CONCISE REPORT

Stimulation of Prostacyclin Synthesis in Vascular Cells by Mononuclear Cell Products

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Supernatants were obtained from human peripheral blood mononuclear cells stimulated with phytohemagglutinin or in a mixed lymphocyte reaction. The effect of mononuclear cell products on vascular prostacyclin (PGI_2) production was measured using cultured rat aortic smooth muscle cells (SMC) or aortic rings. PGI_2 was measured by radioimmunoassay of its metabolite, 6-keto-$PGF_{1\alpha}$. Supernatants containing mononuclear cell products induced PGI_2 production in vascular tissue. Supernatant-induced PGI_2 production of SMC was relatively slow, requiring more than six hours of incubation with supernatants, and was completely prevented by aspirin, a cyclooxygenase inhibitor. The regulation of arachidonic acid metabolism by products of stimulated mononuclear cells, which is critical to the physiology and pathology of blood vessels, may be an important aspect of the interaction between immunocompetent cells and vascular tissue.

THE INFLUENCE OF CELLS of the immune system on vascular cells has received only limited attention (briefly reviewed by Baldwin[1]). At sites of cell-mediated reactions such as delayed hypersensitivity or graft-*v*-host disease, proliferation of capillary endothelial cells has been documented.[2] In vitro products of lymphocytes or macrophages regulate various functions of vascular cells, such as proliferation, migration, production of colony-stimulating factors (CSF), and expression of class II histocompatibility (Ia) antigens.[3-7] Prostacyclin is the major product of arachidonic acid (AA) metabolism in vascular cells and may play a key role in the physiology and pathology of blood vessels.[8]

The present investigation was designed to elucidate whether or not products of blood mononuclear cells influence the AA metabolism of the vessel wall. We found that supernatants of stimulated human peripheral blood mononuclear cells induced prostacyclin (PGI_2) synthesis in vascular cells and tissues.

MATERIALS AND METHODS

Supernatants

Supernatants of stimulated mononuclear cells were prepared essentially as previously described.[9] Briefly, mononuclear cells were separated from the heparinized venous blood of healthy laboratory donors by sedimentation on Ficoll-Hypaque. Mononuclear cells were washed and resuspended in RPMI 1640 medium with 10% fetal bovine serum (GIBCO-Biocult, Glasgow, Scotland) at a concentration of 2 to 5 × 10^6/mL; 10 mL of the cell suspension was cultured in plastic tubes (2070, Falcon, Oxnard, Calif) with 10 μg/mL phytohemagglutinin (PHA; HA17, Wellcome Research Laboratories, Beckenham, England). After 20 hours at 37 °C in air with 5% CO_2, cells were washed three times with 50 mL of culture medium, resuspended in the original volume, and further cultured for 24 hours at 37 °C. Control cultures consisted of mononuclear cells cultivated without PHA. In some experiments (see Results), PHA was added to the control medium and did not affect PGI_2 production.

One supernatant was also generated by a mixed lymphocyte reaction (MLR).[9] Mononuclear cells from two histoincompatible donors (2 × 10^6/mL) were cultured for three days at 37 °C. The control medium consisted of mononuclear cells of each of the two donors cultured alone. Before assessing their effect on the vessel walls, the capacity of supernatant fluid from stimulated mononuclear cells to augment the tumoricidal capacity of human monocytes was tested, as previously described.[9]

Isolation and Culture of Rat Aortic Smooth Muscle Cells

Rat smooth muscle cells were obtained from thoracic aortas according to the method of Travo et al.[10] The cells were grown in Eagle's minimal essential medium on Hanks' balanced salt solution buffered with HEPES (20 mmol/L), supplemented with 10% bovine serum (5% newborn, 5% fetal) and with the addition of penicillin (100 U/mL) and streptomycin (100 μg/mL) (Flow Laboratories, Inc, Rockville, UK). Rat smooth muscle cells were subcultured in a ratio of 1:3 by brief exposure to a mixture of 0.25% to 0.125% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS). The experiments reported here were carried out with cells between the sixth and 22nd passages at confluence. Medium was removed the day before the experiment. Previous experiments using thin-layer chromatography and mass spectrometry showed that these cells produce PGI_2 as the main AA metabolite and that the AA metabolic profile did not change during cell doublings.

