Short Communication

Involvement of the Receptor for Formylated Peptides in the *in Vivo* Anti-Migratory Actions of Annexin 1 and its Mimetics

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**An innovative avenue for anti-inflammatory therapy
is inhibition of neutrophil extravasation by potentiating the action of endogenous anti-inflammatory mediators. The glucocorticoid-inducible protein annexin
1 and derived peptides are effective in inhibiting neutrophil extravasation. Here we tested the hypothesis
that an interaction with the receptor for formylated
peptide (FPR), so far reported only *in vitro*, could be
the mechanism for this *in vivo* action. In a model of
mouse peritonitis, FPR antagonists abrogated the anti-migratory effects of peptides Ac2-26 and Ac2-12,
with a partial reduction in annexin 1 effects. A similar
result was obtained in FPR (knock-out) KO mice. Binding of annexin 1 to circulating leukocytes was reduced
(>50%) in FPR KO mice. *In vitro*, annexin binding to
peritoneal macrophages was also markedly reduced in
FPR KO mice. Finally, evidence of direct annexin 1
binding to murine FPR was obtained with HEK-293 cells
transfected with the receptor. Overall, these results indicate a functional role for FPR in the anti-migratory
effect of annexin 1 and derived peptides.   *(Am J Pathol
2001, 158:1969–1973)*

The process of leukocyte movement outside the blood
vessels is a hallmark of the host inflammatory reaction.
Cell migration into inflamed tissues is finely regulated
such that several soluble or cell-anchored mediators are
involved in promoting this process. These include adhesion molecules,[1,2] and leukocyte activators: they act in
concert to induce and promote the time-dependent process of cell rolling, adhesion and emigration (or diapedesis) that characterize the initial steps of cell egress
from vessels into tissues.[3] The overall process of cell
migration into inflamed tissues must be finely regulated
and controlled, such that overshooting may lead to tissue
injury and chronic inflammation.[4,5] It is therefore not surprising that a series of check-points are placed at different levels of the path that takes a leukocyte from an
environment of rapid flowing blood to the almost static
extravascular space. For instance, only a fraction of leukocytes rolling on the inflamed endothelium will become
firmly adherent.[6] Similarly, a relevant proportion of adherent cells that are apparently ready to begin the emigration or diapedesis process, actually detach from the endothelium and return to the blood flow.[7,8] Therefore,
endogenous mediators able to counteract the action of
the pro-inflammatory mediators exist and operate at different phases of the leukocyte extravasation process.
These include adenosine, nitric oxide, lipoxin A_4, and
annexin 1 (ANXA1).[9,10]

ANXA1 (previously referred to as lipocortin 1) is a
37-kd member of the annexin superfamily of proteins[11]
particularly abundant in neutrophils, where it represents
up to 4% of the cytosolic proteins.[12] On adhesion to
monolayers of endothelial cells *in vitro* ANXA1 is externalized onto the cell surface of neutrophils with the function
to down-regulate cell emigration through the endothelial
cells.[13] Several laboratories, including our own, have
demonstrated the anti-migratory action of exogenous
and, more importantly, endogenous ANXA1 both in
acute[14] and chronic[15] models of inflammation. The anti-migratory property of the full-length protein is retained by
peptides drawn from the N-terminus region, such as peptide Ac2-26.[16] The target of endogenous ANXA1 and

exogenously administered ANXA1 or peptides seems to be the adherent leukocyte: the net result of their action is leukocyte detachment from the vessel wall rejoining the blood stream.[8,17]

The cellular mechanism for the anti-migratory action displayed by ANXA1 and its mimetic has been until recently elusive. In a recent *in vitro* study, Walther and colleagues[18] reported the existence of a functional interaction between ANXA1-derived peptides and the receptor for formylated peptides (FPR) on human neutrophils, as measured with calcium flux assay and L-selectin shedding. FPR belongs to the group of seven transmembrane domain G-protein-linked receptors, and it is activated by formylated peptides: the downstream effect is neutrophil or monocyte/macrophage activation.[19,20] Importantly, FPR is relatively up-stream of several other receptors for leukocyte activators, and *in vitro* FPR activation can cause their rapid desensitization.[21] Peptide Ac2-26 did not compete with FMLP, however FPR antagonists block its *in vitro* effects.[18] In the present study we have addressed the question of FPR involvement in the inhibitory action of ANXA1-derived peptides on the process of neutrophil extravasation *in vivo*. FPR knock-out (KO) mice[22] were used to test the efficacy and binding capacity also of full-length ANXA1 protein.

