Glucocorticoid-induced annexin 1 secretion by monocytes and peritoneal leukocytes

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1 We have studied the ability of the glucocorticoid, dexamethasone, to induce annexin 1 secretion by either human blood monocytes or rat peritoneal leukocytes.

2 The *in vivo* treatment of rats with dexamethasone (1.25 mg kg⁻¹) selectively induced secretion of annexin 1 by peritoneal leukocytes, as assessed by incubating these cells in culture medium. Annexin 1 secretion was also induced in human cultured monocytes, *in vitro*, by 10⁻⁶M dexamethasone.

3 Annexin 1 secretion was inhibited in the presence of 20 mM NH₄Cl or by conducting the experiments at 18°C. In contrast, it was not inhibited by monensin, nocodazole or brefeldin A.

4 The time necessary for annexin 1 synthesis and secretion was less than 15 min.

5 These data indicate that glucocorticoids induce annexin 1 secretion by monocytes or peritoneal leukocytes. Because it is not inhibited by monensin, nocodazole or brefeldin A and it is rapid, annexin 1 secretion seems to occur by the secretory pathway similar to that used by several cytosolic proteins such as interleukin-1β.

Keywords: Annexin 1; lipocortin 1; glucocorticoids; secretion; monocytes; peritoneal leukocytes

Introduction

Annexins form a family of structurally homologous proteins that are synthesized in the cell cytosol and to which different biological functions have been attributed (reviewed in Flower & Rothwell, 1994; Raynal & Pollard, 1994). Among them, it has been suggested that annexin 1 (or lipocortin 1) is the messenger of part of the anti-inflammatory action of glucocorticoids. Indeed, mRNA and protein levels of annexin 1 can be increased by glucocorticoids (Wallner *et al.*, 1986; Goulding *et al.*, 1990; Browning *et al.*, 1990; Solito *et al.*, 1991; Ambrose *et al.*, 1992; Peers *et al.*, 1993; Flower & Rothwell, 1994; Raynal & Pollard, 1994). In addition, extracellular locations of annexin 1 were reported in body fluids, cell surfaces or cell supernatants, some of them being glucocorticoid-inducible (Goulding *et al.*, 1990; Browning *et al.*, 1990; Ambrose & Hunninghake, 1990; Smith *et al.*, 1990; Christmas *et al.*, 1991; Solito *et al.*, 1991; Peers *et al.*, 1993; Serres *et al.*, 1994; Flower & Rothwell, 1994; Raynal & Pollard, 1994). Finally, annexin 1 has extracellular anti-inflammatory properties which have been observed either *in vitro* or *in vivo* (Cirino *et al.*, 1987; 1989; Fradin *et al.*, 1988; Maridonneau-Parini *et al.*, 1989; Browning *et al.*, 1990; Flower & Rothwell, 1994; Raynal & Pollard, 1994). It reproduces several anti-inflammatory actions of glucocorticoids such as inhibition of cellular PLA₂ activity and neutrophil migration. However, this potential annexin 1 function has been the subject of controversy for several reasons. First, the glucocorticoid-induction of annexin 1 synthesis or release is restricted to certain cells or certain culture conditions (Northup *et al.*, 1988; Raynal & Pollard, 1994). Second, the physiological relevance of this extracellular function implies that annexin 1 is secreted. However, as opposed to most of the secreted proteins, annexin 1 is synthesized in the cytosol of cells and does not have the signal peptide that targets proteins to the endoplasmic reticulum and the classical secretory pathway (Raynal & Pollard, 1994). A newly discovered secretory route, exporting cytosolic proteins, has been reported and it was suggested this might be used by annexin 1 (Muesh *et al.*, 1990; Christmas *et al.*, 1991).

In order to assess the potential extracellular anti-inflammatory role of annexin 1 and its relation to glucocorti-

coids, we studied if the latter induced a physiological annexin 1 secretion in cells that are involved in inflammation. We show that glucocorticoids induce annexin 1 secretion in normal human blood monocytes or rat peritoneal leukocytes. The annexin 1 secretory pathway is characterized and shows close similarity with the one used by interleukin-1β (IL-1β).

