Human Alveolar Macrophages Express Elafin and Secretory Leukocyte Protease Inhibitor

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Alveolar Macrophage, Elafin, Secretory Leukocyte Protease Inhibitor

Elafin and secretory leukocyte protease inhibitor (SLPI) are two structurally related serine protease inhibitors present in the lung. The cellular origin of elafin in the alveolar space is unknown. It has been suggested that at least one alveolar leukocyte population express elafin. We therefore postulated that the alveolar macrophage, as the most numerous leukocyte in the alveolar space, express elafin. On the other hand, it is unclear whether human alveolar macrophages are a source of SLPI. In the present study, we showed by RT-PCR that human alveolar macrophages, but not peripheral blood monocytes, express elafin and SLPI transcripts. Elafin, but not SLPI, mRNA expression was increased time dependently in alveolar macrophages stimulated with *Saccharopolyspora rectivirgula* antigen (50 μg/ml), a causative agent of hypersensitivity pneumonitis, but not LPS (10 μg/ml). Intracellular or cell-associated elafin protein accumulated after 24 h of culture only in *S. rectivirgula* antigen-stimulated alveolar macrophages as shown by Western blot. In contrast, alveolar macrophages release 50 ± 6 pg/ml of SLPI in culture medium with no increase in function of time. Alveolar macrophages could be a source of elafin in the lung. In addition to lung structural cells, SLPI could also be derived from alveolar macrophages.

Introduction

Elafin and secretory leukocyte protease inhibitor (SLPI) are two structurally related low molecular weight serine protease inhibitors that share a homologous four-disulfide bridge core harboring the antiprotease domain (Fritz, 1988; Molhuizen and Schalkwijk, 1995; Dijkman, 1995; Schalkwijk *et al.*, 1999). Elafin is a 6-kD elastase-specific inhibitor arising from the proteolytic cleavage of a 12-kD fully active precursor termed pre-elafin or trappin-2. SLPI, also known as mucous protease inhibitor or antileukoprotease, is a broad-spectrum serine protease inhibitor of 11.7 kD.

Both elafin and SLPI are present in the peripheral lung where, together with α_1-antitrypsin, they contribute to tightly regulate the destructive action of potent proteolytic enzymes, especially neutrophil elastase (Dijkman, 1995; Tremblay *et al.*, 1996; McElvaney and Crystal, 1997; Senior and Anthonisen, 1998). Beside their antiprotease activity, elafin and SLPI are considered as important modulators of the host defence and inflammatory response. SLPI has antibacterial, antiviral, antifungal and anti-inflammatory properties (Tomee *et al.*, 1998; Grobmyer *et al.*, 2000). Elafin also has antimicrobial activity (Simpson *et al.*, 1999) and could be involved in defence mechanisms against airborne bacteria (Suzuki *et al.*, 2000). Interestingly, the expression of elafin and SLPI is differentially regulated in the peripheral lung. For instance, we have shown that elafin, but not SLPI, is overexpressed in farmer's lung, a form of hypersensitivity pneumonitis (Tremblay *et al.*, 1996).

The cellular origin of elafin in the alveolar space is unknown (Schalkwijk *et al.*, 1999). *In vitro* studies suggest that elafin is produced by lung epithelial- and Clara-like cells (Sallenave *et al.*, 1993), but this is not supported by immunohistochemical and in situ hybridisation studies on normal lung tissue (Molhuizen and Schalkwijk, 1995). Recently, Griese and colleagues suggested that at least one alveolar leukocyte population express elafin (Griese *et al.*, 1997). We postulated that alveolar macrophages, the most numerous leukocytes in the alveolar space (McLennan *et al.*, 1996), express elafin. The fact that alveolar macrophages are in a high state of activation in hypersensitivity

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pneumonitis (Selman, 1998) could explain the increased levels of elafin in bronchoalveolar lavage fluid from subjects with farmer's lung (Tremblay *et al.*, 1996). The concept that alveolar macrophages express serine protease inhibitors is not new. Indeed, these cells have been known for a while to express α_1-antitrypsin (Mornex *et al.*, 1986; Yuan *et al.*, 1992) and α_1-antichymotrypsin (Nagareda *et al.*, 1991).

