

Endothelins, Peptides with Potent Vasoactive Properties, Are Produced by Human Macrophages

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Summary

Endothelins are peptides, originally isolated from endothelial cells, with potent vasoactive and mitogenic properties. In this study, we demonstrate that human macrophages synthesize and secrete endothelins. Cultured human macrophages were found by immunocytochemistry to stain positively for endothelin 1 and endothelin 3. Their capability to produce and release these peptides was confirmed by a combination of reverse-phase high-performance liquid chromatography and radioimmunoassays, specific for endothelin 1 and 3, respectively. Immunoreactive peptides were identified both in cellular extracts and in macrophage-conditioned medium. The secretion of endothelin 1, but not of endothelin 3, from macrophages could be stimulated 6–10-fold by lipopolysaccharide or phorbol myristate acetate (PMA). Northern blot analysis of total macrophage RNA using an endothelin 1 cDNA probe revealed induction of endothelin mRNA in PMA-treated macrophages. Furthermore, immunoreactive endothelin 1 and 3 were found in U937 cells, a human promonocytic line, and in freshly isolated human monocytes. In contrast, no immunoreactive endothelin was detected in cell extracts from human neutrophils and lymphocytes. The expression of endothelins in tissue macrophages was demonstrated in paraffin sections of human lung using immunohistochemistry. In conclusion, the finding that human macrophages produce endothelins suggests an important role for these peptides in the microenvironment of tissue macrophages. Macrophage-derived endothelins may have an essential function in blood vessel physiology, and aberrant production may contribute to vessel pathology.

The endothelins, a recently discovered family of peptides, are potent vasoconstrictors of mammalian blood vessels (1). Endothelin 1 was originally isolated and sequenced from the supernatant of cultured porcine aortic endothelial cells (2), while the existence of endothelin 2 and 3 was predicted after the isolation of genes related to endothelin 1 (1). Endothelins can affect blood vessels of all sizes and types (3), including lymphatic vessels (4), and can also act on other smooth muscle cells, such as those in the bronchial tree (5). In addition, endothelins are mitogenic for vascular smooth muscle cells and fibroblasts, and potentiate growth factor-stimulated DNA synthesis in these cells (6–11). These properties, along with the observation, both in vivo and in vitro, that blood vessels with intact endothelial lining are responsive to the vasoactive effects of endothelins only upon their application to the adventitia (12, 13), seem to favor a role for endothelins in the regulation of a certain microenvironment rather than as circulating hormones. Moreover, they provoke the search for a source of endothelins other than endothelial cells.

We describe here the expression of endothelins by human macrophages, cells that, by virtue of their ability to present antigen, their secretion of soluble products, and their phagocytic capabilities, are involved in the physiological regulation of their microenvironment and often reside within or in close proximity to blood vessels. These findings further illustrate the potentially far reaching role of endothelins in cell biology.

Materials and Methods

Cells. Human monocytes/macrophages were obtained from PBMC that had been separated from freshly drawn blood using Ficoll-Hypaque. Cells were seeded at a density that allowed them to grow to confluence in T75 flasks ($\sim 4 \times 10^6$ cells/ml; for extraction procedures) or to stay distinctly subconfluent on glass slides ($\sim 2 \times 10^6$ cells/ml; for immunohistochemistry). Monocyte/macrophage starting and maintenance media, which contain a proprietary formulation of granulocyte/macrophage CSF and macrophage CSF, were used according to the manufacturer's recommendation (Pan Data Systems, Rockville, MD). In brief, cells were initially diluted in starting medium, added to the culture dishes,