Methods

Cell preparation

Mononuclear cells Peripheral blood mononuclear leukocytes (about 20% monocytes and 80% lymphocytes) from healthy donors were provided by the Blood Transfusion Centre of the Hôtel-Dieu (Paris). These cells were isolated by erythrocyte sedimentation using Dextran T500 (Pharmacia Biotech, Saint-Quentin, France) and mononuclear cells were separated from polymorphonuclear cells by centrifugation through Ficoll-Hypaque (Pharmacia Biotech) according to Britigan *et al.* (1988). The mononuclear leukocytes were washed three times in PBS (phosphate buffered saline, calcium- and magnesium-free) by 10 min centrifugation at 300 g and resuspended in RPMI 1640, medium (Flow Laboratories, Puteaux, France), containing 0.02% Ultrosor (steroid-free, IBF, Villeneuve la Garenne, France), 1 mM L-glutamine, 100 u ml⁻¹ of penicillin-streptomycin and 20 mM HEPES at 37°C. Monocytes were counted by non-specific esterase staining (Contreras *et al.*, 1980) and were dispensed at 2 to 5 × 10⁶ monocytes/well in 12 or 24 well culture plates. They were allowed to adhere at 37°C with 5% CO₂ for 45 to 60 min and non-adherent cells were removed by three washes with PBS. Adherent monocytes were incubated in RPMI buffer at 37°C and 5% CO₂ in the presence or in the absence of 10⁻⁶ M dexamethasone (Sigma Chemical, Saint-Quentin-Fallavier, France).

Rat peritoneal cells: Male wistar rats (250 to 300 g) were treated for 2 or 24 h with an intraperitoneal injection of saline (0.9% NaCl) or 1.25 mg kg⁻¹ phospho-dexamethasone (Merck Sharp & Dohme-Chibret, Paris, France) in saline (2 ml kg⁻¹). These conditions were used to study annexin 1 secretion because they were previously reported to induce up to 6 fold the

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amount of annexin 1 mRNA in these cells (Wallner *et al.*, 1986). Rats were killed with ether and peritoneal cells were harvested by washing the cavity with 20 ml of PBS containing 1 mM final EDTA (ethylene diamine tetraacetic acid) to remove potential extracellular annexins, bound on cell surfaces. They were centrifuged for 10 min at 300 g, resuspended in RPMI buffer at 1 to 2×10^7 cells ml^{-1} and incubated for 30 min at 37°C .

Cell and supernatant treatment and analysis

At the end of the incubation periods, extracellular annexin 1 bound to the cell surface was washed off by adding 2 or 5 mM EDTA to the medium for a 2 min incubation period. Cells were separated from the medium by a short centrifugation of 20 s at 13,000 r.p.m. This centrifugation did not induce any cell lysis and allowed rapid separation of the cells from the medium. In experiments with adherent monocytes, cells and supernatants were separated by directly aspirating the medium. The latter was separated from any remaining cells by centrifugation at 13,000 r.p.m. for 20 s.

Analysis of annexin content by Western-Blot experiments

Cells were homogenized in 25 mM Tris/HCl, 0.25% Nonidet P-40 containing $1 \mu\text{g ml}^{-1}$ leupeptin, $1 \mu\text{g ml}^{-1}$ antipain, $1 \mu\text{g ml}^{-1}$ pepstatin A, $15 \mu\text{g ml}^{-1}$ benzamidine, 4 mM phenylmethylsulphonyl fluoride and 1000 iu ml^{-1} aprotinin followed by sonication. Aliquots of cell homogenates and supernatants were reserved for measuring the lactate dehydrogenase (LDH) activity. The remaining supernatant proteins were precipitated with 10% final of trichloroacetic acid and centrifuged for 10 min at 13,000 r.p.m. and 4°C . Cell homogenates and supernatant proteins were equilibrated in Laemmli's sample buffer, boiled for 5 min and subjected to SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and Western-blot analysis as previously described (Laemmli, 1970; Coméra *et al.*, 1989). Monospecific rabbit polyclonal antibodies directed against either annexin 1, 2 (a generous gift from J.C. Cavadore, INSERM U249, Montpellier), 5 or 6 were used. Some anti-annexin 1 antibodies were immunoaffinity purified with an annexin 1-sepharose 4B column that we made using 3.6 mg of human recombinant annexin 1 (kindly provided by Biogen, Boston, MA, U.S.A.) bound to 720 mg of CNBR-activated sepharose 4B, previously swollen and washed in HCl 1 mM, as described by the manufacturer (Pharmacia Biotech). Recombinant annexin 1 was allowed to bind to CNBR-activated sepharose 4B in buffer A (NaHCO_3 0.1 M, pH 8.3, NaCl 0.5 M) for 16 h at 4°C , followed by an incubation period of 2 h at room temperature in blocking buffer (1 M ethanolamine, pH 8) and sequential washes in buffer A, buffer B (0.1 M acetate, pH 4, 0.5 M NaCl), then buffer A. Protein concentrations were determined as described by Bradford (1976).