In the lung, SLPI has been localised in structural cells of the large conducting and small peripheral airways (Dijkman, 1995). However, it is unclear whether human alveolar macrophages are also a source of SLPI (Griese *et al.*, 1997). While it is generally accepted that human macrophages (Song *et al.*, 1999; Böhm *et al.*, 1992) do not express this inhibitor, SLPI has been immunolocalised in macrophages of ovarian endometriomas (Suzumori *et al.*, 1999). In contrast to human SLPI, there is no doubt that this inhibitor is expressed in mouse (Jin *et al.*, 1997; Jin *et al.*, 1998; Kawai *et al.*, 1999) and rat (Song *et al.*, 1999; Gipson *et al.*, 1999) cells of the monocytic lineage.

In the present study, we show that alveolar macrophages express elafin and SLPI at both the mRNA and protein level. In contrast to SLPI, the expression of elafin is increased in alveolar macrophages stimulated with *Saccharopolyspora rectivirgula*, a causative agent of farmer's lung. We conclude that alveolar macrophages could be a source of elafin in the lung. In addition to lung structural cells, SLPI could also be derived from alveolar macrophages.

Materials and Methods

Isolation and stimulation of human alveolar macrophages

Alveolar macrophages were obtained by bronchoalveolar lavage performed on normal non-smoker volunteers using standard procedure (McLennan *et al.*, 1996). All subjects gave a written informed consent form and the local ethics committee approved the study. After centrifugation of bronchoalveolar lavage fluid, the cell pellet was washed with Hanks' balanced salt solution (HBSS) and resuspended in RPMI 1640 medium with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA). Total cell counts were determined with a hemocytometer and differential counts on Diff-Quik (Dade Diagnostics, Aguada, Puerto Rico) stained cytocentrifuge cell preparations. Alveolar macrophages were purified by adherence. Briefly, one million macrophages/well were dispensed into 24-well culture plates and incubated for 1.5 h in RPMI 1640 medium (Gibco) containing 10% FBS (Gibco) at 37 °C under 95% air/5% CO_2. Non-adherent cells were removed by washing 4 times with HBSS. Purified alveolar macrophages were either unstimulated or stimulated with 10 μg/ml LPS (Sigma Chemical Co., St. Louis, MO, USA) (Sallenave *et al.*, 1997a) or 50 μg/ml *S. rectivirgula* antigen (Tremblay *et al.*, 1991) in RPMI 1640 medium with 10% FBS for up to 48 h at 37 °C under 95% air/5% CO_2.

Isolation of human peripheral blood leukocytes

Heparinised venous blood was collected from healthy volunteers. After centrifugation and sedimentation on 6% Dextran (Amersham Pharmacia Biotech, Uppsala, Sweden), monocytes/lymphocytes and granulocytes were purified from each other after a centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech). Monocytes were purified by adherence as for alveolar macrophages. Eosinophils were separated from neutrophils by negative selection using a bead-conjugated monoclonal anti-CD16 antibody and a magnetic cell sorter (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) as previously described (Laviolette *et al.*, 1993).

Reverse transcription – polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from 6–10 X 10^6 purified peripheral blood leukocytes and 1–2 X 10^6 alveolar macrophages using TRIzol reagent (Gibco) and a RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA), respectively. The cDNA was synthesized by reverse transcription reaction of 1 μg of RNA using Moloney Murine Leukemia Virus reverse transcriptase (Gibco). Expression of elafin, SPLI and control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was then analyzed by PCR. The following mRNA-specific primer pairs (Medicorp, Keystone Labs, Camarillo, CA, USA) were used: Elafin, sense: 5′-CACGGGAGTTCCTGTTAAAGG-3′ and antisense: 5′-GAACGAAACAGGCCATCCCG-3′;

SLPI, sense:
5′-CATGAAGTCCAGCGGCCTCTT-3′
and antisense
5′-GGCAGGAATCAAGCTTTCACAG.
The following GAPDH-specific primers were used as a positive control for each PCR reaction: sense: 5′-AGTCAACGGATTTGGTCGTAT-3′, antisense: 5′-TCTCGCTCCTGGAAGATGGTG-3′. The PCR reaction was allowed to proceed for 30 cycles, each consisting of 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C and finally a 10-min extension at 72 °C. Aliquots of the PCR products were separated on an 1% agarose gel. A 100-bp DNA ladder was included as a size marker. The elafin and SLPI PCR products were cloned into the pCR®2.1 vector (Invitrogen, Carlsbad, CA, USA) and their identity confirmed by sequencing (Service de séquence d'ADN, Université Laval, Quebec City, Canada).