Materials and Methods

Animals and Model of Inflammation

FPR KO mice, recently described,[22] have been backcrossed with C57BL/6 for six generations, and C57BL/6 mice (purchased from Banton and Kingsman, Hull, UK) were used as wild-type controls in the experiments. All animals were fed on a standard chow pellet diet with free access to water, maintained on 12-hour light/dark cycles, and housed for 1 week before experimentation. Animal work was performed according to Home Office regulations (guidance on the operation on animals was from the Scientific Procedures act 1986).

Zymosan peritonitis was induced as previously reported.[23] Briefly, mice were injected intraperitoneally with zymosan A (1 mg in 0.5 ml of sterile saline). Animals were sacrificed 4 hours later by carbon-dioxide exposure and peritoneal cavities were lavaged with 3 ml of phosphate-buffered saline (PBS) containing 3 mmol/L ethylenediaminetetraacetic acid. Aliquots of the lavage fluid were then stained with Turk's solution (0.01% crystal violet in 3% acetic acid) and differential cell counts performed using a Neubauer hemocytometer and a light microscope (Olympus B061, JENCONS-PLS, Leighton Buzzard, UK).

Reagents

Human recombinant ANXA1, peptide Ac2-26 (Ac-AMVSEFLKQAWFIENEEQEYVQTVK) or Ac2-12 (Ac-AMVSE-FLKQAW) were obtained as described.[17] The doses used were chosen from a previous study.[17] The FPR antagonists Boc1 (*N-t*-butoxycarbonyl-MLP) and Boc2 (*N-t*-butoxycarbonyl-PLPLP)[18] and other materials were

obtained from Sigma-Aldrich Co. (Poole, UK). *In vivo*, compounds were administered intravenously 15 minutes before zymosan.

ANXA1 Binding

In Vitro

Mice received 20 μg of ANXA1 intravenously at time 0, and were bled by cardiac puncture 5 minutes later. ANXA1 bound on the cell surface was measured using a whole blood staining protocol using 10 μg/ml of monoclonal antibody (mAb) 1B.[24–26] Flow cytometry analysis allowed the identification of the monocyte and polymorphonuclear cell population, and the measurement of fluorescence intensity (green channel) associated with either population.

In Vivo

Because radiolabeling protocols cause ANXA1 degradation, we developed an indirect method to assess ANXA1 binding to leukocytes.[25] An estimation of binding affinity was made using a flow cytometric approximation of Scatchard analysis, in which free ANXA1 is calculated from total ANXA1 added to each tube less the amount bound to the cells.[26] The protocol used has already been described.[25] The macrophage population was identified by flow cytometry for the higher values in forward and side scatter characteristics.[23] HEK 293 cells expressing mouse FPR have already been fully characterized for their response to FMLP.[27,28] The ANXA1 binding assay was performed as described above, and the effect of mouse FPR expression on the binding capacity displayed by the cells was determined.

Statistical Analysis

Comparisons between groups were made using one-way analysis of variance followed by Bonferroni posthoc test. A P value <0.05 was considered significant.

Results

Effect of FPR Antagonism or FPR Deficiency on the Anti-Migratory Actions of ANXA1 and ANXA1-Derived Peptides

Figure 1A shows that the intense 4 hours polymorphonuclear leukocyte (PMN) peritoneal infiltration induced by zymosan was inhibited by peptide Ac2-26 and full-length ANXA1, as previously reported.[16] Co-injection of the FPR antagonist Boc1 (50 μg) abrogated the inhibition exerted by peptide Ac2-26, and significantly attenuated that afforded by ANXA1 (Figure 1A). The FPR antagonist Boc2 was as active as Boc1 on peptide Ac2-26 (Figure 2A).

Whereas there was no difference in the extent of PMN accumulation into the inflamed peritoneal cavities of wild-type and FPR KO mice, the dose-dependent inhibition
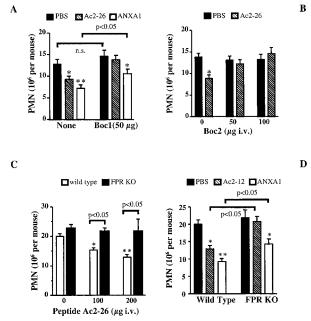
Figure 1. Analysis of ANXA1 and derived peptides anti-migratory activity after treatment with FPR antagonists or in FPR KO mice. **A:** Mice were treated intravenously with 200 μg of peptide Ac2-26, 10 μg of ANXA1, or 100 μl of PBS, alone or together with 50 μg of Boc1, 15 minutes before zymosan. Data are means $\pm$ SEM of 10 to 12 mice per group. **B:** Mice received peptide Ac2-26 (200 μg i.v.) or PBS (100 μl) alone or with the reported doses of Boc2. Data are mean $\pm$ SEM of six mice per group. Wild-type or FPR KO mice were treated intravenously with the reported doses of peptide Ac2-26 (**C**), or with 100 μg of peptide Ac2-12 or 10 μg of ANXA1 (**D**). Controls received PBS alone. In all cases, after 15 minutes mouse peritoneal cavities were inflamed with zymosan (1 mg) and cell influx was quantified 4 hours later. *, $P < 0.05$; **, $P < 0.01$ *versus* respective PBS group.

