Mono-ADP-ribosyltransferases in human monocytes: regulation by lipopolysaccharide

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ADP-ribosyltransferase activity was shown to be present on the surface of human monocytes. Incubating the cells in the presence of BSA leads to an increase in enzyme activity. The acceptor amino acid mainly responsible for the ADP-ribose bond was identified as a cysteine residue. An increase in ADP-ribosyltransferase activity was observed when cells were treated for 16 h with bacterial lipopolysaccharide (LPS). Possible candidates for catalysing the reaction are mono-ADP-ribosyltransferases (ARTs). When measuring expression of the mRNA of ART1, 3, 4 and 5, only ART3 mRNA was detected in unstimulated monocytes. Upon stimulation for 16 h with LPS, lipoteichoic acid or peptidoglycan, ART4 mRNA was found to be expressed. No ART4 signal appeared after a 4 h exposure of the cells to LPS. Cell-surface proteins were labelled when incubating monocytes with [^{32}P]NAD$^+$. Their molecular masses were 29, 33, 43, 45, 60 and 82 kDa. In response to LPS an additional protein of 31 kDa was found to be labelled. The bound label was resistant to treatment with NH_2OH but sensitive to $HgCl_2$, characteristic of a cysteine-linked ADP-ribosylation.

Key words: ADP-ribosylation, cell activation, target amino acid.

INTRODUCTION

Mono-ADP-ribosylation is a post-translational modification of proteins in which the ADP-ribose moiety of NAD$^+$ is transferred to specific amino acid residues in target proteins [1]. The best-characterized mono-ADP-ribosyltransferases (ARTs) are bacterial toxins. Cholera toxin and pertussis toxin interfere with signal transduction in human host cells by ADP-ribosylating regulatory G-proteins [1,2], and diphtheria toxin ADP-ribosylates a diphthamide residue in elongation factor 2, thereby inhibiting mammalian protein synthesis [3]. There is increasing evidence that endogeneous ARTs also play important roles in vertebrates [4,5]. Some of these enzymes have been cloned and biochemically characterized as a family of cell-surface ARTs that are related in structure and function to bacterial ADP-ribosylating toxins [6,7]. Based on similarities in their deduced amino acid sequences and conservation of gene structure, five mammalian ARTs (ART1–5) have been identified so far [8].

In lymphocytes the glycosylphosphatidylinositol (GPI)-linked ART1 and ART2 are associated with immune functions [9–12]. In several other immune responses the catalytic activity of ARTs has been implicated but the molecular identity has not been elucidated so far. ADP-ribosyltransferase activity was detected on the surface of human neutrophils [13], and indirect evidence suggests a role for the enzyme in mediating cytoskeletal realignment during chemotaxis [14]. Data from our laboratory also implicate involvement of ADP-ribosylation in several biological responses of human monocytes. A panel of structurally unrelated inhibitors of ART were found to inhibit lipopolysaccharide (LPS)-induced reactive oxygen intermediate and tumour necrosis factor-α production [15,16] and to reduce the capability of LPS to mediate maximal F-actin assembly [17]. Furthermore, we have reported previously that ADP-ribosyltransferases play a role in phosphorylation of β/γ-actin in monocytes [18].

The aim of the present study was to identify ARTs on human monocytes and to investigate the possible effect of LPS on these enzymes. We show ADP-ribosyltransferase activity on the surface of monocytes and found ART4 to be regulated by LPS at the mRNA level. Furthermore, treatment of intact cells with LPS resulted in ADP-ribosylation of a 31 kDa protein.

MATERIALS AND METHODS

Materials

[^{32}P]NAD$^+$ (1000 Ci/mmol) was obtained from Amersham Buchler (Braunschweig, Germany). LPS of *Escherichia coli* (serotype 055: B5) and lipoteichoic acid were from Sigma (Taufkirchen, Germany) and peptidoglycan was from Fluka (Buchs, Switzerland). Oligonucleotides were synthesized by MWG Biotech AG (Ebersberg, Germany). Soluble peptidoglycan was a gift from Dr U. Zähringer (Research Centre Borstel, Borstel, Germany).

Cell separation and monocyte purification

Peripheral blood mononuclear cells from healthy volunteer donors were obtained by Ficoll-Isopaque (Pharmacia, Freiburg, Germany) density-gradient centrifugation [19]. After repeated washing in PBS containing 0.3 mM EDTA, the monocytes were isolated by counterflow elutriation using the JE-6B elutriation system (Beckman Instruments, Palo Alto, CA, U.S.A.) as described previously [20]. The cell preparations were $> 94\%$ monocytes as determined by morphology and immunofluorescence staining with a monoclonal antibody (mAb) against CD14 (MEM-18). For nested PCR analyses, monocytes were purified further by applying the MACS monocyte isolation kit (Miltenyi

Abbreviations used: ART, mono-ADP-ribosyltransferase; GPI, glycosylphosphatidylinositol; LPS, lipopolysaccharide; RT, reverse transcriptase; mAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glycerophosphatidylinositol.