Measure of elafin and SLPI in alveolar macrophage culture supernatant

Elafin in alveolar macrophage culture supernatant was measured with a home-made ELISA as we described previously (Tremblay *et al.*, 1996). SLPI was measured with an ELISA kit from R& D Systems (Minneapolis, MN, USA).

Electrophoresis and immunoblotting of elafin

Alveolar macrophages were directly homogenised in non-reducing loading buffer. Protein analysis by SDS-PAGE and immunoblotting on nitrocellulose membranes (Amersham Pharmacia Biotech) were performed using standard methods with a rabbit anti-human elafin antiserum (Peptides International, Inc., Louisville, KY, USA) as the first antibody as we described previously (Bourbonnais *et al.*, 2000). Chemiluminescence was detected using ECL reagent (Amersham Pharmacia Biotech).

Results

The expression of elafin and SLPI mRNA was evaluated by RT-PCR using mRNA-specific primers in alveolar macrophages. Both elafin and SLPI mRNA were constitutively expressed in alveolar macrophages from four different healthy subjects (Fig. 1). The identity of the amplified PCR products was confirmed by an independent DNA sequencing service (data not shown). The next series of experiment was performed to determine if this expression is upregulated in stimulated cells. To do so, alveolar macrophages were exposed to either LPS (10 µg /ml) or *S. rectivirgula* antigen (50 µg/ml) for increasing periods of time. As shown in Fig. 2, elafin mRNA expression was increased time dependently in alveolar macrophages stimulated with *S. rectivirgula* antigen compared to unstimulated cells. In contrast, LPS did not significantly upregulate elafin expression. For identical experimental conditions, there was no evident change in the expression of SLPI (data not shown).

Since *S. rectivirgula* antigen stimulated the expression of elafin mRNA in alveolar macrophages, we next investigated if this was also the case at the

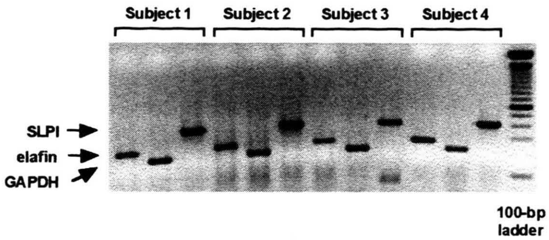

Fig. 1. Electrophoretic analysis of the RT-PCR products of elafin and secretory leukocyte protease inhibitor (SLPI) transcripts in alveolar macrophages from four healthy subjects. Alveolar macrophages express both elafin and SLPI mRNA. GAPDH was used as a control for each PCR reaction and loading.

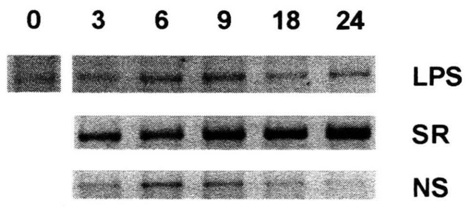

Fig. 2. An example of the effect of LPS and *S. rectivirgula* antigen on elafin mRNA in alveolar macrophages. Alveolar macrophages were either non-stimulated (NS) or stimulated with 10 µg/ml LPS (LPS) or 50 µg/ml *S. rectivirgula* antigen (SR) in RPMI 1640 medium with 10% FBS for up to 24 h before extracting RNA. Equal amounts of total cellular RNA (1 µg) were used pour each reaction RT-PCR.

protein level. While unstimulated and LPS-stimulated alveolar macrophages showed undetectable levels of elafin protein as shown by Western blot, intracellular or cell-associated elafin protein accumulated after 24 hours of culture in presence of *S. rectivirgula* antigen (Fig. 3). As control, IL-1β-stimulated A549 human alveolar epithelial cells (Sallenave *et al.*, 1994) showed detectable levels of elafin protein. Despite having shown the presence of intracellular or cell-associated elafin in *S. rectivirgula*-stimulated alveolar macrophages, we were unable to detect the protein even in concentrated cell culture supernatant. In contrast, unstimulated and *S. rectivirgula*-stimulated alveolar macrophages released the same quantity of SLPI, namely 50 ± 6 pg/ml, in culture medium with no change in function of time.

