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Monocyte/Macrophage-Derived Microparticles Up-Regulate Inflammatory Mediator Synthesis by Human Airway Epithelial Cells

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Cell-derived microparticles (MP),3 also referred to as ectosomes or microvesicles, are cell membrane fragments shed by eukaryotic cells upon activation and/or during apoptosis (1). MP were first discovered in platelets (2), but the existence of MP derived from other cell types has been demonstrated subsequently (3, 4). Originally considered in vitro artifacts or, at most, cell debris generated in vivo but devoid of physiological significance, MP have been since shown to play significant roles in different processes, including blood coagulation and inflammation (5, 6). Besides the relatively well-characterized role of platelet MP as a source of the negatively charged phospholipids essential for the assembly of the multimolecular complexes involved in blood coagulation (7), experiments in vitro have shown that also human monocytes shed MP capable of disseminating procoagulant activity (3). These observations were extended subsequently in vivo models to show that circulating MP participate in thrombus formation (8). Studies aimed at investigating the role of cell-cell interaction in inflammation have demonstrated that endothelial cell activation by polymorphonuclear cells (PMN) in coculture is in part mediated by PMN-derived MP (9).

Once perceived as a passive barrier, the airway epithelium is in fact a complex, integrated structure that participates in a number of physiological phenomena. As the first point of contact for inhaled substances, it is not surprising that the epithelium lining the airways contributes to the innate immune system (10). Among a variety of molecules expressed as a first line of defense, an array of adhesion molecules and soluble mediators involved in the recruitment and activation of inflammatory leukocytes play a critical role to this end (11, 12).

The aim of this study was to investigate whether cell-derived MP carry the potential to contribute to the innate immune system of the airways. We demonstrate that human monocytes/macrophages shed MP that can up-regulate the synthesis of proinflammatory mediators by human airway epithelial cells.

Materials and Methods

Reagents and kits

RPMI 1640 medium, penicillin, streptomycin, l-glutamine, trypsin, trypsin inhibitor, trypan blue, formaldehyde, BSA, o-phenylenediamine, PBS, Ficoll-Hypaque, dextran, calcium ionophore A23187, FBS, histamine, ethidium bromide, and the dye PKH67 were obtained from Sigma-Aldrich. The substrate 3,3′,5,5′-tetrathiybenzidine was obtained from Calbiochem. All other chemicals were obtained from the hospital pharmacy and were of the best grade available.

Abs, cytokines, and chemokines

The mouse anti-human-ICAM-1 mAb (clone 15.2) was obtained from Ancell. Peroxidase-conjugate anti-mouse IgG (γ-chain specific), and IFN-γ were obtained from Sigma-Aldrich (Milan, Italy). Human rTNF-α was

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Abbreviation used in this paper: MP, microparticle; PMN, polymorphonuclear cell; TF, tissue factor.
obtained from Alexis Italy. Human MCP-1 used as the reference standard for the ELISA was obtained from Inalco.

**Cell culture**

Human A549 cells (ATCC CCL-195) were provided by Dr. R. Danesi (University of Pisa, Pisa, Italy). A549 cells were maintained in RPMI 1640 supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 95% air-5% CO2 atmosphere at 37°C. The immortalized bronchial epithelial cells, BEAS-2B (ATCC CRL-9609) were provided by Dr. S. Carnevali (University of Modena, Modena, Italy). BEAS-2B cells were maintained in 50% RPMI 1640 and 50% bronchial immortalized bronchial epithelial cells, BEAS-2B (ATCC CRL-9609) contained biologically active MP (see Results). Nonfluorescent events (shown in black) may either represent membrane fragments that are too small to contain enough fluorescent dye or may have a different origin. Flow cytometry analysis

The MP-containing supernatant of A23187-stimulated monocytes/macrophages was analyzed using a FACScan flow cytometer (BD Biosciences). The light scatter was set at logarithmic gain. The forward light scatter setting was E-01, with a threshold of 16. A total of 10,000 events were analyzed. Based on the results of experiments in which MP were filtered through a 0.22-μm filter, a region (R1) was defined on forward vs side angle light scatter intensity dot plot representation that we deem contains biologically active MP (see Results and Fig. 1).