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Biotech, Auburn, CA, U.S.A.), resulting in 99 % CD14-positive cells.

Stimulation of the cells

Monocytes (2×10^6/ml) were suspended in RPMI 1640 supplemented with 10 % (v/v) fetal calf serum, 2 mM glutamine and antibiotics in Falcon test tubes (Becton Dickinson, Heidelberg, Germany) and incubated with LPS, lipoteichoic acid or peptidoglycan at 37 °C.

Antibodies and immunofluorescence analysis

Mouse mAb 1G4 (IgG2a), which is specific for etheno-adenosine [21], was provided kindly by Dr R. Santella (Columbia University, New York, NY, U.S.A.). The anti-CD14 mAb MEM-18 (IgG1) was a gift of Dr V. Horejsi (Academy of Sciences of the Czech Republic, Prague, Czech Republic). Isotype-control antibodies (IgG2a, IgG1) were from Sigma.

Before cells were incubated in the presence of the indicated mAb and the respective isotypes for 45 min at 4 °C they were preincubated for 30 min at 4 °C with 10 % human AB serum to saturate Fc receptors. Cells were then washed in PBS containing 10 % Haemaccel® (Hoechst, Frankfurt, Germany) and 0.1 % sodium azide, and incubated for a further 30 min at 4 °C with FITC-labelled goat anti-mouse antibody (SIFIN, Berlin, Germany). After washing and fixation in 1 % formaldehyde, cells were analysed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

Cloning and expression of recombinant ART2.2

Procedures for cloning and transfection of plasmid pME. CD8LF-ART2.2 to express N-terminally FLAG-tagged ART2.2 in mammalian cells were as described previously [22]. The structural gene encoding mouse ART2.2 represents one member of a tandem duplication and is denoted *Art2b*.

Treatment of cells with etheno-NAD^+

Monocytes (5×10^7/ml) were incubated in RPMI 1640 for 30 min at 37 °C with 100 μM etheno-NAD^+. After centrifugation at 800 ***g*** for 5 min, cells were washed with PBS, stained with the mAb 1G4 and subjected to FACS analyses as described above.

Chemical stability of protein-ADP-ribosyl linkages

After incubating monocytes (1.6×10^7/ml) with 12.5 μM [^{32}P]-NAD^+ (20 μCi/assay) in the presence of BSA (250 μg) for 60 min at 37 °C in a volume of 125 μl, cells were pelleted. Aliquots (5 μl) of the supernatant were incubated in the presence of 1 M NH_2OH, pH 7.0, or 10 mM $HgCl_2$ in a volume of 50 μl at 37 °C. After 2 h BSA was precipitated with methanol, dissolved in SDS/PAGE sample buffer, analysed by SDS/PAGE and detected by autoradiography.

To determine the nature of the linkage of the radiolabel to the 31 kDa protein, cells (4×10^7/ml) stimulated for 16 h with LPS (100 ng/ml) were incubated with 12.5 μM [^{32}P]NAD^+ (100 μCi/assay) in RPMI 1640. After 60 min cells were washed four times with ice-cold PBS, resuspended in ice-cold permeabilizing buffer (without β-mercaptoethanol), left on ice for 15 min and sonicated (10 strokes; output, 70 %; duty cycle, 60 %; Bandelin Sonopuls GM70, Bandelin, Berlin, Germany). Aliquots (80 μg) of the homogenate were incubated in the presence of 1 M NH_2OH, pH 7.0, or 10 mM $HgCl_2$ at 37 °C. After 2 h the homogenate was precipitated with 10 % trichloroacetic acid (w/v), washed with ice-cold acetone and treated as described above.

ADP-ribosylation assay

Monocytes (2×10^6/ml) were incubated for various times in the presence or absence of LPS (100 ng/ml), centrifuged at 400 ***g*** for 8 min at 4 °C and washed twice in ice-cold PBS. To disrupt the cells, the cell pellet was resuspended in ice-cold permeabilizing buffer containing 10 mM Tris/HCl, pH 7.8, 1 mM EDTA, 4 mM $MgCl_2$, 30 mM 2-mercaptoethanol, 1 mM PMSF and 0.01 % leupeptin, left on ice for 15 min and sonicated as described above. To aliquots of the homogenate containing equal amounts of protein in a final volume of 50 μl of permeabilizing buffer, 25 μl of an ADP-ribosylation reaction mixture was added. The mixture contained 100 mM Tris/HCl, pH 7.8, 120 mM $MgCl_2$, 12.5 μM [^{32}P]NAD^+ (2 μCi/assay), 0.1 mM GTP, 0.1 mM ATP, 10 mM NaF, 0.54 mM $NADP^+$ and 0.4 mM isobutylmethylxanthine.