and allowed to sit undisturbed for 3 h at 37°C. Plates were then gently swirled to resuspend nonadherent cells before the medium was removed and replaced by maintenance medium (supplemented with 15% FCS and 2 mM L-glutamine), which was not changed during the first week of culturing. In 2–3-d intervals, when the medium began to turn yellow, fresh medium was added to the old medium. After 1 wk, the maintenance medium was exchanged every other day. Cells were used for experiments between day 10 and 16 after seeding. At this time, one T75 flask contained a confluent layer of $\sim 3\text{--}4 \times 10^7$ cells, with some variance in numbers from donor to donor. For each experiment, only cells derived from one donor were used. The purity of our cultures, as determined with nonspecific esterase stain (Technikon, Tarrytown, NY) and/or anti-Leu-M5 (Becton Dickinson & Co., Mountain View, CA), was usually >95%. At least 12 h before each experiment, cells were transferred into serum-free medium (RPMI 1640, containing 2 mM L-glutamine). For stimulation experiments, 10 $\mu\text{g}/\text{ml}$ of LPS (*Escherichia coli*; Calbiochem-Behring Corp., La Jolla, CA), 10^{-7} M PMA (Sigma Chemical Co., St. Louis, MO), or 5 ng/ml of TGF- β , kindly provided by Dr. Michael Sporn, National Cancer Institute, were added to the medium. Six to eight flasks were treated in parallel with one stimulant and always compared with an equal number of nontreated flasks. Monocytes were obtained by elutriation of PBMC. T lymphocytes were purified from peripheral blood and tonsil mononuclear cells using the SRBC rosette technique (14). B lymphocytes were obtained from tonsil mononuclear cells by negative selection via repeated rosetting with SRBC (14). The cell populations were >98% pure as assessed by FACS analysis. Neutrophils were obtained by Percoll gradient centrifugation, and platelets from NIH blood bank platelet concentrates. U937, a human myelomonocytic cell line (15), was maintained in RPMI 1640, supplemented with 10% FCS, 2 mM L-glutamine, and 0.02 M Hepes. Sheep choroid plexus cells were purchased from American Type Culture Collection (Rockville, MD).

Immunocytochemistry of Cultured Cells. Macrophages grown on glass slides to <80% confluence were fixed in methanol (-20°C , 10 min) and treated with 10% normal goat serum (room temperature [RT],¹ 20 min) to reduce nonspecific background staining. Rabbit anti-endothelin 1 and, alternatively, anti-endothelin 3 antibody (Peninsula Laboratories, Inc., Belmont, CA) (1:100, RT, 180 min) were used as the first antiserum and rhodamine-labeled goat anti-rabbit IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) (1:1,000, RT, 30 min) as the second antiserum. To test the specificity of the immunohistochemical reaction, normal rabbit serum or antiserum, preabsorbed with synthetic endothelin 1 or 3 (Peninsula Laboratories, Inc.), were substituted for the primary antiserum.

Immunohistochemistry of Paraffin Sections from Human Lung Tissue. Human lung tissue for histological examinations was obtained from open lung biopsy, surgery, or autopsy material. After rehydration, the immunogold silver staining method was performed according to the manufacturer's protocol (Janssen Biotech N.V., Olen, Belgium). Anti-endothelin 1 and 3 antisera, respectively, were used as primary antiserum (Peninsula Laboratories, Inc.) (1:100; RT, overnight). For negative controls, normal rabbit serum was substituted for the primary antisera. Sections were counterstained with Harris's hematoxylin. For comparison, hematoxylin and eosin staining was done on representative sections of each specimen.

Extraction and Chromatographic Procedures. The serum-free macrophage-conditioned medium was centrifuged (2,000 g, 10 min,

2°C) and concentrated by extraction through a Sep-Pak C18 cartridge (Waters Associates, Milford, MA), pretreated with 60% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The retained material was washed with 0.1% TFA, eluted with 60% acetonitrile/0.1% TFA, and speed-vac dried. For HCl extraction, cells were washed two times in cold PBS. Hot 0.1 N HCl (~ 3 ml/ 10^8 cells) was added to the cell pellet. The suspension was mixed, boiled in a waterbath for 4 min, and immediately put on ice. The cells were sheared with a polytron, and the suspension was centrifuged (3,000 g, 15 min, 2°C). The supernatant was collected and lyophilized. The extracts were analyzed by reverse-phase liquid chromatography using a 3.9-mm \times 30-cm, 300- \AA pore, 15- μ particle, C18 Delta-Pak column (Waters Associates). The peptides were eluted with a linear gradient of 5–50% at 70 min and 100% at 80 min with acetonitrile (Burdick & Jackson Laboratories, Inc., Muskegon, MI) containing 0.1% TFA (Pierce Chemical Co., Rockford, IL) at a flow rate of 1 ml/min. Data were collected and analyzed with a photodiode array detector (990; Waters Associates) and chromatographic work station. Fractions (1 ml) were collected, split into two equal aliquots, speed-vac dried, redissolved in RIA buffer, and analyzed by either an endothelin 1 or 3 RIA (Peninsula Laboratories, Inc.). The detection limit of both assays was 1 pg/tube. The endothelin 1 RIA showed a crossreactivity with human big endothelin (the endothelin 1 precursor) of nearly 20%. Recoveries of standard endothelins (Peninsula Laboratories, Inc.) from extracted cells and media were 75–80% and 65–75%, respectively, with a slightly better recovery of endothelin 1 from extracted media as compared with endothelin 3. For screening of other selected hematopoietic cells for endothelin immunoreactivity, lyophilized HCl extracts were redissolved in RIA buffer, pH adjusted, and exposed directly to RIA.