For thin-layer chromatography, smooth muscle cells at confluence were labeled with 0.2 μCi/2 × 10^5 cells 1-^{14}C-arachidonic acid (59 mCi/mmol; Amersham International Ltd, Buckinghamshire, England) for four hours at 37 °C. Approximately 50% of the total radioactivity added was incorporated. After labeling, the cells were repeatedly washed, then incubated for 12 hours in the culture medium. At the end of incubation, the medium was extracted and

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arachidonic acid metabolites were separated by thin-layer chromatography and counted, as previously described.[11] This procedure was applied to the cells at the 6th, 10th, 12th, 18th, 20th, and 22nd passages.

Arachidonic acid metabolites were analyzed by mass spectrometry on confluent monolayers of SMC incubated with 25 μmol/L AA for 30 minutes. After incubation, the medium was extracted and processed for mass-spectrometry analysis of prostaglandins and thromboxane, as described previously.[12] This procedure was applied to cells at the sixth and 22nd passages.

Smooth Muscle Cell Stimulation With Supernatants

After removing the growth medium, the monolayers of intact confluent smooth muscle cells (2.5 to 3.5 × 10^5 cells in a 2-mm^2 culture well) were washed once with 2 mL of PBS, after which 300 μL of culture medium alone, or supernatants of stimulated mononuclear cells, or conditioned medium at the required concentrations, were layered onto the cells. In some experiments, aspirin (Flectadol, Maggioni, Italy) was added to the cells at a concentration of 500 μmol/L for the entire incubation period with mononuclear cell products.

After selected incubation periods at 37 °C, the supernatants were removed from the culture well and stored at −20 °C until tested for 6-keto-$PGF_{1\alpha}$, which was measured by a radioimmunoassay as described.[13] The background of immunoreactive 6-keto-$PGF_{1\alpha}$ in the supernatants of stimulated mononuclear cells and conditioned medium preparations tested before addition to the smooth muscle cells ranged from 0.38 to 1.96 pmol/100 μL and 0.34 to 1.90 pmol/100 μL, respectively. Background values of 6-keto-$PGF_{1\alpha}$ were measured for each supernatant preparation and subtracted from the total 6-keto-$PGF_{1\alpha}$ value measured after incubation of the cells with supernatants.

"Exhausted" Rat Aortic Rings

Rat aortic rings were prepared and "exhausted" as described previously.[14] After this procedure, the vessels produced undetectable antiaggregating activity and only negligible amounts of immunoreactive 6-keto-$PGF_{1\alpha}$ when incubated for ten minutes in 1 mL 0.05 mol/L TRIS buffer (pH 9.0) at room temperature. The "exhausted" rings were incubated at 37 °C with 400 μL conditioned medium or the supernatants of stimulated mononuclear cells. After 30 minutes, PGI_2 activity was checked biologically as described.[14] The capacity of test samples to stimulate PGI_2 activity was expressed as a percentage of the activity of appropriate control vessels tested simultaneously.

PGI_2 activity was also measured by radioimmunoassay of 6-keto-$PGF_{1\alpha}$.[13]

RESULTS

Figure 1 shows the results of a typical experiment in which vascular smooth muscle cells were exposed to supernatants of stimulated mononuclear cells for various times in culture, and PGI_2 production was measured as immunoreactive 6-keto-$PGF_{1\alpha}$, its stable metabolite. Control conditioned medium of unstimulated mononuclear cells did not induce PGI_2 formation in supernatants of smooth muscle cells under these conditions, whereas supernatants of PHA-stimulated mononuclear cells induced appreciable PGI_2 production. Some effect of mononuclear cell supernatants was already apparent after 12 hours of incubation (0.87