The reaction mixtures were incubated for 30 min at 25 °C. The reactions were terminated by adding 50 μl of ice-cold BSA (5 mg/ml) followed by 1.2 ml 25 % (w/v) trichloroacetic acid. After 45 min at 4 °C the resulting precipitates were recovered by centrifugation (10 min at 3000 ***g***). The pellets were resuspended in 250 μl of 2 M KOH, precipitated again and collected on Whatman GF/C glass-fibre filters. After washing, filters were counted by liquid scintillation spectrometry.

To measure ADP-ribosyltransferase activity on intact cells, monocytes (4×10^6/ml) were incubated in RPMI 1640 containing 12.5 μM NAD^+ (15 μCi/assay) and 1 mM ADP-ribose in a volume of 125 μl at 37 °C for 60 min. The cells were washed three times with ice-cold PBS and, after adding 50 μl of ice-cold BSA (5 mg/ml), the samples were treated as described above. When measuring ADP-ribosyltransferase activity on intact cells in the presence of BSA, the washing steps were omitted before the addition of trichloroacetic acid. Control assays contained either cells or BSA alone.

ADP-ribosylation of monocyte surface proteins

Monocytes (4×10^7/ml) were incubated in RPMI 1640 containing 12.5 μM [^{32}P]NAD^+ (100 μCi/assay) and 1 mM ADP-ribose in a volume of 125 μl at 37 °C. After 60 min the samples were washed four times with ice-cold PBS, suspended in ice-cold permeabilizing buffer and sonicated as described above. Equal amounts of protein were precipitated with methanol according to the method of Wessel and Flügge [23]. After centrifugation for 5 min at 9000 ***g*** the pellets were solubilized in SDS sample buffer and boiled for 5 min. Proteins were separated by SDS/PAGE (12 % polyacrylamide gel). The gels were stained with Coomassie Brilliant Blue G250 (Serva, Heidelberg, Germany), dried and autoradiography was performed using a Sterling Cronex film 4 (Newark, DE, U.S.A.) or the phosphorimaging system FLA-3000 (Fuji Film, Duesseldorf, Germany).

RNA isolation and reverse transcription

Total RNA was isolated from 1×10^7 cells using the RNeasy mini kit® (Qiagen, Chatsworth, CA, U.S.A.) according to the manufacturer's instructions. RNA was eluted with 35 μl of RNase-free water. A total of 10 μl was used to prepare cDNA by annealing RNA with 1 μl of oligo-$dT_{(20)}$ (500 μg/ml) for 10 min at 70 °C. Reverse transcription was then performed using Superscript™ II reverse transcriptase (RT; Life Technologies, Gaithersburg, MD, U.S.A.) at 37 °C for 60 min, followed by an inactivation phase of 4 min at 95 °C, in a final volume of 19.5 μl.

To obtain RNA from human tissues as positive controls, the tissues (tonsil, testis and skeletal muscle) were frozen in liquid nitrogen. Thin pieces (approx. 1 cm × 1 cm) were then disrupted and homogenized in lysis buffer. RNA was isolated and reverse transcribed as described above.

PCR primers and reactions

PCR analysis was carried out using cDNA from monocytes normalized with respect to the transcript level of the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR reactions were performed in reaction volumes of 33.1 μl. The reaction mixture contained 1.5 mM $MgCl_2$, 200 μM dNTP, 1 unit of recombinant *Taq* DNA polymerase (Life Technologies) and 85 ng of primers derived from two separate exons. After addition of an aliquot of each RT sample, the reactions were performed in a Crocodile III DNA thermal cycler (Oncor Appligene, Heidelberg, Germany) under the following conditions. An initial denaturation step for 2 min at 95 °C followed by a varied number of cycles of 60 s at 95 °C, 60 s at varied annealing temperatures and 90 s at 72 °C with a prolongation of 10 s/5 cycles. The final extension phase was 15 min at 72 °C.