**Epithelial cell activation by MP**

 Supernatants from stimulated and untreated monocytes were incubated with A549 and BEAS-2B cells grown to confluence in 200 μl/well (final volume) in 96-well plates for 24 h at 37°C. Following an 18-h incubation, the conditioned medium was harvested, cleared by centrifugation for 5 min at 12,000 rpm, and analyzed for IL-8 and MCP-1 content. Cells were fixed for analysis of surface ICAM-1 expression. In some experiments, monocellular cell supernatants were filtered (0.22 μm), ultrafiltered (Amicon ultra-4 centrifugal filter devices; 100 kDa m.w. cut-off; Millipore), or submitted to ultracentrifugation (160,000 × g, 40 min) before incubation with A549 and BEAS-2B cells.
MP originated by monocytes/macrophages up-regulate IL-8 secretion by airway epithelial cells

A549 cells constitutively express an average of 800 pg/ml IL-8 under our experimental conditions. Overnight incubation with the supernatant of unstimulated monocytes/macrophages causes an increase in IL-8 expression. An additional 4-fold increase in IL-8 expression is obtained when A549 cells are incubated with the supernatant of monocytes/macrophages stimulated with A23187 (Fig. 3A). To investigate whether such up-regulation could be solely explained by the release of soluble mediators by monocytes/macrophages upon stimulation with calcium ionophore, the MP-containing supernatant was filtered through a 0.22-μm filter and through an ultrafilter with a nominal m.w. cut-off of 100 kDa. Filtration through a 0.22-μm filter caused a small, not reproducible reduction in the stimulatory effect of the supernatant that did not reach statistical significance after seven consecutive experiments (Fig. 3B). However, ultrafiltration almost completely abolished this effect (Fig. 3B). As a control, ultrafiltration of a paradigmatic soluble molecule involved in A549 stimulation, TNF-α, did not impair its stimulatory effect (Fig. 4). To confirm that the effect of the supernatant required the presence of particulate material rather than of soluble molecules large enough to be retained by a 100-kDa filter, the material was submitted to ultracentrifugation. Ultracentrifugation abolished the stimulatory effect of the supernatant; furthermore, we were able to reconstitute such effect by resuspending the pellet in an equal amount of buffer (Fig. 3C), thus confirming that cell-derived MP, rather than large soluble molecules, are involved. The variations in IL-8 secretion were paralleled by variations in IL-8 mRNA (Fig. 5A). Control experiments using A23187 to directly stimulate A549 cells were performed to rule out the possibility that the stimulatory effect was due to A23187 that remains in the MP fraction (pellet and filter retentate) due to its lipid solubility, rather than to MP. The concentration of IL-8 in the conditioned medium of A549 cells went from 4,105 pg/ml in baseline conditions to 6,393 pg/ml upon direct stimulation with 12 μM A23187 to 52,835 pg/ml upon incubation with MP derived from monocytes/macrophages stimulated with 12 μM A23187 (data from one experiment representative of three).

FIGURE 3. A, IL-8 secretion by A549 cells in baseline conditions and upon overnight incubation with the supernatant of unstimulated monocytes/macrophages or of A23187-stimulated monocytes/macrophages. *, p < 0.05 compared with baseline; n = 7. B, IL-8 secretion by A549 cells upon overnight incubation with the supernatant of A23187-stimulated monocytes/macrophages unfiltered and filtered through a 0.22-μm filter or through an ultrafilter with a cut-off of 100 kDa. *, p < 0.05 compared with unfiltered supernatant; n = 7. C, IL-8 secretion by A549 cells upon overnight incubation with the supernatant of A23187-stimulated monocytes/macrophages before and after ultracentrifugation and with the pellet resuspended in an equal volume of medium; n = 3.
Effect of MP originated by monocytes/macrophages on the expression of other inflammatory mediators by airway epithelial cells

We then sought to determine whether the stimulatory effects of MP were restricted to IL-8, or were part of a broader proinflammatory response. To this end, we investigated the expression of MCP-1, a member of the CC chemokine family, and of ICAM-1, a member of the Ig-like family of adhesion molecules.

