrafiltration with continuous replacement of the filtered volume with 10 vol of fresh media) across an Amicon UM_2 membrane (Amicon Corp., Lexington, Mass.), which has a nominal mol wt cut-off of 1,000 daltons. Restoration of OAF production indicates that prostaglandin synthesis by the leukocyte cultures is necessary for OAF to be produced.

A number of studies have indicated that prostaglandins may be endogenous modulators of cell-mediated immune responses and lymphokine production. Prostaglandins have been shown to both stimulate and inhibit the production of different lymphokines. Most of the described effects of prostaglandins on lymphokine production have been inhibitory. Large concentrations of prostaglandins have been shown to inhibit macrophage migration inhibition factor production by guinea pigs (10, 11), mitogen-induced stimulation of murine lymphocytes (12) and human leukocyte inhibitory factor production (13). Goodwin et al. have suggested that prostaglandin production by glass-adherent cells inhibits T-cell mitogenesis (14). In contrast, prostaglandins of the E series appear to be necessary for the production of a factor by activated leukocytes which increases vascular permeability (15). Now we have shown that prostaglandins are also necessary for the production of another lymphokine, OAF.

The cell source of prostaglandins necessary for OAF production in our experiments is likely to be the monocyte. The monocyte is the principal PGE-producing cell in human peripheral blood (16). The presence of monocytes in the leukocyte population is necessary for OAF production, but not for leukocyte activation (17). Nonadherent lymphocytes devoid of monocytes which are incubated with PHA synthesize DNA and protein, but do not release OAF (17). T cells alone require the presence of monocytes in order to release OAF after activation with PHA (18). Our data reported here suggests that the synergy between the monocyte and the lymphocyte in the production of OAF may be mediated by prostaglandins.

Regardless of the mechanism of the interaction between prostaglandins and leukocytes in OAF production, our data indicate that prostaglandins are necessary for

the production of OAF by stimulated leukocytes, but they are not essential for leukocyte activation as assessed by DNA or protein synthesis. These data also clearly show that responses to indomethacin and related drugs do not prove a prostaglandin-mediated mechanism of bone resorption in animals or in patients with hypercalcemia.

Summary

The production of osteoclast-activating factor (OAF) by normal human peripheral blood leukocytes stimulated by phytohemagglutinin was inhibited by a series of structurally unrelated inhibitors of prostaglandin synthetase. Inhibition of OAF production by these agents was reversed by adding prostaglandins of the E series back to the leukocyte suspension. These results indicate that prostaglandin synthesis is necessary for OAF production.

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