The PCR amplification step was performed with an aliquot of each RT sample and forward and reverse primers as follows. Forward primers: GAPDH, 5′-AAC AGC GAC ACC CAC TCC TC-3′; ART1, H11, 5′-CAG ATG CCT GCT ATG ATG TC-3′; ART3, T03, 5′-GAA GAG AAA AAT GAA GAC GGG AC-3′; ART4, L00, 5′-CCT CCT GCA ACG ATG AGA ATC TGG CTC C-3′; ART5, N55, 5′-CTT CCG AAG CTT CCA AGG ATG GCG CTG G-3′; and reverse primers: GAPDH, 5′-GGA GGG GAG ATT CAG TGT GGT-3′; ART1, H33, 5′-TCA AAG GAG GCC TGG ACC AT-3′; ART3, T99, 5′-GAT AAA CAA TGC ATC AAA CTA CAG AGC-3′; ART4, L99, 5′-GGA GCC ACA AGA TTT CTT TAT ACT CTG C-3′; ART5, N71, 5′-GTG GGC CAC TGC CTT ATC CAG-3′. Different cycles and annealing temperatures were used: GAPDH, 16 cycles at 60 °C; ART1, 40 cycles at 62 °C; ART3, 40 cycles at 60 °C; ART4, 40 cycles at 60 °C; ART5, 40 cycles at 64 °C.

Nested PCRs

The first PCR amplification step was performed with an aliquot of each RT sample and forward (ART1, H11; ART3, T03; ART4, L00; ART5, N55) and reverse (ART1, H33; ART3, T99; ART4, L99; ART5, 5′-CGC GCT CCT TGG GAA AGA CAG AGA-3′) primers under the conditions described above (ART1, 40 cycles at 62 °C; ART3, 20 cycles at 60 °C; ART4, 30 cycles at 60 °C; ART5, 20 cycles at 64 °C).

A second PCR was done with 2 μl of the first PCR reaction product, with nested forward (ART1, H12, 5′-AGC CAA TGG CAG GAG CGT CA-3′; ART3, 5′-GCA ACC ATG ATT CTA GTG GAC-3′; ART4, 5′-TCT TTT GAT GAT CAG TAC CAA-3′; ART5, N55) and reverse (ART1, H33; ART3, T99; ART4, 5′-GAG AGA GAT GCA ATA GCT ATA-3′; ART5, N71) primers under the conditions described above (ART1, 20 cycles at 62 °C; ART3, 40 cycles at 60 °C; ART4, 30 cycles at 58 °C; ART5, 40 cycles at 64 °C).

The PCR products were separated by electrophoresis on 1.8 % (w/v) agarose gels (FMC Bioproducts, Rockland, MA, U.S.A.) containing 1.25 μg/ml ethidium bromide and visualized under UV light. Correct product lengths of the different PCR products (ART1, 981 bp, nested 735 bp; ART3, 1132 bp, nested 1083 bp; ART4, 911 bp, nested 722 bp; ART5, 597 bp and GAPDH: 258 bp) were verified using the 100 bp DNA ladder (Life Technologies) as a molecular-mass standard.

The ART4 fragments obtained were cloned using the TOPO TA Cloning® kit (Invitrogen, San Diego, CA, U.S.A.) according to the manufacturer's instructions. After DNA preparation using a QIAprep plasmid preparation kit (Qiagen) the clones were sequenced by MWG Biotech AG. A BLAST genebank search was used to find corresponding sequences.

RESULTS

LPS leads to an increase in ADP-ribosyltransferase activity

By the use of ADP-ribosyltransferase inhibitors we have shown previously that ADP-ribosyltransferases are involved in LPS-induced biological responses in monocytes [15–17]. In the present study, we asked whether LPS has a direct influence on the activity of ADP-ribosyltransferases in these cells. Initially, we measured the overall ADP-ribosyltransferase activity in cell homogenates. As seen in Table 1, incubating monocytes in the presence of LPS for 16 h lead to an increase in enzymic activity. The increase was less consistent after shorter incubation times

Table 1 Effect of LPS and BSA on ADP-ribosyltransferase activity in monocytes

Monocytes (2×10^6/ml) were incubated in the presence or absence of LPS (100 ng/ml) for 16 h. Thereafter, ADP-ribosylation was carried out with either homogenates or intact cells in the presence or absence of BSA (2 mg/ml) as described in the Materials and methods section. Values are means ± S.D. ($n = 3$).

