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Annexin 1 is overexpressed and specifically secreted during experimentally induced colitis in rats

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Annexin 1 is a protein induced by glucocorticoids endowed with extracellular anti-inflammatory properties. In this study, the local expression and secretion of annexins 1–6, in rat proximal colon, were studied at different times after intracolonic administration of 30 mg trinitrobenzenesulfonic acid in 50% ethanol. Secretion was identified by incubating colonic tissues in a culture medium. The expression of annexins was detected by immunoblotting in tissue homogenates and incubation media. Inflammatory stages were evaluated by measuring myeloperoxidase activity. Annexin 1 expression in colons increased after trinitrobenzenesulfonic acid treatment and was maximal between days 1 to 9, during the cellular stage of the inflammation that corresponded to maximal myeloperoxidase activity. From 12 h to 9 days after trinitrobenzenesulfonic acid/ethanol treatment, annexin 1 was specifically secreted. Annexin 3 was also overexpressed during the cellular stage, but the expression of annexins 2, 4, 5, and 6 was unchanged; none of these annexins were secreted. Annexin 1 was shown to be physiologically secreted because its release was specific, abundant, and not correlated with cellular lysis. Annexin 1 may be considered as a putative candidate in the control of the gut inflammatory processes.

Keywords: colitis; annexin 1; lipocortin 1; secretion; inflammation.

Glucocorticoids are the most effective anti-inflammatory drugs. They inhibit the expression of various proteins that enhance the production of mediators involved in inflammatory processes [1–5]. In the late 1970s, it was also shown that glucocorticoids stimulate both synthesis and secretion of an anti-inflammatory protein, named lipocortin [6]. Later on, this protein was found to be identical to annexin 1 (or lipocortin 1), which was purified and cloned in 1986 [7, 8], and belongs to a family of homologous proteins known as annexins [9].

Annexin 1, or some of its fragments, has biological properties identical to those of lipocortin [9–11]. First, annexin 1 was shown to be synthesized or released in response to glucocorticoids although this is restricted to certain cells or tissues [9, 12–17]. Secondly, it displays extracellular properties reproducing various glucocorticoid actions such as inhibition of the production of pro-inflammatory mediators both *in vitro* and *in vivo* [9–11, 18–26]. In this regard, *in vitro* studies showed that annexin 1 inhibits cellular phospholipase A₂ (PLA₂) and, consequently, the production of arachidonic acid, eicosanoids, platelet-activating factor, and oxygen free radicals [18–21]. *In vivo* studies reported that natural or recombinant annexin 1, and some of its fragments, reduces inflammatory processes, eicosanoid production and neutrophil migration when administered either locally or systemically [9–11, 13, 22–26]. In addition, in some models, local or systemic administration of antibodies directed

to annexin 1 blocks the anti-inflammatory action of glucocorticoids or annexin 1, suggesting that annexin 1 is a natural mediator involved in glucocorticoid actions [24–26].

However, to exert a physiological extracellular anti-inflammatory action, annexin 1 should be secreted. The occurrence of such a secretion is subject to controversy [9]. First, it has been established that annexins are primarily intracellular proteins and are devoid of the signal peptide that is necessary in the conventional protein secretory pathway [9]. Secondly, annexin 1 and other annexins have been identified in extracellular locations, such as on cell surfaces, in cell culture media or in body fluids [8, 12–17, 27–31]. However, their low extracellular concentration led to the hypothesis that they were non-specifically released from damaged or dead cells rather than actually secreted. Nevertheless, the huge amount of annexin 1 detected in seminal plasma demonstrated a real secretion [29] and suggested that at least part of the annexins found in extracellular locations were probably secreted by a non-conventional secretory mechanism, as recently described for several other cytosolic proteins [32].

In addition, other annexins share similar properties with annexin 1. These properties include the inducibility of synthesis or secretion by glucocorticoids [9, 16] and anti-inflammatory properties *in vitro* or *in vivo* [19, 33, 34]. An overexpression of annexin 1 or other annexins has been reported in some pathological processes [9, 10, 35–37]. Annexin 1 was found to be down-regulated in non-perforating Crohn's disease, whereas it was present at control levels in perforating Crohn's disease [38]. Nevertheless, neither the chronology of the appearance of annexin nor the occurrence of a real secretion with respect to inflammation has been well defined.