Northern Blot Analysis. Total cellular RNA was extracted from nontreated and PMA-treated (20 h) macrophages and from human lung tissue (obtained from a patient with Wegener's granulomatosis who underwent lung surgery) using the guanidinium thiocyanate/cesium chloride method (16). 12 μg of RNA was separated on a denaturing formaldehyde 1% agarose gel, transferred to nylon-supported nitrocellulose membranes, UV crosslinked, and hybridized with a cDNA probe for endothelin 1 (kindly provided by Dr. Kenneth D. Bloch, Boston, MA) (17), which was labeled with ^{32}P -dCTP via a random priming kit (Pharmacia Fine Chemicals, Uppsala, Sweden). Northern blots were washed twice in $2\times$ SSC; 0.1% SDS at room temperature, and twice in $0.1\times$ SSC, 0.1% SDS at 52°C for 20 min each. Autoradiography was performed at -70°C by exposing the blots to Kodak X-omat AR films for 24 h.

Results

Endothelins in Cultured Human Macrophages. Immunocytochemistry of cultured macrophages revealed a positive signal for endothelin 1 and 3 in >95% of the cells (Fig. 1). The cellular signal distribution pattern was similar for the two antisera with pronounced perinuclear staining. Replacement of the antisera by normal rabbit serum or antisera that had been preabsorbed with synthetic endothelins resulted in a negative staining pattern (Fig. 1).

The presence of endothelin 1 and 3 in extracts of cultured human macrophages and in macrophage-conditioned media was confirmed utilizing a combination of HPLC and RIA specific for endothelin 1 and 3, respectively (Fig. 2). Reverse-phase HPLC profiles of endothelin 1-like immunoreactivity

¹ Abbreviations used in this paper: BAL, bronchoalveolar lavage; RT, room temperature; TFA, trifluoroacetic acid.

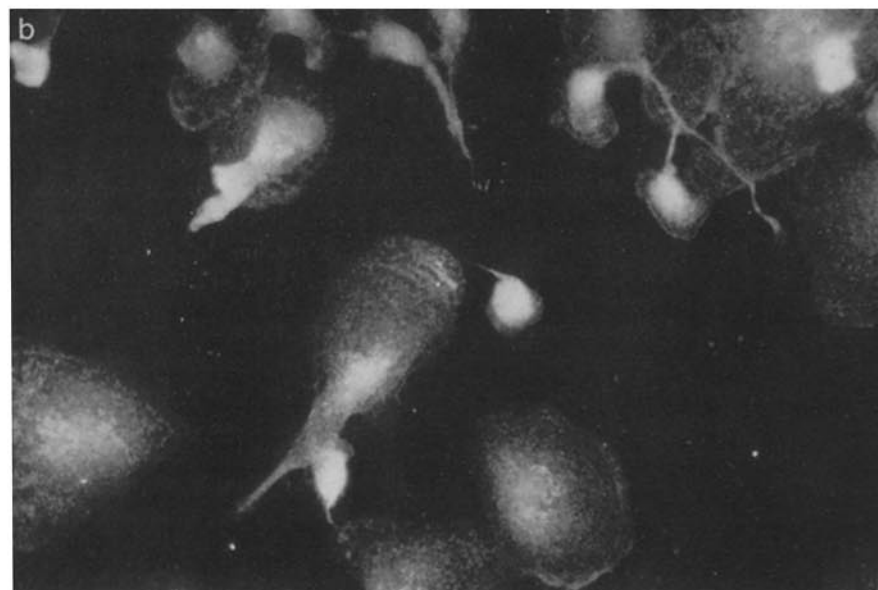
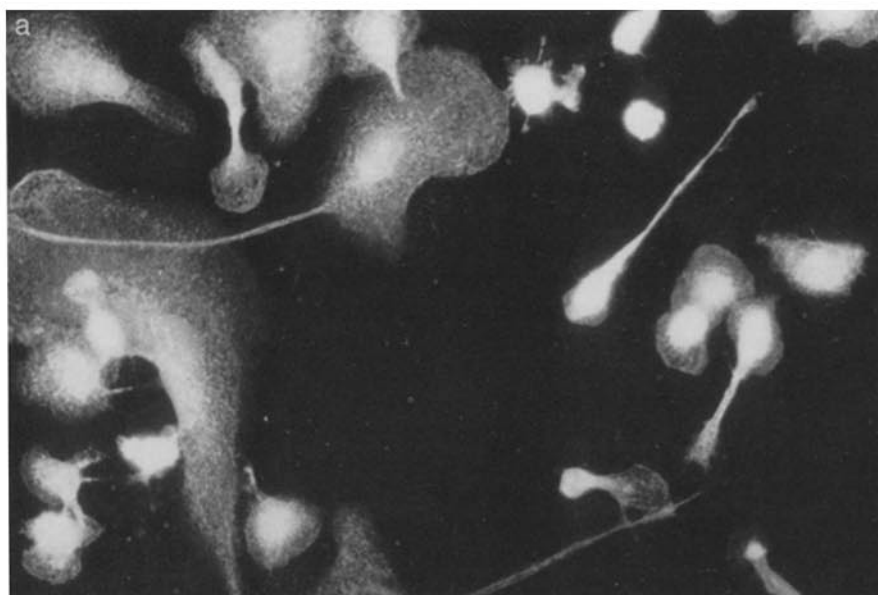