	ADP-ribosyltransferase activity	
Treatment	Homogenate (pmol/min per mg of protein)	Intact cells (pmol/h per 10^6 cells)
None	0.46 ± 0.06	0.049 ± 0.007
LPS (100 ng/ml)	1.62 ± 0.48	0.111 ± 0.034
None		0.28 ± 0.09
BSA (2 mg/ml)		0.97 ± 0.16
BSA (2 mg/ml) + LPS (100 ng/ml)		0.81 ± 0.19

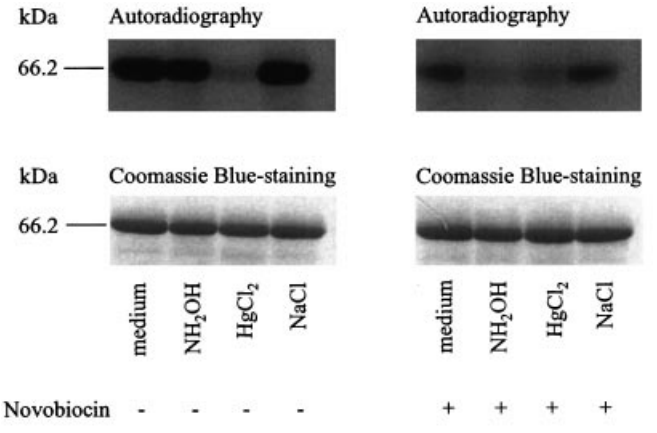

Figure 1 Effect of $HgCl_2$ and NH_2OH on the stability of the ADP-ribose bond

Monocytes (4×10^6/ml) were incubated with [^{32}P]NAD^+ and BSA (2 mg/ml) in the presence or absence of 2.5 mM novobiocin at 37 °C. After 1 h cells were pelleted and aliquots of the supernatant were incubated in the presence of 10 mM NaCl, 1 M NH_2OH (pH 7.0) or 10 mM $HgCl_2$. After 2 h at 37 °C BSA was precipitated with methanol and samples were analysed by SDS/PAGE followed by autoradiography. Similar results were obtained in three separate experiments.

Table 2 Detection of ADP-ribosylated proteins by FACS analysis

Monocytes (5×10^7/ml) that had been incubated for 16 h with or without LPS (100 ng/ml) and ART2.2-transfected EL4 lymphoma cells (5×10^7/ml) were incubated in the presence or absence of etheno-NAD^+ (100 μM) as described in the Materials and methods section. FACS analysis was performed using the etheno-adenosine-specific mAb 1G4. Shown are the median fluorescence intensity values. Numbers in parentheses are the median fluorescence intensity values of the isotype-matched control mAb. Similar results were obtained in two separate experiments.

	Median fluorescence intensity	
Cell type and treatment	No etheno-NAD^+	Etheno-NAD^+
ART2.2-transfected EL4 cells	42 (26)	129 (20)
Monocytes	3 (3)	3 (3)
Monocytes + LPS	3 (3)	4 (4)

(4 h), so all further experiments were carried out at the later time point.

To test whether LPS had the same effect on ADP-ribosyltransferase activity on the surface of human monocytes the enzymic assay was carried out with intact cells instead of cell homogenates. Under the assay conditions described, cell viability was consistently $> 97\%$ after 1 h of incubation as determined by Trypan Blue exclusion. The experiments were performed in the presence of ADP-ribose to minimize the contribution of the non-enzymic addition of free [^{32}P]ADP-ribose to protein acceptors. ADP-ribose may be generated rapidly by hydrolysis of NAD^+ in the presence of NAD^+ glycohydrolase. As shown in Table 1, LPS not only lead to an increase in enzymic activity in cell homogenates but also on intact cells.

The possibility was considered that the low level of ART activity observed in these experiments was due to the absence of a respective target protein. We therefore added BSA as an acceptor protein to the intact monocytes and measured the transfer of the label from [^{32}P]NAD^+ to the BSA protein. The enzyme activity increased from 0.28 to 0.97 pmol/h per 10^6 cells but LPS was without effect (Table 1). The higher basal enzyme activity measured in this set of experiments (compare 0.049 with 0.28 pmol/h per 10^6 cells) might be explained by the fact that the cells were precipitated directly after incubation without the preceding washing steps.

To test whether ADP-ribosylation of BSA was caused by modifications of arginine or cysteine residues, BSA, after being incubated in the presence of monocytes, was treated with NH_2OH or $HgCl_2$. SDS/PAGE analysis (Figure 1) revealed that BSA protein incubated with $HgCl_2$ lost label, whereas incubation in medium, NaCl or NH_2OH had no effect, indicating that cysteine residues were modified. When the incubation was carried out in the presence of novobiocin, an inhibitor of ARTs [24], there was a general decrease in the incorporation of labelled ADP-ribose, which was more pronounced when cells had been treated with NH_2OH. Most probably, part of the labelling was caused by enzymic incorporation of [^{32}P]ADP-ribose and the incorporation that was not inhibited by novobiocin may have occurred via arginine bonds.