The aim of this work was therefore to determine whether an experimental colitis in the rat induces local expression and secretion of annexins and, if so, to investigate this phenomenon in relation to the time course of the inflammatory response.

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Abbreviations. IL-1 β , interleukin-1 β ; N₃ph-SO₃H, trinitrobenzenesulfonic acid; PLA₂, phospholipase A₂; LDH, lactate dehydrogenase; MPO, myeloperoxidase.

Enzymes. Phospholipase A₂ (EC 3.1.1.4); lactate dehydrogenase (EC 1.1.1.27); myeloperoxidase (EC 1.11.1.7).

MATERIALS AND METHODS

Animal preparation and colitis induction. Colitis induction on the proximal colon was adapted from the model of rectocolitis previously described by Morris et al. [39]. 103 male Wistar rats weighing 225–300 g were used. After an overnight fast, rats were anesthetized by intraperitoneal injection of 0.5 mg/kg acepromazine and 100 mg/kg ketamine (Imalgene 1000, Rhône-Mérieux). The early effects of trinitrobenzenesulfonic acid ($\text{N}_3\text{ph-SO}_3\text{H}$)/ethanol were studied in 31 conscious animals. They were fitted with a polyethylene catheter (0.9 mm in external diameter) inserted into the proximal colon 1 cm from the cecocolonic junction and exteriorized at the back of the neck. Five days later, the animals were fasted overnight and received 0.3 ml $\text{N}_3\text{ph-SO}_3\text{H}$ (30 mg) in 50% ethanol in the proximal colon, through the catheter. The animals were sacrificed 4, 8, 12, 16, 20 or 24 h after the treatment; four rats were used each time except for the time points 12 h and 16 h where eight and three rats were treated, respectively. The remaining rats were used for the study of later effects of $\text{N}_3\text{ph-SO}_3\text{H}$ /ethanol. They were fasted overnight, anesthetized and received the same $\text{N}_3\text{ph-SO}_3\text{H}$ /ethanol treatment by direct injection into the proximal colonic lumen. They were sacrificed 1, 3, 6, 9, 12, 15, 18 or 21 days later (eight rats for each time-point). The control animals fitted with a catheter received 0.3 ml saline (0.9% NaCl) and were sacrificed 24 h later while the other control animals were sacrificed 6 days later the 0.3 ml injection of saline. 1 day after the $\text{N}_3\text{ph-SO}_3\text{H}$ /ethanol treatment, no difference was observed between the two modes of administration for all the parameters considered.

Preparation of tissue samples. Animals were sacrificed by cervical dislocation and proximal colons were rapidly collected and rinsed with saline. Colons were cut into equivalent pieces to study either myeloperoxidase (MPO) activity or annexin expression and secretion. MPO activity and annexin expression were determined in all the tissues obtained. Secretion experiments were studied for eight proximal colons from control rats and from animals sacrificed at 12 h and on days 1–21 after the $\text{N}_3\text{ph-SO}_3\text{H}$ /ethanol treatment.

Study of annexin expression. Freshly isolated proximal colons were homogenized using a Polytron for 30 s at 0°C and sonicated for 30 s at 0°C in a lysis buffer, containing 25 mM Tris/HCl, pH 7.4, 5 mM EDTA, 0.25% (by vol.) Nonidet P-40, 1 µg/ml pepstatin, 15 µg/ml benzamidine, 1 µg/ml antipain, 1 µg/ml leupeptin, 1000 IU/ml aprotinin and 4 mM phenylmethylsulfonyl fluoride. Homogenates were equilibrated in an electrophoresis sample buffer [60 mM Tris/HCl, pH 6.8, 10% (by vol.) glycerol, 5% (by vol.) 2-mercaptoethanol, 2% (mass/vol.) sodium dodecyl sulfate (SDS), 0.01% (mass/vol.) bromophenol blue], boiled for 5 min, and stored at –80°C. Protein concentrations were measured according to Bradford [40].