Fig. 7 shows that MCP-1 expression by A549 cells is increased significantly by incubation with the supernatant of A23187-stimulated monocytes/macrophages. The effect is inhibited by ultrafiltration (Fig. 7) and ultracentrifugation (data not shown). RT-PCR shows a parallel between protein expression and mRNA (Fig. 5B).

Direct stimulation of A549 cells with A23187 does not modulate MCP-1 expression (data not shown).

Similar results are obtained when surface expression of the adhesion molecule, ICAM-1, is measured (Fig. 8). Again, RT-PCR shows a parallel between protein expression and mRNA (Fig. 5C).

Effects of histamine on MP production by monocytes/macrophages

To investigate whether agonists known to be involved in inflammatory reactions in humans could elicit a response similar to that elicited by A23187, we stimulated monocytes/macrophages with histamine. Fig. 9 shows that the supernatant of monocytes/macrophages stimulated with histamine up-regulates MCP-1 expression by A549 cells. The effect is abolished by filtration. Direct stimulation of the A549 cells with histamine does not cause any detectable up-regulation of MCP-1 expression (data not shown). Similar results were obtained for IL-8, albeit to a lesser degree compared with the effect of the supernatant of A23187-stimulated monocytes/macrophages (data not shown).

Discussion

The airway epithelium participates actively in the defense against airborne pathogens, as well as in the orchestration of the inflammatory reactions that characterize virtually all pulmonary diseases. The recruitment of blood-borne leukocytes into the airways represents a critical step in these processes and requires the combined action of chemokines and cell-cell adhesion molecules, including IL-8, MCP-1, and ICAM-1. We investigated whether monocyte/macrophage-derived MP have the potential to contribute to the
innate immune properties or the airway epithelium by modulating the synthesis of these molecules. Our data show that monocytes/macrophages can be induced to generate MP with a proinflammatory potential for human airways through their ability to up-regulate IL-8, MCP-1, and ICAM-1 synthesis by bronchial and alveolar epithelial cells.

Activation of fluorescently labeled monocytes/macrophages with the calcium ionophore A23187 induces the shedding of fragments that can be detected by FACS and retain their fluorescent lipid intercalating label, thus confirming that they are derived from cell membranes. The effect is dose dependent. FACS analysis shows fluorescent events in two distinct regions. Filtration of the medium through a 0.22-μm filter causes the disappearance of one of these regions, whereas the other (R1) remains virtually unmodified. The conditioned medium of A23187-stimulated cells up-regulates the synthesis of proinflammatory mediators by airway epithelial cells measured at both the protein and mRNA level. The biological activity of the medium is not affected significantly by filtration. This observation suggests that most of the biological activity is associated with events in R1. On the contrary, ultrafiltration through a filter with a cut-off of 100 kDa (which did not affect the stimulatory properties of the cytokine TNF-α, used as a control) and ultracentrifugation of the material totally abolish the biological activity; furthermore, following ultracentrifugation, such activity can be reconstituted by resuspension of the pellet. These data confirm that particulate matter, rather than soluble molecules, is responsible for the biological activity. Control experiments in which A23187 was added directly to airway epithelial cells showed negligible effects on cytokine production, thus ruling out the possibility that calcium ionophore that might have remained in the MP fraction due to its lipid solubility is directly responsible for the effect. Because A23187 is not a physiological mediator, some of the experiments were also performed with agonists known to be involved in inflammatory responses in humans. Stimulation of monocytes/macrophages with histamine caused the generation of particulate matter capable of modulating IL-8 and MCP-1 expression by airway epithelial cells. Preliminary experiments suggest that histamine requires longer incubations compared

**FIGURE 7.** MCP-1 secretion by A549 cells in baseline conditions and upon overnight incubation with the supernatant of untreated monocytes/macrophages, or of A23187 stimulated monocytes/macrophages, or with the supernatant of A23187-stimulated monocytes/macrophages after filtration through an ultrafilter with a cut-off of 100 KD. *, p < 0.05 compared with baseline; **, p < 0.05 compared with unfiltered supernatant; n = 4.