Figure 1. Immunofluorescent staining of cultured human macrophages ($\times 400$) with anti-endothelin 1 antiserum (a), anti-endothelin 3 antiserum (b), and normal rabbit serum (c).

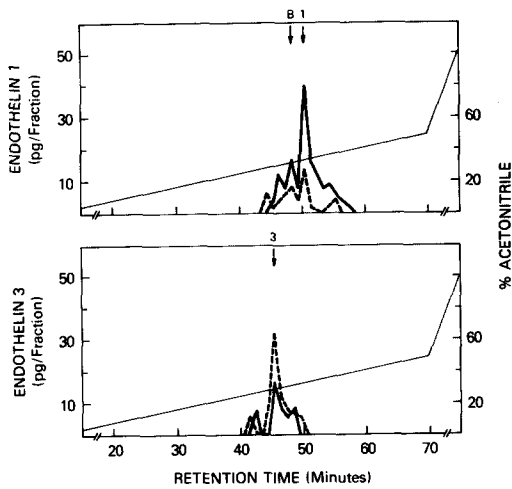


Figure 2. Reverse-phase HPLC profiles of endothelin 1 (*top*) and endothelin 3 (*bottom*) immunoreactivity in extracts of cultured human macrophages (equivalent of a confluent cell layer in one T75 flask; *dotted line*) and macrophage-conditioned medium (from the same T75 flask; 20 h, *solid line*). The arrows indicate the elution positions of synthetic endothelin 1 (1), endothelin 3 (3), and human big endothelin (B), respectively.

resulted in two major peaks eluting with the same retention time as standard endothelin 1 and its precursor form, big endothelin, respectively. Endothelin 3-like immunoreactivity showed a distinct peak at the elution position of standard endothelin 3 and a second peak, occurring earlier, whose nature remains to be determined. Since many macrophage-derived peptides and proteins are increased after stimulation, we used

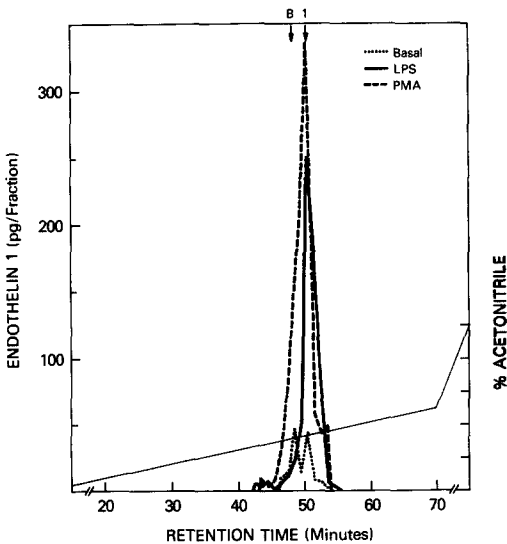


Figure 3. Reverse-phase HPLC profiles of endothelin 1 immunoreactivity in macrophage-conditioned media (medium equivalent of one T75 flask/condition; 26 h) under basal and stimulated (PMA, 10^{-7} M; LPS, $10 \mu\text{g/ml}$) conditions. The arrows indicate the elution position of synthetic endothelin 1 (1) and human big endothelin (B), respectively.