Study of annexin secretion. Annexin secretion was studied by washing colonic fragments followed by incubation in cell culture medium. Tissue washes were performed on ice, to block secretory processes, and in the presence of 2 mM EGTA, to remove potential extracellular annexins that could be bound to the cell surface by calcium-dependent interaction. Proximal colonic tissues were cut into small pieces and washed four times for 20 min in NaCl/P_i (150 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4) three times in the presence of 2 mM EGTA and one time without EGTA. Thereafter, tissue incubation was performed following two stages, in a culture medium [RPMI 1640 (Eurobio) plus 1 mM glutamine, 0.02 mM EGTA, 20 mM Hepes, pH 7.4]. First, colons were incubated for 30 min at 37°C to allow protein secretion. Secondly, samples were adjusted to 2 mM final EGTA, rapidly cooled to 0°C, and

further incubated for a 15-min period on ice, allowing extracellular annexins to be released into the media. After either washes or incubations, tissues were separated from supernatants, homogenized in the lysis buffer, and subsequently equilibrated in the electrophoresis sample buffer as previously described. Supernatants were centrifuged at 13000×g for 20 s, to eliminate cells or tissue fragments. Supernatant proteins were precipitated by a 10-min incubation on ice with 10% (mass/vol.) trichloroacetic acid (final concentration), centrifuged at 13000×g for 10 min, suspended in the electrophoresis sample buffer, boiled for 5 min, and stored at –80°C. Aliquots of homogenates in the lysis buffer and fresh supernatants were kept at 4°C to measure lactate dehydrogenase (LDH) activity.

Study of the annexin 1 extracellular form. Two secretion experiments were modified to establish if annexin 1 in the culture medium was in a soluble form. After the 20-s centrifugation at 13000×g, to eliminate cells or tissue fragments, culture supernatants were supplemented with protease inhibitors (1 µg/ml pepstatin, 15 µg/ml benzamidine, 1 µg/ml antipain, 1 µg/ml leupeptin, 1000 IU/ml aprotinin, and 4 mM phenylmethylsulfonyl fluoride; final concentrations). Samples were centrifuged for 2 h at 100000×g. Pellets, containing the vesicular protein fraction, were resuspended in the electrophoresis sample buffer; supernatant soluble proteins were precipitated as described above and resuspended in the electrophoresis sample buffer.

Detection of annexins by SDS/PAGE and immunoblotting. Proteins from homogenate and supernatant samples equilibrated in the sample buffer were separated by SDS/PAGE in 10% polyacrylamide gels according to Laemmli [41], and electrophoretically transferred onto nitrocellulose membranes as described by Towbin et al. [42] in 25 mM Tris/HCl, 192 mM glycine, 0.01% (by vol.) SDS, 15% methanol. Immunoblottings were performed using different monospecific rabbit polyclonal antibodies. Antibodies were specific to either annexins 1, 3 or 6 [used at 1:1000, 1:100 and 1:1000 dilutions, respectively, a gift from F. Russo-Marie, Institut National de la Santé et de la Recherche Médicale (INSERM) U332, Paris, France], to annexin 1 or 2 from (both used at 1:1000, provided by L. A. Pradel, Centre National de la Recherche Scientifique UA 1112, Paris, France), to annexin 1 or 5 (both used at 1:500, provided by H. Chap, INSERM U326, Toulouse, France), to annexin 4 (used at 1:250, from C. E. Creutz, Charlottesville, USA) and to annexin 2 (used at 1:1000, a gift from J. C. Cavadore, INSERM U249, Montpellier, France). All the antibodies used were tested for their monospecificity by immunoblotting with pure annexins 1–6 (gifts from F. Russo-Marie) and different cell and tissue homogenates. Immunoblottings for annexin 1 detection were performed with polyclonal antibodies affinity purified on a human recombinant annexin-1–Sepharose 4B column kindly provided by F. Russo-Marie and J. F. Browning for the recombinant annexin 1 (Biogen Corp.). Incubations were performed in buffer A (25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 1 h with 5% dried milk, to saturate non-specific binding sites, followed by 1 h in the presence of anti-annexin antibodies. Nitrocellulose sheets were then washed five times for 5 min in buffer A, incubated for 30 min in the presence of anti-rabbit antibodies made by goat and linked to horseradish-peroxidase (1:10000 dilution, Sigma) and washed three times for 5 min in buffer A. Immunolabeled bands were revealed by fluorography, using the ECL reagents (Enhanced Chemiluminescence, Amersham) followed by exposure from 1 min to 1 h to Hyperfilm MP (Amersham). For densitometric quantifications, films were pre-flashed before exposure for 1 ms with a Sensitize pre-flash unit (Amersham).