As monocytes possess a strong ecto-NAD^+ glycohydrolase activity [25] we were concerned that the low ART activity on

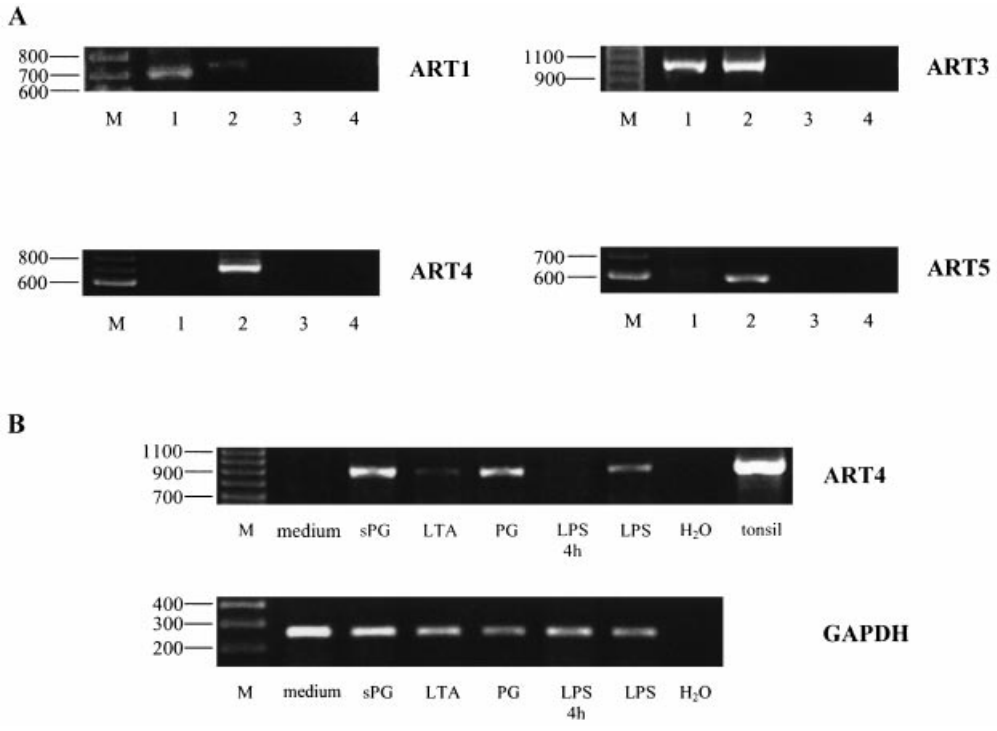

Figure 2 Expression of ART3 and ART4 mRNA in human monocytes

(**A**) The mRNA of monocytes was isolated. Following reverse transcription, PCR was carried out with nested specific primers for ARTs 1, 3, 4 and 5. Lanes 1, monocytes; lanes 2, positive control (ART1, skeletal muscle; ART3 and ART5, testis; ART4, tonsil); lanes 3, no cDNA first step; lanes 4, no cDNA second step; lanes M, mass marker, 100 bp DNA ladder. (**B**) Monocytes (2×10^6/ml) were incubated with LPS (100 ng/ml), lipoteichoic acid (LTA; 10 μg/ml), soluble peptidoglycan (sPG; 25 μg/ml) or peptidoglycan (PG; 10 μg/ml). After 16 h, unless otherwise indicated, mRNA was isolated. After reverse transcription, PCR was performed with specific primers for ART4. Data show one representative experiment out of three.