**FIGURE 8.** ICAM-1 expression by A549 cells in baseline conditions and upon overnight incubation with the supernatant of unstimulated monocytes/macrophages or of A23187-stimulated monocytes/macrophages. *, p < 0.05 compared with baseline; n = 4.

**FIGURE 9.** MCP-1 secretion by A549 cells in baseline conditions and upon overnight incubation with the supernatant of untreated monocytes/macrophages, or of A23187-stimulated monocytes/macrophages, or with the supernatant of A23187-stimulated monocytes/macrophages after filtration through an ultrafilter with a cut-off of 100 KD; *, p < 0.05 compared with baseline; **, p < 0.05 compared with unfiltered supernatant; n = 5.
with A23187; additional studies will be required to investigate whether these differences reflect different activation pathways.

Over the past several years, numerous studies have confirmed that MP derived from different cells exert physiologically relevant effects. Monocytes have been shown to generate MP with a procoagulant potential. This activity has at least two components: first, negatively charged phospholipids, mainly phosphatidylserine, essential for the assembly of the blood clotting enzymatic complexes and normally located to the inner leaflet of the resting cell membrane, are exposed on the outer leaflet of MP membranes. Second, tissue factor (TF), an integral membrane protein that represents the trigger of the so-called extrinsic pathway of blood coagulation, is transferred from the parental cell to the MP membrane (3). More recently, in vivo experiments have shown that MP carry simultaneously on their membrane both TF and an adhesion receptor for P-selectin, P-selectin glycoprotein ligand-1 (8). A new model for the initiation of blood coagulation has been proposed in which circulating MP are recruited to the site of vascular damage and platelet activation, where P-selectin is overexpressed, via the interaction with P-selectin glycoprotein ligand-1, and deliver the procoagulant potential of TF and phosphatidylserine (5). Finally, ex vivo data show that MP derived from platelets, leukocytes, and endothelial cells are present in human plasma and that their number increases in clinical conditions in which the coagulation system has been activated (14).

Several authors have investigated the potential role of leukocyte-derived MP in innate immunity and inflammation. Hess et al. (15) have shown, for example, that fMLP-activated PMN release MP that express a selected set of proteins such as complement receptor 1, myeloperoxidase, and elastase and that may therefore represent a functional unit with antimicrobial properties. PMN-derived MP have been shown to possess both pro- and anti-inflammatory properties, depending on the experimental model. As mentioned before, MP derived from fMLP-stimulated PMN upregulate IL-6 and IL-8 synthesis by human endothelial cells (9). By contrast, MP obtained in a similar fashion down-modulate cellular activation in human macrophages as assessed by the reduction of TNF-α, IL-8, and IL-10 synthesis by zymosan-activated cells (16). Ex vivo data confirm a potential role for MP in inflammatory diseases in humans. The MP number has been shown to be increased in the synovial fluid of inflamed joints of patients with rheumatoid arthritis (17); a potential link between the presence of such MP and the inflammation that represents the hallmark of the disease has been provided by the demonstration of the induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts upon incubation with T-cell- and monocyte-derived MP (18), as well as with autologous synovial MP (19). Whether MP are also present in other body compartments besides plasma and joints is still under investigation. Of particular interest in the context of the present study is the demonstration of the presence of leukocyte-derived MP in the airway epithelium lining fluid in humans (20).

In conclusion, we demonstrate that MP derived from human monocytes/macrophages have the potential to sustain the innate immunity of the airway epithelium, as well as to contribute to the pathogenesis of inflammatory disease of the lungs characterized by increased expression of chemokines and adhesion molecules. Additional studies will be necessary to investigate whether this potential mechanism plays an actual role in pathological conditions in humans.

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Disclosures
The authors have no financial conflict of interest.

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