Densitometric quantification of annexin 1 secretion. The extracellular and intracellular contents of annexin 1 from secretion studies were assessed by SDS/PAGE and immunoblotting using different dilutions of both homogenates and supernatants and different concentrations of pure human annexin 1 (from F. Russo-Marie). To quantify annexin 1 contents, densitometric scans of the fluorographs were performed with a Shimadzu CS930 chromoscan (Kyoto, Japan) with a linear correlation between densitometric values and the amounts of annexin 1 for 20–100 ng. The percentage of extracellular annexin 1 was calculated by dividing the amount detected in supernatants (38 kDa and 33 kDa) by the sum of the amount detected in supernatants and homogenates multiplied by 100.

Measurement of cellular lysis. Cell viability was determined by spectrophotometric measurement of LDH activities as described by Bergmeyer and Bernt [43], in both fresh supernatants and tissue homogenates from the secretion experiments. The percentage of cellular lysis is equivalent to the percentage of LDH released into supernatants and was calculated by dividing the activity detected in supernatants by the sum of the activities detected in supernatants and in tissue homogenates multiplied by 100.

Measurement of MPO activity. Proximal colonic tissues were homogenized twice for 30 s and sonicated three times for 30 s at 0°C in 0.5% hexadecyltrimethylammonium bromide in 50 mM sodium phosphate, pH 6.0, according to Bradley et al. [44]. The homogenates were clarified by centrifugation at $13000\times g$ for 15 min, at 4°C, and were assessed for MPO activity in 3 ml 50 mM sodium phosphate, pH 6.0, containing 16 mM aqueous guaiacol and 5.9 mM H_2O_2 as previously described [45]. The increase in absorbance was measured for 2 min at 470 nm using a Uvikon 860 spectrophotometer (Kontron Instruments). Protein concentrations were determined using the Bio-Rad DC protein assay. MPO activities were expressed in U/mg protein with 1 U hydrolyzing $1\ \mu\text{mol } H_2O_2/\text{min}$.

Statistics. Statistics were established using a one-way analysis of variance followed by the Student's *t* test. Differences were considered significant for *P* values ≤ 0.05 .

RESULTS

Kinetics of tissue damage and MPO activities. During the first 24 h following $N_3\text{ph-SO}_3\text{H}$ /ethanol treatment, mucosa had a continuous yellow to dark orange colour from 4 h after treatment, and a hyperemia that increased up to 24 h. From 1 to 6 days after $N_3\text{ph-SO}_3\text{H}$ /ethanol administration, proximal colons showed a strong inflammation with at least 80% of mucosal necrosis over 1–5 cm, a dark red to brown colour, and apparent hyperemia and ulcers. On day 9, necrotic tissues were dissociated from the bowel wall by slabs and recovered in distal colonic contents. At days 12–21 after $N_3\text{ph-SO}_3\text{H}$ /ethanol treatment, no more necrotic tissues were present. The wall thickness increased from day 3 to, at least, day 12, followed by a gradual decrease. However, the colonic diameter remained larger than in the control rats until day 21 after treatment. No bowel perforation was found at any time.

MPO activity of the tissues increased from days 1–9 after $N_3\text{ph-SO}_3\text{H}$ /ethanol administration, with a factor in the range 15–26 compared to control values (Fig. 1). From days 12–21, this activity decreased rapidly. All values were significantly different from control values ($P < 0.05$) except at day 21. Maximal MPO activities corresponded to the cellular stage of inflammation with the presence of numerous leukocytes.

Annexin expression. Annexin 1 expression in the proximal colon was increased from 4 h to 21 days after $N_3\text{ph-SO}_3\text{H}$ /ethanol

Fig. 1. Effect of $N_3\text{ph-SO}_3\text{H}$ /ethanol treatment on MPO activity in proximal colon tissues: a time course study. MPO activity in colonic homogenates was determined by spectrophotometric measurement and expressed in U/mg protein with 1 U hydrolyzing $1\ \mu\text{mol } H_2O_2/\text{min}$. The time labeled 0 corresponds to control rats. Data are expressed as means $\pm$ SD with $n = 8$ for each time. Compared to controls, values are significantly increased (* for $P \leq 0.001$ and • for $P \leq 0.005$) at any time after $N_3\text{ph-SO}_3\text{H}$ /ethanol treatment except on day 21.