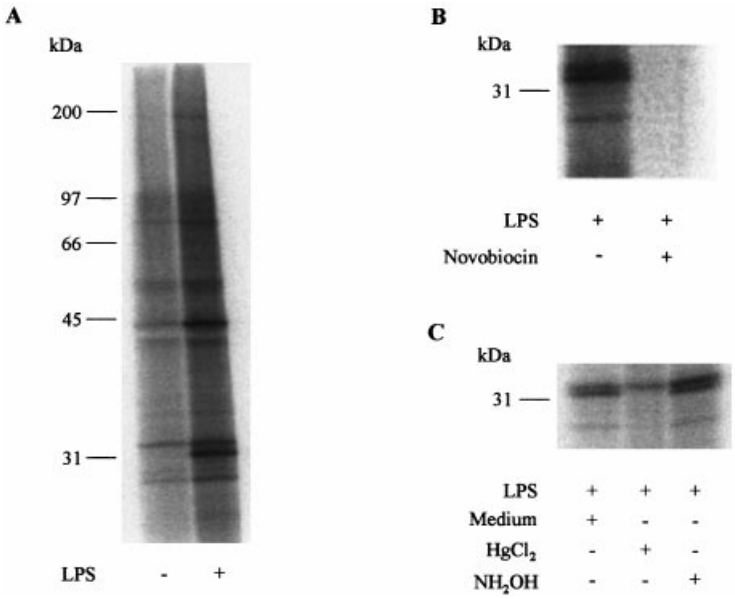

Figure 3 Effect of LPS on ADP-ribosylation of cell surface proteins

Monocytes (2×10^6/ml) were incubated in the presence or absence of LPS (100 ng/ml) for 16 h. After washing, cells were incubated further with 12.5 μM [^{32}P]NAD$^+$ (100 μCi/assay) for another hour in the absence (A) or presence (B) of 5 mM novobiocin. Some of the cells that had been incubated with LPS were homogenized and treated with either $HgCl_2$ or NH_2OH (C). Proteins were precipitated and separated by SDS/PAGE. The autoradiographs are representative of three experiments.

monocytes may have been due to insufficient substrate availability for ART-mediated ADP-ribosylation. Therefore we used a FACS assay, which allows the increase of NAD$^+$ availability. The cells were incubated with 100 μM etheno-NAD$^+$, resulting in etheno-ADP-ribosylated cell-surface proteins that were detected by the etheno-adenosine-specific mAb 1G4. Whereas incubation of ART2.2-transfected EL4 lymphoma cells with etheno-NAD$^+$ resulted in 1G4 staining of most cells, no staining was observed on monocytes (Table 2).

Expression of ART4 in stimulated monocytes

Having shown an increased ADP-ribosyltransferase activity on monocytes in response to LPS, we investigated the expression of ARTs 1, 3, 4 and 5 in these cells. The ART2 gene appears not to be expressed in humans since it contains three premature stop codons [6]. To detect mRNAs of the different ARTs we applied RT-PCR. As positive controls for the expression of ARTs 1, 3, 4 and 5, cDNAs from the following tissues were used: skeletal muscle (ART1), testis (ARTs 3 and 5) and tonsil (ART4). In addition, we used nested primers to increase the selectivity towards specific PCR products. As seen in Figure 2(A), transcript levels could only be detected for the ART3 gene. RT-PCR yielded an ART3-specific amplification product of 1083 bp.

Stimulation of the cells with LPS (100 ng/ml) for 16 h resulted in the expression of ART4 mRNA (Figure 2B). The PCR product, which had a length of 911 bp, was cloned and nucleotide sequencing followed by database searching identified the encoded product as ART4 mRNA. At shorter incubation times (4 h) no ART4 mRNA was detectable. To test whether other ligands known to activate monocytes would affect ART4 expression, cells were incubated with the Gram-positive bacterial cell-wall components lipoteichoic acid [26] and peptidoglycan [27]. As seen in Figure 2(B), stimulation with either compound resulted in the appearance of ART4 mRNA, indicating that transcription of the ART4 gene seems to be a common event associated with monocyte activation.

LPS induces labelling of a 31 kDa protein

It was of interest to know whether ART activity on monocytes was accompanied by detectable ADP-ribosylation of cell-surface proteins. As seen in Figure 3, incubation of intact monocytes with [^{32}P]NAD$^+$ caused weak labelling of proteins with molecular masses of approx. 29, 33, 43, 45, 60 and 82 kDa. Upon stimulation of monocytes with LPS prior to labelling with [^{32}P]NAD$^+$, one labelled protein band appeared with a molecular mass of approx. 31 kDa. The presence of an 80-fold excess of unlabelled ADP-ribose over [^{32}P]NAD$^+$ had no effect on the label of the 31 kDa protein. Furthermore, in the presence of novobiocin the label of the 31 kDa protein disappeared, indicating that ADP-ribosylation of the 31 kDa protein was likely to be due to an ART.

Analysis of the chemical stability of the ADP-ribosyl–protein bond revealed that the label was almost detached from the protein in the presence of 10 mM $HgCl_2$, suggesting that the predominant acceptors were cysteine residues.