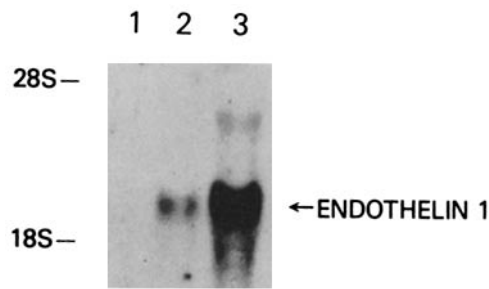


Figure 4. Northern blot analysis of total RNA from PMA-treated (lane 2) and nontreated (lane 1) macrophages, and from human lung tissue (lane 3) using an endothelin 1 cDNA probe.

the combination HPLC/RIA to specifically measure the amounts of endothelin 1- and 3-like immunoreactivity in macrophage-conditioned media after treatment of the cells with either LPS ($10 \mu\text{g/ml}$), PMA (10^{-7} M), or TGF- β (5 ng/ml) for 20 h. Both LPS and PMA treatment resulted in a marked increase in endothelin 1 secretion (Fig. 3). Of note is the fact that the big endothelin peak, which is distinct under basal conditions, could not be distinguished after stimulation of the cells with LPS or PMA. From a basal level of 11 pg/ml , LPS induced a sixfold increase of endothelin 1 to 65 pg/ml . The rise after PMA treatment was even more striking, approaching 10-fold over baseline. In contrast, TGF- β had no effect on the baseline production of endothelin 1. En-

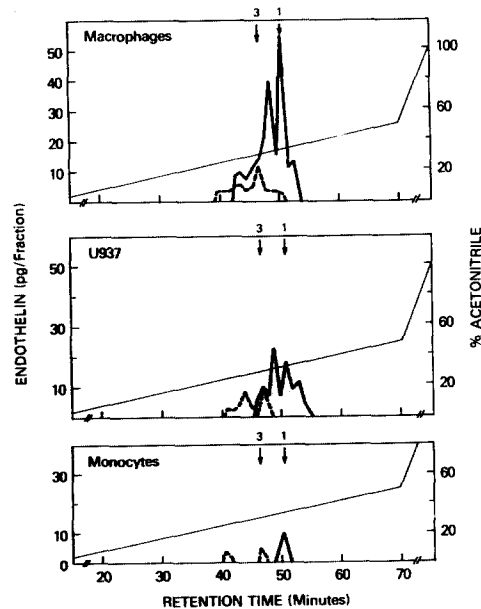


Figure 5. Reverse-phase HPLC profiles of endothelin 1 (*solid line*) and endothelin 3 (*dotted line*) immunoreactivity in extracts of cultured human macrophages (confluent cell layer in one T75 flask), U937 cells (4×10^7 cells), and human peripheral blood monocytes (4×10^7 cells). The arrows indicate the elution positions of synthetic endothelin 1 (1) and endothelin 3 (3), respectively.