treatment with maximal levels at 1–9 days post-treatment (Fig. 2A and B). From 12 h until day 6, colonic annexin 1 was always expressed in two forms: the major one had an apparent molecular mass of 38 kDa and corresponded to the intact protein, the minor one migrated slightly faster at 33 kDa. The two forms of annexin 1 were also detected occasionally at 8 h post-treatment (one animal out of four) and at day 9 (four animals out of eight). All the other colonic homogenates expressed only the 38-kDa form of annexin 1. These two forms were confirmed to be annexin 1 because they were both recognized by monospecific anti-annexin 1 polyclonal antibodies from different sources. Moreover, the 38-kDa protein was recognized by a monoclonal antibody directed to the N-terminal end of human annexin 1 (Zymed; data not shown). Annexin 3 expression was also increased during $N_3\text{ph-SO}_3\text{H}$ /ethanol-induced inflammation, especially at days 3–9 post-treatment (Fig. 2B). In contrast, colonic expression of annexins 2 and 6 was similar to that of the control rats throughout the time period after $N_3\text{ph-SO}_3\text{H}$ /ethanol administration (Fig. 2B). Annexins 4 and 5 were not detected in any colonic homogenate.

Annexin secretion. Supernatants from incubated tissues obtained from rats at 12 h to 6 days after $N_3\text{ph-SO}_3\text{H}$ /ethanol treatment contained annexin 1 (Fig. 3A, not shown for 12 h). At day 9, four of the eight tissues tested released this protein. Annexin 1 was not found in supernatants of colonic tissues from controls or in treated rats from days 12–21, even after extensive film exposure. All the tissues that expressed the two forms of annexin 1 in homogenates from freshly isolated colons, released the protein in these two forms when incubated in the RPMI medium (Figs 2 and 3). Annexins 2, 3 and 6, which are expressed in proximal colons, were not found in supernatants (Fig. 3B). When tissues that expressed the two forms of annexin 1 were washed (three times for 20 min in NaCl/P_i plus 2 mM EGTA), or washed the same way and incubated in RPMI medium (for 30 min at 37°C followed by 15 min at 0°C in the presence of 2 mM EGTA), the whole 33-kDa protein and part of the 38-kDa annexin 1 were recovered in supernatants. The corresponding colonic homogenates contained only the 38-kDa protein (Fig. 4). Extraction from the tissues of both annexin 1 forms required the presence of 2 mM EGTA (data not shown).

To determine if the release of annexin 1 is due to a physiological secretion, the amounts of annexin 1 present in both homogenates and supernatants were quantified by immunoblotting and densitometric analysis for four samples from either control

Fig. 2. Immunoblotting analysis of the annexin expression in homogenates of freshly isolated proximal colons at different times after $N_3\text{ph-SO}_3\text{H}$ /ethanol administration. (A) Early effect of $N_3\text{ph-SO}_3\text{H}$ /ethanol on the annexin 1 expression. Colons were recovered at 0 (control rats), 4, 8, 12, 16, 20 and 24 h after treatment. (B) Late effects of $N_3\text{ph-SO}_3\text{H}$ /ethanol on the expression of annexins 1 (An 1), 2 (An 2), 3 (An 3), and 6 (An 6) expression. Colons were isolated at 0 (control rats), 1, 3, 6, 9, 12, 15, 18 and 21 days after treatment. Equivalent amounts of proteins were loaded for each sample. The expressions of annexins 1 and 3 are increased by $N_3\text{ph-SO}_3\text{H}$ /ethanol treatment with a maximum from days 1–9 after administration. The expressions of annexins 2 and 6 remain similar at any time.

Fig. 3. Immunoblotting analysis of annexin secretion by proximal colons during incubation in RPMI medium. Tissues were isolated at different times after $N_3\text{ph-SO}_3\text{H}$ /ethanol administration (0 for control rats, 1, 3, 6, 9, 12, 15, 18 and 21 days) and washed (four washes of 20 min in NaCl/P_i ; three times with 2 mM final EGTA and subsequently once without EGTA). Thereafter, tissues were incubated in RPMI medium for 30 min at 37°C followed by 15 min at 0°C after adding 2 mM final EGTA. The presence of annexin 1 (An 1), 2 (An 2), 3 (An 3), and 6 (An 6) is detected by immunoblotting in homogenates (H) and supernatants (S) after RPMI incubation. Equivalent amounts of homogenate proteins were used for each sample. Supernatant volumes loaded represent five times those of the corresponding homogenates. Annexin 1 is the only one of the annexins detected in the colon that is released into the medium. Its release occurs selectively at days 1, 3, 6, and 9 after $N_3\text{ph-SO}_3\text{H}$ /ethanol treatment.