produced by peptide Ac2-26 was diminished in FPR KO mice (Figure 1C). In this set of experiments, the effect of the shorter peptide Ac2-12 was also tested. Both Ac2-12 and full-length ANXA1 significantly reduced PMN influx in wild-type mice: the shorter peptide was no longer active in FPR KO mice, whereas the inhibition afforded by ANXA1 was again reduced (Figure 1D).

ANXA1 Binding to Circulating Leukocytes

The existence of saturable binding sites for ANXA1 on human[29] and mouse[30] leukocytes has long been known, however the nature of these sites has so far remained elusive.[25] ANXA1 injection into the tail vein of wild-type mice resulted in a detectable binding to circulating monocytes and PMN, as measured *ex vivo* (Figure 2, A and B). There was a marked and significant reduction (40 to 60%) in the extent of binding measured after intravenous injection in FPR KO mice. Figure 2A shows a representative histogram with ANXA1 bound on circulating monocytes, whereas Figure 2B shows the cumulative data for four distinct mice.

ANXA1 Binding to Mouse FPR

Because residual binding and anti-migratory activity were displayed by ANXA1 in FPR KO mice, binding studies were performed *in vitro* on peritoneal macrophages.
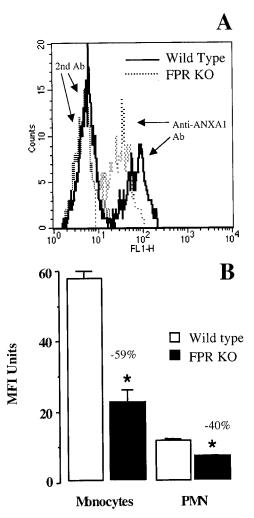

Figure 2. *In vivo* ANXA1 binding to circulating leukocytes. **A:** Representative histograms showing monocyte-associated fluorescence as detected 5 minutes after intravenous treatment with 20 μg of ANXA1 in wild-type and FPR KO mice. Fluorescence because of the second antibody alone is also shown. **B:** As in **A**, but data are means $\pm$ SEM of four distinct mice. Results obtained for the PMN population are also shown. No binding to lymphocytes was detected (not shown). *, $P < 0.05$ *versus* respective wild-type group.

Figure 3A confirms the existence of a saturable binding to these cells,[30] and shows a pronounced reduction in cells taken from FPR KO mice. Nonetheless residual-binding activity is present. Out of four distinct experiments, approximate kd 110 $\pm$ 20 nmol/L and 250 $\pm$ 20 nmol/L could be calculated in wild-type and KO mice. Finally, we sought direct evidence for ANXA1 interaction with mouse FPR. Importantly, HEK-293 cells transfected with mouse FPR displayed higher binding capacity for the protein than control cells (Figure 3B).

Discussion

The glucocorticoid-inducible protein ANXA1 is an endogenous mediator able to down-regulate the process of leukocyte extravasation, and therefore it is one of the mediators responsible for anti-inflammation.[9,10] Identification of the molecular mechanism(s) responsible for ANXA1 anti-migratory activity may lead to the identifica-
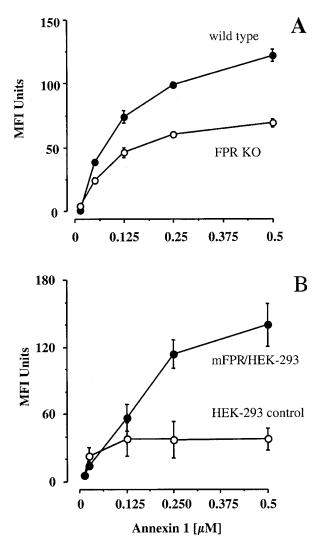
Figure 3. Characterization of ANXA1 binding *in vitro*. **A:** Reported is a saturation concentration-response curve for ANXA1 binding to peritoneal MØ collected from wild-type or FPR KO mice (pool of four animals), as determined *in vitro*. Data are means ± SEM of triplicate determinations, and are representative of three distinct experiments. **B:** ANXA1 binding to 293 control cells and to 293 cells transfected with mouse FPR is shown. Data are from three experiments performed in duplicate.

tion and design of novel anti-inflammatory therapeutics. In our previous studies we have identified in the ANXA1 N-terminus as the region retaining this property.[9,16] It has recently been shown that ANXA1-derived N-terminus peptides activate FPR in *in vitro* settings.[18] In the present study we demonstrate that FPR is functionally linked to the *in vivo* anti-migratory actions displayed not only by ANXA1-derived peptides, but also in the actions of the full-length protein.