Determination of cell viability

Cell viability was determined by trypan blue exclusion or by spectrophotometric measurement of LDH activity in both supernatants and cell homogenates, as described by the manufacturer (Boehringer Mannheim, Meylan, France, kit: 1 087 592). The trypan blue exclusion method was used for peritoneal leukocyte samples which contained low numbers of cells. Peritoneal samples contained less than 3% dead cells as measured by trypan blue and less than 5% dead cells when measured by LDH activity.

Metabolic labelling of human monocytes and immunoprecipitation of annexin 1

Adherent monocytes, 4.5×10^6 /well, were incubated overnight in RPMI buffer in the presence or absence of 10^{-6} M dexamethasone followed by 45 min incubation in the same con-

ditions but in a medium devoid of methionine; 250 μCi /well of [^{35}S]-methionine (Amersham, Les Ulis, France) was then added for a further incubation of 15 or 30 min. Subsequently, 5 mM EDTA was added for 2 min and supernatants were isolated as previously described. Annexin 1 was immunoprecipitated from supernatants by incubation at 4°C with anti-annexin 1 antibodies for 2 h. After addition of 450 μg of protein-A sepharose (Pharmacia Biotech, Saint-Quentin, France) and a 1 h-incubation, the mixtures were washed 6 times with 20 mM triethanolamine/HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS buffer followed by centrifugation 5 min at 10,000 g. An additional wash was performed in the above buffer but in the absence of detergents. The samples were analysed by SDS-PAGE, protein fixation, a 30 min incubation in Amplify solution (Amersham), drying and autoradiography.

Results

Glucocorticoid-induced annexin 1 secretion

Dexamethasone-induced annexin 1 release was observed from either peritoneal leukocytes isolated from *in vivo* treated rats (Figure 1) or human cultured blood monocytes (Figure 2). However, dexamethasone did not change the intracellular annexin 1 content of peritoneal leukocytes (Figure 1b). In both cell types, the release of annexin 1 was inhibited in the presence of 20 mM NH_4Cl in the medium. Annexin 1 release by peritoneal leukocytes was very abundant: whereas cell death was very low ($< 5\%$), 50% of the total annexin 1 amount had been

Figure 1 Western-blot analysis of annexin secretion from supernatants (S) and cells (C) of rat peritoneal leukocytes incubated for 30 min in RPMI medium. Rats were previously treated with saline for 2 h (1) or with 1.25 mg of dexamethasone for 2 h (2) or for 24 h (3). (a) Effect of the presence (+) or the absence (-) of 20 mM NH_4Cl in the incubation media on annexin 1 release in supernatants. (b) Analysis of the presence, in cells and supernatants, of annexin 1 (An 1), 2 (An 2), 5 (An 5) and 6 (An 6). Cell homogenates (30 μg proteins) and equivalent volumes of supernatant samples were loaded.

released from peritoneal cells during the 30 min incubation period (Figure 1b, quantified by densitometric scanning in two separate experiments). Peritoneal cells also expressed annexin 2, 5 and 6 but none of these proteins was detected in the medium (Figure 1b).