Fig. 4. Immunoblotting analysis of annexin 1 extraction from one proximal colon isolated 1 day after $N_3\text{ph-SO}_3\text{H}$ /ethanol administration. H1, homogenate of freshly isolated colon; H2 and S2, homogenate and pooled supernatants, respectively of colon washes (four washes of 20 min in NaCl/P_i ; three times with 2 mM final EGTA and subsequently once without EGTA); H3 and S3, homogenate and supernatant, respectively, of colon incubation in RPMI (tissue was washed as previously described, subsequently incubated in RPMI medium for 30 min at 37°C followed by 15 min at 0°C and in the presence of 2 mM final EGTA). Equivalent amounts of proteins were loaded for all homogenates; S2 and S3 volumes loaded represent two and five times more than H2 and H3 volumes, respectively. Migration positions of molecular mass standards are noted on the left (kDa). The whole 33-kDa form and part of the 38-kDa form of annexin 1, which are expressed in the freshly isolated tissue (H1), can be removed in supernatants S2 and S3 in the presence of 2 mM EGTA.

animals or rats after 1, 3, 6 or 9 days of $N_3\text{ph-SO}_3\text{H}$ /ethanol colonic administration (Table 1). The results are expressed as amounts of intracellular annexin 1 (present in homogenates) and as percentages of extracellular annexin 1. The latter were compared to the percentage of cell lysis. Whereas all tissues displayed identical cell lysis, which remained inferior to 5.5% at all the times tested, the percentage of extracellular annexin 1 increased up to 24.4% on day 3 (Table 1). Moreover, there was no correlation between the percentage of cell lysis and the percentage of extracellular annexin 1 ($r = 0.109$, $n = 8$). The percentage of extracellular annexin 1 released by rat proximal colons varied with time after $N_3\text{ph-SO}_3\text{H}$ /ethanol administration. It increased from days 1–3 ($P < 0.01$), and subsequently decreased on days 6 and 9. On days 1–6, $N_3\text{ph-SO}_3\text{H}$ /ethanol treatment increased annexin 1 intracellular expression up to 25–36 times compared to initial values. On day 6, it reached a mean value as high as 7.5% of the total protein, which is much more than the previously estimated cellular contents of this protein [8, 46].

The extracellular form of annexin 1. After centrifugation of culture media for 2 h at $100\,000\times g$, the pellets contained vesicular proteins, whereas the soluble proteins remained in the supernatants. As detected (Fig. 5) and quantified by immunoblotting, 30% of the total secreted annexin 1 was found in the pellets, in the 38-kDa form. Supernatants contained the two forms of soluble annexin 1 (33 kDa and 38 kDa), which constituted 70% of the total secreted protein.

DISCUSSION

As a main conclusion, our study shows that annexins 1 and 3, but not annexins 2, 4, 5, and 6, are overexpressed during $N_3\text{ph-SO}_3\text{H}$ /ethanol-induced rat colitis. Moreover, annexin 1 appears to be selectively secreted during the cellular stage of the colitis. In this model, proximal colons exhibit significantly high levels of MPO activity until day 9, and these high levels corre-

Table 1. Time course comparison of the amounts of intracellular annexin 1, extracellular annexin 1 and cellular lysis during secretion experiments. The amounts of intracellular annexin 1 and the percentages of extracellular annexin 1 were determined by densitometric quantification after immunoblotting in supernatants and colonic homogenates obtained from secretion experiments. Annexin 1 amounts were calculated as ng/ μg total proteins in homogenates. The percentage of extracellular annexin 1 was calculated from the amounts of extracellular (present in supernatants) and intracellular (present in the corresponding homogenates) annexin 1. The percentage of cellular lysis was estimated by LDH activity measurement in homogenates and supernatants. Data are expressed as the mean $\pm$ SD ($n = 4$). This experimental colitis increases annexin 1 intracellular expression and release, with no correlation between the percentage of extracellular annexin 1 and the percentage of cellular lysis.