Since the alveolar macrophage expresses elafin and SLPI, we were interested to show if its precursor, i.e. peripheral blood monocyte, also expresses these serine protease inhibitors. Peripheral blood monocytes did not show detectable levels of either elafin or SLPI mRNA (Fig. 4). As a positive control, we showed the expression of elafin and SLPI in peripheral blood neutrophils from the same subjects since these cells are known to express both inhibitors (Sallenave *et al.*, 1997a). Simultaneously, no elafin or SLPI transcripts were detected in eosinophils from the same subjects. These observations were consistently repeated in peripheral blood leukocytes obtained from four healthy subjects.

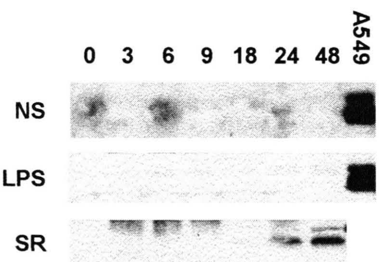

Fig. 3. An example of elafin protein expression in alveolar macrophages in function of time. Alveolar macrophages were either non-stimulated (NS) or stimulated with 10 μg/ml LPS (LPS) or 50 μg/ml *S. rectivirgula* antigen (SR) in RPMI 1640 medium with 10% fetal bovine serum (FBS) for up to 48 h before the cells were directly homogenised in non-reducing loading buffer. Proteins, corresponding to 2.5 X 10^5 alveolar macrophages, were separated by SDS-PAGE as described in the Materials and Methods section. Cell culture supernatant from IL-1β-stimulated A549 cells was used as a positive control.

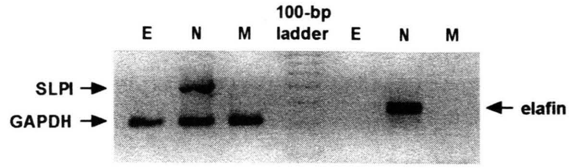

Fig. 4. Electrophoretic analysis of the RT-PCR products of elafin and SLPI transcripts in peripheral blood eosinophils (E), neutrophils (N) and monocytes (M) from one healthy subject. Only neutrophils expressed elafin and SLPI mRNA glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for each PCR reaction and loading. This typical set of results was repeated with cells obtained from three additional healthy subjects.

Discussion

Elafin and SLPI are two low molecular weight serine protease inhibitors that, together with α_1-antitrypsin, protect the fragile lung architecture from proteolytic, especially elastolytic, damage (Dijkman, 1995; Tremblay *et al.*, 1996; McElvaney and Crystal, 1997; Senior and Anthonisen, 1998). While α_1-antitrypsin is mainly synthesized by hepatocytes (Perlmutter, 1993) and SLPI is locally produced by airway structural cells (Dijkman, 1995), the cellular origin of elafin in the lung is yet unknown (Schalkwijk *et al.*, 1999). As far as we know, all published *in vivo* studies failed to show elafin expression in lung structural cells. In light of a recent observation showing elafin expression in unpurified total bronchoalveolar lavage leukocytes (Griese *et al.*, 1997), we postulated that alveolar macrophages are the leukocyte expressing elafin in the peripheral lung. At the same time, we wanted to definitively settle the controversy whether human macrophages express (Suzumori *et al.*, 1999) or not (Griese *et al.*, 1997; Song *et al.*, 1999; Böhm *et al.*, 1992) SLPI.

We showed that alveolar macrophages constitutively express both elafin and SLPI mRNA. However, elafin and SLPI expression is differently regulated in these cells. Elafin, but not SLPI, mRNA levels were increased in alveolar macrophages stimulated with *S. rectivirgula*. This thermoactinomycete is a causative agent of farmer's lung, a form of hypersensitivity pneumonitis (Cormier and Schuyler, 1992). Interestingly, we already showed that, in contrast to SLPI, elafin levels are increased in bronchoalveolar lavage fluid of subjects with this lung disease (Tremblay *et al.*, 1996). This strongly suggests that alveolar macrophages, but not alveolar epithelial cells, are the cellular source of elafin in farmer's lung. Supporting this concept is our observation that elafin expression in A549 lung epithelial cells, a cell line known to express this inhibitor *in vitro* (Sallenave *et al.*, 1993), is not increased by *S. rectivirgula* (Bingle C. D., Bingle L. and Tremblay G. M., unpublished results). A discordant note is that we only showed intracellular or cell-associated elafin protein expression in alveolar macrophages being unable to detect any elafin protein in cell culture supernatant. This suggests that an additional triggering signal is required to make alveolar macrophages externalise elafin.