As shown previously (Browning *et al.*, 1990), the ability of dexamethasone to induce annexin 1 secretion was variable. It was observed in 8 out of 20 experiments for human blood monocytes and in 3 out of 12 experiments for rat peritoneal leukocytes. Moreover for both cell types, the time of dexamethasone treatment necessary to induce annexin 1 secretion was variable. For instance, in one experiment (Figure 1a) annexin 1 secretion by peritoneal leukocytes was induced 2 h after dexamethasone treatment of the rats while in another experiment (Figure 1b), no secretion was seen after 2 h but was seen 24 h after dexamethasone. In this latter study annexin 1 release was either very abundant or not detectable at all.

Modulation by chemicals or temperature of annexin 1 secretion by monocytes

This study was done on human cultured monocytes responding to *in vitro* dexamethasone induction that was performed for 3 or 16 h (Figure 2). Different chemicals were used that block the transport of proteins at different steps along the classical secretory pathway which secrete proteins with a signal peptide. This type of protein secretion is inhibited by (i) brefeldin A which prevents the egress from the endoplasmic reticulum (ER), (ii) the monovalent ionophore monensin which blocks the transport in the late Golgi, (iii) nocodazole which depolymerizes microtubules and inhibits the migration of vesicles and (iv) amines (ammonium chloride, methylamine or others) which stop vesicle or granule transport by increasing their intracellular pH (Tartakoff, 1983; Dean *et al.*, 1984; Klausner *et al.*, 1992). We also analysed the effect of incubation at 18°C which inhibits intracellular trafficking (Rubartelli *et al.*, 1990). NH_4Cl was used at 20 mM which is the suboptimal dose that does not induce cellular lysis and is consistent with the concentration of methylamine (10 mM) used to block interleukin- β secretion (Rubartelli *et al.*, 1990). Monensin and brefeldin A were used at 10 μM and 0.5 $\mu\text{g ml}^{-1}$ respectively, according to Rubartelli *et al.* (1990) and nocodazole at 33 μM , the suboptimal dose to avoid cytotoxicity. This latter was reported to depolymerize monocyte microtubules and to induce inter-

leukin- β secretion when used at a concentration as low as 3.3 μM (Newton, 1990).

Cellular lysis was from 5 to 7% for every sample shown in Figure 2a and was from 8 to 10% except in the presence of monensin and brefeldin A where they were, 19 and 13%, respectively, for the samples shown in Figure 2b. Annexin 1 release was inhibited in the presence of 20 mM NH_4Cl and at 18°C. In contrast, this release was not inhibited but rather increased in the presence of either 10 μM monensin, 33 μM nocodazole or 0.5 $\mu\text{g ml}^{-1}$ brefeldin A. Except for the effect of monensin in Figure 2b (but not in Figure 2a), there was no correlation between the rates of cellular lysis and annexin 1 release. Annexin 1 sometimes appears as three different peptides which have been shown to be extracellular annexin 1 proteolytic fragments (Smith *et al.*, 1990; Christmas *et al.*, 1991).

Quantification of annexin 1 secretion time in monocytes

In order to determine the time required for the translated annexin 1 to be secreted, metabolic protein labelling using [^{35}S]-methionine was performed (Figure 3). This secretion was rapid since the time between annexin 1 translation and secretion was less than 15 min. It should be noted that, in agreement with our previous results, radiolabelled annexin 1 release was induced by dexamethasone.

Discussion

Our study establishes that annexin 1 is secreted. This is demonstrated in the experiment where intact rat peritoneal leukocytes released up to 50% of the total cellular annexin 1 in 30 min. Indeed, both abundance and selectivity of this release suggest that it is a genuine secretion. Such an abundant release of annexin 1 was previously reported in seminal fluids (Christmas *et al.*, 1991). Annexin 1 secretion in human blood mononuclear cells, monocytes or in rat peritoneal leukocytes is probably physiological because it is inhibited by 20 mM NH_4Cl or at 18°C and there is no apparent correlation between the rates of annexin 1 release and cellular lysis. These results support the concept that dexamethasone induces annexin 1 secretion as shown in previous studies (Goulding *et al.*, 1990; Browning *et al.*, 1990; Solito *et al.*, 1991; Peers *et al.*, 1993; Flower & Rothwell, 1994; Raynal & Pollard, 1994). It is interesting to note that other annexins were reported to be secreted with no apparent glucocorticoid inducibility except for annexin 5 in differentiated U937 cells (Pfäffle *et al.*, 1988; Christmas *et al.*, 1991; Solito *et al.*, 1991; Raynal & Pollard, 1994). Further studies remain necessary to determine to what extent secretion of annexin 1 and possibly other annexins contribute to physiological glucocorticoid action.