Time after $N_3\text{ph-SO}_3\text{H}$ treatment	Intracellular annexin 1	Extracellular annexin 1	Cellular lysis
days	ng/ μg total protein	%	
0 (control)	2.1 ± 0.8	not detected	5.1 ± 1.3
1	53.9 ± 23.0^a	11.7 ± 3.2	4.9 ± 0.7
3	59.6 ± 37.4^a	24.4 ± 5.6	5.4 ± 0.4
6	75.4 ± 35.0^a	18.5 ± 3.7	4.4 ± 1.7
9	29.8 ± 20.9^a	12.4 ± 4.8	5.3 ± 1.3

^a Values significantly different from control ($P \leq 0.003$).

Fig. 5. Immunoblotting analysis of the annexin 1 extracellular form in one proximal colon isolated 1 day after $N_3\text{ph-SO}_3\text{H}$ /ethanol administration. H and ST, homogenate and total supernatant, respectively, of colon incubation in RPMI as previously described. SS, supernatant of colon incubation in RPMI after a 2-h centrifugation at $100\,000\times g$; SP, pellet of colon incubation in RPMI after a 2-h centrifugation at $100\,000\times g$. The volume loaded for each supernatant represents five times the volume of the homogenate. The whole 33-kDa form and the major part of the 38-kDa form of annexin 1 were detected in soluble forms in the supernatant of the colon incubation in RPMI after a high-speed centrifugation; a minor part of the 38-kDa form was found in the pellet.

spond to the presence of necrotic mucosa. Subsequently, MPO activities decreased rapidly, from day 12 until day 21 after $N_3\text{ph-SO}_3\text{H}$ treatment. The activities were significantly different from control values except at day 21. This rapid recovery of normal MPO values after $N_3\text{ph-SO}_3\text{H}$ /ethanol administration differs from the findings of several reports [39, 47] although one study reached an identical conclusion [48].

Other investigators previously reported a local increase of the expression of either annexin 1 alone, or various annexins, in pathological conditions [9, 10, 35–37]. For instance, annexin 1 overexpression has been detected in resident keratinocytes from thick skins, or in reactive astrocytes that migrate in kainate-lesioned rat cerebellum [36, 37]. In addition, annexins 1 and 3 were reported to be abundant in inflammatory leukocytes such

as macrophages (annexin 1) [13] and neutrophils (annexins 1 and 3) [49, 50]. In our model, the maximal expression of both annexins 1 and 3 occurs during the cellular inflammatory stage, corresponding to maximal MPO activities. This suggests that such overexpressions may be attributed, at least in part, to local leukocyte invasion of inflamed colons, but the cells overexpressing annexin 1 in this colitis remain to be identified.

This experimental colitis induces intracellular expression of both annexins 1 and 3, but the concept that annexins can have an intracellular anti-inflammatory activity is a subject of controversy [9]. Among the various hypotheses put forward for the intracellular role of annexins, an action in secretory processes and in signal transduction, including PLA_2 activation, has been proposed [9, 50, 51]. However, the pathophysiological consequence of annexin intracellular overexpression during inflammation is still unknown.

In contrast, the extracellular anti-inflammatory properties of annexins are well documented [9–11]. Our results show that annexin 1 is specifically released into the medium of the incubated colon during the cellular stage of colitis. Two forms of annexin 1 (38 kDa and 33 kDa) were detected in inflamed colonic tissues. Only part of the 38-kDa annexin 1 was extracted from colons, and extensive washes with longer incubation times or higher EGTA concentrations did not improve its release (data not shown). This suggests that part of the 38-kDa protein is extracellular because it can be removed from tissues by chelating calcium ions. The non-extractible part is presumed to remain intracellular. The 33-kDa fragment of annexin 1 was completely removable from tissues by washes in the presence of 2 mM EGTA, indicating an exclusively extracellular location. In agreement with our results, it was shown that annexin 1 has a molecular mass of 38 kDa inside the cells and is partially proteolyzed at its N-terminal end, in the extracellular medium [12, 17, 28, 29]. Such a degradation was suggested to downregulate its anti-inflammatory activity [28]. Monoclonal antibodies directed against the N-terminal part of human annexin 1 recognized the 38-kDa rat annexin 1 but not the 33-kDa form, whereas all the polyclonal antibodies tested recognized both forms. This observation strongly suggests that the 33-kDa annexin 1 is a fragment lacking the N-terminus. Moreover, our results support the finding that proteolysis occurs in the extracellular milieu, on the soluble 38-kDa annexin 1. Indeed, after a high-speed centrifugation of culture medium, the 33-kDa form is detected in supernatants, but not in pellets.