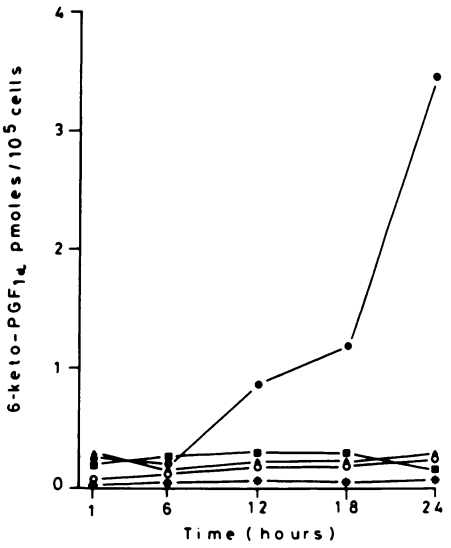

Fig 1. Time course of PGI_2 production by rat smooth muscle cells in the presence of mononuclear cell products (30% of total incubation volume) (●—●), conditioned medium (○—○), medium with PHA added (▲—▲), mononuclear cell products with aspirin added (✱—✱), or medium alone (■—■). Values are means of quadruplicate experiment (SD was 5% to 8% of the mean). The figure illustrates typical results, which were similar in six other experiments.

pmol/10^5 cells *v* 0.15 pmol/10^5 cells for conditioned medium) and was maximal after 24 hours (3.49 pmol/10^5 cells). Because further incubation (up to 48 hours) did not consistently augment the levels of 6-keto-$PGF_{1\alpha}$ (data not shown), the 24-hour exposure time of smooth muscle cells to supernatants was used routinely.

The supernatant preparations used in most experiments were obtained by pulsing mononuclear cells with PHA for 24 hours, followed by extensive washing (see Materials and Methods). PHA alone did not induce PGI_2 synthesis in vascular smooth muscle cells (Fig 1). However, a supernatant preparation obtained by mixed lymphocyte reaction did induce PGI_2 synthesis, thus further excluding any significant role of possible residual contamination of supernatants with trace amounts of lectin.

Exposure of smooth muscle cells to aspirin during incubation completely prevented the rise in 6-keto-$PGF_{1\alpha}$ levels induced by mononuclear cell products, thus further indicating that the radioimmunoassay was measuring an AA metabolite of the cyclooxygenase pathway.

The results shown in Fig 1 were obtained at a 30% supernatant concentration in the culture medium. Different supernatants varied considerably in potency (ie, minimal active dilution), with activity usually first detectable at a 5% to 15% concentration (Fig 2). For experiments on smooth muscle cells, four preparations

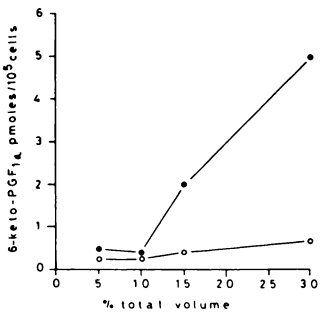

Fig 2. PGI_2 production by rat smooth muscle cells as a function of mononuclear cell product concentration. The cells were incubated for 24 hours with mononuclear cell products (●—●) or conditioned medium (O—O). Values are means of quadruplicate experiments (SD never exceeded 10% of the mean). This figure illustrates typical results that were similar in four other experiments.

were tested; all induced PGI_2 production, with a mean level of 2.25 ± 0.5 (range 1.53 to 3.49) pmol/10^5 cells of 6-keto-$PGF_{1\alpha}$ at a 30% concentration (24-hour incubation) compared to 0.34 ± 0.15 (range, 0.24 to 0.5) for control conditioned media.