The results of the present study shed a new light on the controversy whether human macrophages express SLPI. There is a common belief that human macrophages do not express SLPI (Griese *et al.*, 1997; Song *et al.*, 1999; Böhm *et al.*, 1992). In contrast to Griese and colleagues, who were unable to detect SLPI transcripts in bronchoalveolar lavage cells (Griese *et al.*, 1997), we found that alveolar macrophages express SLPI mRNA. The discrepancy between the two studies could be explain by the fact that the cells used in the former study (Griese *et al.*, 1997) were obtained from preterm neonates of less than 30 weeks gestation, while we used cells from adult subjects. This could mean that SLPI expression in human alveolar macrophages is developmentally regulated as for other functional activities (Sherman, 1997). Not only alveolar macrophages from normal volunteers express SLPI mRNA, but these cells in culture release about 50 pg/ml/10^6 cells/24 h, a value that is the same as the one recently reported as an abstract by Takeyabu and colleagues (Takeyabu *et al.*, 2000).

Having shown that alveolar macrophages express elafin and SLPI, we performed a RT-PCR on purified peripheral blood monocytes from normal subjects. Like others (Böhm *et al.*, 1992; Griese *et al.*, 1997; Song *et al.*, 1999), we did not observe any detectable level of either elafin of SLPI transcripts in these cells. Our results and those from others mentioned above suggest that SLPI expression is the privilege of macrophages, but not monocytes. Compellingly, a thorough reading of the literature shows that SLPI expression has been demonstrated in rat and mouse macrophages, but not monocytes, in all (Jin *et al.*, 1997; Jin *et al.*, 1998; Kawai *et al.*, 1999; Gipson *et al.*, 1999) but one (Song *et al.*, 1999) study. Even in this latter study, rat SLPI mRNA was present at very high levels in peritoneal macrophages, but close to absent in resting peripheral blood monocytes. In contrast to monocytes, purified neutrophils from the same subjects clearly express both inhibitors as previously reported by others (Böhm *et al.*, 1992; Sallenave *et al.*, 1997a), while eosinophils do not.

To the best of our knowledge, the present study provides the first evidence that human alveolar macrophages express elafin and SLPI. Such expression may be physiologically highly relevant. First, expression of elafin and SLPI in alveolar macrophages may constitute a first wave of elastase defence, while inhibitor generation by lung structural cells, including SLPI (Dijkman, 1995) and α_1-antitrypsin (Sallenave *et al.*, 1997b), act as a second wave as suggested by a recent study (Gipson *et al.*, 1999). Second, elafin and SLPI may be considered as members of the human alveolar macrophage armamentarium against airborne pathogens considering their antimicrobial properties (Tomee *et al.*, 1998; Simpson *et al.*, 1999). Third, elafin and SLPI expression may suppress alveolar macrophage inflammatory functions by an autocrine mechanism. Indeed, SLPI upregulates the production of IL-10 and TGFβ, two anti-inflammatory cytokines, in macrophages (Sano *et al.*, 2000), interferes with the prostaglandin E_2-dependent production of metalloproteases in monocytes (Zhang *et al.*, 1997), and suppresses the production of nitric oxide and TNFα in SLPI-transfected macrophages (Jin *et al.*, 1997). We are currently investigating whether elafin also shows such anti-inflammatory activities on macrophages.

Regulatory mechanisms orchestrating the expression of serine protease inhibitors in alveolar macrophages and lung structural cells are, most than likely, complex. For instance, alveolar macrophages respond to *S. rectivirgula* antigen by increasing elafin, but not SLPI, expression (this study), while oncostatin M specifically stimulates α_1-antitrypsin in alveolar epithelial cells (Sallenave *et al.*, 1997b). Further studies are under way to identify specific triggering signals for elafin and SLPI expression in alveolar macrophages.

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