Our study supports the view that annexin 1 is also secreted *in vivo*. The colonic expression of both 33-kDa and 38-kDa annexin 1 in freshly isolated homogenates was restricted to tissues that release annexin 1 in secretion experiments. The fact that extracellular 33-kDa annexin 1 was completely removed from tissues upon washing with EGTA prior to incubation in RPMI, leads to the conclusion that annexin 1 was further secreted into the medium during this *in vitro* incubation. Moreover, several observations suggest that annexin 1 release in RPMI medium is a physiological secretion by living cells: (a) this release is specific for annexin 1 since we never detected annexins 2, 3 and 6 in the medium; (b) the percentage of cell lysis is identical whether annexin 1 is released or not; (c) the large amounts of annexin 1 found in supernatants, especially on day 3 after N_3 ph- SO_3H treatment, exclude a release by dead or damaged cells.

Several cytoplasmic proteins, devoid of a secretory peptide signal, have been reported to be released and different alternative secretory pathways have been proposed. One hypothesis is the post-translational/translocation of proteins across the membranes of several organelles. For example, cytosolic interleukin- 1β (IL- 1β) is supposed to translocate within recycling endo-

somes, and thus the exocytotic fusion of vesicles with the plasma membrane should deliver IL- 1β to the milieu in a soluble form [52]. Since the secreted annexin 1 in seminal fluid is totally soluble, it could be released by such a pathway. However, in our model, 30% of extracellular annexin 1 is detected in pellets after a high-speed centrifugation, despite the presence of EGTA. This suggests that the protein is either externally bound to vesicles in a Ca^{2+} -independent manner, or is trapped within them. Indeed, two other secretory pathways, where vesicles containing annexin 1 are released, can be suggested. First, annexin 1 has been localized in late endosomes and has been supposed to mediate inward vesiculation in multivesicular bodies [53]. In the case of a fusion between multivesicular bodies and the plasma membrane, vesicles containing annexin 1 could be released into the extracellular milieu. Secondly, in another pathway, now referred to as ectocytosis [54], evagination of the chondrocyte plasma membrane appears to be responsible for the formation of bone matrix vesicles [55] that are enriched in certain cytoplasmic proteins, including annexin 1 [56]. Such mechanisms can be proposed in our model, but, in order to explain the presence of soluble extracellular annexin 1, vesiculation of the plasma membrane would be followed by a rapid lysis of vesicles.

Using this model, we found that annexin 1 secretion is restricted to the cellular stage of the inflammation, from 12 h to 9 days after N_3 ph- SO_3H administration. This supports the argument that annexin 1 secretion is an induced event related to cell activation in the inflamed gut. Other studies have suggested that annexin 1 secretion and its extracellular action do not occur during all the inflammatory period and models. For instance, peritoneal inflammatory macrophages isolated from thioglycolate-treated animals do not secrete annexin 1, even when incubated in the presence of glucocorticoids [13]. Moreover, local anti-inflammatory action of annexin 1 was not observed in all the tested models, but it can depend on the major inflammatory mediators involved at the time of its administration [22, 26]. For instance, its action in rat paw edema depends on the inflammatory agent used and is not observed during the early stage (1 h after carrageenan administration) that does not involve prostaglandin production but histamine release [22].

Taken together, our data indicate that annexin 1 secretion occurs *in vivo* and *in vitro* in inflamed proximal colon. Moreover, we show that the major part of the extracellular annexin 1 (70%) is in a soluble form. In view of the known anti-inflammatory extracellular properties of this protein, it could be suggested that it is involved in the control of the physiopathology of experimental colitis. The local action of annexin 1 could modulate either the production of eicosanoids and other inflammatory mediators, or leucocyte migration. Indeed, both events are induced during the time of its *in situ* secretion [47, 48], but whether these are annexin-1-induced effects remains to be demonstrated.

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