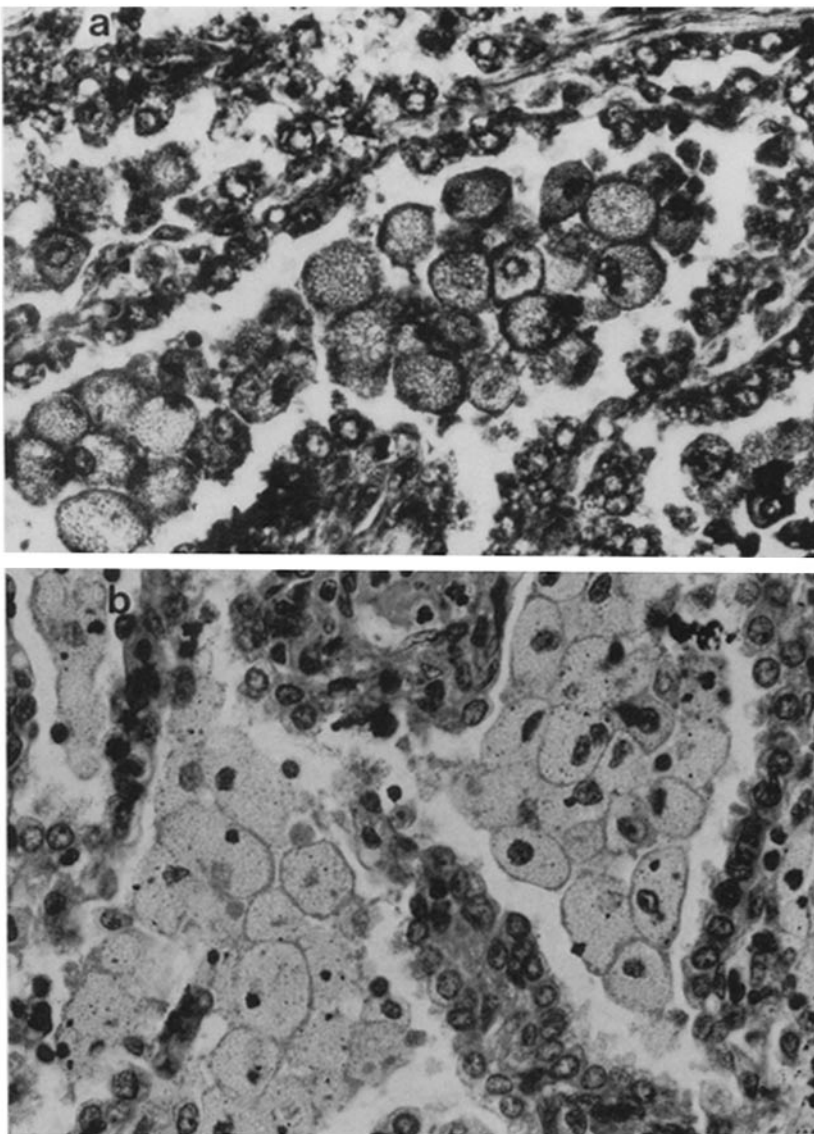


Figure 6. Immunogold-silver staining of paraffin sections of human lung tissue (obtained from a patient with Wegener's granulomatosis; $\times 500$) with anti-endothelin 3 antiserum (*a*), and normal rabbit serum (*b*). While *a* shows positive staining of both intraalveolar macrophages and alveolar epithelial cells, no staining is seen in the control specimen (*b*).

dothelin 3 in the macrophage-conditioned media was not clearly affected by any of the compounds tested (data not shown). Northern blot analysis of total macrophage RNA using an endothelin 1 cDNA probe showed a distinct 2.5-kb band in PMA-treated cells that was not visible in unstimulated cells (Fig. 4).

Endothelins in Other Hematopoietic Cells. In addition to cultured macrophages, endothelins were also detected in cell extracts from freshly isolated monocytes and from U937 cells, a human promonocytic line. Reverse-phase HPLC profiles of endothelin 1- and 3-like immunoreactivity in these extracts displayed a pattern very similar to that seen in cultured macrophages (Fig. 5). Table 1 gives the results of a screening by RIA of other hematopoietic cells for endothelin 1 and 3 immunoreactivity. While PBMC, purified monocytes, and platelets displayed endothelin immunoreactivity, neutrophils, as well as T and B lymphocytes, were negative. Sheep choroid

plexus cells had been found previously to contain immunoreactive endothelins (Ehrenreich et al., unpublished observations) and were used as an additional positive control in this assay.

Examination of Human Lung Tissue. Using immunohistochemistry, alveolar macrophages showed distinctly positive staining for endothelin 1 and 3 (Fig. 6). In agreement with recent reports on a number of animal species (18, 19), human airway epithelium was found to stain positively for both peptides (Fig. 6). Northern blot analysis of total RNA from human lung tissue detected a very strong 2.5-kb band and a weaker band of ~ 3.7 kb (Fig. 4). In rat lung, the presence of both a 2.5- and a 3.7-kb form of endothelin mRNA has been reported previously and has been ascribed to the transcription of endothelin 1 and 3, respectively (20). If this was the case in the human system as well, it would indicate a degree of crosshybridization of the endothelin 1 cDNA probe with endothelin 3 mRNA.