DISCUSSION

The results presented here show the presence of ADP-ribosyltransferase activity on the surface of human monocytes. The enzyme activity is relatively low (0.049 pmol/h per 10^6 cells) compared with that reported previously for human polymorphonuclear neutrophil leucocytes (1.4 ± 0.2 pmol/h per 10^6 cells) [13]. A 2-fold increase was observed upon addition of LPS (100 ng/ml) to the cells. In the presence of BSA, the basal activity increased but LPS had no additional effect. These results indicate that the enzyme present on monocytes (probably ART3) is capable of ADP-ribosylating BSA and that the LPS-induced activity (probably ART4) may depend on a substrate other than BSA. The ADP-ribose–BSA bond was split by $HgCl_2$ (10 mM) but not by NH_2OH (1 M), indicating that the modification of BSA probably occurred at a cysteine residue.

ADP-ribosylation of cysteine has been reported in bovine erythrocytes [28] and an NAD$^+$:cysteine ADP-ribosyltransferase that modified $G_{\alpha i}$ has been purified from human erythrocyte and platelet membranes [29]. ADP-ribosylation of cysteine can also occur non-enzymically via the reaction of ADP-ribose, generated from NAD$^+$ by NAD$^+$ glycohydrolases, with cysteine, resulting in the formation of ADP-ribosylthiazolidine, which is sensitive to both $HgCl_2$ and NH_2OH [30]. This linkage is different from the thioglycoside formed by pertussis toxin-catalysed ADP-ribosylation of a cysteine in the heterotrimeric G-proteins [30]. In the present study BSA was ADP-ribosylated efficiently in the presence of an 80-fold excess of ADP-ribose and only to a minor extent in the presence of novobiocin, indicating that ADP-ribosylation occurs as an enzymic reaction rather than a non-enzymic transfer of ADP-ribose to BSA. As the fraction of the label that had not been detached from the protein in the presence of novobiocin was removed by NH_2OH, it seems likely that, in addition to enzymic modification at a cysteine residue, a minor part of the protein might have been modified non-enzymically at an arginine residue.

Using a new FACS assay for monitoring etheno-ADP-ribosylation of cell-surface proteins [31], we were unable to demonstrate ADP-ribosylated proteins on either stimulated or unstimulated monocytes.The reason for this is uncertain. It is possible that the

amount of ADP-ribosylated protein is too low to be detected by FACS analysis or that the antibody recognizing etheno-adenosine has no access to the modified protein.

Possible candidates for catalysing the ADP-ribosylation of surface proteins are the mammalian ARTs 1, 3, 4 and 5. These enzymes are GPI-anchored proteins, with the exception of ART5, which possesses a hydrophobic N- but not C-terminal signal sequence and may be secreted instead of becoming GPI-linked [8]. So far, hardly any target proteins specific for the known mammalian cell-surface ARTs have been identified. In C2C12 mouse myoblasts ART1 activity, which appeared on differentiation of myoblasts to myotubes, has been shown to catalyse the ADP-ribosylation of integrin α_7 [32], and ART1, by attaching ADP-ribose groups to arginine residues of T-cell co-receptors, has been implicated in the inhibition of T-cell functions, such as cytolytic activity and cell proliferation [10,11]. The ART2 enzyme has been associated with the development of autoimmune disease [11]. Its defective expression on mouse T-cells has been linked to increased susceptibility to the disease [12]. When trying to associate ADP-ribosyltransferase activity on monocytes with specific ARTs we measured the expression of ART mRNA in stimulated and unstimulated cells. Of the four ARTs tested we only detected ART3 mRNA in unstimulated cells, and stimulation with LPS led to the expression of ART4 mRNA. Similarly to LPS, other monocyte activators, such as lipoteichoic acid and peptidoglycan, initiate the expression of ART4, indicating that regulation of this enzyme, at least at the mRNA level, is a general intracellular event in a process of monocyte activation by a variety of extracellular stimuli. Expression of ART4 mRNA seems to be a rather late event in stimulus-induced metabolic processes. It certainly is not associated with rapid signal-transduction mechanisms.

With respect to the possible function of ART4 in monocyte activation it will be of great interest to identify its physiological target protein. We found a 31 kDa protein ADP-ribosylated in response to LPS. As the adduct was sensitive to mercuric ions, it is likely that a cysteine residue participates in the linkage. Whether ART4 is a possible candidate for catalysing the ADP-ribosylation of p31 deserves further investigation. So far, little is known about ART4. The protein is different from other ARTs in its catalytic regions I and III, which lack amino acids thought to be essential for ADP-ribosylation of guanidino compounds [8,33,34]. The acceptor amino acid modified by ART4 has not yet been identified.

In summary, we show for the first time the stimulus-induced regulation of ART gene expression and cell-surface ART activity in human monocytes. Furthermore, we have identified a 31 kDa protein that is a specific target of the external enzyme on activated monocytes. These results provide further supportive evidence for the involvement of ADP-ribosyltransferases in regulating the biological responses of monocytes.

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