In some experiments, the smooth muscle cells were stimulated with arachidonate (25 μmol/L) 24 hours after incubation with the 30% stimulated mononuclear cell supernatant. Mean 6-keto-$PGF_{1\alpha}$ production was 8.6 ± 0.7 pmol/10^5 cells for the cells incubated with supernatants of stimulated mononuclear cells and 7.6 ± 0.8 pmol/10^5 cells for the cells incubated with conditioned medium. These values were not statistically different by the Student's *t* test.

The results indicate that supernatant fluid from stimulated mononuclear cells contains products capable of inducing PGI_2 production in cultured smooth muscle cells. It was of interest to see whether or not such effects were also observed using whole vessel walls. As shown in Table 1, stimulated mononuclear cells produced substances that consistently stimulated PGI_2 production from "exhausted" rat aortic rings.

DISCUSSION

The results reported here show that supernatants of stimulated mononuclear cell cultures induce PGI_2 synthesis in vascular tissues. The effect of supernatants was observed on whole aortic tissue and cultured smooth muscle cells and was detected by two different PGI_2 assay methods. Induction of PGI_2 production in smooth muscle cells by mononuclear cell supernatants was dependent on the integrity of the cyclooxygenase pathway of arachidonic metabolism, as indicated by the fact that aspirin completely blocked PGI_2 production.

Table 1. Prostacyclin-Stimulating Activity of Mononuclear Cell Products on "Exhausted" Rat Aortic Rings

	6-Keto-$PGF_{1\alpha}$ (pmol/mL)	PGI_2 Activity (% Inhibition of Platelet Aggregation)
Conditioned medium	7.8 ± 6.1	12.4 ± 1.7
Mononuclear cell products	43.3 ± 4.4*	71.0 ± 15.5*

Values are means ± SD of six specimens.
*$P < .01$, Student's *t* test.

The induction of PGI_2 synthesis by mononuclear cell products required a long interaction with smooth muscle cells (> six hours). In contrast, induction of PGI_2 synthesis in endothelial cells or smooth muscle cells, by AA, thrombin, or the ionophore A23187 occurs rapidly, requiring only a few minutes.[15,16] However, stimulation of fibroblast PGE_2 synthesis by lymphocyte-monocyte products requires at least 12 hours to be apparent.[17]

Products of lymphocytes or monocytes reportedly affect various functions of vessel wall cells (endothelial and/or smooth muscle cells), such as proliferative capacity, migration, and production of colony-stimulating factors.[3-7] Except for interferon (IFN), which has been shown to modulate the expression of class II histocompatibility antigens in endothelial cells,[7] the nature of the mediator(s) involved in these effects has not been defined. Identification of the product of stimulated mononuclear cells that induces PGI_2 synthesis in vascular tissue was beyond the scope of the present investigation, which was aimed only at assessing whether or not an unfractionated stimulated mononuclear cell supernatant affected PGI_2 synthesis—a critical aspect of the physiology of the vessel wall. Experiments designed to elucidate the nature of the product(s) and the producing cell(s) are now under way. Preliminary data suggest that monocytes are required for the generation of active supernatants. Gel filtration experiments on Sephacryl S200 suggest a molecular weight range of 15,000 to 40,000. For instance, in one experiment, pooled Sephacryl S200 fractions in the 15,000 to 40,000 range caused a fivefold stimulation of PGI_2 synthesis at 24 hours, while all other fractions were completely inactive with respect to control medium. Various species of human IFN (recombinant α-type A and A/D; natural β; recombinant γ) have so far shown no effect on PGI_2 synthesis, although a preliminary communication suggests that IFN does modulate PGI_2 production under different experimental conditions.[18] A partially purified preparation containing interleukin 1 (IL 1) proved extremely effective.

Whatever the nature of the product(s) of stimulated mononuclear cells, the results reported here show that mediators of cells of the immune system can affect PGI_2 secretion by vascular tissue. This arachidonate

metabolite plays a major role in the regulation of important biologic functions, such as platelet aggregation and vascular tone. It is tempting to speculate, for instance, that PGI_2 secretion induced by mononuclear cell products might contribute to vasodilation at sites of delayed hypersensitivity reactions.

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