Table 1. Comparison of Endothelin 1 and 3 Immunoreactivity in Human Monocytes and Other Selected Hematopoietic Cells

Cell type	Cell number	Immunoreactive endothelin 1	Immunoreactive endothelin 3
	$\times 10^6$	pg	pg
Platelets	4,000	12	2.1
Neutrophils (PBMC)	12.5	ND	ND
T lymphocytes (tonsil)	7	ND	ND
T lymphocytes (PBMC)	10	ND	ND
B lymphocytes (tonsil)	7.5	ND	ND
Monocytes PBMC	12.5 7	15 9	3.2 ND
Sheep choroid plexus cells	10	12	4.8

Experiments were repeated two to three times each with consistent results. The data are from one representative experiment.

Discussion

The present study demonstrates the presence of immunoreactive endothelin 1 and 3 in cultured human macrophages and macrophage-conditioned medium, as well as in cellular extracts from human peripheral blood monocytes and U937 cells, a human promonocytic line. In contrast, no immunoreactive endothelin was detectable in lymphocytes or neutrophils; however, platelets showed positive immunoreactivity. The absolute concentrations of endothelins in monocytes and macrophages seem to vary somewhat among individual healthy donors, which could well be related to the varying state of activation of monocytes in different individuals.

Incubation of macrophages with LPS and PMA resulted in an increase of endothelin 1 secretion by 6–10-fold. In addition, Northern blot analysis of total macrophage RNA using an endothelin 1 cDNA probe revealed a distinct 2.5-kb mRNA transcript upon stimulation of the cells with PMA. Consistent with this observation, the 5' flanking region of the human endothelin 1 gene contains consensus sequences for the phorbol ester-responsive element (21). In endothelial cells, however, endothelin 1 secretion was not increased by PMA (22). In contrast, TGF- β , which did not affect macrophage-derived endothelin 1 in our study, represents a potent stimulator of endothelin 1 message in endothelial cells (23). This suggests that the signals for production of this peptide may vary among cell types. In contrast to our findings with endothelin 1, en-

dothelin 3 levels in macrophage-conditioned medium were not significantly modulated by any of the compounds tested. Since the two peptides are coded for by different genes (1), they may be independently regulated. In fact, their different potencies with respect to their vasoconstrictive or mitogenic effects, and their different affinity for and biologic activity at a particular receptor (3, 5, 24–26), point to different physiological roles for these peptides.

In contrast to recent findings of others and ourselves (27, 28) showing the simultaneous expression of endothelin and endothelin receptors in primary astrocytes, implying the possible existence of an autocrine control mechanism in these cells, cultured human macrophages did not specifically bind either endothelin 1 or endothelin 3 (Ehrenreich et al., unpublished observations). Furthermore, T and B lymphocytes from peripheral blood or tonsils that are devoid of endothelin immunoreactivity also failed to show any specific endothelin binding. Therefore, at least under basal conditions, lymphocytes are not likely to be major sources of or targets for endothelins. Given the fact that under normal physiologic conditions, and even more so in inflammatory states, macrophages are found in close proximity to vascular smooth muscle cells, fibroblasts, or mesangial or glial cells, all of which possess endothelin receptors (6–11, 27–29), these cells are potential targets for the actions of macrophage-derived endothelins.

To delineate the potential in vivo relevance of our findings with monocytes/macrophages, we examined human lung tissue and noted distinct immunohistochemical staining of alveolar macrophages for endothelin 1 and 3. Since the lungs apparently play a major role in the clearance of endothelins from the circulation (30, 31), alveolar macrophage endothelin might represent a peptide that has been adsorbed by these cells. The fact, however, that cultured macrophages express endothelin 1 mRNA and can be specifically stimulated to secrete endothelins, together with the identification of endothelins in monocytes and U937 cells, strongly argues that endothelin detected in tissue macrophages is at least in part synthesized in situ. Interestingly, in a recent pilot study, we detected immunoreactive endothelin 1 in bronchoalveolar lavage (BAL) samples of patients with Wegener's granulomatosis and lung involvement, as well as in human pleural and peritoneal fluid samples from patients with a variety of disorders (Ehrenreich et al., unpublished observations). While the presence of immunoreactive endothelin 1 in human BAL was reported recently (32), pleural and peritoneal fluid have not yet been described to contain endothelins. Despite preliminary evidence for a correlation between endothelin levels and macrophage counts in these body fluids, the source of endothelins is still not clear. Endothelial cells in these richly vascularized areas, as well as the respiratory epithelium (18, 19) in the case of BAL, could contribute to the detectable levels of endothelins. Nonetheless, it is likely that macrophage-derived endothelins do contribute significantly to the total quantity of these peptides in conditions where large numbers of these cells are present.

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