Some studies, on human monocytes or on leukocytes, reported that dexamethasone induces either an annexin 1 mRNA or protein synthesis or its secretion whereas others did not detect any of these events (Wallner *et al.*, 1986; Northup *et al.*, 1988; Browning *et al.*, 1990; Goulding *et al.*, 1990; Ambrose *et*

Figure 2 Western-blot analysis of the effect of chemicals or 18°C on annexin 1 release in supernatants of human adherent monocytes. Cells were incubated for 16 h (a) or 3 h (b) in the presence (+) or in the absence (-) of 10^{-6}M dexamethasone (Dex) at 37°C with either no product added (-), 20 mM NH_4Cl (N), 10 μM monensin (M), 33 μM nocodazole (No) or 0.5 $\mu\text{g ml}^{-1}$ brefeldin A (B) and at 18°C (18). Equivalent volumes of supernatants were loaded.

Figure 3 Detection of newly synthesized annexin 1 in human adherent monocyte supernatants. Cells were stimulated with (+) or without (-) 10^{-6}M dexamethasone (Dex) and were incubated for 15 min or 30 min in the presence of [^{35}S]-methionine (250 $\mu\text{Ci}/4.5 \times 10^6$ cells). Annexin 1 was immuno-precipitated from supernatants and detected by SDS-PAGE and autoradiography.

et al., 1992; Peers *et al.*, 1993). In our study, annexin 1 secretion by both monocytes or peritoneal leukocytes in response to dexamethasone was very variable. This agrees with previous reports which relate differences in the dexamethasone response concerning annexin 1 synthesis or secretion by cells, depending on the cell type, the state of cellular activation or differentiation or on the requirement for factors in addition to glucocorticoids (Browning *et al.*, 1990; Solito *et al.*, 1991; Ambrose *et al.*, 1992; reviewed in Raynal & Pollard, 1994). This could most certainly modulate the *in vivo* occurrence of this phenomenon and make it difficult to predict in which state of differentiation/activation circulating monocytes and peritoneal leukocytes are in and, therefore, whether they will respond to glucocorticoids; indeed, we have found that annexin 1 promoter responds differently to glucocorticoids according to the type of cells and to the presence of other transcription factors (E. Solito, unpublished results).

Annexin 1 is secreted via a pathway independent of the endoplasmic reticulum-Golgi complex

The fact that monensin, nocodazole and brefeldin A do not inhibit annexin 1 secretion strongly suggests that annexin 1 is not secreted by the classical pathway (ER/Golgi) but by an alternative route. Indeed, secretion of interleukin 6 and tumour necrosis factor α (TNF α) by this classical pathway were found to be inhibited by monensin and brefeldin A in human

monocytes (Rubartelli *et al.*, 1990). This is further supported by the fact that the time elapsed between annexin 1 biosynthesis and secretion, in monocytes, is shorter than 15 min and much faster than the time required for protein secretion by the classical pathway. Annexin 1, IL1- β , basic fibroblast growth factor and thioredoxin secretions share similar properties for modulation of their secretion, suggesting they could be secreted by a common secretory pathway (Rubartelli *et al.*, 1990; Newton, 1990; Rubartelli & Sitia, 1991; Mignatti *et al.*, 1992). This is consistent with the fact that all these proteins do not have any signal peptide and are synthesized in cell cytosol. Another study reported a similar inhibition of annexin 1 secretion by epidermal cells using the amine chloroquine or an incubation at 18°C (Serres *et al.*, 1994).

It is not yet known why eukaryotic organisms display this alternative secretory pathway in addition to the classical one. It was reported that many of the mammalian secreted proteins lacking a signal sequence were related to inflammation, coagulation or tissue damage (Rubartelli & Sitia, 1991). Our study shows that annexin 1 secretion can be very abundant and be rapidly processed by cells.

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