This study started by testing the effect of two FPR antagonists in an *in vivo* model of PMN extravasation. Both Boc1 and Boc2 exerted a potent antagonism on the anti-migratory action displayed by peptide Ac2-26. Although these antagonists are relatively selective for FPR (*in vitro* they do not alter the effect of platelet-activating factor on human and mouse neutrophils; data not shown), this finding was validated by using FPR KO mice. These mice are more susceptible to *Listeria monocytogenes* in-

fection than wild-type controls, and succumb at a higher rate after bacterial infection. In addition, their acute inflammatory reaction to thioglycollate is not different from that of control animals.[22] Here, the response to peritoneal zymosan injection was not different between wild-type and FPR KO mice. Overall these data provide *in vivo* relevance to the finding of Walther and colleagues,[18] who tested peptide Ac2-26 on human neutrophils and HeLa cells transfected with human FPR. However, *in vitro* the activity was retained by the region spanning amino acids 19 to 25 (EYVQTVK). In addition, data for full-length ANXA1 were not shown.[18] In our hands, peptide Ac2-12 (given at a dose equimolar to 200 μg of peptide Ac2-26) was able to inhibit (~40%) neutrophil extravasation, and a more pronounced effect was measured with recombinant ANXA1. Both these responses were significantly altered in FPR KO mice, with a significant residual activity being measured for ANXA1. The efficacy of the shorter peptide validated our previous study in which direct observation of mesenteric microvessels was conducted.[17] Future studies are required to address this discrepancy on the sequence of the ANXA1 N-terminus required for FPR activation.

The fact that ANXA1 retained some degree of efficacy in Boc-treated and FPR KO mice clearly indicated the existence of another receptor or mechanism of action. This may not be surprising in view of the length of ANXA1 (346 amino acids; molecular weight, 37 kd).[31] The existence of specific and saturable ANXA1 binding sites on human monocytes and neutrophils was originally described by Goulding and colleagues[25] reporting the existence of a binding protein with an apparent molecular weight of 15 kd. ANXA1 binding to mouse circulating leukocytes and peritoneal macrophages has also been characterized.[16] To test the hypothesis formulated above we performed ANXA1 binding assay to leukocytes of FPR KO mice. *In vivo* administration of ANXA1 resulted in a detectable binding to peripheral circulating leukocytes. As expected,[26] the highest binding was observed to monocytes with a lower but still above basal binding to circulating neutrophils. No binding to lymphocytes was measured (data not shown). A significant reduction in ANXA1 binding was measured on circulating monocytes and neutrophils of FPR KO mice. These data are of importance as they link ANXA1 binding to FPR *in vivo* to the anti-migratory action of the protein. Importantly, no changes in neutrophil response to the chemokine macrophage inflammatory protein-1α[22] or to the FPR2 agonist serum amyloid protein type A[27,28] have been reported in FPR KO mice, suggesting (in absence of specific antibodies) that at least the receptors used by these chemoattractants are not altered by deletion of the mouse FPR gene.

The apparently modest binding to PMN is expected because adhesion to endothelial cells augments ANXA1 binding capacity to this leukocyte type.[26] Importantly FPR can be exported on the neutrophil cell surface on adhesion because it is contained in few cytoplasmic granules and vesicles.[32] Because ANXA1 is predominantly contained in gelatinase granules,[33] we propose that PMN adhesion to the endothelium triggers externalization of ANXA1 and FPR, and therefore activates the entire pathway. The *in vivo* data here presented suggest that

interaction with FPR is the mechanism responsible for the leukocyte detachment phenomenon promoted by endogenous and exogenous ANXA1, and peptides Ac2-26 and Ac2-12.[8,17]

In conclusion, this study provides the first *in vivo* evidence that FPR is functionally involved in the *in vivo* inhibitory effects of ANXA1 and derived peptido-mimetics. We also report for the first time reduced ANXA1 binding in the absence of a specific receptor. On the other hand, strong indication that other receptor(s) and/or mechanism(s) must be involved in the action of full-length ANXA1 is also provided. For instance direct interaction between ANXA1 and very late antigen-4 on a human monocytic cell line has recently been demonstrated.[34] In view of the lack of direct competition between peptide Ac2-26 and formyl-Met-Leu-Phe *in vitro* binding assays,[18] and the fact that formyl-Met-Leu-Phe and ANXA1 are clearly able to produce opposite effects in inflammation, it is likely that we have so far only scraped the surface of a complex and fascinating biological system.

Acknowledgments

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